ISSN 0378-4347

VOL. 277 OCTOBER 14, 1983
(Biomedical Applications, Vol. 28)
COMPLETE IN ONE ISSUE

JOURNAL OF CHROMATOGRAPHY
BIOMEDICAL APPLICATIONS

ELSEVIER

JOURNAL OF CHROMATOGRAPHY

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0378-4347/83/$03.00

Printed in The Netherlands

VOL. 277 JOURNAL OF CHROMATOGRAPHY OCTOBER 4, 1983

(Biomedical Applications, Vol. 28)

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(Abstracts/Contents Lists published in Analytical Abstracts, Biochemical Abstracts, Biological Abstracts, Chemical Abstracts, Chemical Titles, Current Contents/Physical, Chemical & Earth Sciences, Current Contents/Life Sciences, Index Medicus, Mass Spectrometry Bulletin and Science Citation Index)

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JOURNAL OF CHROMATOGRAPHY

Vol. 277 (1983)

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JOURNAL
of
CHROMATOGRAPHY

INTERNATIONAL JOURNAL ON CHROMATOGRAPHY,
ELECTROPHORESIS AND RELATED METHODS

BIOMEDICAL APPLICATIONS

Vol. 28 1983

ELSEVIER
AMSTERDAM — OXFORD — NEW YORK

0378-4347/83/$ 03.00

Printed in The Netherlands

Journal of Chromatography, 277 (1983) 1—24
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1809

REVIEW

CHROMATOGRAPHIC ANALYSIS OF GLUTAMIC ACID DECARBOXYLASE IN BIOLOGICAL SAMPLES*

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(First received March 25th, 1983; revised manuscript received June 3rd, 1983)

CONTENTS

1. INTRODUCTION

L-Glutamic acid (GA) is formed from the deamidation of L-glutamine by the enzyme L-glutamine aminohydrolase (EC 3.5.1.2., glutaminase) [1, 2]. The product of this reaction is then α-decarboxylated by L-glutamic acid decarbox-

*Portions of this manuscript have been previously presented at the 38th Georgia Academy of Sciences, Mercer University at Macon, Georgia (April 1980) and the 181st American Chemical Society National Meeting in Atlanta, Georgia (April 1981).

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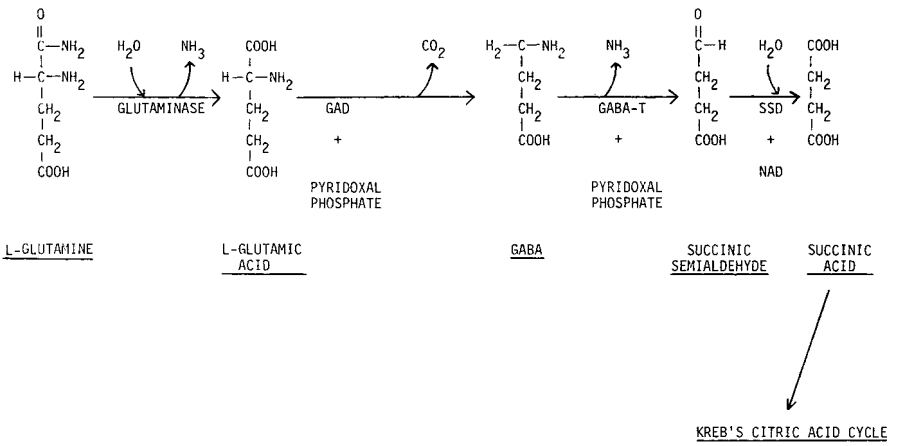

Fig. 1. Overall reaction scheme for conversion of L-glutamine to GABA and its metabolism to succinic acid. The enzyme GAD is believed to be the rate-limiting step which produces steady-state levels of GABA.

ylase (EC 4.1.1.15., GAD) to form γ-aminobutyric acid (GABA). GAD is a highly specific enzyme that requires pyridoxal phosphate as a coenzyme and is believed to be the rate-limiting step that produces steady-state concentrations of GABA in tissues [3—5]. GABA is metabolized by 4-aminobutyrate-2-ketoglutarate transaminase (EC 2.6.1.19., GABA-T) to succinic semialdehyde and further by succinic semialdehyde dehydrogenase (EC 1.2.1.16., SSD) to succinic acid which can enter the Krebs citric acid cycle. This overall scheme of the reactions is presented in Fig. 1. An excitatory effect is observed by GA in the central nervous system (CNS) whereas its α-decarboxylation product (GABA) is established as a principal inhibitory neurotransmitter in the CNS of vertebrates [6—8]. GABA is considered as a hyperpolarizing inhibitory neurotransmitter and lately data indicate GABA mediated depolarization of afferent nerve terminals suggesting a presynaptic inhibitory function [9—11].

GAD is indicated to be the rate-limiting enzyme that determines steady-state levels of GABA in the nervous system. The concentration of GABA correlates well with the GAD activity in tissues. However, GAD is found solely within the neurons whereas GABA is distributed in glial cells as well as within neurons. Therefore, the activity of this enzyme is used as a more precise marker for GABAnergic neurons than GABA due to the latter's distribution and possible metabolism during tissue preparation [12].

In the CNS, GABA projections from cortex to neostriatum to globus pallidus (GP), entopeduncular nucleus (EP) and substantia nigra (SN) [13, 14] as well as from SN to ventromedial thalamus (VM) [15] are reported. In structures associated with the extrapyramidal system high concentrations of GABA and GAD are observable. In several mammalian species the highest levels of these two components are found in the SN and GP [16, 17].

Assay procedures for this enzyme and its major product have their greatest utility in clinical practice for postmortem studies of CNS pathology. Reduced levels of GABA are found in brain regions of some patients with schizophrenia [18] and dominantly inherited cerebellar disorders [19]. Postmortem brains of

Huntington's chorea patients (these patients presently account for 1% of all chronically hospitalized individuals in mental institutions) contain reduced content of GABA and GAD in cortex, striatum and SN [20—23]. Cerebral spinal fluid from live Parkinson's disease patients contains significantly decreased quantities of GABA when compared with normal controls [24] and published autopsy studies present data of low concentrations of GAD and GABA in striatum and SN [20, 25]. Recently, however, Perry et al. [26] have published data that mean GAD activity in Parkinson's disease patients (when matched with better control subjects for agonal status) is lower in the putamen than that of controls, but that the difference is not significant; also, increased levels of GABA are found. This recent study cast doubt on the hitherto traditional assumption that GABAnergic neurotransmission is decreased in the striatum of Parkinson's disease [26].

To date, there are four different types of procedures utilized for analysis of GAD in tissue samples. Of these methods only one directly measures the enzyme's concentration whereas the others measure the enzymatic activity. The first technique is that of mannometric determination of carbon dioxide (CO_2) evolved by α-decarboxylation of GA. Subsequently, via use of L-[^{14}C]GA as substrate, radiochemical methods are now employed for measurement of $^{14}CO_2$ evolution with picogram sensitivities. The second procedure consists of analysis of GABA formed by the coupled reactions of GABA-T and SSD which gives rise to an equivalent number of moles of NADPH. Fluorometric quantitation of NADPH by this enzymatic cycling process approaches the femtomole range. A third method utilizes the measurement of GABA formation from tissue homogenates incubated with GA as substrate. GABA is separated from other components in the mixture chromatographically and with the use of different analytical techniques the limits of detection range from nanograms to picograms. The last methodology requires the use of immunocytochemical and immunological techniques with nanogram quantitation. These procedures involve the direct measurement of the enzyme's concentration and not its activity. Antibodies are produced against GAD and its concentration is determined fluorometrically, radiochemically or microscopically.

In this article all four methodologies used for GAD analysis will be discussed. However, the major focus will be upon those that involve or rely upon chromatographic procedures for separation and analysis. For more detailed information concerning the other non-chromatographic procedures the reader is referred to the references of Roberts et al. [7], Wu [12] and Krogsgaard-Larsen et al. [27]. Chromatographic methods have become increasingly important for quantitation and specificity of analysis. Most involve the measurement of the enzyme's activity via GABA formation following incubation of the tissues. Also discussed in this review will be some of the common problems that arise and introduce errors in activity calculations such as alternative pathways and post-mortem changes.

2. ASSAY ERRORS

2.1. Alternative pathways

In 1972, Wilson et al. [28] reported an observation of differences in GAD activity measurement between $^{14}CO_2$ evolution and [^{14}C]GABA formation in tissue cell cultures. The radiolabeled gas was analyzed by radiochemical methods and [^{14}C]GABA was separated from the cell culture mixture after incubation with [^{14}C]GA via thin-layer chromatography (TLC) and quantitated by scintillation counting. GAD activity measured by [^{14}C]GABA formation was as much as 10% less than that estimated by $^{14}CO_2$ production. It was concluded that excess $^{14}CO_2$ was produced from glutamate by pathways other than GAD. Drummond and Phillips [29] later published similar data concerning GAD activity in non-neural tissues. [^{14}C]GABA was separated and analyzed with an amino acid analyzer from crude tissue homogenates. The differences observed with greater $^{14}CO_2$ production than [^{14}C]GABA was attributed to the alternative CO_2 producing pathway of the coupled reactions of glutamic acid dehydrogenase (EC 1.4.1.2.) and lipoate acetyltransferase (EC 2.3.1.12.) of the α-ketoglutarate dehydrogenase complex.

Another problem that has arisen with CO_2 methods has been associated with impurities that have been found in commercially labeled GA. These impurities gave rise to additional CO_2 production without concurrent stoichiometric production of GABA [29—31]. It was noted that a carbonyl-trapping agent, aminooxyacetic acid, stimulated excess formation of $^{14}CO_2$ in brain homogenates when unpurified [^{14}C]GA was utilized [31]. However, upon purification of the substrate, significantly less $^{14}CO_2$ was formed. The impurity 2-pyrolidone-5-carboxylate isolated from commercial L-glutamate preparations has been suggested to cause increased CO_2 production [32]. Using impure radiolabeled L-glutamate, Wu et al. [33] analyzed GAD activity in various tissues by five different assay techniques. One method utilized the trapping of radiolabeled $^{14}CO_2$ whereas the other four involved the quantitation of radiolabeled GABA formation by various chromatographic means. In brain tissue all five techniques gave similar results; however, when samples of non-neural tissues were tested (heart, liver, kidney) only the methods utilizing GABA formation for activity determination had similar results. The activity measured via the $^{14}CO_2$ technique was three to four times greater than that of the other procedures and lead the authors to suggest that GAD measurement in non-neural tissue did not give valid information for activity measurements by CO_2 trapping techniques.

Wu et al. [34] demonstrated that mouse brain GAD will also utilize L-aspartic acid as a substrate and identified the α-decarboxylation products as β-alanine. This compound possessed approximately 3—5% of the activity of L-glutamate as substrate. Also, recent evidence suggests that GABA may be produced from 4-aminobutyraldehyde in mammalian brain by 4-aminobutyraldehyde dehydrogenase [35]. Therefore, it is possible to have GABA production from alternative pathways. However, at this time information is limited and further evidence must be acquired before any conclusive statements can be made concerning alternate GABA producing pathways.

2.2. Postmortem changes

Extremely rapid GABA formation is observed in rat brain tissues if it is not frozen within minutes of death [36—41]. The increase in GABA formation begins about 1—2 min following death and reaches levels of 140% of initial values within 5 min [36, 37]. These increases in GABA concentration occur in both solid tissue or tissue suspensions that are not cooled immediately postmortem. A possible explanation for these observed increases in GABA content is that there is a 9% increase in the activity of the GAD enzyme in rats for the first 2 h after decapitation followed by a slow decline to zero activity. After a period of time the GA content would be expected to be reduced and not as much GABA, as is found experimentally, produced. However, hydrolysis of proteins, peptides and reduced glutathione may serve as a possible source of GA [42]. Also, the degradative enzyme of GABA, GABA-T, apparently loses its activity for metabolism of GABA and compounds such as aminooxyacetic acid have been demonstrated to specifically inhibit GABA-T and not GAD in rat brain tissue [36]. In Table 1 are presented data [38] of rapid postmortem changes observed fluorometrically in temperature preserved and non-preserved rat brain tissue. The left hemispheric samples were immediately cooled postmortem whereas the right hemisphere samples were allowed to stand at room temperature (22°C) for 10 min before being treated for analysis. In the GP and SN (medial and lateral) over 100% increases in GABA were noted in the samples left at room temperature.

TABLE 1

POSTMORTEM CHANGES IN GABA OBSERVED IN LEFT AND RIGHT HEMISPHERES OF RAT BRAIN

Values expressed as μg GABA.

Brain region	Left hemisphere*	Right hemisphere**
GP	0.64	1.39
SN medial	2.45	5.12
SN lateral	2.07	4.28

*Samples immediately cooled postmortem.
**Samples left at room temperature 10 min postmortem.

A number of techniques have been developed to preserve the enzyme's activity and reduce the rapid postmortem GABA increases. These techniques include such methods as dropping rats head first into liquid nitrogen or decapitation followed by liquid nitrogen treatment or ice water [36, 37]. A method reported by Holdiness et al. [43] has proved useful for analysis of GAD activity in micropunches (< 2 mg) of rat brain tissue. In this procedure the rat was decapitated with a guillotine and the brain rapidly removed and frozen in dry ice. The brain samples were transferred to a temperature controlled microtome where micropunches were obtained of appropriate slices. Since GABA production was analyzed for the enzyme's activity, the sample was homogenized and half was transferred to a test tube containing 10% trichloroacetic acid for en-

zyme inactivation. This sample served as the blank and its concentration of GABA was subtracted from that of non-denatured incubated sample to obtain the correct GAD activity.

3. ASSAY PROCEDURES (NON-CHROMATOGRAPHIC)

3.1. Mannometric and radiolabeled carbon dioxide methods

Roberts and co-workers [4, 44] developed the first mannometric technique for GAD activity analysis which involved the measurement of CO_2 evolution following α-decarboxylation of GA. In 1959 Albers and Brady [45] reported the first radiometric procedure utilizing L-[1-^{14}C] GA as substrate leading to the formation of $^{14}CO_2$. The limit of detection of this particular method was 10^{-11} mol of radiolabeled carbon dioxide. Later Sisken et al. [46] and Roberts and Simonsen [47] made improvements in gas-trapping procedure and apparatus used for gas collection. It was noted that the atmosphere in which the crude homogenates were incubated could effect the results of the assay. Sisken et al. [46] reported that higher concentrations of $^{14}CO_2$ were recorded in an oxygen atmosphere as opposed to a nitrogen atmosphere. Others have also observed increased CO_2 production in an oxygen containing atmosphere as compared with inert atmospheres even after impurities had been carefully removed from commercial preparations of GA [47, 48]. This excess production of $^{14}CO_2$ was greatly reduced when an inert atmosphere, Triton X-100, or mitochondrial electron transport inhibitors were utilized during incubation of the homogenates [48, 49].

3.2. GABA-transaminase and fluorometric procedures

In Fig. 2 the enzymatic cycling procedure of the coupled reactions of GABA-T and SSD is presented [12, 50]. This reaction scheme combines the GABA assay of Jakoly and Scott [51] with the enzymatic cycling procedure of Lowry et al. [52]. GABA and α-ketoglutarate are converted to succinate semialdehyde (SSA) and glutamate by GABA-T and this product (SSA) is further converted to succinate in a second reaction by succinate semialdehyde dehydrogenase. Equivalent moles of $NADP^+$ in the second reaction are reduced to NADPH. In the third step of the reaction NADPH is oxidized to $NADP^+$ by glutamate dehydrogenase (GDH) and then reduced back to NADPH by glucose-6-phosphate dehydrogenase (EC 1.1.1.49., G-6-PD) in the fourth reaction. An amplification of 10,000 fold is achieved in 1 h. Therefore, for every GABA or NADPH molecule added, 10,000 molecules of 6-phosphogluconate are formed. The 6-phosphogluconate (6-PG) in reaction 4 is converted to ribose-5-phosphate (R-5-P) by 6-phosphogluconate dehydrogenase (EC 1.1.1.44., 6-PGD) and $NADP^+$ is subsequently reduced to NADPH which is determined fluorometrically. The production of NADPH is equivalent to the amount of GABA formation. Amplifications up to 100,000,000 are possible with a detection limit of 10^{-18} mol GABA [12]. This technique can be applied to the analysis of GAD activity in single isolated nerve cell bodies in the CNS [53]. However, this method is limited in crude extracts by the effects of inhibitors or activators on

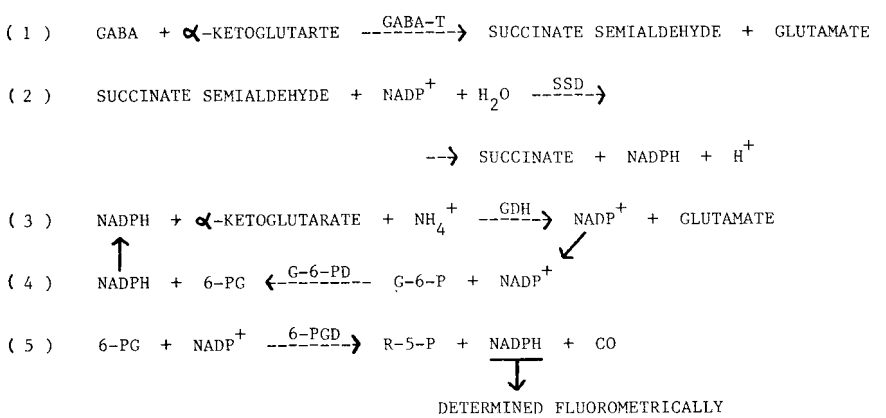

Fig. 2. Principle of microdetermination of GABA by a combination of enzymatic cycling and GABAase system (GABAase refers to the coupled enzymes: GABA-T and SSD). (Reproduced with permission from ref. 12.)

the coupling reactions and by the inadequate purity of available enzymes.

The first fluorometric procedure was reported by Lowe et al. [54] in 1958. This method relied upon measurement of GABA formation in order to calculate GAD activity. A tissue homogenate was incubated with L-glutamate in sodium phosphate buffer at pH 6.4 and pyridoxal-5-phosphate. The limit of detection of the procedure was 10^{-7} mol GABA. Later MacDonnel and Greengard [48] compared Lowe's method [54] with that of a $^{14}CO_2$ technique. When Triton X-100 was utilized in an air atmosphere both procedures gave similar results of all tissues analyzed. Triton X-100 increased the fluorometric assay activity but a decrease in the isotopic assay activity was recorded in all cases. The detergent was believed to activate GAD and concurrently inhibit non-GAD CO_2 release from glutamate. The authors of this paper suggested that it was more reliable to quantitate GAD activity via GABA formation than CO_2 evolution since Triton X-100 may not completely inhibit non-GAD dependent decarboxylation of glutamate in all tissue homogenates. Holdiness et al. [43] reported improvements in the method of Lowe et al. [54]. In this procedure an improved sample collection procedure was described and the reaction of GABA with ninhydrin for fluorometric analysis had a detection limit of $0.20 \cdot 10^{-6}$ mol GABA. Activities reported in sub-regions of rat brain were 7.91 ± 1.47 (GP), 6.87 ± 2.07 (EP), 3.83 ± 0.69 (VM), 13.80 ± 2.14 (SN_m) and 8.23 ± 2.26 (SN_l) μg GABA per h per mg protein. Protein was measured by the method of Lowery et al. [55] and the anatomical tissue punch placement can be observed in Fig. 3 (atlas of Pellegrino and Cushman [56]).

3.3. Immunochemical assays

Immunochemical methods provide a powerful means for quantitation of the enzyme's concentration and anatomical location rather than its activity as previously discussed. The techniques use radioimmunoassay, microcomplement fixation and enzyme-labeled immunoassay procedures. These methods employ the making of a specific antibody against GAD [57]. Red blood cells are sensi-

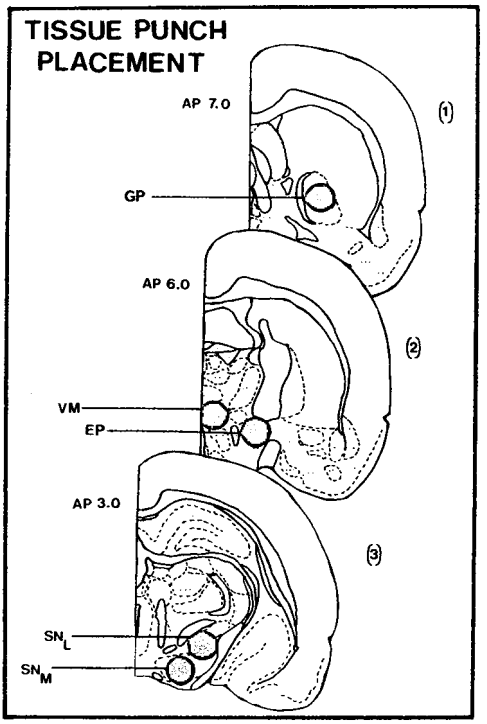

Fig. 3. Tissue punch placement. The first punch was from a 1-mm thick slice containing the globus pallidus (GP). The second slice contains the entopenduncular nucleus (EP) and ventromedial thalamus (VM). The third slice contains the substantia nigra medial (SN_M) and lateral (SN_L) punches. The numbers on the left refer to the anterior—posterior axis coordinates in the brain atlas of Pellegrino and Cushman [56]. Punch diameter, 1.30 mm.

tized with an antigen and reacted with anti-GAD immunoglobins. The amount of complement fixed after the antibody is added is proportional to the amount of antigen. This microcomplement fixation test can detect nanogram levels of GAD [58]. Radioimmunoassay and enzyme immunoassay procedures involve incubation of known quantities of the labeled enzyme antigen with unlabeled antigen. Binding is established by a standard curve and the concentration of the unknown antigen is determined. Also, peroxidase-labeled antibody is utilized for visualization of GAD at the light and electron microscopic levels in brain tissue [59—61].

4. CHROMATOGRAPHIC PROCEDURES

All chromatographic methods utilize the formation of GABA from L-GA as a means for GAD activity measurement. The product (GABA) is chromatographically separated from the homogenate incubation mixture before analysis. If radiolabeled GABA is formed, following separation from its radiolabeled substrate, it can be generally analyzed without further purification of the sample. However, if non-radioactive methods are employed a sample of tissue homogenate should be analyzed for GABA content prior to incubation. Following incubation, the original content of GABA present in tissues is subtracted from

that of the incubation mixture and a more precise measurement of GAD activity is acquired.

The chromatographic procedures to be described are finding wide utility in many laboratories. These methods offer similar sensitivity and generally better specificity for estimation of GAD activity in various biological media.

4.1. Paper and thin-layer chromatography

In 1959, Wilson et al. [62] reported what seems to be the first paper chromatographic procedure for separation of GABA. By this method [^{14}C]GA was incubated with a suspension of lyophilized cells of *Clostridium welchii*. The [^{14}C]GABA formed was separated by paper chromatography and identified via ninhydrin reaction and R_F values. GAD activity was not estimated; instead, the [^{14}C]GABA generated was utilized for further studies in rats and determined to be further metabolized to succinate in vivo. Kravitz [63] in 1962 incubated L-[3,4-$^{14}C_2$]GA and L-[U-^{14}C]GA with central ganglia and peripheral nerve bundles of lobsters. These incubations were lyophilized and separated by paper chromatography. The author observed with L-[U-^{14}C]GA that $^{14}CO_2$ and not radioactive GABA was formed; however, using L-[3,4-$^{14}C_2$]GA as substrate [^{14}C]GABA and not radioactive CO_2 was produced. Also noted in this experimentation was that GABA caused a reduction in the release of transmitters from excitatory nerve terminals at crayfish neuromuscular junctions. Prabhakaran and Braganca [64] published results indicating the presence of GAD activity from leprosy skin lesions. *Mycobacterium leprae* were separated from the skin nodules and incubated with and without L-GA. The preparations were able to decarboxylate GA and GABA was separated and identified via paper chromatography using ninhydrin for visible detection. DL-[3-$^3H_1$]GA in addition to DL-[U-^{14}C]GA have been used as substrates for enzymatic analysis [65]. In this case either substrate was incubated with lobster nerve cord and the reaction stopped by pipetting the mixture onto a Dowex-1 acetate chloride column. The GA was absorbed while GABA and other neutral amino acids passed through the column. The ^{14}C- or ^{3}H-radiolabeled GABA was then separated and identified by ascending paper chromatography. A Packard Strip Scanner was used to scan the radioactive chromatograms with nanogram levels of detection.

High-voltage paper electrophoretic procedures have been devised for GAD activity assays [66, 67]. As mentioned previously, Wu et al. [33] compared five different techniques of GAD analysis in different tissues one of which was electrophoretic. By this method the GABA concentration was determined by the ratio of total area of the electrophoresis to the area of the GABA peak. This method of analysis of GAD activity was found to be in agreement with the other procedures used except for the $^{14}CO_2$ method when tested in non-neural tissues.

In 1967 Homola and Dekker [68] observed the α-decarboxylation of a number of radiolabeled analogues of GA. The incubation mixtures were separated by two-dimensional TLC and the products quantitated radiometrically. Using this technique of separation and identification it was noted that the threo isomer of γ-hydroxy-L-glutamate could serve as a substrate for GAD with approximately 20% of the activity as that of L-glutamate. Wilson et al. [28] compared

the evolution of $^{14}CO_2$ from L-[U-^{14}C]GA with that of γ-amino[U-^{14}C]butyric acid formation in glial cell cultures. Using two-dimensional TLC for separation of radiolabeled GABA they observed up to 10% difference between the $^{14}CO_2$ evolution method as compared with [^{14}C]GABA. It was concluded that the increased $^{14}CO_2$ was being formed via alternative pathways other than that of GAD in these cell cultures. Miller and Martin [30] demonstrated by TLC that commercial L-[^{14}C]GA contained an impurity that produced excess CO_2. Aminooxyacetate, an inhibitor of GAD, was found to clearly stimulate $^{14}CO_2$ production from unpurified GA as when compared with production of [^{14}C]GABA from purified [^{14}C]GA in an incubation mixture. By this procedure, following incubation with purified [^{14}C]GA, the reaction products were separated by passing the contents through an AG1-X2 resin to retain GA and allow [^{14}C]-GABA to pass. The radiolabeled GABA was then further separated and purified on cellulose TLC plates and the spots quantitated by audioradiography. Wood et al. [69] also used cellulose thin-layer sheets for separation of GABA from mouse brain homogenates utilizing prepurified [^{14}C]GA as substrate. The solvent system consisted of chloroform—methanol—ammonia (2:2:1) and the chromatographic procedure required 35 min for development via a one-dimensional separation. The samples were analyzed with a scintillation counter and recovery was found to be 97% following deproteinization. Sellstrom et al. [70] and Prostenik et al. [71] both applied one-dimensional TLC for determination of GAD activity in brain homogenates. Radiolabeled GABA formed from the incubation mixture was identified by ninhydrin reaction and quantitated via scintillation counting in the former method [70]. The latter method of Prostenik et al. [71] used densitometry for quantitation of GABA. Pahuja and Reid [72] have published the latest known ion-exchange TLC method for analysis of GAD activity in bovine sub-retinal intercellular fluid and retina. The substrate (5 m*M*) of L-GA was incubated in the above mentioned homogenates (50 μl) with 50 m*M* potassium phosphate, pH 6.8, 1 m*M* EDTA, and 0.5 m*M* pyridoxal phosphate. The enzymatic reaction was stopped by immersion in ice water and 5 μl of the incubation mixture spotted on Ionex SB-Ac plates. Ascending TLC was carried out using water—ethyl acetate (92:8) and following chromatography the strips were cut out and analyzed by ninhydrin reaction. Separation of GABA (R_F 0.95) from glutamic acid (R_F 0.06) and other reaction products was obtained in 30 min. Fig. 4 presents the complete separation of radiolabeled GABA formed from the use of L-[3,4-$^3H_1$]glutamic acid as substrate when incubated with crude homogenates. Under the same conditions as described, incubation of the homogenate and radiolabeled GA without the enzyme for 2 h resulted in absolutely no formation of GABA by other enzymatic or non-enzymatic means. This present method was able to detect GAD activity in 1 μg of crude extracts when tritiated labeled GA (2000 cpm/nmol) was used and separation of the GA from GABA was not affected by changes in ionic strength or pH of the reaction mixture [72].

GAD activity has also been determined by the measurement of dansylated GABA. The reaction product from the incubation mixture was reacted with 5-dimethylamino-1-naphthalene sulfonyl chloride (dansyl chloride, Dns-Cl) via the procedure of Roberts et al. [73]. The samples were then separated on polyamide TLC plates and quantitated spectrophotometrically. Radiolabeled

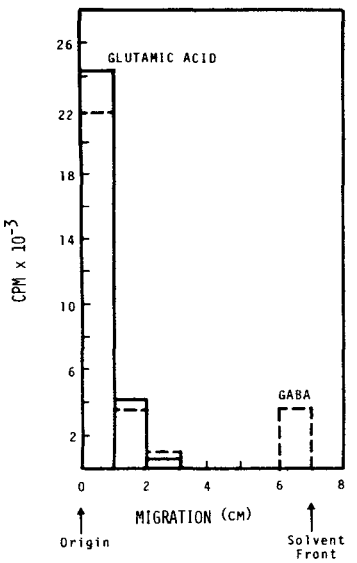

Fig. 4. Separation of [^{3}H]GABA (———) from [^{3}H]glutamic acid. After development, the strip was dried, cut into 1-cm pieces and radioactivity counted. The assay mixture was incubated with 180 μg of crude enzyme preparation at 37°C for 60 min. The control (—) contained the assay mixture mixed with enzyme at 37°C followed immediately by cooling at 0°C. The composition of the assay mixture has been described in the text. (Reproduced with permission from ref. 72.)

GA was also tested by this procedure and the [^{14}C]GABA formed was chromatographed under the same conditions as a test for the specificity of the analytical procedure. Morum and Wasterlain [74] reported the comparison of TLC separated dansylated [^{14}C]GABA with $^{14}CO_2$ production in tissue homogenates. Again the GAD values calculated by the CO_2 method were higher than the GABA method which lead the authors to suggest that the lack of stoichiometric agreement was probably due to alternative pathways and impurities in the substrate preparations. Osborne [75] reported the use of [^{14}C]dansyl chloride for derivatization of GABA from tissue homogenates. The fluorescent product was readily isolated by polyamide one-dimensional TLC and quantitated radiometrically. Later Osborne [76] introduced [^{3}H]dansyl chloride and reported detection limits of 1 pmol of GABA in nervous tissue. Strang [77] isolated GABA as the Dns-γ-butyralactone derivative with silica gel H. The solvent system used for chromatographic development was 18% ethanol in chloroform. The samples were analyzed radiometrically by $^{14}CO_2$ evolution and fluorescence and both gave comparable agreement.

4.2. Amino acid analyzers and ion-exchange chromatography

Wu et al. [34] demonstrated that L-[U-^{14}C]aspartic acid possessed about 3—5% of the activity found with L-glutamate as substrate in mouse brain extracts. The evidence came directly from α-decarboxylation and identification of the reaction products β-alanine and CO_2. These products were shown not to be due to impurity contamination in the L-glutamate. The reaction products were

identified with an amino acid analyzer on a cation-exchange resin and derivatized with ninhydrin for detection. Others had previously reported that bacterial GAD could decarboxylate α-methyl-DL-glutamic acid and L-glutamine in addition to L-glutamate [78]. Landcaster et al. [79, 80], Kravitz and Potter [81] and Moore and Stein [82] have described the analysis of GAD activity via amino acid analyzers. In the case of Landcaster et al. [79, 80] [^{14}C]GA was incubated with human kidney homogenates and the [^{14}C]GABA produced was separated from the substrate and homogenation mixture and analyzed by elution on an ion-exchange resin. The products were quantitated via scintillation counting. It was noted in these papers that renal GABA may play a role in acid—base metabolism of the kidneys.

Various ion-exchange methods have been used for GAD activity analysis [65, 81, 83, 84]. One procedure previously mentioned was that of Miller and Martin [30] in which a cation-exchange resin was used for partial purification of an incubation mixture prior to final analysis of GABA production via TLC and audioradiography. Chude and Wu [31] prepurified L-glutamate (radiolabeled) via ion-exchange chromatography prior to incubation. The substrate was incubated with GAD and the mixture separated by a Bio Rad AG1X column. The incubation mixture consisted of 0.1 ml of 0.1 *M* potassium phosphate, pH 7.2 with 0.2 m*M* pyridoxal phosphate, 1 ml 0.05 *M* potassium phosphate, pH 6.5 and 1 m*M* 2-aminoethylisothiouronium bromide, 0.2 m*M* pyridoxal phosphate and 0.2 ml 0.5 *N* sulfuric acid. The [^{14}C]GABA was completely eluted and GA was retained on the column by suction filtration. Radiolabeled GABA was measured by scintillation counting. This method offered advantages of rapid analysis and was more direct since CO_2 was not involved for analysis.

4.3. High-performance liquid chromatography

High-performance liquid chromatography (HPLC) methods for GABA and GAD activity analysis have become available only in recent years. These methods possess low detection limits and increased specificity for product measurement. Ion-exchange HPLC coupled with post-column derivatization and fluorescence detection has been applied for GABA analysis (GAD activity was not measured) by various investigators [85—89]. The limits of detection for these methods range from 1—50 pmol. Griesmann et al. [90] devised a fluorometric HPLC method for detection of dansylated GABA in rabbit cerebellum. This procedure involves pre-column derivatization with dansyl chloride and separation was effected with a reversed-phase column. The limit of detection was 100 pmol and this assay was compared with established TLC [91] and amino acid analyzer methods [82] using radiolabeled GABA. The HPLC procedure differed by +1.0% and −5.5% when compared with the TLC and amino acid analyzer methods, respectively [80]. Pahuja et al. [92] have also applied reversed-phase column separation of dansylated GABA from retinal cells. In this particular case GAD activity was measured via use of [^{3}H]GA as substrate. Following incubation of the homogenate with the radiolabeled substrate the products were derivatized in reaction vessels and lyophilized before HPLC injection. Ultraviolet (UV) absorption at 206 nm was used for detection (Fig. 5) and fractions were collected and analyzed via scintillation counting (Fig. 6A

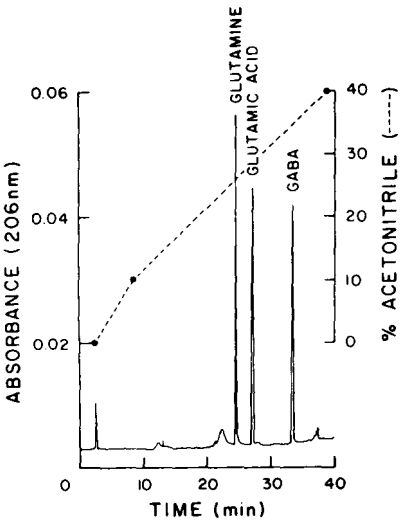

Fig. 5. Chromatogram showing the separation of 0.5 μg of dansylated glutamine, glutamic acid and GABA. The dashed line indicates the mobile phase gradient (% acetonitrile) used. (Reproduced with permission from ref. 92.)

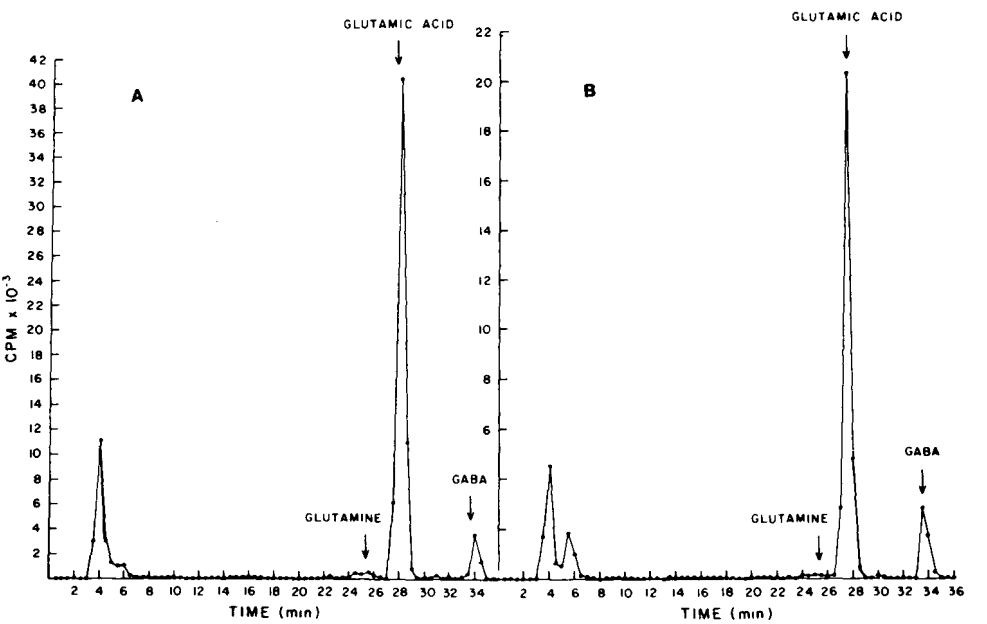

Fig. 6. Chromatograms of the reaction mixture of (A) crude brain extract under GAD assay conditions, 0.17 mg of crude enzyme protein plus phosphate buffer plus pyridoxal phosphate incubated for 1 h; (B) crude retinal extract under GAD assay conditions, 0.30 mg crude enzyme protein and phosphate buffer plus pyridoxal phosphate incubated for 2 h. If 0.5 m*M* pyridoxal phosphate was not added to either incubation mixture, GABA formation was not observed. (Reproduced with permission from ref. 92.)

and B). If pyridoxal phosphate was not added to the incubation mixture GABA formation was not observed. Gradient elution with acetonitrile was required for chromatographic separation of the derivatized products. As little as 100 ng of the product could be detected by this procedure using UV detection.

Recently electrochemical detection coupled with HPLC separation has been applied for GABA analysis [93]. GABA and the internal standard, 5-aminovaleric acid (5-AVA), were derivatized with 2,4,6-trinitrobenzenesulfonic acid and the components detected by electrochemical reduction with an annealed pyrolytic graphite electrode held at −0.8 V vs. saturated calomel electrode. Either a strong cation-exchange or a reversed-phase column were used for separation; limits of detection of GABA extracted from rat brain were 4 pmol. This method of analysis has yet to be applied for measurement of GAD activity in biological tissues; however, it should provide a very sensitive and specific technique for small sample size such as specific nuclei in brain tissues.

Post-column derivatization with *o*-phthalaldehyde using cation-exchange and reversed-phase columns have been applied to fluorometric GABA analysis in biological tissue [86, 94—97]. These methods were not used to measure GAD activity. Probably the first reported HPLC procedure used for GAD activity analysis was that of Meek [98]. Brain homogenates were incubated at 37°C for 30 min with GA as substrate and following denaturization of the enzyme via precipitation of the supernate was separated on a strong cation-exchange column before analysis. In this particular case an internal standard was not used for quantitation. Holdiness [99] reported a fluorometric procedure for analysis of GAD activity in sub-regions of rat brain using a cation-exchange column. The sample collection procedure has been previously described [43] and the

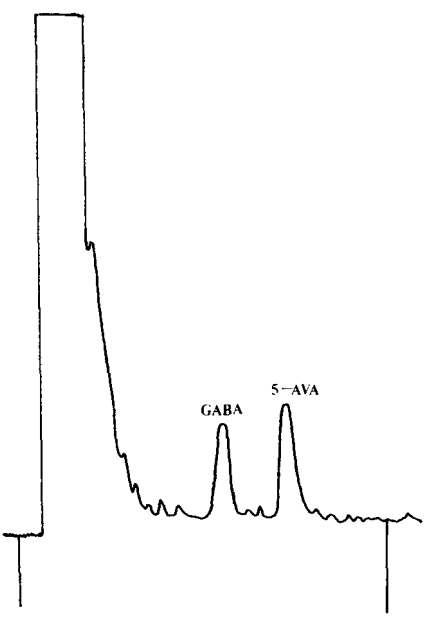

Fig. 7. HPLC chromatogram of GABA and 5-aminovaleric acid (5-AVA internal standard) extracted from rat brain tissue. The retention times of GABA and internal standard are 4.70 and 6.86 min, respectively.

tissue punch placement can be seen in Fig. 3. From each 1-mm thick brain tissue slice, two tissue punches were taken from symmetrical locations in the left and right hemispheres. Each set of punches was immediately transferred to an eppendorf tube containing 100 μl of internal standard (5-AVA) and homogenized for 10 sec under low-power sonication. Following sonication, 50 μl of this solution was transferred to an identical tube containing 20 μl of 3% trichloroacetic acid (TCA). This second tube served as a blank and its concentration of GABA was subtracted from the original sample. The substrate—buffer was prepared as previously described [43] and 50 μl of this solution was added to both the sample and blank tubes before they were incubated for 2 h at 38°C. The enzyme was inactivated by addition of 20 μl of 3% TCA to the original sample tube and all tubes were centrifuged at 950 *g* for 20 min. Injections of 40 μl of the supernate were made into the chromatograph. The precipitate was analyzed for protein as described by Lowery et al. [55] and GAD activity was reported in μg GABA per h per mg protein. Fig. 7 shows a typical chromatogram of GABA and internal standard isolated from rat brain. Positive identification was achieved by peak superimposition, i.e., by addition of GABA and 5-AVA standard to the extracts and observing increased peak height at the corresponding retention times. The lower limit of detection (signal-to-noise ratio = 2:1) of this procedure was 11 ng GABA. The average GAD activities (± S.D.) found by this method in each brain region (n = 5) were 7.81 ± 1.08 (GP), 6.73 ± 1.58 (EP), 3.75 ± 0.71 (VM), 13.70 ± 1.75 (SN_M) and 8.17 ± 1.68 (SN_L).

4.4. Gas chromatography—mass spectrometry

A number of gas chromatographic (GC) procedures have been developed for analysis of GABA content in biological media without necessarily being applied for GAD activity measurements. Many of these procedures have provided very sensitive and specific means of detection and quantitation. Flame ionization detection (FID) methods include the procedures of Shimada et al. [100] who made the N-trifluoroacetyl-*n*-butyl ester of GABA and the N-trifluoroacetyl-methyl esterification method for GABA by Carlyle [101]. Also direct injection of GABA into the gas chromatograph following extraction has been utilized [102]. The lactam product of GABA, 2-pyrrolidone, resulting from dehydration of GABA from a Carbowax column, was identified by mass spectrometry (MS). Electron-capture gas chromatography (EC—GC) has been employed for greater sensitivity and specificity of analyses. Wilk and Orlowski [103] and Pearson and Sharman [104] have demonstrated the use of combinations of 2,2,3,3,3-pentafluoropropionyl-2,2,3,3,3-pentafluoropropyl and 1,1,1,3,3,3-hexafluoroisopropyl-trifluoroacetyl derivatives, respectively, for GABA derivatization. The latter reagent formed easily at room temperature and possessed excellent EC—GC properties. The limit of detection was 10 pmol GABA in rat brain tissue. The extraction procedure of Pearson and Sharman [104] was later improved by Schmid and Karobath [105]. The same derivatization agents were used for GC—MS identification of the product and its internal standard (5-AVA). Both EC—GC and GC—MS procedures gave similar results for GABA analyzed from brain tissue. Tago et al. [35] applied an EC—GC method to the analysis of GABA by enzymatic activity in brain tissue. The derivatization

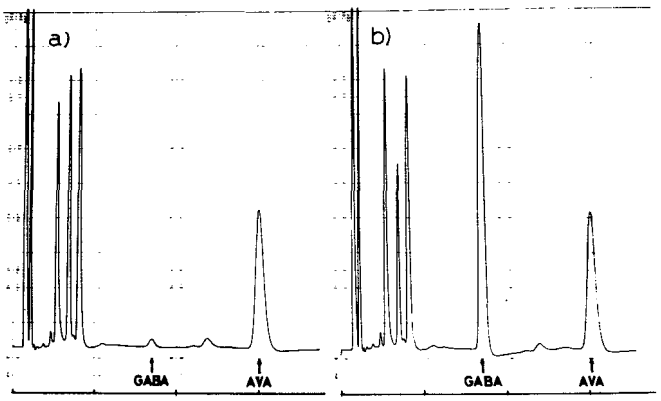

Fig. 8. Gas chromatograms of the acylated GABA and 5-aminovaleric acid (AVA). GABA formed from 4-aminobutyraldehyde dehydrogenase was acylated with trifluoroacetic anhydride and hexafluoroisopropanol, together with the internal standard. (a) represents the control and (b) represents the formation of GABA following a 10-min incubation period. (Reproduced with permission from ref. 35.)

agents were trifluoroacetic anhydride (TFAA) and hexafluoroisopropanol (HFIP) with 5-AVA as internal standard. Fig. 8 presents a typical chromatogram from their data. This method of analysis has been utilized for demonstration of evidence of synthesis of GABA from 4-aminobutyraldehyde dehydrogenase [35].

A number of GC—MS techniques have also been applied for GABA measurement in brain tissue [102, 105—112]. Cattabeni et al. [113] and Holdiness et al. [106] developed mass fragmentographic methods utilizing hexamethyldisilane, trimethylchlorosilane, and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) as the derivatizing agents forming trimethylsilyl (TMS) derivatives of the amine and carboxylic acid moieties of this molecule. The internal standard was 5-AVA and separation was performed on a 3% OV-17 column (Gas-Chrom Q), 100—120 mesh at 140°C. These two GC—MS procedures detected GABA in rat brain tissue with nanogram sensitivity. Employing the technique of selective ion monitoring (SIM) ions *m/e* 174 and 304 (GABA) and *m/e* 174 and 318 (5-AVA) were utilized for quantitation in brain tissue.

Huizinga et al. [114] and Bertilsson and Costa [115] both utilized GC—MS methods for GABA analysis in cerebral spinal fluid and brain tissue, respectively. Both procedures used a deuterated internal standard [$^2H_2$]GABA and pentafluoroproprionic anhydride (PFPA) and HFIP as derivatizing agents. The ions selected for quantitation by Huizinga et al. [114] were *m/e* 204 (GABA) and *m/e* 206 (deuterated internal standard). Monitoring ions *m/e* 232 (GABA) and *m/e* 234 (deuterated internal standard) gave essentially the same standard curve and quantitative results. Ions *m/e* 204 (GABA) and *m/e* 206 (internal standard) were utilized for SIM by Bertilsson and Costa [115] for their mass fragmentographic analysis.

To date, only two mass fragmentographic techniques have been developed for measurement of GAD activity in biological tissues. Cattabeni et al. [116] described a procedure in which rat cerebellum tissue homogenate was incubated with [$^2H_5$]GA as substrate and measured the formation of [$^2H_5$]GABA. The

internal standard 5-AVA was also added to the homogenate for comparison of measurement and ions *m/e* 304 (GABA), *m/e* 309 ([$^2H_5$]GABA) and *m/e* 318 (5-AVA) were monitored for quantitation. The derivatization procedure and chromatographic conditions have been previously described [113]. The standard curves for the two different internal standards revealed essentially the same data for GABA content in rat cerebellum. However, it was not determined whether any isotopic discrimination occurred with the enzyme and the deuterated substrate.

Holdiness et al. [117, 118] have described the latest known mass fragmentographic method of analysis of GAD activity in sub-regions of rat brain tissue. The internal standard was [$^2H_2$]GABA and the derivatization procedure utilized Sylon HTP [hexamethylchlorosilane—trimethylchlorosilane—pyridine (3:1:9)] and BSTFA for 90 min at room temperature. Separation was performed on a 3% OV-17 column (under the conditions described [106]) at 160°C. Ions *m/e* 304.1 (GABA) and *m/e* 306.2 ([$^2H_2$]GABA) were selected for quantitation. The limit of detection for this particular GC—MS method was 15 ng GABA per mg tissue. The sample collection procedure and substrate—buffer preparations have been previously described [43, 99]. Following homogenization the sample was divided into two fractions; one was inactivated with TCA and served as blank while the other portion was incubated under standard conditions as previously stated for the fluorometric HPLC method [99]. The concentration of GABA from the blank was subtracted from that of the incubated mixture. Following enzyme inactivation with TCA and centrifugation an 80-μl aliquot

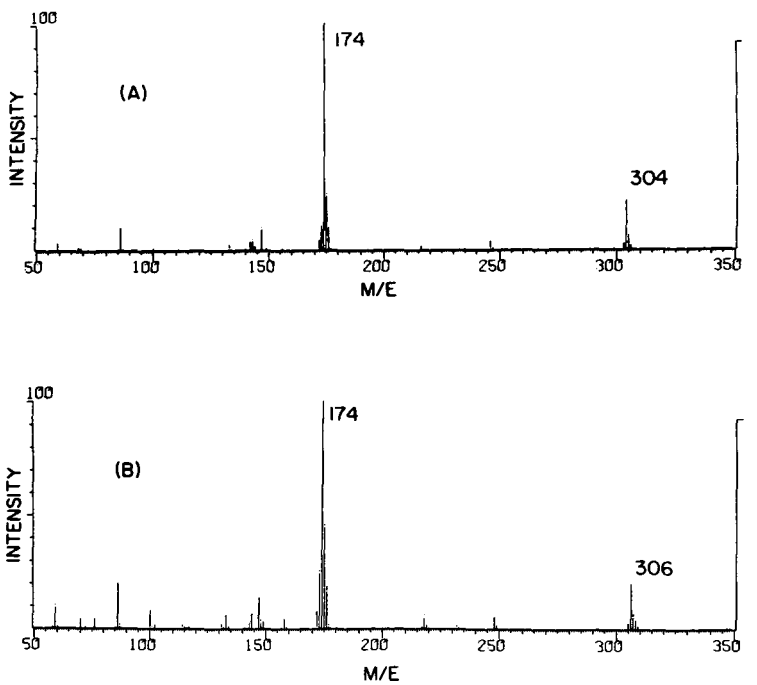

Fig. 9. Mass spectra of the trimethylsilyl derivatives of (A) GABA and (B) [$^2H_2$]GABA at 70 eV.

of the sample was transferred to a small 1.5-ml eppendorf tube and evaporated to dryness under reduced pressure. The samples were then derivatized as stated above and analyzed by GC—MS.

A mass spectrum of the TMS derivative and deuterium labeled derivative is presented in Fig. 9. The base peak in both spectra is *m/e* 174. The ions monitored $(M - 15)^+$ by electron impact at 70 eV were *m/e* 304.1 (GABA) and *m/e* 306.2 ($[^2H_2]$GABA). The relative abundancies of *m/e* 304.1 and *m/e* 306.2 are 22% and 20%, respectively. From mass spectra analysis, $[^2H_2]$GABA gave a small contribution to *m/e* 304.1 of 0.22% relative abundance while GABA has an ion of 12.83% relative abundance at *m/e* 306.2. The signal intensities of the above mentioned ions are 2.5 times stronger at 70 eV than at 30 eV.

To test whether greater sensitivity could be achieved, spectra of pure standards of each compound were obtained using chemical ionization with methane as reagent gas. In both samples the base peak was *m/e* 174. The $(MH)^+$ ions of GABA (*m/e* 320) and $[^2H_2]$GABA (*m/e* 322) were both present at 7% relative abundance. At 70 eV the $(M - 15)^+$ relative abundancies of *m/e* 304.1 (GABA) and *m/e* 306.2 ($[^2H_2]$GABA) were 75% and 73%, respectively. It was found that the chemical ionization signal intensities of these two ions were 4.25 times less than the signal intensities at 70 eV by electron impact. Therefore, electron impact was used in all GABA measurements.

Fig. 10 presents a mass fragmentogram of one of the tissue samples. The chromatographic retention time of the endogenous compound was identical to that of its deuterated analogue. The average GAD activity values (± S.D.) found by this method in each brain region ($n = 5$) were 7.75 ± 0.93 (GP), 6.70 ± 1.02 (EP), 3.68 ± 0.70 (VM), 13.66 ± 1.06 (SN_M) and 8.12 ± 0.95 (SN_L). These

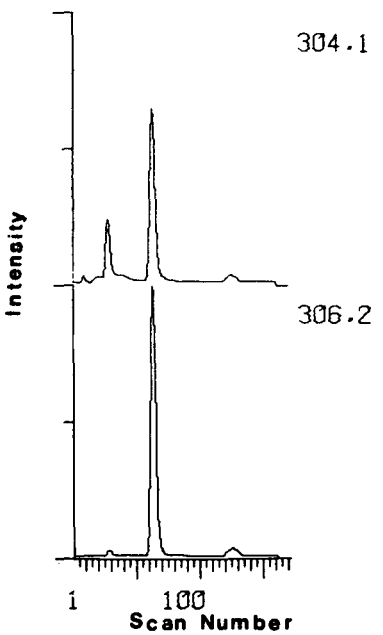

Fig. 10. Mass fragmentogram of *m/e* 304.1 of GABA and *m/e* 306.2 of $[^2H_2]$GABA from a tissue sample.

brain punch samples weighing approximately 2.50 mg each were taken from the same locations as presented in Fig. 3. By measurement of a characteristic ion of GABA and comparison of its retention time to that of a deuterated internal standard, this assay method represents a very specific, selective and sensitive method for GAD activity measurement.

5. CONCLUSION

Numerous chromatographic and non-chromatographic methods for analysis of GAD activity in biological tissues have been discussed in this manuscript. Methods are generally devised or selected for use according to the investigator's expertise and possible prior experience with either radioactive materials, fluorometric or chromatographic techniques and the type of instrumentation that is available in their laboratory. Therefore, of the procedures available major consideration must be given to the reliability, specificity, selectivity and reproducibility among the different instrumental techniques and their comparison with different tissues. As has been described in this paper differences of up to 10% have been noted between $^{14}CO_2$ and [^{14}C]GABA methods utilized for GAD analysis due to alternate pathways of production of carbon dioxide [28, 46], impurities in the enzyme and substrate preparations, and reaction vessel atmospheres [29—31, 33]. MacDonnel and Greengard [48] compared a $^{14}CO_2$ production method with that of a fluorometric procedure. They demonstrated that the addition of Triton X-100 (utilized in an air atmosphere) modified the $^{14}CO_2$ method in such a way that it inhibited non-GAD CO_2 release from glutamate. Following modifications both methods produced similar GAD activities in both neural and non-neural tissues. Wu et al. [33] measured GAD activity in brain and non-neural tissues by five different analytical techniques ($^{14}CO_2$, column separation, electrophoretic separation, a filtration method and by amino acid analysis). The last four procedures involved analysis of GABA production as opposed to carbon dioxide formation. Purified L-[U-^{14}C]GA was utilized as substrate. All five methods gave similar GAD activities in brain tissue; however, for tissues as heart, kidney and liver, the $^{14}CO_2$ assay technique revealed GAD activities approximately three to four times that of the GABA formation methods. This suggested that the CO_2 method does not give valid quantitation of GAD activity in crude non-neural tissue preparations [12, 33].

This author has developed three analytical instrumental techniques (fluorometric [43], HPLC [99] and GC—MS [117, 118]) for analysis of GAD activity in rat brain tissue. The same collection procedure, strain of rats and tissue punch locations (see Fig. 3) were utilized for all three methods. In Fig. 11 is presented a bar graph of the GAD activity found at each location by each analytical method. As can be seen by the data each analytical technique gave approximately the same GAD activity in each sub-region of rat brain; therefore, demonstrating the reliability, selectivity and reproducibility of each different method. The slight decrease in average GAD activity at each sub-region from fluorometric to HPLC to GC—MS is attributed to the specificity of the technique used. The GC—MS method is considered to be the most specific since a characteristic ion fragment is monitored for analysis.

The GAD activities found by this author in the above three listed procedures

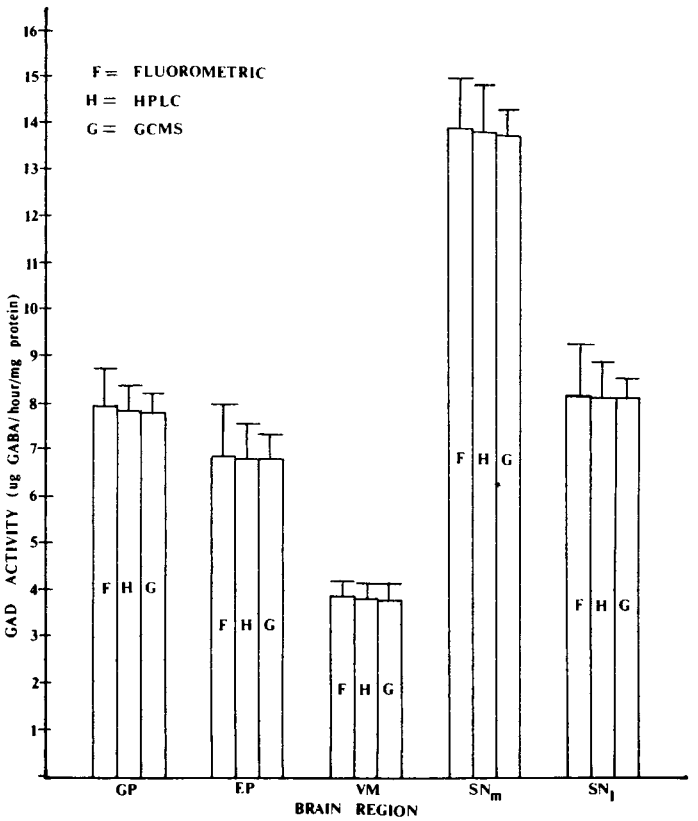

Fig. 11. Bar graph of GAD activity versus sub-region in rat brain for three different analytical techniques. (F) is a fluorometric method [43], (H) is an HPLC method [99], and (G) is a GC—MS procedure [117, 118].

TABLE 2

A COMPARISON OF RATIOS OF GAD ACTIVITY IN SUB-REGIONS OF RAT BRAIN BY VARIOUS ANALYTICAL METHODS

Analytical technique	GP/EP sub-region	GP/VM sub-region	Reference
Fluorometric	1.15	2.06	Holdiness et al. [43]
Fluorometric HPLC	1.16	2.08	Holdiness [99]
GC—MS	1.16	2.11	Holdiness et al. [117]
$^{14}CO_2$	1.80	—	Nagy et al. [14]
$^{14}CO_2$	1.93	—	Walaas and Fonnum [8]
$^{14}CO_2$	—	2.03	Tappaz et al. [119]

are in agreement with those found by Nagy et al. [14], Walaas and Fonnum [8] and Tappaza et al. [119] in the GP, EP and SN. A comparison of regional GAD activities has been made with these data and that in the previously cited literature and is presented in Table 2. The author choose what he considered the best brain tissue comparisons (regardless of analytical technique) with respect

to anatomical location. Due to the different ways activity values are reported (i.e., per mg tissue, mg protein dry weight, wet weight) ratios of activities between regions have been used for comparison. Although a direct comparison of these ratios is not ideal, the ratios should be relatively consistent from method to method. As can be seen by the data in Table 2 there are comparable results reported by this author and that of the previously cited literature. Possible discrepancies among these activity values could be due to extraneous $^{14}CO_2$ production from alternative pathways, impurities in substrate mixtures, variations in anterior—posterior axis location in brain regions, differences in protein content among sub-regions, comparison of whole dissected regions as opposed to micropunches and differences in strains of rats tested.

As previously mentioned there seems to exist evidence for GABA production from an alternative pathway of 4-aminobutyraldehyde dehydrogenase (4-ABALD) in rat brain [35, 120, 121]. Also a synthetic pathway for GABA formation from putrescine has been proposed by Seiler et al. [122] in fish brain and Seiler and Al-Therib [123], Seiler et al. [124] and Sugahara et al. [125] in mammalian brain although it has not been fully clarified. It is possible that 4-ABALD may be involved in the conversion of putrescine to GABA in mammalian brain [35]. Although alternative routes of GABA formation may exist in neural tissue, their incubation conditions are different from those of the established conditions for GAD analysis; therefore, it seems unlikely that extraneous GABA would be produced to any significant extent by alternative pathways under these stated reaction conditions. This fact also seems to be verified by the method of Pahuja and Reid [72] who demonstrated that crude retinal incubation homogenates did not produce GABA without the presence of the GAD enzyme in the reaction vessel.

6. SUMMARY

A number of non-chromatographic and chromatographic methods for analysis of GAD activity in biological tissues have been described. The majority of these chromatographic methods utilize the analysis of GABA formed from incubation homogenates. Depending upon the analytical technique selected, limits of detection range from nanogram to picogram levels of GABA. Also discussed have been some of the commonly associated problems and their resolution with sample collection, postmortem changes and alternative pathways of CO_2 and GABA production which can lead to errors in accurate determination of GAD activity in biological samples.

7. ACKNOWLEDGEMENTS

The author wishes to express his appreciation to the authors and publishers of the following articles who provided permission and photographs for the figures reproduced in this manuscript: (1) Physiology Reviews, Dr. J.Y. Wu (ref. 12); (2) Journal of Liquid Chromatography, Drs. S.L. Pahaja and T.W. Reid (ref. 72); (3) Journal of Chromatography, Drs. S.L. Pahuja, J. Albert and T.W. Reid (ref. 92); and (4) Journal of Neurochemistry, Drs. K. Tago, S. Kurioka and M. Matsuda (ref. 35).

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Journal of Chromatography, 277 (1983) 25—39
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1785

GAS CHROMATOGRAPHIC—MASS SPECTROMETRIC ANALYSIS OF POLYOLS IN URINE AND SERUM OF UREMIC PATIENTS

IDENTIFICATION OF NEW DEOXYALDITOLS AND INOSITOL ISOMERS

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(First received February 11th, 1983; revised manuscript received May 6th, 1983)

SUMMARY

A gas chromatographic—mass spectrometric method was applied to a study of polyols in urine and serum of normal subjects and uremic patients. 4-Deoxythreitol, 4-deoxyerythritol, 5-deoxyxylitol, 5-deoxyarabitol, 2-deoxyribitol, 6-deoxymannitol, 6-deoxygalactitol, neoinositol and chiroinositol were identified in normal urine as well as in uremic urine for the first time. In uremia the urinary excretion of myoinositol and chiroinositol was significantly increased. The serum levels of myoinositol, chiroinositol and scylloinositol were increased in the uremic patients, whereas the serum level of 1-deoxyglucose (1,5-anhydroglucitol) was significantly decreased in the uremic patients as compared with the normal subjects. These findings suggest the altered metabolism of chiroinositol and 1-deoxyglucose in the uremic state.

INTRODUCTION

Polyols in human physiological fluids and tissue have recently drawn attention, and the polyol concentrations have been reported to change in various

0378-4347/83/$03.00

diseases. Pitkänen [1] reported that the serum and urinary levels of myo-inositol were increased in the uremic state, and that the urinary excretion of mannitol and myoinositol was increased in diabetes mellitus. Servo et al. [2] reported that the 1-deoxyglucose level in the cerebrospinal fluid was decreased in uremia and in diabetes mellitus. The derangements of the polyol metabolism were considered as possible causes of clinical symptoms. For example, retention of myoinositol was considered to be a cause of the uremic polyneuropathy [3, 4]. The increased production of sorbitol in the lens causes cataracts in diabetes mellitus [5], and sorbitol also plays an important role in the formation of diabetic complications in nervous tissue [6].

Detailed studies of polyols in urine and serum were performed to clarify the exact derangement of the polyol metabolism in uremia using high-resolution gas chromatography—mass spectrometry. In our study several new deoxyalditols were demonstrated to be present in human urine, and the abnormal metabolism of chiroinositol and scylloinositol was also demonstrated in the uremic state.

MATERIALS AND METHODS

Chemicals

Erythritol, xylitol, arabitol, L-arabinose, 2-deoxyribose, L-rhamnose and L-fucose were the products of Tokyo Kasei Kogyo Co. (Tokyo, Japan). Ribitol, mannitol, sorbitol, myoinositol, D-fructose, D-arabinose, D-glucose and D-galactose were the products of Yoneyama Chemical Industries Ltd. (Osaka, Japan). Threitol, xylulose and 6-deoxyglucose were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

4-Deoxythreitol was synthesized by the sodium borohydride reduction of 4-deoxythreose, which was synthesized from D-xylose according to the methods of Leven and Compton [7] and Hough and Taylor [8]. 4-Deoxyerythritol was synthesized by the sodium borohydride reduction of 4-deoxyerythrose, which was synthesized from D-arabinose according to the method of Sugimoto and Matsuura [9]. 5-Deoxyxylitol was synthesized by the sodium borohydride reduction of 5-deoxyxylose, which was synthesized from D-xylose according to the method of Leven and Compton [7]. 5-Deoxyarabitol was synthesized by the sodium borohydride reduction of 5-deoxyarabinose, which was synthesized from D-arabinose according to the method of Zinner et al. [10]. 2-Deoxyribitol was synthesized by the reduction of 2-deoxyribose with sodium borohydride. 6-Deoxymannitol was synthesized by the reduction of L-rhamnose with sodium borohydride, 6-deoxysorbitol, by the reduction of 6-deoxyglucose with sodium borohydride, and 6-deoxygalactitol by the reduction of L-fucose with sodium borohydride.

Samples

Twenty-four-hour urine samples were obtained from five healthy adults and ten patients with chronic renal failure. Four of ten uremic patients were on 5-h hemodialysis three times a week. The serum creatinine level of ten uremic patients averaged 8.3 ± 3.7 mg/dl, ranging from 3.1 to 16.2 mg/dl.

Serum samples were obtained from eight healthy adults and twelve patients

with chronic renal failure. Nine of twelve uremic patients were on hemodialysis treatment. The serum creatinine level of the twelve uremic patients was 10.7 ± 4.0 mg/dl, ranging from 4.9 to 15.4 mg/dl.

The urine and serum samples were kept at −20°C prior to analysis.

Sample preparation

Serum was filtered through a cone membrane filter (CF 25, Amicon, Lexington, MA, U.S.A.). One milliliter of serum ultrafiltrate, or a volume of urine equivalent to 1 mg of creatinine, was applied to a Dowex 50W-X8 (H^+) column (5 cm × 0.8 cm I.D.) after the addition of 50 μg of ribitol as an internal standard. Anion and neutral substances were eluted with 30 ml of distilled water. The eluate was applied to Amberlite IRA 400 ($HCOO^-$) column (5 cm × 0.8 cm I.D.). Neutral substances were eluted with 30 ml of distilled water. The eluate was dried on a lyophilizer. The neutral substances were dissolved with 9 ml of hot methanol, then transferred to a test tube, and concentrated into about 2 ml with a nitrogen stream. The concentrate was transferred to a sample glass vial and dried with a nitrogen stream. The sample was then trimethylsilylated with 90 μl of N,O-bis(trimethylsilyl)trifluoroacetamide and 10 μl of trimethylchlorosilane at 60°C for 20 min; 2 μl of the sample were subjected to gas chromatography—mass spectrometry.

Gas chromatography—mass spectrometry

Derivatized samples were analyzed with a Hewlett-Packard 5710A gas chromatograph combined with a double-focussing mass spectrometer (JMS D-300, JEOL). The data were stored and processed by a JMA 2000 data system of JEOL. The gas chromatograph was equipped with an OV-101 open-tubular glass capillary column (30 m × 0.25 mm I.D.) and a splitless injector. The column temperature was programmed from 120°C to 260°C at 3°C/min.

Electron-impact ionization (EI) mass spectra were recorded at an ionizing energy of 70 eV, an ionization current of 300 μA, and an accelerating voltage of 3 kV. Chemical ionization (CI) mass spectra were recorded with ammonia as a reactant gas. Ionizing energy was 260 eV and the other conditions were the same as for EI.

Quantitation of polyols in urine and serum

Standard curves were obtained with the OV-101 capillary column (30 m × 0.25 mm I.D.) using standard solutions with concentrations ranging from 1 μg to 1 mg per 1 ml of distilled water. After addition of 50 μg of ribitol as an internal standard, these solutions were processed as described in the section for sample preparation. Standard curves relating the concentrations of threitol, erythritol, xylitol, arabitol, mannitol, sorbitol and myoinositol to the ratios of peak area of the polyols to ribitol were obtained from the gas chromatograms. Since the concentration of ribitol in normal and uremic urine was less than 5 μg per urine volume equivalent to 1 mg of creatinine, and since ribitol was not detected in normal or uremic serum, 50 μg of ribitol were used as an internal standard.

To compare the daily urinary excretion of neoinositol, chiroinositol, epi- or cisinositol and scylloinositol in uremic patients with that in normal subjects,

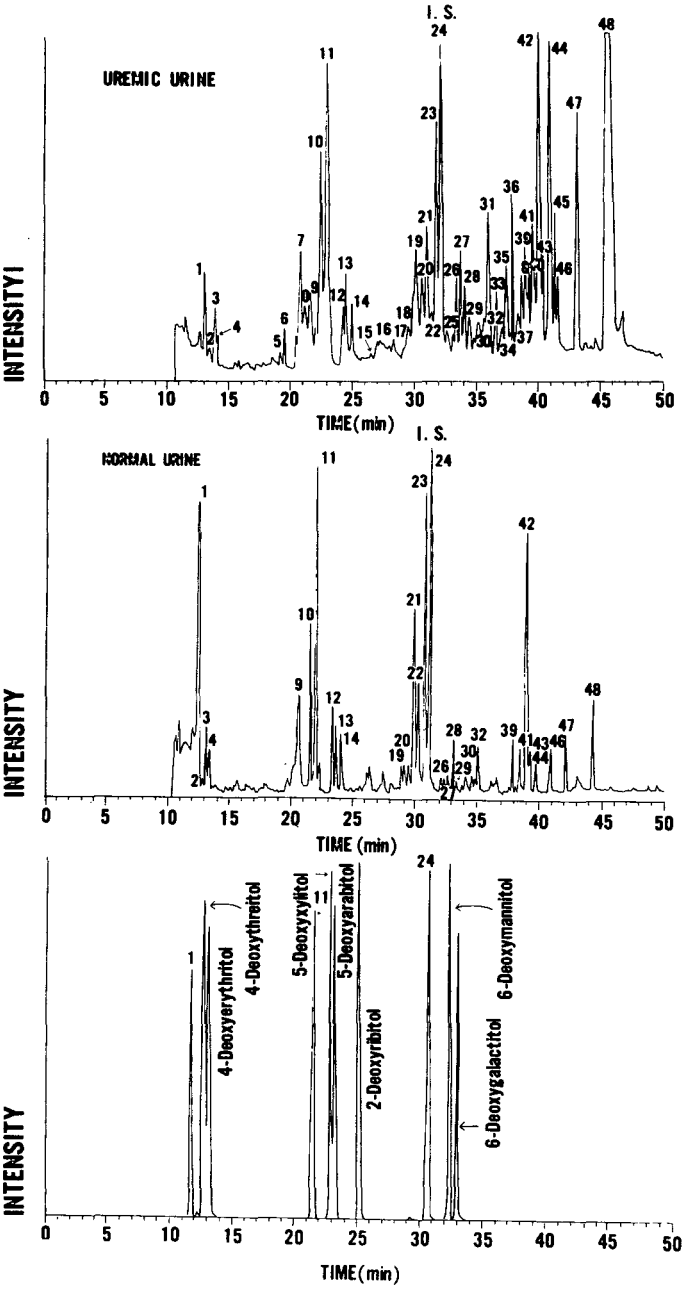

Fig. 1. Gas chromatograms of polyols in urine of a patient with chronic renal failure (upper chromatogram), a healthy subject (middle chromatogram) and of synthesized polyols (lower chromatogram). Peak identifications: 1 = glycerol, 3 = 4-deoxythreitol, 4 = 4-deoxyerythritol, 10 = threitol, 11 = erythritol, 12 = 5-deoxyxylitol, 13 = 5-deoxyarabitol, 15 = 2-deoxyribitol, 19 = xylulose, 22 = xylitol, 23 = arabitol, 24 = ribitol (internal standard), 26, 28 = isomers of 6-deoxyhexitol, 27 = 6-deoxymannitol, 29 = 6-deoxygalactitol, 30 = fructose, 31 = 1-deoxyglucose, 36 = α-glucose, 37 = β-galactose, 39 = neoinositol, 42 = mannitol, 43 = sorbitol, 44 = chiroinositol, 45 = β-glucose, 46 = epi- or cisinositol, 47 = scylloinositol, 48 = myoinositol.

peak area ratios of the inositol isomers to ribitol, corresponding to the concentrations per urine volume equivalent to 1 mg of creatinine, were obtained from the gas chromatograms and the values obtained were multiplied by the daily amount of creatinine excreted in the urine (mg/day).

Serum levels of 1-deoxyglucose, chiroinositol and scylloinositol were obtained from the mass chromatograms, because the peaks of these compounds were difficult to separate from the peaks of glucose furanoside, β-glucose and an unknown substance, respectively, in the gas chromatograms. The ion 259, $(M - 193)^+$, was used for 1-deoxyglucose. The ion 318, $(M - 294)^+$, was used for chiroinositol, scylloinositol and other inositol isomers. The ion 422, $(M - 90)^+$, was used for ribitol. Peak height ratios of 1-deoxyglucose, chiroinositol, and scylloinositol to ribitol were obtained from the mass chromatograms to compare the serum levels of the compounds in uremic patients with those in normal subjects.

Recoveries of polyols

Quintuplicate estimations of recoveries were carried out using 50-μg amounts of polyols, which were added to 1 ml of distilled water. The solution was applied to Dowex 50W-X8, the eluate then applied to Amberlite IRA-400, the polyols were subsequently eluted with distilled water and quantitated. Recoveries of threitol, erythritol, xylitol, arabitol, mannitol, sorbitol and myoinositol were as follows: 104.4 ± 3.0% (mean ± S.D., $n = 5$), 105.4 ± 8.5%, 94.7 ± 13.7%, 109.6 ± 6.8%, 96.7 ± 9.5%, 92.1 ± 8.7%, and 89.3 ± 14.8%, respectively.

EXPERIMENTAL RESULTS

Polyol profiles of uremic urine and serum

Fig. 1 shows the gas chromatograms of polyols in urine of a patient with chronic renal failure (upper chromatogram) and in normal urine (middle chromatogram). Identification of the peaks was performed by comparing their mass spectra and retention times with those of authentic compounds or from reference to the literature. In the uremic urine the peaks of chiroinositol, scylloinositol and myoinositol were higher than in normal urine.

Fig. 2 shows the gas chromatograms of polyols in the serum of a patient with chronic renal failure (upper chromatogram) and in normal serum (lower chromatogram). In the uremic serum the peaks of threitol, erythritol, arabitol, mannitol, sorbitol, chiroinositol, scylloinositol and myoinositol were higher than in normal serum.

Identification of deoxyalditols in urine

EI mass spectra of peaks 3 and 4 presented similar spectra, as shown in Figs. 3 and 4 (lower spectra), respectively. Molecular ions of the two compounds were found to be 322 by recording CI mass spectra. The intense peaks at *m/z* 117 and *m/z* 219 in addition to the peaks at *m/z* 103 and *m/z* 205 suggested the structure of 4-deoxytetriol by comparison with the mass spectrum of glycerol (M^+ 308). Since the trimethylsilyl (TMS) derivative of 4-deoxythreitol and peak 3 showed identical EI mass spectra (Fig. 3) and identical relative retention times (Fig. 1), peak 3 was identified as 4-deoxythreitol.

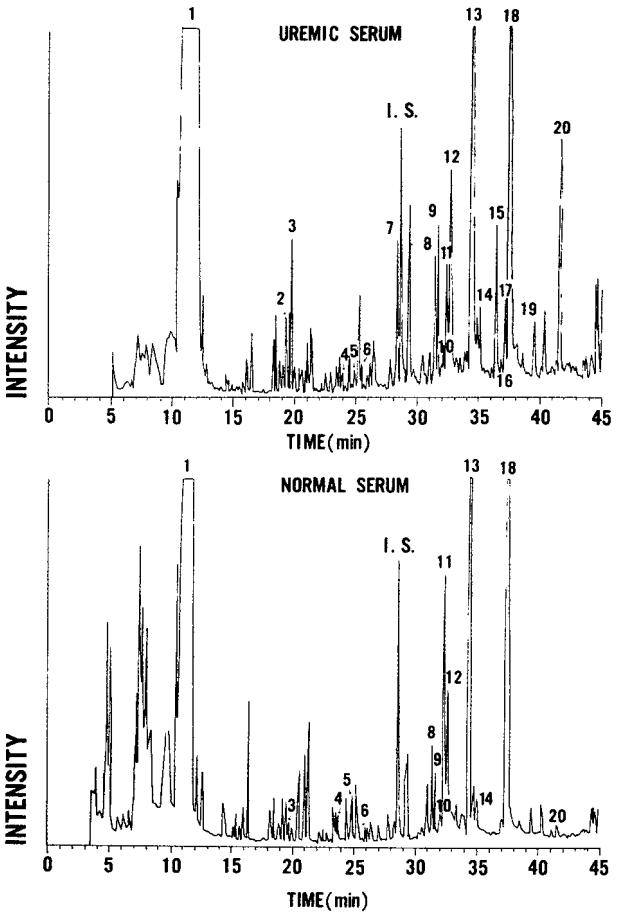

Fig. 2. Gas chromatograms of polyols in the serum of a patient with chronic renal failure (upper chromatogram) and a healthy subject (lower chromatogram). Peak identifications: 1 = glycerol, 2 = threitol, 3 = erythritol, 4, 5 = arabinose, 7 = arabitol, 8, 9, 10 = fructose, 11 = 1-deoxyglucose, 12 = glucose furanoside, 13 = α-glucose, 14 = β-galactose, 15 = mannitol, 16 = sorbitol, 17 = chiroinositol, 18 = β-glucose, 19 = scylloinositol (minor component), 20 = myoinositol.

The TMS derivative of 4-deoxyerythritol and peak 4 also showed identical mass spectra (Fig. 4) and identical relative retention times (Fig. 1). Peak 4 was then identified as 4-deoxyerythritol.

EI mass spectra of peaks 12 and 13 presented similar spectra as shown in Figs. 5 and 6 (lower spectra), respectively. CI mass spectra of the peaks indicated the molecular ions at *m/z* 424. The intense peaks at *m/z* 117 and *m/z* 219 in addition to the peaks at *m/z* 103 and *m/z* 205 suggested the structure of 5-deoxypentitol by comparison with the mass spectrum of erythritol (M^+ 410). Since the TMS derivative of 5-deoxyxylitol showed identical EI mass spectra (Fig. 5) and identical relative retention times (Fig. 1), peak 12 was identified as 5-deoxyxylitol. The TMS derivative of 5-deoxyarabitol and peak 13 also showed identical mass spectra (Fig. 6) and identical relative retention times (Fig. 1). Peak 13 was then identified as 5-deoxyarabitol.

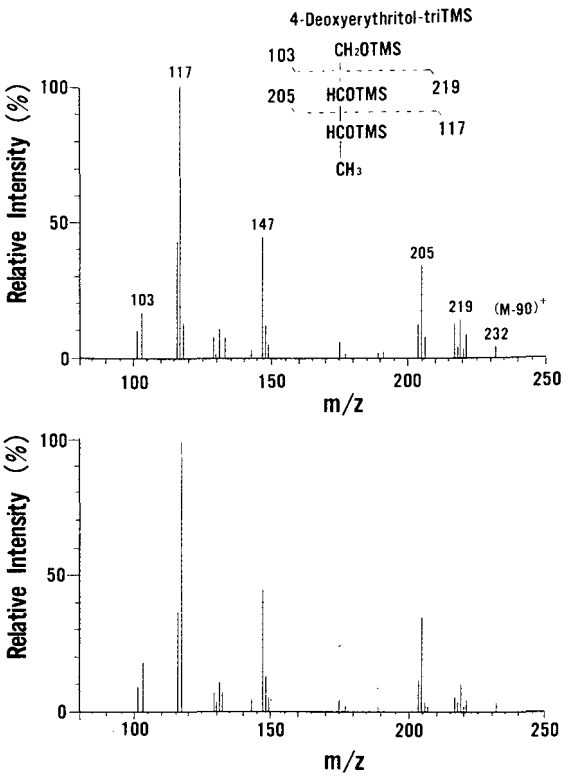

Fig. 3. EI mass spectra of TMS derivative of 4-deoxythreitol (upper spectrum) and of peak 3 (lower spectrum) in the gas chromatograms of Fig. 1.

The EI mass spectrum of peak 15 is shown in Fig. 7 (lower spectrum); the CI mass spectrum of the peak indicated the molecular ion at *m/z* 424. The intense peak at *m/z* 219, the low peak at *m/z* 117 and the base peak at *m/z* 103 suggested the structure of 2-deoxypentitol by comparison with the mass spectrum of erythritol (M^+ 410). Peak 15 was identified as 2-deoxyribitol since the TMS derivative of 2-deoxyribitol and peak 15 showed identical mass spectra (Fig. 7) and identical relative retention times (Fig. 1).

The EI mass spectrum of peak 27 is shown in Fig. 8 (lower spectrum). The molecular ion was found to be 526 by recording the CI mass spectrum. The base peak at *m/z* 117 and the intense peak at *m/z* 219 together with the peaks at *m/z* 103, 205 and 307 suggested the structure of 6-deoxyhexitol by comparison with the mass spectrum of ribitol (M^+ 512). Synthesized 6-deoxymannitol showed an identical EI mass spectrum (Fig. 8) and identical relative retention time (Fig. 1) with those of peak 27. Although synthesized 6-deoxysorbitol showed an almost identical relative retention time with that of peak 27, the mass spectrum of 6-deoxysorbitol-penta-TMS revealed a ten-fold increase in the intensity of the *m/z* 409 ion as compared with that of 6-deoxymannitol-penta-TMS. Peak 27 was then identified as 6-deoxymannitol.

The EI mass spectrum of peak 29 is presented in Fig. 9 (lower spectrum). Peak 29 and peak 27 showed similar mass spectra, suggesting an isomeric

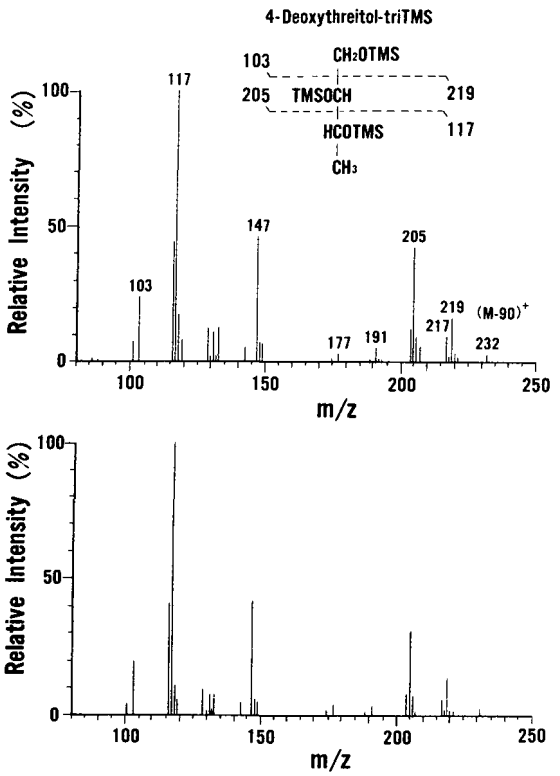

Fig. 4. EI mass spectra of TMS derivative of 4-deoxyerythritol (upper spectrum) and of peak 4 (lower spectrum) in the gas chromatograms of Fig. 1.

relationship. The molecular ion of peak 29 was 526 from the CI mass spectrum. Since the TMS derivative of 6-deoxygalactitol and peak 29 showed identical EI mass spectra (Fig. 9) and identical relative retention times (Fig. 1), peak 29 was identified as 6-deoxygalactitol.

Peaks 26 and 28 showed EI mass spectra similar to that of peak 27, suggesting that these peaks are isomers of 6-deoxyhexitol.

4-Deoxythreitol, 4-deoxyerythritol, 5-deoxyxylitol, 5-deoxyarabitol, 6-deoxymannitol, and 6-deoxygalactitol were identified in normal urine as well as in uremic urine for the first time.

Identification of inositol isomers in urine and serum

Five inositol isomers were found in normal and uremic urine (Fig. 1). The EI mass spectra of the five isomers are shown in Table I. Each relative intensity (%) indicated represents the mean of five measurements. Peak 48 was identified as myoinositol by comparing the EI mass spectrum and the relative retention time with those of the authentic compound.

Peak 39 in the gas chromatogram of Fig. 1 had a base peak at *m/z* 318. The relative intensity of the *m/z* 305 ion was as low as 30% and the relative intensities of *m/z* 432 and 433 were as high as 29% and 16%, respectively. By

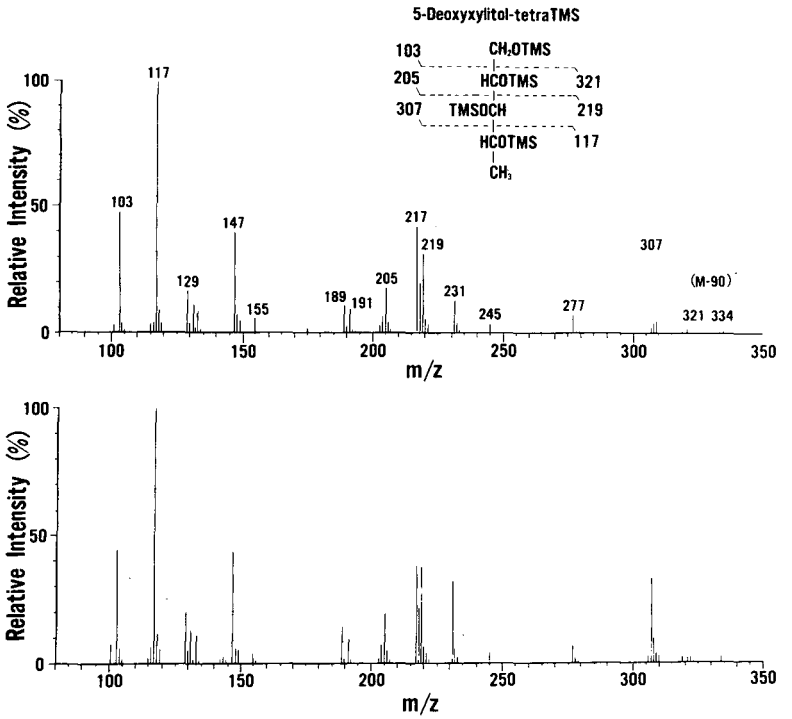

Fig. 5. EI mass spectra of TMS derivative of 5-deoxyxylitol (upper spectrum) and of peak 12 (lower spectrum) in the gas chromatogram of Fig. 1.

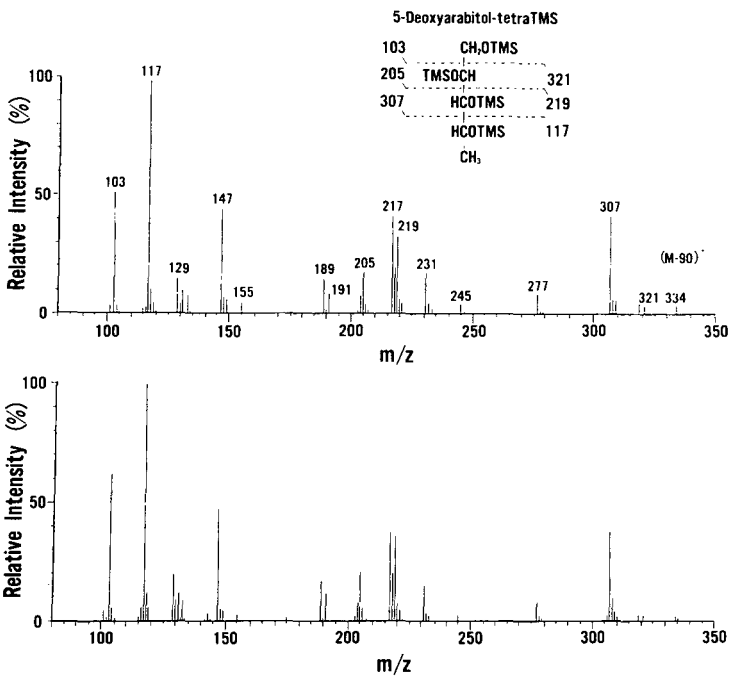

Fig. 6. EI mass spectra of TMS derivative of 5-deoxyarabitol (upper spectrum) and of peak 13 (lower spectrum) in the gas chromatogram of Fig. 1.

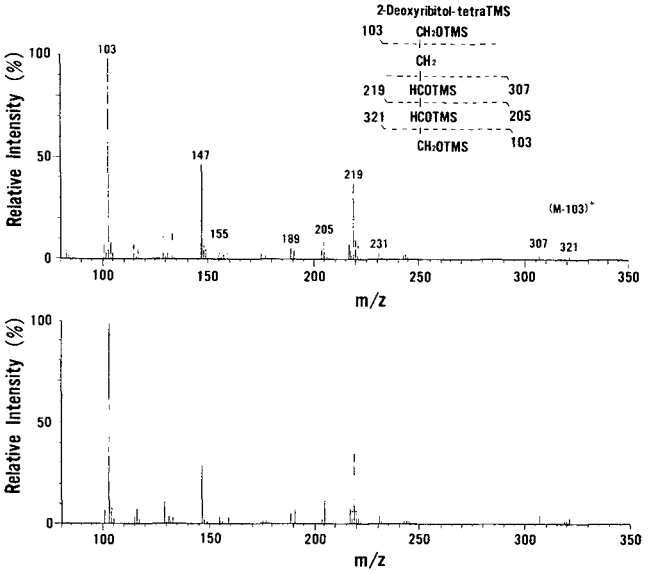

Fig. 7. EI mass spectra of TMS derivative of 2-deoxyribitol (upper spectrum) and of peak 15 (lower spectrum) in the gas chromatogram of Fig. 1.

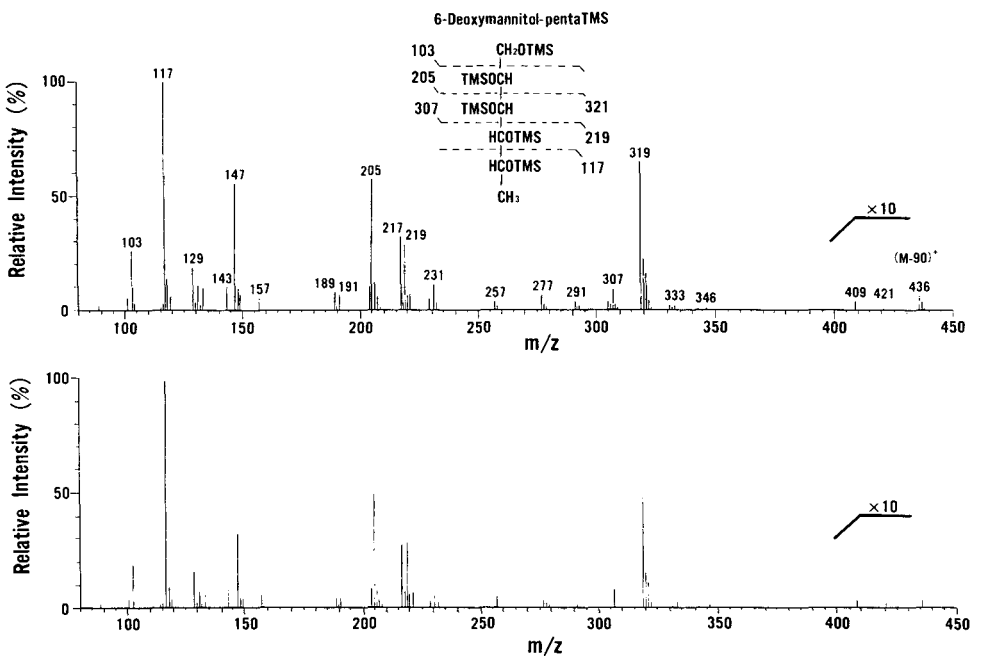

Fig. 8. EI mass spectra of TMS derivative of 6-deoxymannitol (upper chromatogram) and of peak 27 (lower spectrum) in the gas chromatograms of Fig. 1.

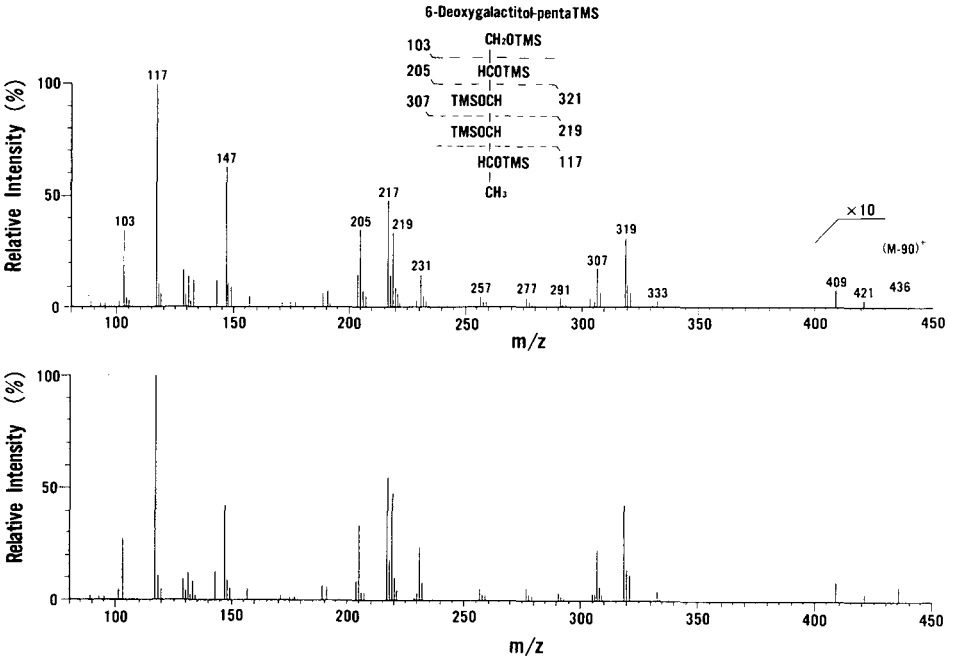

Fig. 9. EI mass spectra of TMS derivative of 6-deoxygalactitol (upper spectrum) and peak 29 (lower spectrum) in the gas chromatograms of Fig. 1.

comparison with the reference spectra [11] these characteristics of peak 39 indicated that this peak is the TMS derivative of neoinositol.

Peak 47 had a base peak at *m/z* 318. The relative intensities of *m/z* 265 and 367 were as low as 7% and 3%, respectively. By comparison with the reference spectra [11], these characteristics indicated that peak 47 is the TMS derivative of scylloinositol.

Peak 44 had a base peak at *m/z* 318. The relative intensity of *m/z* 305 was 82%. The relative intensities of *m/z* 432 and 433 were 9% and 10%, respectively. By comparison with the reference spectra [11], these characteristics indicated that peak 44 is chiroinositol.

Peak 46 had a base peak at *m/z* 217. The relative intensity of *m/z* 305 is higher than that of *m/z* 318. By comparison with the reference spectra [11], these characteristics suggested that peak 46 is epiinositol or cisinositol.

In normal serum only one inositol isomer, myoinositol, was detected, whereas in uremic serum chiroinositol, scylloinositol and myoinositol were detected (Fig. 2). Neoinositol and epi- or cisinositol could not be detected in normal serum or in uremic serum.

Urinary polyol excretion in uremic patients

Table II shows the daily urinary excretion of polyols in patients with chronic renal failure and of healthy adults. The daily urinary excretion of creatinine in uremic patients was 0.61 g, which was 44% of the daily creatinine excretion in healthy adults. The daily urinary excretion of erythritol, xylitol and arabitol

TABLE I

EI MASS SPECTRA OF INOSITOL ISOMERS IN THE GAS CHROMATOGRAMS OF FIG. 1

Each relative intensity (%) indicated represents the mean of five measurements.

m/z	Peak No.				
	39 Neoinositol	44 Chiroinositol	46 Epi- or cisinositol	47 Scylloinositol	48 Myoinositol
103	12	12	22	13	12
129	18	17	20	11	21
133	13	13	41	11	18
147	75	70	82	70	74
189	3	2	2	2	3
191	32	41	46	48	52
204	20	22	28	41	34
217	64	83	100	81	100
221	6	5	6	6	10
265	22	27	10	7	25
291	12	7	4	6	13
293	4	5	5	6	5
305	30	82	57	74	96
318	100	100	30	100	60
343	2	2	2	1	2
367	10	11	5	3	11
393	5	6	2	8	7
417	0.5	2	0.2	0.4	1
419	0.5	2	0.4	0.8	1
432	32	9	2	3	18
433	18	10	7	3	14
507	4	8	0.5	4	6
612	0.1	0.7	0.1	0.5	2

TABLE II

DAILY URINARY EXCRETION OF POLYOLS IN UREMIC PATIENTS

	Normal (n=5)	Uremic (n=10)	Uremic/normal
Threitol (mg/day)	5.7 ± 3.3	4.0 ± 3.3	0.70
Erythritol (mg/day)	66 ± 15	20 ± 13***	0.33
Xylitol (mg/day)	6.9 ± 4.8	2.0 ± 2.4**	0.29
Arabitol (mg/day)	46 ± 9.5	16 ± 12***	0.35
Mannitol (mg/day)	81 ± 37	94 ± 85	1.2
Sorbitol (mg/day)	8.6 ± 2.1	12 ± 14	1.4
Myoinositol (mg/day)	100 ± 71	430 ± 290**	4.3
Neoinositol*	55 ± 44	29 ± 36	0.53
Chiroinositol*	130 ± 140	750 ± 270***	5.8
Epi- or cisinositol*	44 ± 53	23 ± 48	0.52
Scylloinositol*	200 ± 140	290 ± 260	1.5
Creatinine (g/day)	1.4 ± 0.3	0.61± 0.28***	0.44

*Peak area ratio to internal standard (ribitol 50 μg) per day.
**$p < 0.05$.
***$p < 0.01$.

in uremic patients was significantly decreased compared with healthy subjects. However, the daily urinary excretion of myoinositol, chiroinositol and scyllo-inositol was increased in uremic patients compared with healthy subjects. The increase of myoinositol and chiroinositol in uremic urine was especially noticeable.

Serum polyol levels in uremic patients

Table III shows the serum concentrations of polyols in patients with chronic renal failure and healthy adults. In uremic serum the concentrations of threitol, erythritol, arabitol, mannitol, sorbitol, myoinositol, chiroinositol and scyllo-inositol were increased as compared with normal serum. Xylitol, neoinositol and epi- or cisinositol could not be detected in uremic serum or in normal serum. The serum concentration of 1-deoxyglucose in uremic patients was significantly decreased compared with that in healthy subjects.

TABLE III

SERUM POLYOL LEVELS IN UREMIC PATIENTS

	Normal (n=8)	Uremic (n=12)	
Threitol (mg/dl)	0	0.099 ± 0.092	
Erythritol (mg/dl)	<0.1	0.98 ± 1.4	
Xylitol	0	0	
Arabitol (mg/dl)	0	1.5 ± 0.90	
Mannitol (mg/dl)	0	2.6 ± 2.5	
Sorbitol (mg/dl)	0	0.31 ± 0.21	
Myoinositol (mg/dl)	<1.0	9.4 ± 6.9	
Neoinositol	0	0	
Chiroinositol*	0	0.79 ± 0.74	
Epi- or cisinositol	0	0	
Scylloinositol*	0	0.28 ± 0.30	
1-Deoxyglucose**	1.0 ± 0.75	0.32 ± 0.34	$p < 0.05$

*Peak height ratio of m/z 318 to m/z 422 (ribitol 50 μg).
**Peak height ratio of m/z 259 to m/z 422 (ribitol 50 μg).

DISCUSSION

In the present profiling analysis, 4-deoxythreitol, 4-deoxyerythritol, 5-deoxyxylitol, 5-deoxyarabitol, 2-deoxyribitol, 6-deoxymannitol, and 6-deoxy-galactitol were identified in normal urine as well as in uremic urine for the first time. Although the metabolism of these deoxyalditols is not yet known, the endogenous reduction of 2-deoxyribose and fucose is a possible cause of the formation of 2-deoxyribitol and 6-deoxygalactitol. The metabolic origin and the physiological significance of these deoxyalditols should be determined.

Neoinositol and chiroinositol were also identified in normal and uremic urine for the first time, and the increased urinary excretion and elevated serum levels of chiroinositol and scylloinositol were first demonstrated in uremic patients. The urinary excretion of neoinositol and epi- or cisinositol was, however, decreased in uremic patients compared with normal subjects. Scyllo-

inositol was detected in the urine of mammals and in human urine following oral administration of myoinositol. Myoinositol, myoinose-2 and scylloinositol were detected in organs of rat and rabbit [12]. Scylloinositol is considered to be formed by the reduction of myoinose-2 which originates from the dehydrogenation of myoinositol. The increased urinary excretion and increased serum level of scylloinositol in the uremic patients in our study may be due to the increased formation of scylloinositol from myoinositol. The increased urinary excretion and increased serum level of chiroinositol as well as myoinositol in the uremic patients suggests that chiroinositol is formed from myoinositol or that the catabolism of chiroinositol takes place exclusively in the kidneys as myoinositol. The decreased urinary excretion of neoinositol and epi- or cisinositol in the uremic patients suggests that the metabolism of these inositols has no relation with myoinositol and that the catabolism of these inositols does not take place in the kidneys.

Myoinositol is an essential growth factor for human cell lines in tissue culture. It is a constituent of phosphoinosides, such as phosphatidylinositol, phosphatidylinositol monophosphate and phosphatidylinositol diphosphate, which may be linked to neural activity in neural tissue. Myoinositol is demonstrated to be synthesized from glucose. Urinary myoinositol excretion is normally a minor mechanism for the disposition of endogenously synthesized myoinositol or dietary intake (300—900 mg/day in man) of myoinositol [13]. The major catabolic pathway for myoinositol requires its initial oxidation to D-glucuronate in the renal cortex [14]. The complete pathway is referred to as the glucuronate—xylose—pentose phosphate pathway [15]. After nephrectomy the production of respiratory $^{14}CO_2$ from ^{14}C-labelled myoinositol injected into rats is virtually abolished [14]. The impaired renal oxidation of myoinositol in uremic patients is thus a major factor for the increased urinary excretion and increased serum level of myoinositol. Retention of myoinositol has been considered a possible cause of uremic polyneuropathy [3, 4].

The urinary excretion of erythritol, xylitol and arabitol was decreased in the uremic patients. This result is in accordance with an earlier study [1]. In the uremic patients the serum levels of threitol, erythritol, arabitol, mannitol and sorbitol were elevated. Since the urinary excretion of threitol, erythritol, and arabitol is decreased it seems likely that these polyols were retained in the blood due to the impaired renal function. The elevated serum levels of mannitol and sorbitol accompanied by the slightly increased urinary excretion suggest that the endogenous production of mannitol and sorbitol is increased in the uremic state. Sorbitol can be converted to mannitol through fructose [16]. The similar behavior of mannitol and sorbitol in the uremic state supports the interrelated metabolism of these two polyols.

The serum level of 1-deoxyglucose in the uremic patients was significantly decreased as compared with that in the normal subjects. 1-Deoxyglucose was first detected in the cerebrospinal fluid by Pitkänen [17]. The level of this compound in the cerebrospinal fluid was reported to be low in diabetic patients and in uremic patients.

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Journal of Chromatography, 277 (1983) 41—51
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1803

AZETIDINE-2-CARBOXYLIC ACID CONTAMINATED DIETARY PROLINE AS A CAUSE OF URINARY EXCRETION OF 4-AMINO-2-(S-CYSTEINYL)BUTYRIC ACID IN PATIENTS ON ORAL TREATMENT WITH A SYNTHETIC DIET

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(First received March 2nd, 1983; revised manuscript received May 25th, 1983)

SUMMARY

Three children with branched-chain ketoaciduria (maple syrup urine disease) were found to excrete an abnormal amino acid when they were on an artifical diet. This substance was identified as 4-amino-2-(S-cysteinyl)butyric acid with the use of column liquid chromatography, gas chromatography—mass spectrometry of various derivatives, and 360 MHz

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0378-4347/83/$03.00

^{1}H-NMR spectroscopy. The same compound was detected in urine samples from subjects undergoing an oral loading test with L-proline. The chromatographic analysis of commercial proline from two sources indicated that one of the batches was contaminated (< 1%) with L-azetidine-2-carboxylic acid (the homologue of proline with a four-membered ring). The latter compound is probably metabolized by the human via ring-opening and addition of a cysteine moiety. It is highly probable that the artificial diet given to the patients contained the impure proline and that the L-azetidine-2-carboxylic acid in the proline gave rise to the excretion of the 4-amino-2-(S-cysteinyl)butyric acid.

INTRODUCTION

Patients with an inherited defect of amino acid metabolism such as phenylketonuria, maple syrup urine disease (MSUD), homocystinuria, tyrosinemia and others are treated by replacement of most of the natural dietary protein by an amino acid mixture, containing the minimal amount of the index essential amino acid necessary for normal growth.

When analyzing the urine of three patients with MSUD on dietary treatment, an abnormal ninhydrin-positive compound was observed. The phenomenon of the abnormal excretion was transient and had disappeared three years after its first observation.

In this paper we describe the identification of the abnormal ninhydrin-positive compound as 4-amino-2-(S-cysteinyl)butyric acid. We could also obtain evidence that azetidine-2-carboxylic acid was its precursor. The latter compound appeared to be a contaminant of commercially available proline. The use of this contaminated proline in the artificial diet most probably caused the biochemical anomaly in the patients' urine.

MATERIALS AND METHODS

General

L-Proline was obtained from two sources: D and J. L-Azetidine-2-carboxylic acid was purchased from Aldrich Europe (Beerse, Belgium).

Micro-scale two-dimensional thin-layer chromatography and column chromatography for routine analysis of ninhydrin-positive compounds in physiological fluids were performed as described previously [1] (see also ref. 2).

Gas—liquid chromatography (GLC) and combined gas—liquid chromatography—mass spectrometry (GLC—MS) of derivatized amino acids were carried out essentially as reported earlier [3]; oven temperatures were dependent on the type of compounds. High-resolution mass measurements and metastable measurements using a defocusing technique according to Barber and Elliott [4] were performed using an AEI MS-902 mass spectrometer (direct inlet system; electron-impact MS).

90-MHz and 360-MHz ^{1}H-NMR spectra were recorded with a Varian EM-390 and a Bruker HX-360 (Fourier Transform mode) spectrometer, respectively. Before analysis, amino acids were exchanged three times in ^2H_2O with intermediate lyophilization. Chemical shifts (δ) at a probe temperature of 25°C are given relative to sodium 5,5-dimethyl-5-silapentane-2-sulphonate in ^2H_2O as solvent (indirectly to acetone: δ = 2.225 ppm).

Isolation of 4-amino-2-(S-cysteinyl)butyric acid (ACBA)

The urine (10 ml) of a patient with MSUD on treatment was filtered and then applied to a small column (5 ml) of Dowex 50W-X8, 50—100 mesh, H^+. The resin was washed with 100 ml of water. Then, the urinary amino acids were eluted with 40 ml of 2 *M* ammonia. After evaporation of the solvent the residue was dissolved in 0.5 ml of 0.1 *M* pyridine—acetic acid, pH 3.50, and fractionated on a column of Aminex-MS fraction B, H^+ (150 × 0.6 cm). The elution was performed with the same buffer as mentioned above, at a flow-rate of 1.0 ml/min and a column temperature of 48.2° C. The unknown amino acid was present in the fractions between 350 and 400 ml of the eluate (ninhydrin detection) and proved to be pure by thin-layer chromatography. The yield was 4 mg.

Desulfurization with Raney nickel

The isolated urinary substance (1 mg) was dissolved in 2 ml of water and heated at 100° C for 2 h with 0.5 ml of a suspension of Raney nickel [5]. After decantation, the catalyst was washed three times with water at 100° C. The combined water phases were evaporated to dryness under reduced pressure and analyzed by two-dimensional thin-layer chromatography, column chromatography and GLC—MS (after trimethylsilylation).

Synthesis of 4-amino-2-(S-cysteinyl)butyric acid (ACBA)

For the synthesis of the title compound a similar strategy was followed as described earlier for isovalthine [6]. 4-Acetamidobutyric acid (1 g; 6.9 mmol) [7] was dissolved in 5 ml of freshly distilled thionyl chloride. The solution was kept at room temperature for 2 h and then warmed to 80° C. After the addition of 1.8 g of bromine (22.5 mmol), the mixture was refluxed for 3 h at 100° C. The excess thionyl chloride and bromine were removed by evaporation under reduced pressure. To the residue a mixture of 15 ml of water and 4 ml of triethylamine was added, followed by 1 g of L-cysteine (8.3 mmol). The suspension was stirred magnetically at room temperature for two days, whereby the pH was kept at 8.5. Subsequently, the precipitate was removed by filtration and the clear filtrate evaporated to dryness. The residue was suspended in 50 ml of 2 *M* HCl and refluxed for 3 h. The dark-brown syrup, obtained after evaporation of the acidic solution, was dissolved in 20 ml of water. The amino acid mixture was applied to a column (30 × 2 cm) of Dowex 50W-X8, 100—200 mesh, H^+. The resin was washed with 200 ml of water and the amino acids eluted with 250 ml of 3 *M* ammonia. The fraction containing the amino acids was evaporated to dryness. After esterification with methanolic HCl and trifluoroacetylation, GLC—MS showed the presence of ACBA, and in addition some contaminants, i.e. 4-aminobutyric acid, cystine, and S-carboxymethylcysteine.

The formed mixture of stereoisomers of ACBA gave rise to two peaks in the column chromatogram of the amino acid analyzer. However, in view of the reaction conditions used, it is tempting to assume that all four possibilities are present. The two peaks were fractionated on a preparative scale with a column (107 × 2.5 cm) of Aminex Q 150S, H^+, using 0.1 *M* pyridine—acetic acid, pH 3.50, at a flow-rate of 7.3 ml/min and a column temperature of 48.2° C. The

pooled fractions between 4700 and 5800 ml of eluate correlated mainly with the first peak of ACBA on the amino acid analyzer (composition: first peak, 82.2%; second peak, 17.8%). The pooled fractions between 6600 and 7100 ml correlated mainly with the second peak on the amino acid analyzer (composition: first peak, 24.6%; second peak, 75.4%). The yields of the lyophilized fractions were 35 and 25 mg, respectively.

Isolation of L-azetidine-2-carboxylic acid (ACA)

ACA-contaminated L-proline source D (100 mg) was fractionated on a column (100 × 2.5 cm) of Aminex-MS fraction B (H^+) with 0.1 *M* pyridine—acetic acid, pH 3.50, at a flow-rate of 5.0 ml/min and a column temperature of 50.0°C. ACA eluted just before proline as a broad peak. To obtain a total yield of 5 mg, the isolation procedure was repeated two times.

For the gas chromatographic determination of the absolute configuration of ACA on SP-1000 as non-chiral stationary phase [8], part of the sample was treated with (—)-2-butanolic HCl and trifluoroacetic anhydride.

Derivatization procedures

Esterification: amino acids (0.5 mg) were treated with methanolic, trideuteromethanolic, 1-butanolic, (±)-2-butanolic or (—)-2-butanolic 3 *M* HCl (1 ml) for 2 h at 100°C [3]. Acylation: alkyl esters of amino acids were acylated with trifluoroacetic anhydride (1 ml) for 4 h at 20°C [3]. Trimethylsilylation: amino acids (0.5 mg) were treated with N,O-bis-(trimethylsilyl)-acetamide—pyridine (1:1, v/v) for 30 min at 70°C.

Patients

D.G., a three-week-old girl, first child of Turkish parents, was admitted after a positive outcome of the Guthrie population screening test for MSUD. She was placed on the special diet, low in branched-chain amino acids. When 8 months old, the intake of contaminated proline was 2.9 g/day*. Urinary ACBA was 2.6 and 3.1 mmol/g creatinine on two different days. The disease of the patient was classified as the intermediate form of MSUD. In spite of metabolic dysregulations the development of the patient was satisfactory.

A.E., male, second child of healthy German parents, was treated for MSUD starting 5 days after birth. The outcome was favourable. At the age of 6 months the intake of contaminated proline was 3.3 g/day*. Urinary ACBA was 3.6 and 4.1 mmol/g creatinine.

G.T., female, third child of Turkish parents, was admitted at the age of 13 days with apathy, muscular hypotonia and feeding difficulties. Acute MSUD was diagnosed and treatment with the same diet was started. In spite of the dietary regimen, mental and motor development were severely retarded. The intake of contaminated proline at the age of 14 months was 3.9 g/day*. Urinary ACBA was 2.5 and 3.2 mmol/g creatinine.

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*As calculated retrospectively.

RESULTS

Identification of urinary 4-amino-2-(S-cysteinyl)butyric acid

Two-dimensional thin-layer chromatography of the urinary amino acids in the three patients on dietary treatment gave rise to an unknown heterogeneous greyish-blue spot, approximately in the position of oxidized cystathionine. Upon oxidation of the sample with H_2O_2 a small shift to the direction of the application point was observed (Fig. 1). On column chromatography the corresponding unknown compound eluted at the position of leucine.

For structural analysis, the compound was isolated by ion-exchange column chromatography. The influence of H_2O_2 on the position of the substance on a thin-layer chromatogram and a low 570/440 nm absorbance ratio of the column chromatographic fraction, suggested the presence of sulphur in the amino acid. Treatment with an iodoplatinate reagent [9] yielded within a few minutes a white spot on a pink background, indicative of sulphur, most probably in a thioether. Desulfurization with hydrogenated Raney nickel gave rise to the formation of alanine and 4-aminobutyric acid. Their identity was verified by GLC—MS of the corresponding trimethylsilyl derivatives [10]. Apparently, alanine and 4-aminobutyric acid were linked to each other via a sulphur bridge.

The sulphur content and the position of the bridge was further investigated by GLC—MS after three types of derivatization of the unkown substance: (1) pertrimethylsilylation (derivative I); (2) treatment with methanolic HCl followed by trifluoroacetylation (derivative II); (3) treatment with trideuteromethanolic HCl followed by trifluoroacetylation (derivative III). The mass

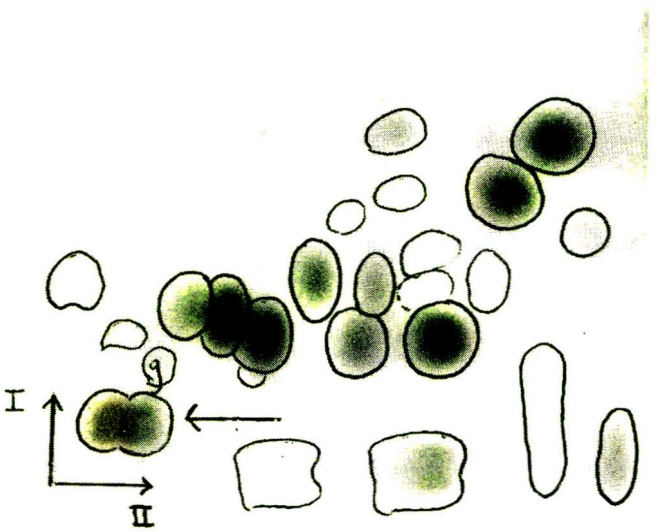


Fig. 1. Two-dimensional thin-layer chromatography on cellulose of urinary amino acids in a patient with MSUD on treatment with a dietary amino acid mixture; the applied material was oxidized with H_2O_2. The sulphur-containing amino acid is indicated with an arrow. Solvent I = *n*-butanol—pyridine—water (1:1:1, v/v), and solvent II = 88% phenol—25% ammonia—water (10:0.05:1, v/v). For further details, see refs. 1 and 2.

spectra of the pertrimethylsilylated amino acid (derivative I) and the N-trifluoroacetyl amino acid methyl ester (derivative II) are presented in Figs. 2 and 3, respectively.

Comparison of the mass spectra of the three derivatives I—III showed that only one sulphur atom is involved in the linkage between alanine and 4-aminobutyric acid (I: *m/z* 567, M_I-CH_3; II: *m/z* 410, measured formula $C_{12}H_{12}N_2O_5SF_6$, $M_{II}-CH_3OH$; III: *m/z* 413, $M_{III}-C^2H_3OH$). The three mass spectra suggested this sulphur atom to be attached at C-3 of alanine, thus making it a cysteinyl residue (I: *m/z* 218; II: *m/z* 184, measured formula $C_5H_5NO_3F_3$; III: *m/z* 187). The determination of the linkage between cysteine and 4-aminobutyric acid was difficult. The presence of *m/z* 174 and 188 in the mass spectrum of I could be explained by the attachment of the sulphur atom of the cysteinyl group at C-2 of 4-aminobutyric acid. The presence of the peaks at *m/z* 126 and 140 in the mass spectra of both II and III supported this con-

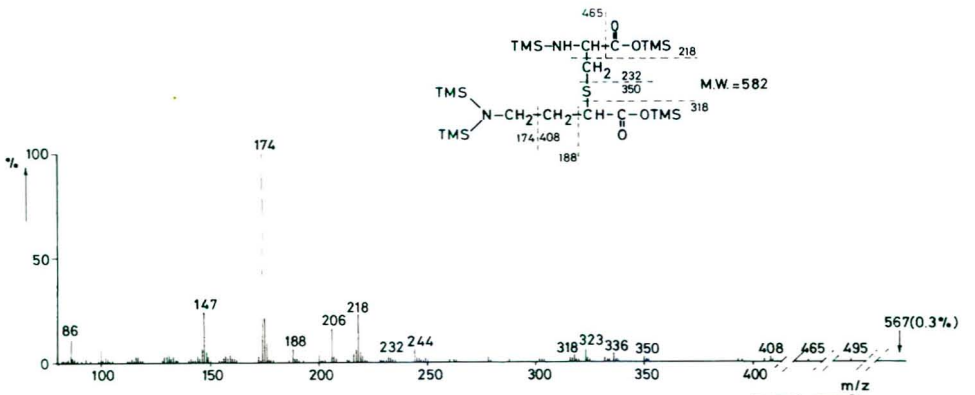


Fig. 2. Electron-impact (70 eV) mass spectrum of pertrimethylsilylated 4-amino-2-(S-cysteinyl)butyric acid.

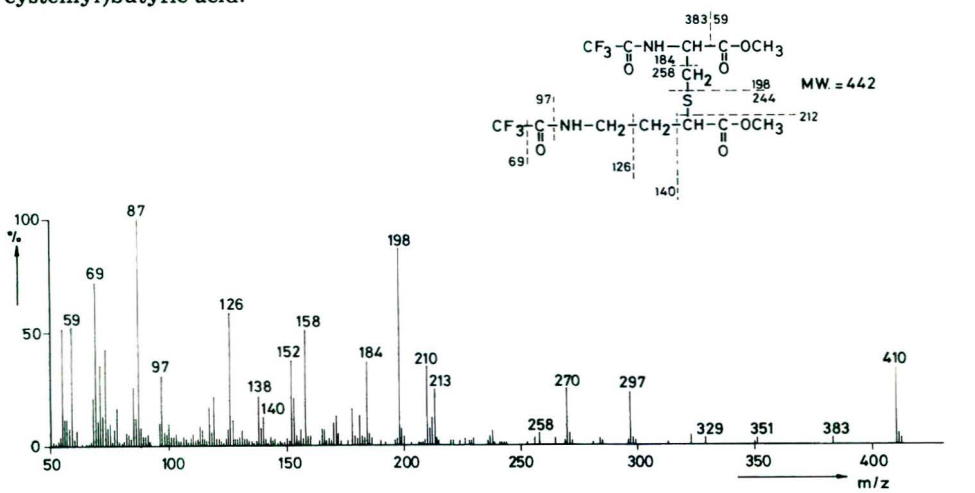


Fig. 3. Electron-impact (70 eV) mass spectrum of N-trifluoroacetyl 4-amino-2-(S-cysteinyl)-butyric acid methyl ester.

clusion. However, the occurrence of *m/z* 87 in the mass spectrum of II and *m/z* 90 in that of III, being in both cases the most abundant ions, suggested a connection with C-4. Because of this contradiction the mass spectrometric origin of *m/z* 87 was studied in more detail. Metastable measurements for II, using the defocusing technique of Barber and Elliott [4], indicated that the most prominent precursor-ion of *m/z* 87 appeared to be *m/z* 213. No evidence was obtained for precursor-ions > *m/z* 213. It is possible to presume that the fragmentation pattern includes formation of *m/z* 213 and the subsequent transition to *m/z* 87 via a simple cleavage. It is reasonable to suppose that the fragment ion *m/z* 87 in the mass spectrum of II (and *m/z* 90 in that of III) does not yield direct structural information. Taking into account the various mass spectrometric data, the most likely position of the sulphur atom of the cysteinyl residue is at C-2 of 4-aminobutyric acid.

To obtain conclusive evidence for the suggested structure, the underivatized amino acid was investigated by 360-MHz ^{1}H-NMR spectroscopy. The ^{1}H-NMR spectrum recorded in ^2H_2O at pH 7 is depicted in Fig. 4. The various signals were assigned by comparison with the ^{1}H-NMR spectra of L-cysteine, 4-aminobutyric acid and 4-amino-2-hydroxybutyric acid [11], recorded under the same conditions (not shown here).

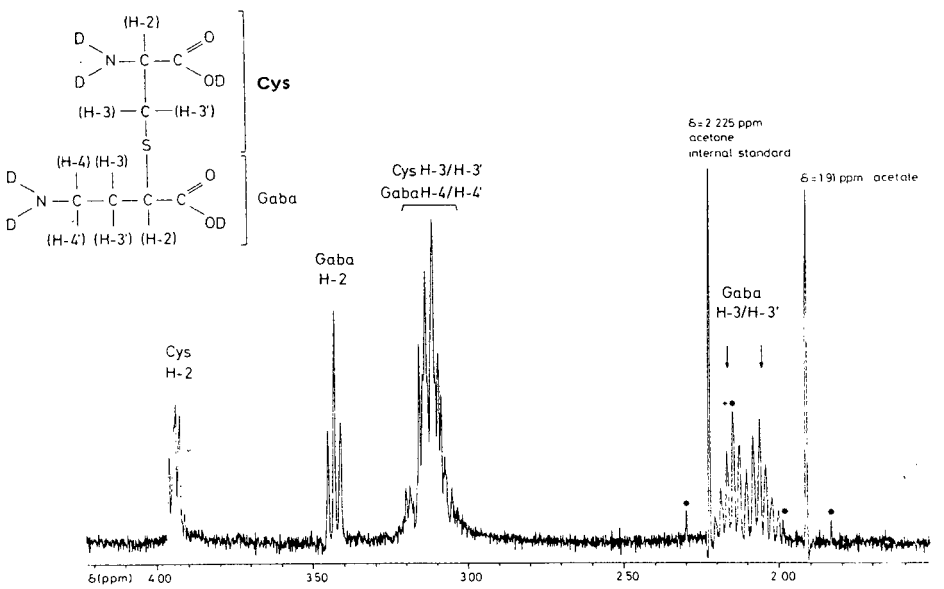


Fig. 4. Resolution-enhanced, 360-MHz ^{1}NMR spectrum of 4-amino-2-(S-cysteinyl)butyric acid, recorded in ^2H_2O at pH 7. Asterisks (*) denote spinning side bands. Gaba = 4-aminobutyric acid.

The cysteinyl part of the molecule was demonstrated by the presence of a double doublet at δ = 3.95 (Cys H-2), and two double doublets in the δ-region 3.05—3.20 (Cys H-3/H-3'). Compared with the spectrum of L-cysteine, no large differences in chemical shift were observed. The 4-aminobutyric acid part of the molecule gave rise to a triplet at δ = 3.45 (GABA* H-2), multiplets at δ =

* 4-Aminobutyric acid.

2.05 and 2.16 (GABA H-3/H-3′) and two multiplets in the δ-region 3.05—3.20 (GABA H-4/H-4′). Compared with the ^{1}H-NMR spectrum of free 4-aminobutyric acid, a relatively high downfield shift was observed for GABA H-2 (δ = 2.29; 2H → δ = 3.43; 1H). A similar phenomenon for GABA H-2 was observed in the spectrum of 4-amino-2-hydroxybutyric acid. The other protons of the 4-aminobutyric acid part of the molecule resonate in the same δ-range as found for 4-aminobutyric acid and 4-amino-2-hydroxybutyric acid. These data clearly indicate that in the isolated substance the sulphur atom is attached to C-2 of 4-aminobutyric acid, leading to the structure of ACBA.

The identity of the urinary ACBA was verified by comparison with the synthetic stereoisomers. It turned out that the first eluting peak of the mixture of stereoisomers on the amino acid analyzer co-chromatographed with the urinary compound.

Identification of L-*azetidine-2-carboxylic acid in commercial* L-*proline*

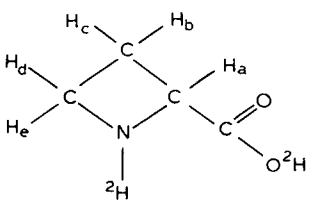
Because of the occurrence of ACBA in the urine of a patient and a healthy control after loading with L-proline source D, it was assumed that this proline sample should contain a precursor of the new sulphur-containing amino acid. Two-dimensional thin-layer chromatography of the commercial L-proline showed, just below the proline spot, an unknown spot that colored yellow with the ninhydrin reagent [2]. On the amino acid analyzer the compound eluted at the position of glutamic acid as a broad peak. The compound was separated from L-proline by preparative ion-exchange chromatography and identified by 360-MHz ^{1}H-NMR spectroscopy and GLC—MS.

The 360-MHz ^{1}H-NMR spectrum corresponded with that of commercial L-ACA. In Table I the various chemical shifts and coupling constants are summarized (these data could not be deduced earlier from the 60-MHz ^{1}H-NMR spectrum reported by Aldrich [12].

TABLE I

360-MHz ^{1}H-NMR DATA OF L-AZETIDINE-2-CARBOXYLIC ACID OBTAINED IN ^2H_2O AT A PROBE TEMPERATURE OF 25°C AND pH 7.95

Chemical shifts δ are given in ppm relative to internal sodium 5,5-dimethyl-5-silapentane-2-sulphonate (indirectly to acetone: δ = 2.225 ppm). Coupling constants J are given in Hz.



Proton	Chemical shift (ppm)	Coupling constants (Hz)
H_a	4.81	J_{ab}, 8.0; J_{ac}, 10.1
H_b	2.55	J_{bc}, −12.2; J_{ba}, 8.0; J_{be}, 9.8; J_{bd}, 8.3
H_c	2.79	J_{cb}, −12.2; J_{ca}, 10.1; J_{ce}, 6.1; J_{cd}, 9.5
H_d	4.10	J_{db}, 8.3; J_{dc}, 9.5; J_{de}, −10.6
H_e	3.93	J_{eb}, 9.8; J_{ec}, 6.1; J_{ed}, −10.6; J_{eN}, 0.8

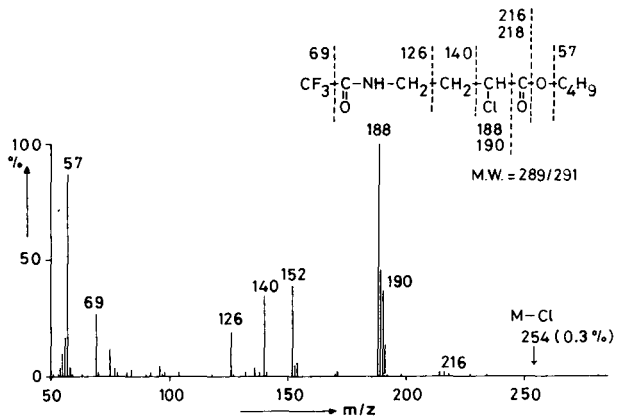


Fig. 5. Electron-impact (70 eV) mass spectrum of N-trifluoroacetyl 4-amino-2-chlorobutyric acid 1-butyl ester.

After treatment with 1-butanolic HCl and trifluoroacetic anhydride, identification of N-trifluoroacetyl 4-amino-2-chlorobutyric acid 1-butyl ester (Fig. 5) was made by GLC—MS. Derivatization of commercial L-ACA gave rise to the same product. Only a trace of intact N-trifluoroacetyl ACA 1-butyl ester was observed. As has been demonstrated previously, ACA is decomposed by hydrochloric acid to yield 4-amino-2-chlorobutyric acid among other degradation products [13].

The absolute configuration of the isolated ACA was determined indirectly by capillary GLC of the N-trifluoroacetyl 4-amino-2-chlorobutyric acid (—)-2-butyl ester on SP-1000 [8]. Commercial L-ACA was derivatized using either (—)- or (±)-2-butanol. The N-trifluoroacetyl 4-amino-2-chlorobutyric acid (—)-2-butyl ester obtained from L-ACA gave rise to one main GLC peak, but owing to the presence of a small amount of the (+)-enantiomer in the commercial (—)-2-butanol sample [(—)/(+) = 94:6], a small peak with a higher retention time was also observable. From this observation it can be concluded that the conversion of L-ACA into the chiral 4-amino-2-chlorobutyric acid occurs stereospecifically. GLC of the (±)-2-butyl ester showed two peaks. The peak with the lowest retention time corresponded with the main peak derived from authentic L-ACA treated with (—)-2-butanol. Therefore, the other peak must belong to L-ACA treated with (+)-2-butanol. As was discussed earlier [14], on non-chiral stationary phases L-enantiomers treated with (+)-2-butanol have the same retention time as D-enantiomers treated with (—)-2-butanol. The (—)-2-butyl ester derivative corresponding with the isolated ACA co-eluted with the (—)-2-butyl ester derivative of the L-enantiomer.

In conclusion, the commercial L-proline used contained L-azetidine-2-carboxylic acid. Quantitative analysis of the proline sample by column chromatography demonstrated the presence of 1.9% of the latter substance.

Recent investigations of new batches of L-proline (source D) showed that L-ACA was no longer detectable. Analysis of L-proline (source J) did not lead to the finding of ACA.

DISCUSSION

The abnormal amino acid ACBA was initially observed in the urine of three patients with MSUD, all on dietary treatment in the same university pediatric hospital. The phenomenon was transient. MSUD patients from elsewhere did not excrete this product. This course of events pointed rather to an exogenous origin of the abnormal compound or its precursor than to hitherto unknown errors of metabolism in MSUD. The accidental finding of ACBA in the urine of a patient who was loaded with commercially available proline led to the recognition that ACA, present as a contaminant by up to 1.9% in this imino acid sample, was the precursor. Also a healthy control subject, who was loaded with this contaminated proline, excreted ACBA.

The formation of ACBA from ACA may proceed via a direct attachment of L-cysteine to L-ACA. Another mode of formation might be via 4-amino-2-chlorobutyric acid, which could arise from ACA with gastric hydrochloric acid. This intermediate could react with L-cysteine in turn, to form ACBA. Now, it is highly probable that the proline used in the special MSUD diet also contained ACA. Later, when proline without ACA was used in the diet, patients no longer excreted ACBA.

Up to now L-ACA has not been found in human physiological fluids. However, this imino acid occurs in the free form in a variety of plants such as *Convallaria majalis* L. (lily-of-the-valley) and *Polygonatum officinale* [15—18]. The compound can act as a proline analogue, being incorporated into proteins [19].

Incorporation of ACA in proteins resulting in impaired biological activity [19] may be harmful to individuals daily exposed to oral loading with this compound. However, there is no evidence of damage in our patients. Two out of three had a good outcome; in one, G.T., with the severe form of MSUD, treatment was less successful, but the clinical picture did not point to causal factors other than can be expected in this disorder. A considerable part, 40% in our control, of the ACA intake was eliminated as ACBA in the first 8 h after administration. Nevertheless, we should be careful when giving a formula diet with free amino acids to patients. The amino acids used should be carefully analyzed for impurities. In general, the daily administration of small amounts of potentially toxic impurities to patients may cause damage that is difficult to recognize afterwards.

ACKNOWLEDGEMENTS

We thank Mr. C. Versluis (Laboratory of Analytical Chemistry, University of Utrecht, The Netherlands) and Mr. D. Ketting for recording the mass spectra; Mrs. G. Blanken-Aarsen, Miss E.E.E. Meuleman and Mr. F. van der Lecq for technical assistance; Dr. L. Dorland and Dr. H. van Halbeek (Department of Bio-Organic Chemistry, University of Utrecht, The Netherlands) for recording the 360-MHz ^{1}H-NMR spectra; Dr. P. Krogsgaard-Larsen (Royal Danish School of Pharmacy, Department of Chemistry BC, Copenhagen, Denmark) for the generous gift of *R,S*-4-amino-2-hydroxybutyric acid.

This study was supported by "Het Praeventiefonds" (S.K.W.) and by the

Netherlands Foundation for Chemical Research (SON)/Netherlands Organization for the Advancement of Pure Research (ZWO) (J.F.G.V.).

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Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1797

DETERMINATION OF POLYAMINES IN HUMAN BLOOD BY ELECTRON-CAPTURE GAS—LIQUID CHROMATOGRAPHY

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(First received February 23rd, 1983; revised manuscript received May 6th, 1983)

SUMMARY

The present work was undertaken to develop a sensitive and selective method for the estimation of putrescine, spermidine and spermine in human blood employing electron-capture gas—liquid chromatography. Polyamines were derivatized with heptafluorobutyric anhydride. The heptafluorobutyric derivatives of polyamines could be well resolved within 15 min under a temperature programme. The detection limit was 0.1 pmol for putrescine and cadaverine, and 0.02 pmol for spermidine and spermine. The method was applied to polyamine determinations in erythrocytes from human blood. For pre-separation of the polyamines from other compounds, a simple clean-up method utilizing an activated Permutit has been devised. Major interfering substances could be removed by the batchwise Permutit treatment. The mean values of spermidine and spermine concentrations, and the spermidine/spermine ratio in erythrocytes obtained from normal subjects (n = 11) were similar to reported values. The analytical procedure is thought to be applicable to various biological materials.

INTRODUCTION

Over the past decade, numerous analytical methods have been devised for the quantitative determination of the naturally occurring di- and polyamines putrescine, spermidine and spermine [1—3]. Among these methods gas—liquid chromatography (GLC) has been shown to be a useful tool for the routine analysis of polyamines in biological materials. The method is fast, convenient, selective and shows good resolution. An additional advantage of GLC seems to be in the application to a structural analysis of unusual natural polyamines [4—7] or identification of metabolic products of polyamines [8, 9] in combination with mass spectrometry. Since putrescine, spermidine and spermine were successfully separated as their trifluoroacetic derivatives [10], trifluoroacetic anhydride has been extensively used as an acylating reagent for polyamine assay by flame ionization GLC. The method has been adopted for

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the estimation of the polyamine contents of urine from normal and cancer patients [11, 12]. However, due to the lack of sensitivity, the application of this method has been limited to the analysis of urine, which usually contains much higher levels of polyamines than other physiological fluids such as blood and cerebrospinal fluid.

Electron-capture detection (ECD) is a highly sensitive detection system for fluorine-containing compounds. Makita et al. [13] first applied this technique to the quantitative assay of polyamines using pentafluorobenzyl chloride for derivatization, but determination of spermine was unsuccessful. More recently Rattenbury et al. [14] developed an ECD method for measuring urinary polyamines as their pentafluoropropionyl derivatives. In spite of its excellent sensitivity which enables the measurement of polyamines at picomole level, little has been reported so far on the analysis of blood polyamines by ECD—GLC. The major difficulty appears to be in the purification step for separating polyamines from other compounds, mainly amino acids, which readily react with acylating reagents and produce serious interfering peaks. With the rapid and accurate determination of polyamines in human blood in mind, we devised a simple method using an activated Permutit for the clean-up of samples containing large quantities of amino compounds.

In our method, heptafluorobutyric anhydride (HFBA) was selected as an excellent acylating reagent for the conversion of the polyamines into volatile and highly ECD-sensitive derivatives. This paper describes the optimal conditions for derivatization and separations of the polyamines. The applicability of the ECD—GLC technique to the quantitative assay of these amines in erythrocytes from human blood is demonstrated.

EXPERIMENTAL

Chemicals

Putrescine, cadaverine, spermidine and spermine were purchased from Nakarai Chemicals (Kyoto, Japan) in the form of their hydrochlorides. Heptafluorobutyric anhydride (HFBA), activated Permutit, and 1,5-diamino-3-azapentane were obtained from Wako Pure Chemical Industries (Osaka, Japan). Dimethylamine was purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.); it was redistilled before use. Other organic and inorganic chemicals were obtained from commercial sources.

Preparation of erythrocytes and extraction of polyamines

Human blood was obtained from healthy volunteers (11 males; age 22—43 years). The blood was collected in glass centrifuge tubes containing 1/10 vol. (v/v) of 3.8% sodium citrate solution and was immediately centrifuged at 1500 *g* for 15 min to pellet the cells. The plasma was removed, the buffy coat aspirated and the cellular pellet was resuspended in 0.9% NaCl and centrifuged again at 1500 *g* for 15 min. Extraction of free polyamines from the erythrocyte fraction was carried out with 10% (w/v) trichloroacetic acid (TCA). Generally, 0.5 ml of packed erythrocytes was used for polyamine analysis. The samples were mixed with 3 ml of 10% TCA, shaken mechanically for 5 min and centrifuged at 1500 *g* for 15 min. The supernatant fluid was placed into a glass

centrifuge tube equipped with a glass stopper and extracted twice with 5 ml of diethyl ether to remove the excess TCA.

Sample pre-separation

The polyamine-containing extracts of erythrocytes were pre-separated by means of activated Permutit. To the sample solution, 1/10 vol. (w/v) of activated Permutit was added and mechanically shaken for 5 min. The supernatant was discarded and the Permutit was washed once with 5 ml of 2% NH_4OH and twice with 5 ml of distilled water. The polyamines were eluted from the Permutit particles by shaking with 5 ml of a 40% solution (w/v) of dimethylamine in methanol for 5 min. This step was repeated two times in order to complete the elution of polyamines. After sedimentation of the Permutit particles, the supernatant was collected and evaporated to dryness in order to remove dimethylamine. The residue was dissolved in 1 ml of 1 *N* HCl. Distilled dimethylamine should be used for the extraction of polyamines, since the commercial reagent always contained significant amounts of interfering materials.

Reaction of polyamines with HFBA

An aliquot of the sample solution was transferred to 3-ml reaction vials equipped with a Teflon-lined screw cap, and an exact amount of 1,5-diamino-3-azapentane was added as an internal standard. The mixture was evaporated to dryness under a stream of pure nitrogen at 90°C. After cooling, 200 μl of acetonitrile and 50 μl of HFBA were added to the dryed residue. The vials were capped and heated at 65°C for 10 min. The reaction mixture was evaporated to dryness under a stream of nitrogen and then redissolved in 1 ml of diethyl ether. The ether solution was washed once with an equal volume of saturated Na_2CO_3 solution. After centrifugation, the aqueous phase was discarded and 1 μl of ether phase was taken for the GLC analysis.

Separation of HFB polyamine derivatives

An Hitachi 163 gas chromatograph fitted with a ^{63}Ni EC detector (pulse interval = 50 μsec) was employed in this study. The GLC column of Pyrex glass (150 cm × 3 mm I.D.) was packed with 3% silicon OV-17 on 80—100 mesh Chromosorb W HP. The glass column was silanized before use with a 10% solution of hexamethylenedisilazane in toluene and washed with methanol and acetone. The initial column temperature of 120°C was maintained for 2.5 min. The temperature was then increased at a rate of 15°C/min to a final temperature of 280°C. The temperature of the EC detector was 300°C. Highly pure nitrogen was used as carrier gas at a flow-rate of 60 ml/min. Mass spectra of the HFB derivatives of the polyamines were obtained with a double-focussing Hitachi M-80 mass spectrometer.

RESULTS AND DISCUSSION

Preliminary experiments were carried out to examine the optimal conditions for derivatization of the polyamines with HFBA. For preparing derivatives at the nanogram level, 50 μl of HFBA were sufficient to complete the reaction.

With the use of acetonitrile as solvent, acylation of putrescine, cadaverine, spermidine and spermine was quantitative within 10 min at 65° C. The reaction at room temperature lowered the yield of HFB derivatives and sometimes resulted in additional peaks on the gas chromatogram, possibly due to partial acylation of the amines. Diethyl ether was a good solvent for dissolving HFB polyamine derivatives. Other organic solvents such as benzene, dioxane, pentane, hexane and heptane did not completely dissolve the polyamine derivatives, especially HFB spermine. Washing the ether phase with saturated Na_2CO_3 solution was effectively removing impurities which interfered in the gas chromatogram. HFB polyamines were stable in ether solution at least for several weeks; no decomposition occurred even in the presence of water.

From the structural analysis of the HFB polyamines by gas chromatography—mass spectrometry, it was confirmed that HFB groups were transferred into both primary and secondary amino groups in each polyamine molecule. Molecular ions (M^+) were obtained at *m/e* 480 for HFB putrescine, *m/e* 494 for HFB cadaverine, *m/e* 733 for HFB spermidine, and *m/e* 986 for HFB spermine. Excess acylation of the primary amino groups did not occur under the experimental conditions.

For the conversion of the polyamines to volatile and EC-sensitive derivatives, other fluorinated acylating reagents have also been studied. Derivatization with trifluoroacetic anhydride was unsuitable for the ECD technique since trifluoroacetic derivatives of putrescine and cadaverine showed extremely low sensitivity to the EC detector. In the case of pentafluoropropionic anhydride, the by-product pentafluoropropionic acid was a serious source of interference as pointed out by Rattenbury et al. [14], because this acid eluted close to spermidine. Pentafluoropropionic imidazole has the advantage of not releasing pentafluoropropionic acid into the reaction mixture, but it was not practical for the purpose of routine analyses of polyamines, since special care is needed in the handling and storage of this reagent due to its sensitivity to moisture. In the case of HFBA, HFB acid was produced by hydrolysis during the reaction, but did not interfere with the elution of the polyamine derivatives under the conditions described in this paper and the HFB polyamine derivatives showed the most excellent EC sensitivity among the acylating reagents.

Fig. 1 shows an elution profile of a standard mixture of the HFB derivatives of putrescine, cadaverine, spermidine and spermine. Each peak represents 1 pmol of polyamine. As might be expected from the numbers of HFB groups incorporated into each polyamine molecule, HFB spermidine and HFB spermine gave higher detector responses than those of the diamines. Symmetrical peaks and good separations of the HFB polyamine derivatives were obtained on a column of 3% silicon OV-17 coated on Chromosorb W HP. Other liquid phases tested, including 2% OV-25, 3% OV-225, 5% SE-30 and 10% Apiezon grease M, were found to be unsuitable because of unsatisfactory separations, or broad and asymmetrical peaks.

Fig. 2 shows calibration curves of the HFB derivatives. Peak height ratios of polyamine to internal standard, 1,5-diamino-3-azapentane, were plotted against the amount of each polyamine added to the reaction mixture. Linear relationships were obtained for up to 2 pmol of putrescine and cadaverine, and 1 pmol

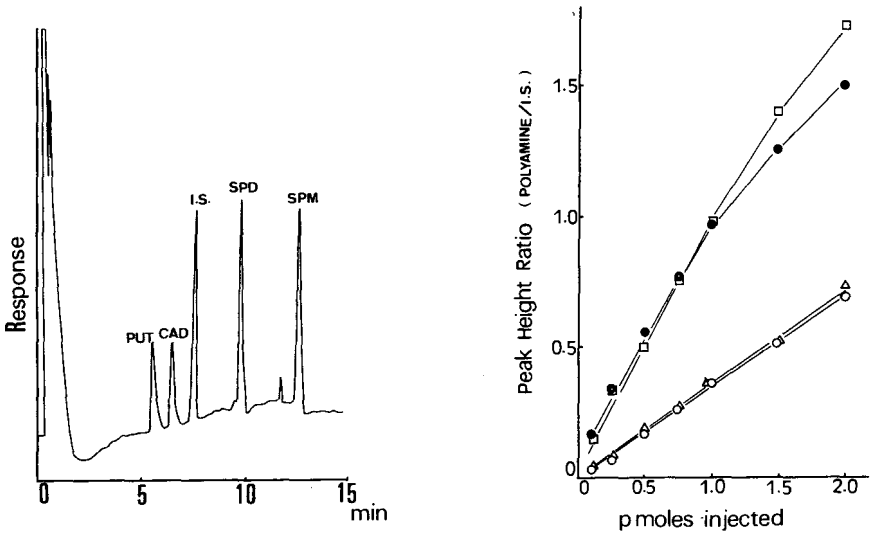

Fig. 1. Gas chromatogram of a standard mixture of polyamines. The polyamines were derivatized with HFBA as described in the text. Column: 3% OV-17 on 80—100 mesh Chromosorb W HP, 150 cm × 3 mm I.D. glass. Nitrogen gas flow-rate: 60 ml/min. Instrumental conditions: initial temperature 120°C, delay 2.5 min, 15°C/min, and final temperature 280°C. Detector: ^{63}Ni electron-capture detector, 300°C at a pulse interval of 50 μsec. Each peak represents 1 pmol of putrescine (PUT), cadaverine (CAD), spermidine (SPD), and spermine (SPM). I.S.: 1,5-diamino-3-azapentane. The peak between SPD and SPM is a spurious peak eluted from the OV-17 column.

Fig. 2. Calibration curves for HFB polyamine derivatives. The analytical conditions of ECD—GLC are given in the legend of Fig. 1. (○), Putrescine; (△), cadaverine; (●), sperimidine; (□), spermine.

of spermidine and spermine. The detector response of HFB spermine declined sometimes because of adsorption of the derivative on the surface of the stationary phase or the glass column. The recovery of HFB spermine was improved by employing a column conditioner Silyl-8 (Pierce Chemical Co., Rockford, IL, U.S.A.) prior to the application of the samples into the GLC column. The detection limit of the method was 0.1 pmol for putrescine and cadaverine, 0.02 pmol for spermidine and spermine.

Generally, a major portion of the free spermidine and spermine of whole blood is found in the erythrocytes [15, 16]. Clinical attention has recently been focussed on the possible usefulness of the analysis of polyamines in this blood component, since in cancer patients abnormally high polyamine concentrations were detected more readily in the blood cells than in plasma [17—19]. In view of the planned medical application of our ECD—GLC technique, the method was applied to polyamine determinations in the erythrocyte fraction of human blood.

In the analysis of human blood, the separation of the polyamines from other compounds, especially amino acids, is a prerequisite, because HFBA readily reacts not only with polyamines but also with compounds such as phenols, alcohols, and amino acids, to form EC-sensitive derivatives. The usual clean-up method using a cation-exchange column [11, 14, 20] was unsuccessful

because of the incomplete removal of EC-sensitive materials in the sample solution. Pre-treatment of the acid extract with an activated Permutit was found to be a useful method. Permutit particles adsorbed polyamines over a wide pH range. The polyamines were easily eluted from the Permutit particles by shaking with a 40% solution of dimethylamine in methanol. Methylamine and trimethylamine solutions were less effective. The recovery of each polyamine during the batchwise Permutit treatment is shown in Table I. Major interfering substances could be removed by this simple procedure.

TABLE I

RECOVERY OF POLYAMINES FROM PERMUTIT WITH 40% DIMETHYLAMINE–METHANOL SOLUTION

	Recovery* (%)
Putrescine	96.9 ± 2.4
Spermidine	95.8 ± 3.5
Spermine	88.7 ± 4.0

*Mean ± S.E.M. (n = 12).

Fig. 3 is a gas chromatogram of a typical blood sample. The concentration of putrescine was considerably lower than that of spermidine and spermine; cadaverine was not detected in healthy subjects. Total recovery of the polyamines was examined using erythrocyte fractions. From the analysis of samples with and without polyamines (putrescine 20 nmol; spermidine 10 nmol; spermine 10 nmol) added to erythrocytes, the recovery of putrescine, spermidine and spermine was determined to be 73–81%, 72–98% and 59–72%, respectively.

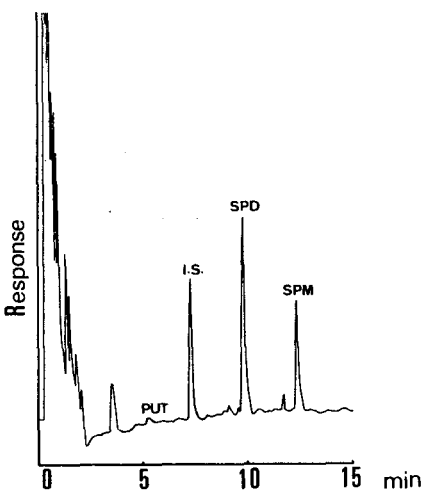

Fig. 3. Gas chromatogram of the polyamines isolated from human erythrocytes. The erythrocyte fraction was prepared from normal human blood. The polyamines were extracted, separated, and converted to HFB derivatives as descibred in the Experimental section. PUT, putrescine; SPD, spermidine; SPM, spermine. I.S., 1,5-diamino-3-azapentane.

The spermidine and spermine concentrations, and spermidine/spermine ratios in the erythrocytes from normal subjects (11 males, age 22—43 years) are listed in Table II. Alterations of the spermidine/spermine ratio in whole blood or erythrocytes were observed in patients with various diseases [21]. The spermidine/spermine ratio is therefore frequently used as an indicator for characterizing abnormalities. In the present investigation, the mean spermidine/spermine ratio in the erythrocytes of healthy subjects was 1.66. Despite the different methods employed for the extraction, pre-separation, and determination of the polyamines, this value was similar to those reported by other investigators [15, 16, 18, 22], indicating the applicability of the present ECD—GLC method to blood polyamine analysis.

TABLE II

SPERMIDINE AND SPERMINE CONCENTRATIONS IN ERYTHROCYTES FROM HEALTHY HUMAN VOLUNTEERS

Sample	nmol/ml packed cells		Spermidine/spermine ratio
	Spermidine	Spermine	
1 Y.T.	8.0	5.6	1.43
2 S.F.	8.4	3.8	2.21
3 T.K.	15.6	12.2	1.28
4 N.Y.	8.2	5.0	1.64
5 N.O.	14.4	11.3	1.27
6 C.T.	11.5	8.9	1.29
7 S.S.	15.5	7.0	2.21
8 T.N.	16.6	8.5	1.95
9 S.F.	18.6	8.6	2.16
10 K.O.	7.6	7.6	1.00
11 H.N.	13.9	7.7	1.81
Mean ± S.D.	12.6 ± 3.8	7.8 ± 2.4	1.66 ± 0.41

The high degree of sensitivity of our analytical method enabled the quantitative determination of polyamines in small blood samples. It should be noted, however, that special care is needed to avoid contamination with EC-sensitive materials from various sources during the experimental procedure. The rapid and accurate analysis of polyamines in extracellular fluids has significant clinical utility in the diagnosis and evaluation of patients with various diseases [23]. The assay procedure presented in this paper is thought to be suitable for routine assays of polyamines in biological fluids. The method has several advantages: a rapid pre-separation method, easy derivatization with HFBA, excellent sensitivity of EC detection, and a fast and reproducible gas chromatographic separation.

ACKNOWLEDGEMENTS

We are grateful to the late Dr. M. Osugi of Wakayama Research Laboratories, Kao Soap Co., for his helpful suggestions and encouragement throughout this work.

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Journal of Chromatography, 277 (1983) 61—69
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1779

DOSAGE DE L'ACIDE PHENYL-2 BUTYRIQUE DANS LE PLASMA PAR CHROMATOGRAPHIE GAZEUSE ET CHROMATOGRAPHIE LIQUIDE

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(Reçu le 1 février 1983; manuscrit modifié reçu le 6 mai 1983)

SUMMARY

Determination of 2-phenylbutyric acid in plasma by liquid and gas chromatography

Two chromatographic methods which allow the measurement of 2-phenylbutyric acid in serum are described: a gas chromatographic, after silylation, and a reversed-phase high-performance liquid chromatographic. The liquid chromatography with a fluorescent detection, after derivatization by 4-bromomethyl-7-methoxycoumarin, is ten times more sensitive than gas chromatography and 50 ng/ml can be measured in biological liquids.

INTRODUCTION

L'acide phényl-2 butyrique (PB) estérifié par des amino alcools diversement substitués permet l'obtention d'un certain nombre de médicaments à propriétés pharmacologiques diverses tel le butamirate antitussif (Ia) [1], le butéthamate anticholinergique et spasmolytique (Ib) et le fenbutrazate anorexique (Ic) [2—4]. Dans l'organisme, la transformation métabolique entraîne la rupture de la liaison ester, comme cela a été montré dans le cas du butamirate [5]. Après absorption, il donne naissance à divers métabolites dont les principaux sont l'acide phényl-2 butyrique (II) et le diéthyl-amino-éthoxy-éthanol qui sont conjugués pour être ensuite éliminés.

Le dosage de l'acide phényl-2 butyrique, dans le plasma et les urines, permet donc de suivre la pharmacocinétique de ce type de molécules.

A cette fin, nous décrivons deux méthodes chromatographiques, l'une en phase gazeuse après silylation de l'acide, l'autre en phase liquide après sa transformation en ester fluorescent par la bromométhyl-4 méthoxy-7 coumarine (BrMmC) (IV) (Fig. 1).

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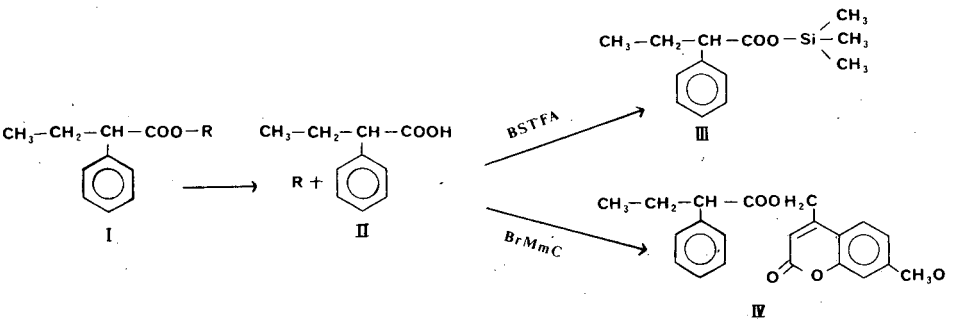


Ia R = $(C_2H_5)_2-N-CH_2-CH_2-O-CH_2-CH_2$ – **(BUTAMIRATE)**

Ib R = $(C_2H_5)_2-N-CH_2-CH_2$ – **(BUTETHAMATE)**

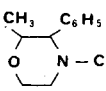
Ic R =  – **(FENBUTRAZATE)**

Fig. 1. Schéma de la réaction.

Ces deux méthodes utilisent le même étalon interne et présentent une excellente corrélation. Elles ont cependant des performances différentes puisque la méthode en chromatographie liquide avec détection fluorimétrique est dix fois plus sensible que la méthode en chromatographie gazeuse et permet d'atteindre des concentrations de 50 ng ml^{-1} dans le sérum ou les urines.

Le choix entre ces deux techniques dépendra donc des disponibilités matérielles du laboratoire et des quantités recherchées.

MATÉRIEL ET MÉTHODES

Chromatographie gazeuse

Un chromatographe Girdel 3000, muni d'un détecteur à ionisation de flamme, est utilisé avec un enregistreur Servo Trace dont le déroulement est de 0.5 cm min^{-1}. La température du four est de 140°C, celle de l'injecteur de 190°C et celle du détecteur de 290°C. Le débit de l'hydrogène est de 25 ml min^{-1}, celui de l'air comprimé de 350 ml min^{-1} et la pression de l'azote 1.5 bar, l'atténuation est de 1 × 8. Une colonne de verre (2 m × 2 mm), préalablement silanisée, est remplie par une phase stationnaire SE-30, 2.4% sur Gas-Chrom Q (80—100 mesh) (Alltech Europe).

Chromatographie liquide

L'ensemble chromatographique est constitué par une pompe Chromatem 380, un injecteur à boucle (20 µl) Rhéodyne 7125, une colonne inox (15 cm × 4.6 mm I.D.) remplie de Sphérisorb ODS Chromatem (5 µm) selon la méthode de Coq et al. [6], d'un spectrofluorimètre Schoeffel GM 970 dont la longueur d'onde d'excitation est fixée à 330 nm et la longueur d'onde d'émission à 418 nm. Le déroulement de l'enregistreur est de 0.5 cm min^{-1}.

Réactifs

Sulfate de soude anhydre, acide anisique (An), acide chlorhydrique, soude en pastille, acide perchlorique, diéthyléther, heptane, méthanol (Normapur, Prolabo, France). N,O-Bis triméthylsilylacétamide (BSA), bromométhyl-4 méthoxy-7 coumarine (BrMmC) (Regis Chemical Co., U.S.A.). 18-Crown-6 ether (Aldrich, U.S.A.). Acide phényl-2 butyrique (PB) (Laboratoire Valpan, France). Acide phényl-2 butyrique (PB) (Laboratoire Valpan): solutions étalons dans l'éther à 10 μg ml^{-1} (CL) et à 150 μg ml^{-1} (CPG). Acide anisique (Prolabo): solutions étalons dans l'éther à 10 μg ml^{-1} (CL) et à 100 μg ml^{-1} (CPG).

Toutes ces solutions sont stables un mois quand elles sont conservées à +4° C.

La phase mobile est constituée par un mélange méthanol—eau—acide perchlorique (65:35:0.075, v/v).

Méthode par chromatographie gazeuse

À 1.5 ml de plasma sont ajoutés 200 μl de la solution éthérée d'acide anisique (100 μg ml^{-1}), 75 μl d'acide chlorhydrique normal et 2 ml d'éther. Après agitation au Vortex pendant 1 min et centrifugation, 10 min à —9° C, à 2000 *g*, la couche éthérée est séparée. Cette opération est renouvelée une fois. Les phases éthérées sont réunies, séchées sur sulfate de sodium anhydre et, après avoir été transvasées dans un autre tube à hémolyse, bouché émeri, évaporées à sec à 30° C, sous courant d'azote.

Le résidu sec est repris par 75 μl d'une solution de BSA dans l'heptane (1:3, v/v) et laissé en contact 30 min. 1—3 μl du mélange sont injectés dans le chromatographe dans les conditions décrites ci-dessus.

Étalonnage. Il est réalisé à partir de plasma de sujet témoin non-traité. Quatre tubes à hémolyse, contenant chacun 1.5 ml de plasma, sont additionnés respectivement de 50, 100, 150 et 200 μl de la solution standard d'acide phényl-2 butyrique (150 μg ml^{-1}). A chacun de ces tubes correspondent des concentrations respectives de 5, 10, 15 et 20 μg d'acide phényl-2 butyrique par millilitre de plasma.

Un blanc est obtenu en traitant un sérum non-surchargé de la même manière.

Méthode par chromatographie liquide

Après acidification par 100 μl d'acide chlorhydrique normal et addition de 75 μl de la solution éthérée d'acide anisique (10 μg ml^{-1}), 0.5 ml de plasma sont extraits deux fois par 2 ml d'éther comme indiqué dans la méthode précédente.

Les phases éthérées sont desséchées sur sulfate de sodium anhydre et placées dans des tubes à hémolyse bouchés émeri, et additionnées de 10 μl de solution de méthanolate de sodium 0.015 *M*. La phase organique est évaporée à sec, sous courant d'azote. Au résidu, sont ajoutés 500 μl de la solution de BrMmC et 250 μl de la solution de 18-Crown-6 éther.

Les tubes sont entourés de papier d'aluminium, hermétiquement bouchés, et placés dans un bloc chauffant à 70° C pendant 15 min. La phase organique est évaporée à sec, sous courant d'azote, et le résidu est repris par 100 μl de

méthanol. Vingt μl sont injectés dans le chromatographe. Le débit de la phase mobile est fixé à 2.5 ml min^{-1}.

Étalonnage. Il est réalisé à partir de plasma de sujet témoin. Quatre tubes à hémolyse contenant chacun 0.5 ml de plasma sont additionnés respectivement de 125, 250, 375 et 500 μl de la solution standard d'acide phényl-2 butyrique (10 μg ml^{-1}) correspndant à des concentrations respectives de 0.25, 0.50, 0.75 et 1 μg d'acide phényl-2 butyrique par millilitre de plasma. Un blanc est obtenu en traitant un sérum témoin non-surchargé de la même manière.

RÉSULTATS

La Fig. 2a et b montre les tracés obtenus par chromatographie en phase gazeuse à partir d'un plasma d'un sujet avant traitement et celui du même sujet contenant 10 μg ml^{-1} d'acide phényl-2 butyrique.

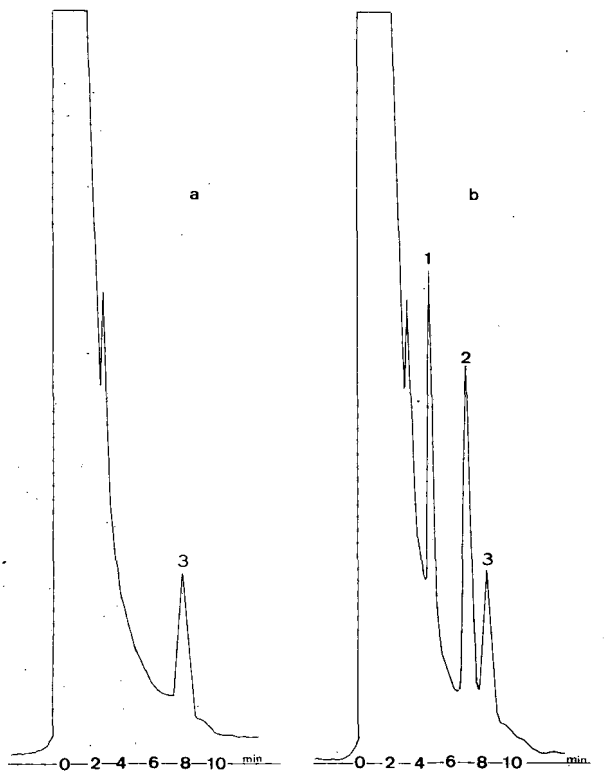


Fig. 2. Chromatographie gazeuse. (a) Chromatogramme d'un extrait plasmatique d'un sujet non-traité. (b) Chromatogramme d'un extrait plasmatique contenant 10 μg ml^{-1} d'acide phényl-2 butyrique (1), l'étalon interne (2) et substance endogène (3).

Le temps de rétention relatif du dérivé silylé de l'acide phényl-2 butyrique par rapport à celui de l'acide anisique est de 0.63 dans les conditions décrites.

La Fig. 3a et b présente les tracés obtenus en chromatographie liquide à partir d'un plasma d'un sujet avant traitement et celui du même sujet contenant 0.5 μg ml^{-1} d'acide phényl-2 butyrique.

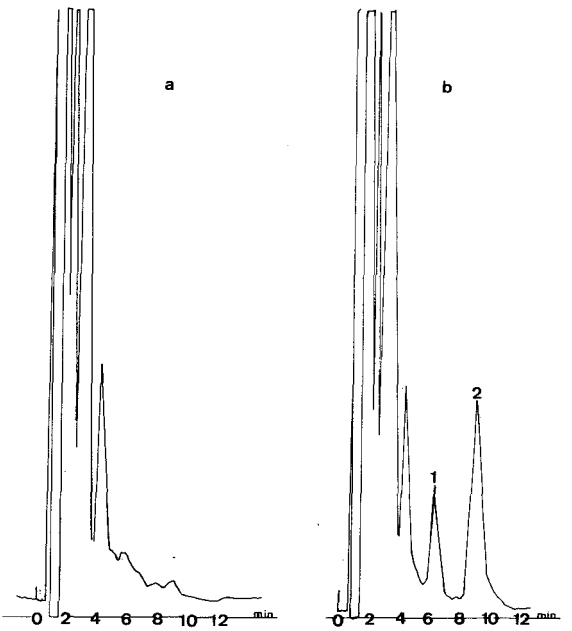


Fig. 3. Chromatographie liquide. (a) Chromatogramme d'un extrait plasmatique d'un sujet non-traité. (b) Chromatogramme d'un extrait plasmatique contenant 0.50 μg ml^{-1} d'acide phényl-2 butyrique (1) et l'étalon interne (2).

Le temps de rétention relatif de l'ester de l'acide phényl-2 butyrique par rapport à celui de l'acide anisique est de 1.45 dans les conditions décrites. On constate que, par rapport aux sujets non-traités, les constituants normaux des plasmas n'interfèrent pas.

Critères de fiabilité des méthodes analytiques

Le Tableau I regroupe les caractéristiques analytiques de ces deux méthodes: rendement d'extraction, linéarité, précision, sensibilité et leur corrélation.

TABLEAU I

CARACTÉRISTIQUES ANALYTIQUES DES DEUX MÉTHODES

	Chromatographie gazeuse	Chromatographie liquide
Linéarité	$y = 0.093x - 0.065$ $r = 0.997$	$y = 0.365x + 1.744$ $r = 0.999$
Précision		
Reproductibilité ($n = 4$)	3.77%	1.51%
Répétabilité	3.6—6.2%	0.77—5.1%
Limite de sensibilité	2 μg ml^{-1}	50 ng ml^{-1}
Corrélation entre les deux méthodes	$y = 0.840x + 0.121$ $r = 0.979$	

Rendement d'extraction et exactitude. Le rendement d'extraction par l'éther en milieu acide a été étudié par spectrophotométrie UV en se plaçant au maximum d'absorption de l'acide phényl-2 butyrique (214 nm) et de l'acide anisique (212 nm). Dans les conditions expérimentales décrites, ces rendements sont de 84 ± 5% (n = 5) pour l'acide phényl-2 butyrique et de 82 ± 9% (n = 5) pour l'étalon interne.

L'exactitude des méthodes a été déterminée en surchargeant des plasmas témoins, avec des quantités connues d'acide phényl-2 butyrique. Les quantités retrouvées sont satisfaisantes (Tableau II).

TABLEAU II

EXACTITUDE ET RÉCUPÉRATION

	Quantité d'acide phenyl-2 butyrique ajoutée (en μg ml^{-1})	Quantité retrouvée (en μg ml^{-1})	Pourcentage récupéré
Chromatographie liquide (n = 4)	0.25	0.29 ± 0.017	116
	0.50	0.53 ± 0.02	96.3
	0.75	0.76 ± 0.02	101
	1	1.02 ± 0.017	102
Chromatographie gazeuse (n = 5)	5	4.9 ± 0.34	98
	10	10.1 ± 0.73	101
	15	15.62 ± 0.48	101.4
	20	20.14 ± 0.91	108.5

Linéarité. La linéarité de la méthode a été étudiée pour des concentrations allant de 5 à 20 μg ml^{-1} en CPG et de 0.25 à 1 μg ml^{-1} en CL. Les courbes d'étalonnage, obtenues à partir des rapports de surface du pic de l'acide phényl-2 butyrique et de l'étalon interne, sont parfaitement linéaires comme l'indiquent leurs coefficients de régression (Tableau I). De plus, la moyenne des courbes standard effectuées pendant un mois donne une excellente reproductibilité: $y = 0.095x - 0.053$, $r = 0.995$ en CPG et $y = 1.744x + 0.365$, $r = 0.998$ en CL.

Précision. La reproductibilité de la méthode a été déterminée en répétant quatre fois, au cours d'un mois, l'analyse des mêmes échantillons: en chromatographie liquide, un plasma renfermant 1.4 μg ml^{-1} a présenté un coefficient de variation de 3.77%, en phase gazeuse, un plasma renfermant 10 μg ml^{-1} a présenté un coefficient de variation de 1.51%.

La répétabilité a été déterminée en répétant plusieurs fois, dans une même journée, l'analyse d'un même échantillon.

En chromatographie liquide, les coefficients de variation (n = 4) ont été de 5.1, 3.42, 1.54 et 0.77% pour des valeurs respectives de 0.25, 0.5, 0.75 et 1 μg ml^{-1}. En chromatographie gazeuse, pour des concentrations de 5, 10, 15 et 20 μg ml^{-1}, les coefficients de variation ont été de 5.8, 6.2, 3.6 et 4.1% (n = 6).

Sensibilité. Les limites de sensibilité, dans les conditions décrites, sont de 2 μg ml^{-1} soit 200 ng injecté dans un volume de 5 μl en chromatographie

gazeuse, et de 50 ng ml^{-1} soit 5 ng injectés dans un volume de 20 μl en chromatographie liquide.

Corrélation des deux méthodes. La droite de régression obtenue pour dix analyses effectuées en chromatographie liquide et chromatographie gazeuse montre une bonne corrélation entre les deux méthodes (r = 0.979).

DISCUSSION

Des procédés de dérivation, pour diminuer la polarité de l'acide phényl-2 butyrique en CPG ou pour augmenter la sensibilité de sa détection en chromatographie liquide, ont été retenus. Il a été nécessaire de rechercher comme étalon interne une substance ayant les mêmes caractéristiques d'extraction, de dérivation et de séparation que l'acide phényl-2 butyrique transformé.

L'acide benzoïque, s'extrayant et se dérivant comme l'acide phényl-2 butyrique, nous a semblé tout d'abord devoir convenir [7], en effet, sur une colonne OV-17 1.5% à 100°C, des temps de rétention de 3.6 min pour l'acide benzoïque et de 8 min pour l'acide phényl-2 butyrique étaient parfaitement acceptables et donnaient une excellente linéarité d'étalonnage.

Le passage en milieu biologique s'avéra moins satisfaisant car, outre ces deux pics, quatre autres pics apparaissaient dont l'un se confondait avec l'acide benzoïque.

La présence de ce pic ayant pu être attribuée à l'action du réactif silylant sur l'extrait biologique éthéré, de nombreux essais ont été effectués pour obtenir la séparation des pics: changement des conditions de température et de pression, changement de colonne, changement de réactif silylant (BSTFA). Des essais sur colonnes remplies SE-30 2.40%, Gas-Chrom Q 80—120 mesh, ont montré que cette phase permettait d'accroître la sensibilité mais il est apparu que l'on ne pouvait pas bien séparer ce pic parasite de l'acide benzoïque. Ceci nous a conduit à rechercher d'autres étalons internes. Parmi les acides testés, l'acide anisique et l'acide hydrocinnamique ont présenté des temps de rétention respectifs de 6 et 4.5 min.

Dunges et al. [8] ayant décrit que l'acide anisique forme facilement avec la bromométhyl-4 méthoxy-7 coumarine un ester fluorescent AnMmC, ce composé a pu être également retenu comme étalon interne en chromatographie liquide.

La formation de dérivés fluorescents a été réalisée en présence de 18-crown-6 éther proposé pur les transformations des sels métalliques dans les solvants aprotiques [9]. Mais, contrairement aux auteurs précédemment cités, l'utilisation de carbonate de potassium comme deuxième catalyseur n'a pas ici amélioré de façon sensible la réaction [10].

Une augmentation du temps de dérivation au-delà de quinze minutes ainsi qu'un plus grand excès de BrMmC n'est pas souhaitable car il apparait des pics parasites. Les conditions opératoires décrites sont les plus appropriées et, l'optimum de cette transformation est obtenue pour une température de 70°C.

Afin de séparer en phase inverse l'acide phényl-2 butyrique et l'étalon interne, les conditions optimales de cette séparation ont été recherchées en utilisant des proportions variables de méthanol et d'eau, de préférence à l'acétonitrile qui inhibe la fluorescence des esters de la méthyl-4 méthoxy-7 coumarine [11].

TABLEAU III

VARIATION DES FACTEURS DE CAPACITÉ DE SÉLECTIVITÉ ET DE RÉSOLUTION EN FONCTION DE LA NATURE DE LA PHASE MOBILE DES ESTERS FLUORESCENTS

	Phase mobile (v/v)																	
	MeOH—H_2O (60:40)			MeOH—H_2O (65:35)			MeOH-H_2O (70:30)			MeOH—H_2O (60:40) + 75 µl $HClO_4$			MeOH—H_2O (65:35) + 75 µl $HClO_4$			MeOH—H_2O (70:30) + 75 µl $HClO_4$		
	pH apparent																	
	7.45			7.24			7.00			2.28			2.22			2.50		
	k'	α	R_s	k'	α	R_s	k'	α	R_s	k'	α	R_s	k'	α	R_s	k'	α	R_s
PbMmC	9.46			6.8			3.81			15			8.75			4.11		
		1.66	6.75		1.54	4.53		1.49	3.49		1.7	11.73		1.54	6.31		1.41	4.03
AnMmC	5.69			4.4			2.45			9			5.75			2.88		

Cette étude a été réalisée pour des valeurs de pH apparent au voisinage de la neutralité ou en milieu acide et les résultats obtenus sont rapportés dans le Tableau III.

L'augmentation de la teneur en méthanol diminue les facteurs de capacité k' ainsi que les facteurs de sélectivité α. La diminution du pH est pratiquement sans action sur les facteurs de sélectivité, mais elle augmente les facteurs de capacité ainsi que l'efficacité et, par ce fait, améliore la résolution R_s [12].

Compte tenu de la nécessité de trouver un compromis entre le temps de l'analyse et la meilleure résolution, le mélange eau—méthanol (65:35, v/v), additionné de 75 μl d'acide perchlorique, a été préféré.

CONCLUSION

Les méthodes proposées se sont révélées simples, rapides et fiables. Elles ont pu être utilisées lors de l'établissement de données pharmacocinétiques. Leur application aux urines, après une hydrolyse préalable par le suc d'Hélix pomatia, a permis d'établir les profils d'étlimination de cet acide après absorption de butamirate [13].

Leur différence réside dans leurs limites de sensibilité et, outre l'appareillage disponible, leur mise en oeuvre devra être fonction des quantités recherchées.

RÉSUMÉ

Deux méthodes chromatographiques permettant de doser l'acide phényl-2 butyrique dans le sérum sont décrites: une méthode par chromatographie en phase gazeuse après silylation et une méthode en chromatographie liquide en phase inverse. La chromatographie liquide met en oeuvre une détection fluorimétrique après formation d'un ester fluorescent avec la bromométhyl-4 méthoxy-7 coumarine. Cette méthode est dix fois plus sensible que la chromatographie gazeuse et permet de déterminer des concentrations de 50 ng/ml dans les milieux biologiques.

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Journal of Chromatography, 277 (1983) 71—77
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1788

CLINICAL ANALYSIS ON STEROIDS

XXV★. ASSAY OF ESTRADIOL 17-SULFATE 2-HYDROXYLASE ACTIVITY BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received February 14th, 1983; revised manuscript received May 6th, 1983)

SUMMARY

A simple method for the assay of the 2-hydroxylation enzyme of estradiol 17-sulfate in rat liver microsomes has been established by using reversed-phase high-performance liquid chromatography with electrochemical detection. The technique devised involves elution with 50 m*M* acetate buffer, pH 5.0—methanol (3:2, v/v) using an ODS SIL column and monitoring the potentials at 1.1 V vs. the silver/silver chloride reference electrode. The calibration curve of the relationship between the amounts of 2-hydroxyestradiol 17-sulfate (5—50 ng) injected and peak heights (cm) of the chromatogram was linear. By this method, some kinetic parameters of estradiol 17-sulfate 2-hydroxylase were measured.

INTRODUCTION

Catechol estrogens produced by hydroxylation at C-2 of female hormone estrogens are now recognized as physiologically potent metabolites in living animals [1—3]. This *ortho*-hydroxylation of phenol is also observed in the metabolism of synthetic estrogens such as diethylstilbestrol, and also in that of therapeutically so-called "antiestrogens" such as tamoxifen.

In a recent investigation [5] we demonstrated the sulfate-specific 2-hydroxylation of estradiol 17-sulfate (E-17-S) by liver microsomes from male rat. In contrast, no such regulating effect was observed in those from female

★Part XXIV: I. Yoshizawa, S. Itoh, K. Nagata and N. Kawahara, Chem. Pharm. Bull. (Tokyo), 31 (1983) in press.

0378-4347/83/$03.00

rat, in which multiple kinds of hydroxylated products including 2-hydroxylated metabolite were formed. To investigate the characteristics of the 2-hydroxylase for E-17-S, it became necessary to establish the assay for the enzyme.

In the present paper we wish to report a simple method for assay of the 2-hydroxylase activity by determining the product, 2-hydroxyestradiol 17-sulfate (2-OH-E-17-S) by high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Materials

2-Hydroxyestradiol [6], 2-OH-E-17-S [7], 2-OH-E-17-S-2-methyl ether [8], 4-hydroxyestradiol [9], 6α- and 6β-hydroxyestradiols [10, 11], and 7α- and 7β-hydroxyestradiols [12], were prepared in this laboratory according to the known methods. E-17-S was prepared from estradiol by the method of Kirdani [13]. Estradiol and estriol were purchased from Steraloids (Wilton, NH, U.S.A.). 4-Nitroestrone and 15α-hydroxyestradiol were generous gifts from Dr. J. Fishman (The Rockefeller University, New York, NY, U.S.A.), to whom our thanks are due. Glucose 6-phosphate (G-6-P), NADP, NADPH, and G-6-P dehydrogenase, were obtained from Oriental Yeast, Inc. (Osaka, Japan). Sep-Pak C_{18} cartridges were obtained from Waters Associates (Milford, MA, U.S.A.). Methanol and water used for elution of steroidal conjugates through cartridges were bubbled with nitrogen to remove any dissolved oxygen, followed by addition of ascorbic acid (5 mg in each 100 ml). Peroxide-free ether was prepared and used for the solvolysis of the conjugates. All other reagents and solvents used were obtained commercially.

High-performance liquid chromatography

HPLC was carried out using a Model 803 chromatograph equipped with an EC-8 electrochemical detector at 1.1 V vs. the Ag/AgCl reference electrode (Toyo Soda, Tokyo, Japan). A stainless-steel column (30 cm × 4.0 mm I.D.) packed with TSK-Gel LS 410 ODS SIL (5 μm) (Toyo Soda) was used and maintained at 40°C in a circulating water bath. Preparative HPLC was carried out by the same machine using a column (30 cm × 7.5 mm, I.D.) packed with the same stationary phase, and a UV detector 1205-T (Toyo Soda) at 280 nm. The following solvent systems were used as mobile phase. System A: acetate buffer (50 m*M*, pH 5.0)—methanol (60 : 40, v/v); system B: acetate buffer (50 m*M*, pH 4.0)—methanol (45 : 55, v/v); system C: acetate buffer (50 m*M*, pH 4.0)—methanol (45 : 36, v/v).

Animals

Wistar rats were fed a synthetic diet. All the animals, weighing 200—300 g, were starved for 18 h prior to sacrifice.

Preparation of microsomes

Rat liver microsomes were prepared by the method described previously [5]. The microsomal protein was determined by the method of Lowry et al. [14] using bovine serum albumin as reference standard.

Assay procedure

The standard incubation was carried out with the following conditions. Ice-cold reaction vessels contained microsomal protein (0.5 ml), an NADPH-generating system (NADP, 0.5 m*M*; G-6-P, 5 m*M*; $MgCl_2$, 5 m*M*; G-6-P dehydrogenase, 0.6 unit/ml), KCl (90 m*M*), EDTA (0.1 m*M*), and E-17-S (0—400 μM). The mixture was diluted with Tris—HCl buffer (pH 7.4, 50 m*M*) to 3.0 ml as a final volume and was incubated at 37°C under aerobic conditions. The mixture was incubated for 30 min except for the study of the time-course. The reaction was terminated by heating the incubation vessels in boiling water for 1 min, followed by addition of ascorbic acid (5 mg) as antioxidant and a known amount of internal standard (approx. 0.5—20 μg), and finally was diluted with 10 ml of water. For the control experiment, the incubation was performed using boiled microsomes (100°C for 1 min) with the same procedure as described above.

The incubation mixtures were centrifuged at 1500 *g* for 20 min, and the precipitates were suspended in water and again centrifuged. The combined supernatants were passed through Sep-Pak C_{18} cartridges. After washing with 2.0 ml of water, the conjugate fraction was obtained by elution with methanol (4.0 ml). The eluates were passed once through the membrane filter (0.8 μm) and the methanolic filtrates were evaporated under a nitrogen stream at 40°C to give the residues, which were subjected to HPLC using system A as mobile phase.

Separation and solvolysis of steroid conjugates

The incubation mixtures of ten experiments (substrate concentration: 200 μM) were passed through Sep-Pak C_{18} cartridges, and the combined methanolic eluate was dried under a nitrogen stream. The residue was subjected to preparative HPLC using solvent system A. Confirmation of the separation of these fractions was done by HPLC with electrochemical detection. Three separated fractions were submitted to solvolysis by the method of Burstein and Lieberman [15], with the modification of adding ascorbic acid to the ether and aqueous layers. After the addition of 4-nitroestrone (about 10 μg) as internal standard, each hydrolyzate was subjected to HPLC using systems B and C.

RESULTS

The methanolic eluates of the incubation mixture through Sep-Pak C_{18} cartridges were applied to HPLC, the results of which are shown in Fig. 1. Two peaks (1 and 2) in both sexes are coincident with those of authentic specimens, E-17-S and 2-OH-E-17-S, respectively. The formation of another peak (3, consisting of three kinds of peaks) was observed only when the female rat was used. Because the hydroxylated product of E-17-S is not available except 2-OH-E-17-S, it is necessary to confirm that peak 2 is composed of only 2-OH-E-17-S. To examine this, the following experiment was undertaken.

An incubation mixture was applied to preparative HPLC to separate the products corresponding to peaks 1, 2, and 3 in Fig. 1. Solvolysis of these fractions gave their hydrolyzates. Only 2-hydroxyestradiol was detected

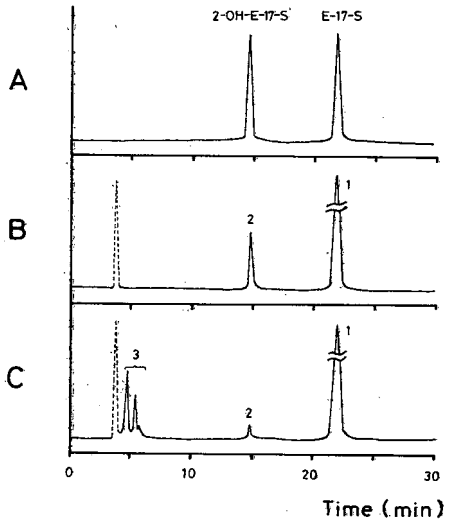

Fig. 1. Comparison of high-performance liquid chromatograms between the authentic conjugates (A) and incubation products of estradiol 17-sulfate with rat liver microsomes of male (B) and female (C) rats. Dotted lines are peaks of ascorbic acid added as antioxidant. E-17-S = estradiol 17-sulfate, 2-OH-E-17-S = 2-hydroxyestradiol 17-sulfate.

TABLE I

COMPARISON OF THE RELATIVE RETENTION TIMES OF AUTHENTIC STEROIDS AND THE HYDROLYZATES OF INCUBATION PRODUCTS OF ESTRADIOL 17-SULFATE

Compound	Solvent system	
	B	C
Estradiol	1.08	0.98
2-Hydroxyestradiol	0.64	0.51
4-Hydroxyestradiol	0.60	0.44
6α-Hydroxyestradiol	0.24	0.13
6β-Hydroxyestradiol	0.33	0.22
7α-Hydroxyestradiol	0.24	0.13
7β-Hydroxyestradiol	0.25	0.14
15α-Hydroxyestradiol	0.23	0.12
16α-Hydroxyestradiol (estriol)	0.31	0.19
Hydrolyzate of peak 1	1.09	0.98
Hydrolyzate of peak 2	0.64	0.51
Hydrolyzate of peak 3	0.25 0.31 0.33	0.14 0.18 0.22
4-Nitroestrone	1.00 (27.40 min)	1.00 (60.80 min)

in the hydrolyzate of peak 2, and only estradiol in that of peak 1, whereas in the hydrolyzate of peak 3, 6β- and 7β-hydroxyestradiols, and estriol were found (Table I). It was confirmed, therefore, that peak 2 is composed of only 2-OH-E-17-S, and peak 3 is a mixture of the 17-sulfates of 6β-, 7β-, and

16α-hydroxylated estradiols. Peak 1 was composed of only the substrate. No 4-hydroxyestradiol was detected in any hydrolyzate, although this catechol is known to be produced from estradiol in human tissues [16].

From the above results, it can be seen that the peak height of peak 2 of the incubation products depends on the amount of 2-OH-E-17-S produced. Thus, the quantification of 2-OH-E-17-S by HPLC became possible. Development of a method for the quantification of 2-OH-E-17-S was then undertaken. The calibration curve was constructed by plotting the peak height of 2-OH-E-17-S to that of the internal standard against the amount of the former, and a satisfactory linearity was observed in the range of 5—100 ng of the catechol. An analogous result was obtained for the substrate, E-17-S.

In order to confirm the validity of the present method for the determination of 2-OH-E-17-S, the recovery test was carried out using authentic sample. A known amount of the conjugates was added to the incubation medium, and the conjugates recovered through the whole clean-up procedure were determined. It is evident from the data in Table II that 2-OH-E-17-S was recovered to a satisfactory extent.

TABLE II

RECOVERIES OF ESTROGEN CONJUGATES* FROM THE INCUBATION MEDIUM AND TAKEN THROUGH THE WHOLE CLEAN-UP PROCEDURE

Each steroid was dissolved in the incubation medium (3.0 ml) and the mixtures were immediately heated for 1 min in boiling water followed by the same treatment as described in the assay procedure

Added amount (μmol)	Recovery (%)	
	E-17-S	2-OH-E-17-S
0.05	88.01 ± 2.94**	89.87 ± 2.31
0.1	87.67 ± 2.61	90.88 ± 2.56
0.5	88.56 ± 1.63	94.02 ± 1.12
1.0	90.72 ± 1.35	91.17 ± 0.94

*E-17-S = estradiol 17-sulfate; 2-OH-E-17-S = 2-hydroxyestradiol 17-sulfate.
**Mean ± S.D. (n = 6).

Some kinetic parameters of E-17-S 2-hydroxylase were also measured by the present method. Fig. 2 shows the influence of the incubation time and that of the enzyme concentration upon the production of 2-OH-E-17-S by male rat liver microsomes. The enzyme activity was linear up to 30 min incubation and up to 2.0 mg of protein. Fig. 3 shows the effect of substrate concentration upon product formation. Analogous results were obtained from the experiment with female rats.

The enzyme kinetics of E-17-S 2-hydroxylase from the microsomes of the male rat follows classical Michaelis—Menten kinetics producing a linear Lineweaver—Burk plot (Fig. 4). Analogous results were obtained when female rat liver microsomes were used. The apparent K_m values for E-17-S under these conditions were 85.5 and 117 μM for the 2-hydroxylase of male and

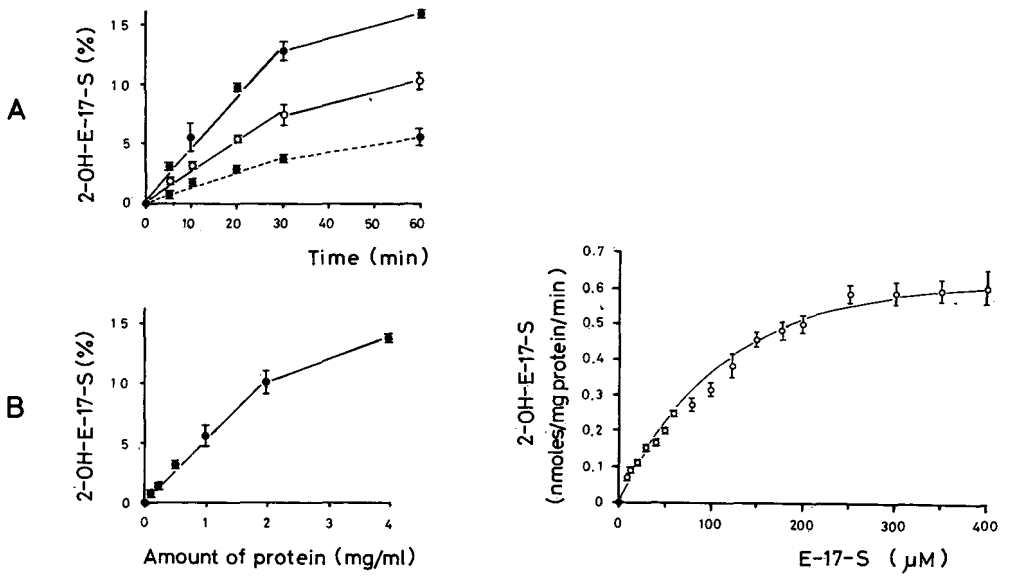

Fig. 2. (A) Effect of incubation time with different microsomal protein concentrations: (•—•), 2 mg/ml; (○—○), 1 mg/ml; (•---•), 0.5 mg/ml. (B) Effect of microsomal protein concentration upon the product formation. Substrate concentration = 200 μM, n = 5. Incubation time in B = 30 min.

Fig. 3. Effect of substrate concentration on estradiol 17-sulfate 2-hydroxylase activity of male rate liver microsomes. E-17-S = estradiol 17-sulfate, 2-OH-E-17-S = 2-hydroxy-estradiol 17-sulfate.

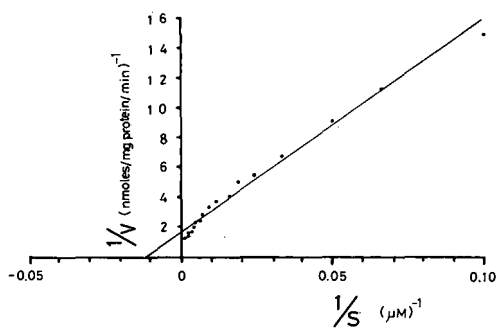

Fig. 4. Lineweaver—Burk plot for estradiol 17-sulfate 2-hydroxylase of male rat liver microsomes.

female rats, respectively. The V_{max} values for freshly prepared microsomes were 0.64 and 0.19 nmol/mg protein per min, for male and female rats, respectively.

DISCUSSION

Many skeletal carbons or even the angular methyl group are oxidized in the metabolism of estrogen in several species including humans, such as at positions 2, 4, 6, 7, 11, 14, 15, 16, and 18 [17, 18]. Hydroxylation at C-2, how-

ever, is the major pathway in estrogen metabolism. Our previous results [5] on rat liver microsomal hydroxylation of E-17-S demonstrated that the hydroxylation was sex-dependent, and that the 2-hydroxylation by male rat was sulfate-specific. Clarification of the enzyme is, therefore, important endocrinologically in estrogen metabolism. In the present study, the assay of the 2-hydroxylase by HPLC became possible. This is due to the fact that the separated peak corresponding to 2-OH-E-17-S was shown to be composed solely of this catechol.

In spite of the numerous reports on the formation of 4-hydroxylated estrogen in human and other animals [16, 19], the present result demonstrates that no detectable amount of 4-hydroxylated product was found.

The apparent K_m value obtained for E-17-S by male rat liver microsomes was 85.5 μM, that is a lower value than that for free estradiol: 2.2 μM [20], 2.0 μM [21], and 39.2 μM [22]. The difference in the value between free and 17-sulfoconjugated estradiols may be reasonable considering the characteristics of the microsomal enzyme.

The sex difference in kinetic parameters, especially the K_m value, as observed in the present paper, indicates that the hepatic 2-hydroxylation enzyme is different between male and female rats. An analogous results has been recently reported, although the result was on the 2-hydroxylation of free estradiol [20].

Several authors have already reported the assay of estradiol 2-hydroxylase using a radioenzymatic method [21], and a radiometric procedure [22, 23]. The present method for estrogen 2-hydroxylase using HPLC may be applied also to free estrogen metabolism because of its simplicity and greater speed.

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Journal of Chromatography, 277 (1983) 79—85
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1771

QUANTITATION OF TESTOLACTONE AND 4,5-DIHYDROTESTOLACTONE IN PLASMA AND URINE USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received January 18th, 1983; revised manuscript received May 6th, 1983)

SUMMARY

A rapid, sensitive, and selective assay is described for the quantitation of both testolactone and its recently identified metabolite, 4,5-dihydrotestolactone, in plasma and urine using high-performance liquid chromatography. The procedure includes a methylene chloride extraction prior to chromatography and quantitation using peak height ratios (ultraviolet absorbance detection, 242 nm) of testolactone and 4,5-dihydrotestolactone to the internal standard, testosterone. A sensitivity of 20 ng/ml for both testolactone and 4,5-dihydrotestolactone is easily achieved using only 0.5 ml of sample. Mean recoveries for testolactone and its metabolite are 95.0% and 81.8%, respectively, and the mean coefficient of variation of the procedure is 3.5% for the drug and 7.1% for the metabolite. This method is currently being used to study the pharmacokinetics of testolactone and 4,5-dihydrotestolactone in male patients. A steady-state plasma concentration versus time profile from a representative patient is included.

0378-4347/83/$03.00

INTRODUCTION

Testolactone (Fig. 1A), is an aromatase inhibitor which effectively blocks the peripheral aromatization of androgens into estrogens. The drug has been used to treat women with breast cancer who are post-menopausal or who have had an oophorectomy [1, 2], and more recently to study sex steroid regulation of pituitary gonadotropin secretion [3, 4]. Testolactone is currently under investigation for treatment of certain disorders in men in which estrogen overproduction is suspected, such as idiopathic oligospermic infertility [5, 6] and pubertal gynecomastia.

Although testolactone has been used therapeutically for about 20 years, to our knowledge there is no published procedure for quantitating testolactone in biological fluids. In this publication we describe a rapid, sensitive, and selective high-performance liquid chromatographic (HPLC) procedure for the quantitation of testolactone in both plasma and urine. The assay method also permits the concurrent detection and quantitation of 4,5-dihydrotestolactone (Fig. 1B), a metabolite of testolactone which has been recently isolated and identified in our laboratory [7]. This procedure is currently being used to study the pharmacokinetics of testolactone and its metabolite in male patients.

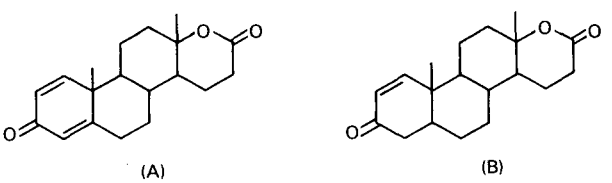

Fig. 1. Chemical structures of (A) testolactone (13-hydroxy-3-oxo-13,17-secoandrosta-1,4-dien-17-oic acid δ-lactone) and (B) 4,5-dihydrotestolactone (13-hydroxy-3-oxo-13,17-seco-androsta-1-en-17-oic acid δ-lactone).

EXPERIMENTAL

Materials and reagents

U.S.P. Reference Standards of testolactone and testosterone were used to prepare plasma and urine standards and internal standard solution, respectively. A metabolite of testolactone, 4,5-dihydrotestolactone, was isolated and purified according to ref. 7. Briefly, urine collected from a testolactone patient was extracted with methylene chloride (glass-distilled, HPLC grade from Burdick and Jackson Labs., Muskegon, MI, U.S.A.). Following separation and concentration, the organic layer was injected onto the HPLC system (conditions described below). A preparative HPLC column (Zorbax® C_8 reversed phase, 25 cm × 9.4 mm I.D., from Dupont, Wilmington, DE, U.S.A.) was used to separate and collect the suspected metabolite in the eluent. The eluent was then extracted with methylene chloride, which was subsequently evaporated to dryness. The isolated metabolite was used in the preparation of plasma and urine standards.

Acetonitrile, methanol, and methylene chloride, glass-distilled and certified

HPLC grade, were obtained from Burdick and Jackson Labs. Water was double-distilled in glass. All other chemicals used were reagent grade. All HPLC solvents were filtered through a 0.45-μm filter (Millipore, Bedford, MA, U.S.A.) and then degassed under reduced pressure prior to use.

Chromatographic system and conditions

A Spectra-Physics liquid chromatograph, Model 3500B (Spectra-Physics, Santa Clara, CA, U.S.A.) was equipped with a Kratos variable-wavelength ultraviolet spectrophotometric detector, Spectroflow 773 (Kratos, Westwood, NJ, U.S.A.) set at 242 nm. The system included a Zorbax C_8 reversed-phase analytical column (25 cm × 4.6 mm I.D., 5-μm particle size) from Dupont. A 7 cm × 2 mm I.D. guard column packed with Co:Pell ODS (30—38 μm) particles (Whatman, Clifton, NJ, U.S.A.) preceded the analytical column. The mobile phase consisted of 40% (v/v) acetonitrile and 0.1% glacial acetic acid in water. The flow-rate was maintained at 1.2 ml/min. The pressure was 110.3 bars and the absorbance units full scale (a.u.f.s.) varied between 0.005 and 0.08.

Procedures

To 0.5 ml of plasma or urine sample or standard was added 0.25 μg testosterone (25 μl of 10 μg/ml in methanol) to serve as internal standard. To each sample were added 4.0 ml of methylene chloride. The samples were mixed for 15 min using an automatic shaking apparatus (Eberbach, Ann Arbor, MI, U.S.A.). They were then centrifuged at 2000 *g* for 5 min. The top (aqueous) layer was removed and discarded, and the bottom (organic) layer was transferred to a clean vial using a pasteur pipet. The methylene chloride layer was then evaporated to dryness using a gentle air stream at 40°C. The residue was reconstituted with 150—300 μl (plasma samples) or 300—500 μl (urine samples) mobile phase and mixed by vortexing. A 100-μl aliquot was injected onto the HPLC system.

Plasma and urine standards were prepared by adding known amounts of testolactone and 4,5-dihydrotestolactone stock solutions (10 or 100 μg/ml in methanol) to human plasma and urine to yield final concentrations ranging from 20 ng/ml to 10 μg/ml. Testolactone and 4,5-dihydrotestolactone were quantitated by comparison of the peak height ratio of drug or metabolite to internal standard using a calibration curve. The peak height ratios were plotted against concentrations of testolactone or 4,5-dihydrotestolactone and analyzed by weighted least squares linear regression [8] to generate daily calibration curves. Weighted regression analysis was necessary to accurately quantitate concentrations of samples which varied over the two orders of magnitude represented by the standard curve. A weighting factor equal to the square of the reciprocal measured concentration was used. Interday variability was determined by the reproducibility of the daily standard curves ($n \geqslant 5$) with respect to both their slopes and the calculated values for testolactone and its metabolite at six different concentrations ranging between 0.019 and 2.9 μg/ml. Intraday variability was assessed from the results of replicate analyses ($n \geqslant 5$) of plasma standards containing 0.025, 0.25 and 2.5 μg/ml of both drug and metabolite. These concentrations were representative of the low, medium

and high range of concentrations used in our standard calibration curves. Aqueous solutions containing known amounts of testolactone, 4,5-dihydrotestolactone, and testosterone were compared with the plasma or urine standards undergoing analysis to calculate the percent recovery at each of the six concentrations of the standard curve. Statistical analyses to determine means, standard deviations, standard errors, and coefficients of variation were performed using the programs available for the Hewlett-Packard 85 computer (Hewlett-Packard, Corvallis, OR, U.S.A.)

RESULTS AND DISCUSSION

HPLC procedure for plasma

Representative chromatograms from assayed samples of control human plasma, a prepared plasma standard, and plasma from a male patient treated with testolactone to which testosterone has been added are shown in Fig. 2. Retention times for testolactone, 4,5-dihydrotestolactone, and testosterone are 9.2, 11.6, and 13.6 min, respectively. No interfering peaks are observed in plasma from untreated patients. There is no interference from endogenous testosterone since, even in male plasma, endogenous testosterone levels (3—12 ng/ml) are only about 1—2% of the exogenously added internal standard testosterone (500 ng/ml).

The calibration curves constructed from daily runs of plasma standards were linear and highly reproducible. Plasma samples up to 10 μg/ml have been assayed using this procedure, and their results indicate that the linearity of response extends at least to this concentration. The mean slope (peak height ratio versus concentration in μg/ml) of six calibration curves for testolactone obtained during a 1-month period was 2.161 ± 0.046 (S.D.), C.V. = 2.1%. For 4,5-dihydrotestolactone, the mean slope was 0.742 ± 0.022 (S.D.), C.V. = 3.0%. The mean coefficient of variation of the procedure for the range of concentrations included in the standard curves is 3.5% for testolactone and 7.1% for 4,5-dihydrotestolactone (Table I). These data indicate the assay procedure is highly reliable and reproducible.

The intraday variability of the method was determined at three different concentrations spanning the range of the standard curve. At 0.025 μg/ml (n = 5), 0.25 μg/ml (n = 6), and 2.5 μg/ml (n = 6) of testolactone, the coefficients of variation of testolactone content were found to be 10.7%, 5.2%, and 3.5%, respectively. For 4,5-dihydrotestolactone content, the coefficients of variation at the low, medium, and high concentrations listed above were, respectively, 6.6% (n = 5), 1.0% (n = 6), and 3.8% (n = 6).

The recoveries of testolactone and 4,5-dihydrotestolactone at all six concentrations used in standard curve calibration averaged 95.0 ± 6.5% (S.D.), C.V. = 6.8% and 81.8 ± 1.5% (S.D.), C.V. = 1.8%, respectively. The recovery of the internal standard, testosterone, was 91.5 ± 2.9% (S.D.), C.V. = 3.2%.

Using the procedure described, 20 ng/ml testolactone and 20 ng/ml 4,5-dihydrotestolactone can easily be detected while maintaining a signal-to-noise ratio of at least 3 (a.u.f.s. = 0.005).

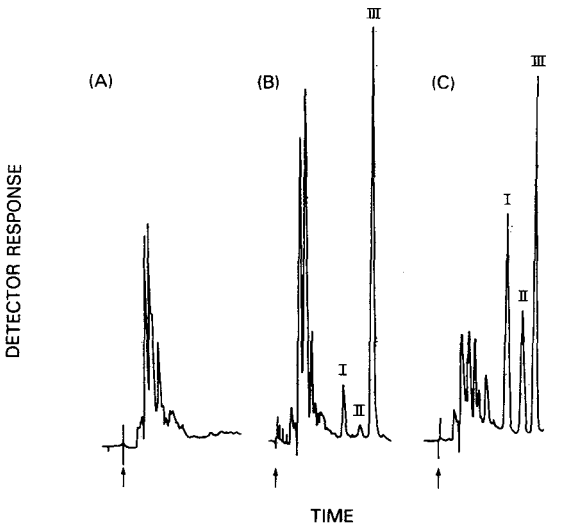

Fig. 2. Representative chromatograms of human plasma samples following sample preparation described in text. (A) Blank human plasma. (B) Human plasma to which was added testolactone (I) and 4,5-dihydrotestolactone (II), 0.05 μg/ml of each, and testosterone (III). (C) Plasma sample from male patient treated with 2 g/day of testolactone with testosterone added (testolactone and metabolite concentrations found to be 0.25 and 0.41 μg/ml, respectively). a.u.f.s. = 0.01.

TABLE I

INTERDAY VARIABILITY OF ASSAY PROCEDURE

Concentration (μg/ml)	*n*	Mean observed concentration (μg/ml)	S.D.	C.V. (%)
Testolactone				
0.019	5	0.019	0.0005	2.4
0.048	5	0.049	0.003	6.1
0.144	6	0.145	0.007	5.1
0.393	6	0.370	0.011	3.0
1.206	6	1.193	0.035	2.9
2.929	6	3.107	0.049	1.6
			Mean	3.5
4,5-Dihydrotestolactone				
0.019	6	0.018	0.002	11.2
0.046	6	0.055	0.009	15.8
0.147	6	0.142	0.010	7.4
0.416	6	0.390	0.008	2.0
1.249	6	1.241	0.044	3.5
2.868	6	3.059	0.092	3.0
			Mean	7.1

HPLC procedure for urine

The assay method described can also be used for the quantitation of testolactone and 4,5-dihydrotestolactone in urine. No interfering peaks were observed in urine samples from untreated patients, and the sensitivity, recovery, and reproducibility are similar for both plasma and urine. In general, higher concentrations of both testolactone and metabolite are found in patient urine than in plasma.

To facilitate quantitation of these higher urine concentrations, it is sometimes helpful to double the amount of internal standard added per sample (0.05 μg testosterone) and increase the reconstitution volume of the extracted urine samples (300—500 μl). With the exception of these minor changes, the sample preparation of urine and plasma samples is identical.

Analysis of plasma from testolactone-treated patients

Sequential blood samples were obtained from male patients receiving 500 mg testolactone or a placebo four times daily for at least 3 months. Samples were drawn at 0, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0, 9.0, and 12.0 h after a morning dose of testolactone. The next dose, normally administered 6 h after the morning dose, was not given. An aliquot of plasma from each sample was analyzed for both testolactone and 4,5-dihydrotestolactone as described. A concentration versus time profile of the results obtained from one patient is shown in Fig. 3. The sensitivity of the assay procedure described was adequate for the quantitation of both testolactone and its metabolite. In no patient sample did drug or metabolite levels fall below 20 ng/ml during the 12-h period monitored, and no detectable levels of testolactone or its metabolite were observed in plasma samples from patients who received placebo.

The assay procedure described for testolactone and 4,5-dihydrotestolactone offers easy and rapid sample preparation, selectivity for both drug and metabolite, and the sensitivity required for pharmacokinetic studies of both agents in humans.

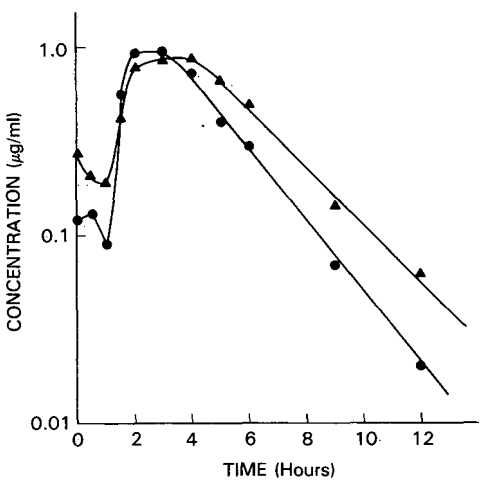

Fig. 3. Plasma concentration versus time profile of testolactone (•) and 4,5-dihydrotestolactone (▲) in a male patient as quantitated by the assay procedure described.

ACKNOWLEDGEMENTS

The authors wish to acknowledge Ms. Alice Rogot for technical assistance and Mrs. Audrey Pellegrino for preparation of the manuscript.

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Journal of Chromatography, 277 (1983) 87—99
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1765

DETERMINATION OF FREE AND TOTAL POLYAMINES IN HUMAN SERUM AND URINE BY ION-PAIRING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING A RADIAL COMPRESSION MODULE

APPLICATION TO BLOOD POLYAMINE DETERMINATION IN CANCER PATIENTS TREATED OR NOT TREATED WITH AN ORNITHINE DECARBOXYLASE INHIBITOR

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(First received June 28th, 1982; revised manuscript received May 5th, 1983)

SUMMARY

A sensitive and rapid determination of free and total polyamines (putrescine, cadaverine, spermidine and spermine) in urine and serum is described. The procedure is based on reversed-phase high-performance liquid chromatographic separation using radial compression module (Radialpak C 8). The samples are purified with a silica gel Sep-Pak cartridge. The polyamines are converted to dansyl chloride derivatives and separated using a linear gradient of triethylammonium phosphate—methanol within 15 min. The lower limits of detection are 10 pmoles for spermine and 5 pmoles for other polyamines.

This method is applied to cancer patients treated by cytotoxic chemotherapy with or without difluoromethylornithine (DFMO). All four polyamines are significantly increased in these patients before treatment. On day 8, after onset of treatment, the levels of polyamines in patients not treated with DFMO are more elevated than on day 1, while in patients treated with DFMO the levels are decreased. However, DFMO does not seem to modify the treatment result. The patients which have a low level of putrescine before and during

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0378-4347/83/$03.00

treatment, do not respond to treatment. Perhaps this low level is the consequence of conjugation of this polyamine?

INTRODUCTION

The natural polyamines (putrescine, cadaverine, spermidine and spermine) are present in all living cells where they have been particularly implicated in growth processes [1, 2]. They are derivatives of ornithine and methionine. Cadaverine was initially suspected as originating solely via bacterial degradation but it has since been shown to be a product of mammalian cell synthesis [3]. Polyamine synthesis is controlled mainly by the enzyme ornithine decarboxylase. Russell [4] has shown the interest of using these cations in the detection of cancer. It should be noted, however, that the cations are not specific to any particular type of cancer with the possible exception of putrescine in medulloblastoma [5]. Since these first studies, polyamine assay has equally been used to evaluate patient response to therapy and eventually to detect relapse [6]. The assay of polyamines is also interesting in certain other pathological conditions such as growth retardation [7], and in mental illness such as schizophrenia [8]. In this latter case it is cadaverine and its conjugated derivatives which are found in large quantities in serum.

For several years now there has been a need to develop and to improve the techniques of polyamine assay. High-performance liquid chromatography (HPLC) coupled with fluorometric detection presents strong sensitivity and good specificity as a technique for polyamine assay [9, 10]. In the studies concerning cancer, the authors have preferred to determine urinary polyamines which are found in greater quantities than blood polyamines. Also there are few methods permitting the determination of serum cadaverine levels [11].

In this paper we describe a fast and reproducible method which permits the assay of the four cations in their free forms, or freed by hydrolysis, in serum and in urine. The method involves HPLC in reversed phase with a radial compression module and a mobile phase (triethylammonium phosphate–methanol) that is used to separate polyamines already dansylated. This technique has been used particularly in evaluating the effect of an ornithine decarboxylase inhibitor [12], difluoromethylornithine (DFMO), coupled with cytotoxic chemotherapy in cancer patients.

MATERIAL AND METHODS

Apparatus

HPLC was performed on a Waters chromatograph composed of two 6000 A pumps, a U_6K injector and a solvent progammer Model 720, a radial compression module RCM 100 equipped with a cartidge Radialpak C 8 (10 μm) with an internal diameter of 5 mm. Detection was accomplished using a spectrofluometer Aminco SPF 500 equipped with a continuous flow cell of 8 μl and an Aminco SPF 500 recorder (excitation wavelength 350 nm, emission 500 nm).

Reagents

Putrescine, cadaverine, spermidine and spermine, and 1,6-hexanediamine (internal standard) were obtained from Fluka. The standard solution contained 10 mmoles/l of each polyamine and 12 mmoles/l of 1,6-hexanediamine in 0.1 *N* HCl.

Mobile phase

The products were of ultrapure quality. Buffer, triethylammonium phosphate (TEAP) 0.25 *N*, pH 3.5, was filtered through a 0.22-μm Millipore membrane before use; methanol was filtered through a 0.45-μm Millipore membrane. TEAP and methanol mixtures were prepared as follows: solution A was TEAP—methanol (50:50), and solution B TEAP—methanol (20:80). The mixtures were degassed with helium or in an ultrasound bath immediately prior to chromatography. A linear gradient curve (curve no. 6) was realized between solutions A and B going from 0 to 100% of B in 10 min with a flow-rate of 1.5 ml/min. The pressure at the head of the column varied from 40 to 60 bar during the gradient.

Purification of the biological samples

The samples were purified using a Sep-Pak cartridge (Waters) [13].

Urine. Twenty-four-hour urine samples were collected in polyethylene bottles containing 10 ml of 1 *N* HCl and kept at —20° C until assay.

For the free polyamines, a 2-ml aliquot to which were added 100 μl of internal standard, was filtered through a GS Millipore membrane (0.22 μm) and the pH was adjusted to 9 with concentrated NH_4OH. The sample was then loaded onto the Sep-Pak; this was washed with 10 ml of water and the polyamines were eluted with 10 ml of 0.1 *N* HCl. The eluent is lyophilized to prevent any damage resulting from a too slow evaporation. The dried residue was dissolved in 200 μl of 0.1 *N* HCl and was ready to be dansylated.

To determine total polyamine concentration, 2 ml of urine were submitted to hot acidic hydrolysis in 6 *N* HCl at 110° C for 12 h. The hydrolysate was centrifuged and evaporated at 40° C in a rota-vapor. The residue was dissolved in 1 ml of 0.1 *N* HCl and purified with Sep-Pak as described above.

Serum. The blood was collected in polyethylene tubes without anticoagulant and immediately centrifuged; the serum is conserved at —20° C until assay.

For the free polyamines, serum is deproteinized according to the procedure of Kai et al. [11]; 100 μl of internal standard, 1 ml of distilled water and 200 μl of 3 *M* $HClO_4$ were mixed with 1 ml of serum. After centrifugation at 4° C (2000 *g* for 10 min), the supernatant was adjusted to pH 7 with 1.5 *M* KOH and left at 4° C for 30 min, then centrifuged again. The supernatant was adjusted to pH 9 with concentrated NH_4OH and was purified as described above using Sep-Pak.

Determination of the total polyamine content was carried out using hydrolysis in the same manner as described for urine.

Formation of fluorescent derivatives

The purified sample was mixed with 200 μl of 0.1 *N* HCl and placed in a siliconized glass tube; 100 μl of a saturated solution of sodium carbonate and

400 μl of dansyl chloride (Dns-Cl) (a 20-mg/ml solution in acetone of 5-dimethylaminonaphthalene-1-sulfochloride) were added. The tubes were stoppered and placed in darkness at 54°C for 1 h. Excess free Dns-Cl was removed by the addition of 100 μl of a proline solution (150 mg/ml) and the tubes were left in darkness for an additional hour. The dansylated derivatives were extracted using 500 μl of ethyl acetate. After centrifugation, 20 μl of extract were directly injected for urine determinations. For serum, 20—80 μl could be injected depending on polyamine concentration.

Calculations

Polyamine level was automatically calculated by the Waters Model 730 data module using the ratio of the area of the polyamine peak to the area of the internal standard peak.

Normal subjects

The method was applied to both serum and urine samples from healthy subjects.

Cancer patients

Twenty-three men with neck and head cancers (mean age 54.5 years ± 7) and 15 women with breast cancers (mean age 59 years ± 11). All these patients had advanced cancers. One group of patients was treated with cytotoxic chemotherapy and difluoromethylornithine (DFMO, Merrel Laboratory) 10 g per 24 h given four times a day, the other group received chemotherapy only. During treatment, patients with neck and head cancers received chemotherapy (cis-platinum + vincristine + adriamycin + bleomycin) 6 days a month. Patients with breast cancers received chemotherapy (adriamycin + vindesine) one day a month.

Treatment was considered as being positive if after a period of two months there was a verifiable amelioration of the patient's general condition and a regression of $\geqslant$ 50% of metastases. The treatment was considered as having failed if during the two months there was no change or there was an increase of tumor mass.

All of the patients had one blood polyamine determination prior to treatment (day 1) and several determinations were performed during the follow-up of their disease (eight dosages were performed for each patient).

Statistical evaluation

We used the non-parametric Mann—Whitney *U* test [14].

RESULTS

Fig. 1 shows a chromatogram of a standard polyamine solution. The dansylated derivatives are well separated in less than 15 min with a linear gradient of TEAP—methanol, pH 3.5, in 10 min and isocratic conditions until 15 min. At pH 4 the capacity factor is less (Fig. 2) but the separation of putrescine and cadaverine is not satisfactory.

Table I gives the retention times measured on ten different days for a

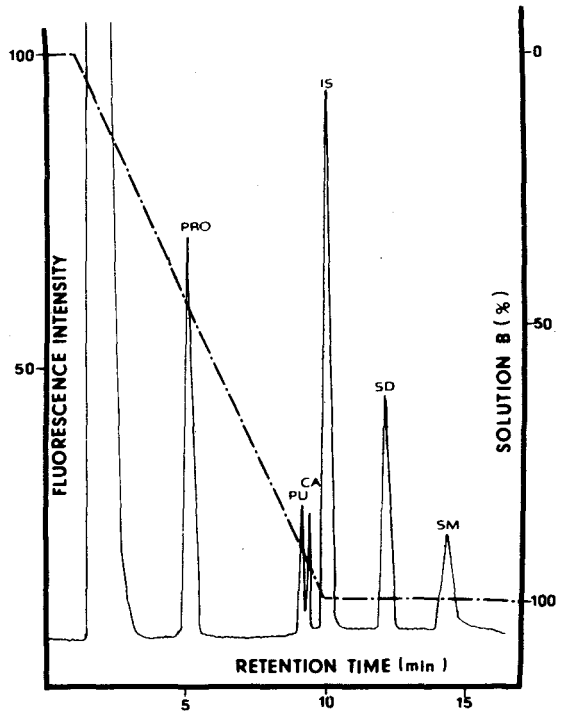

Fig. 1. Reversed-phase HPLC separation of the dansylated derivatives of a standard polyamine solution (100 pmoles per 20 μl), using a Radialpak C 8 column. Mobile phase: 0.25 *N* TEAP—methanol pH 3.5 (solution A = TEAP—methanol, 50:50; solution B = TEAP—methanol, 20:80). Flow-rate = 1.5 ml/min. PRO = proline, PU = putrescine, CA = cadaverine, IS = internal standard, SD = spermidine, SM = spermine.

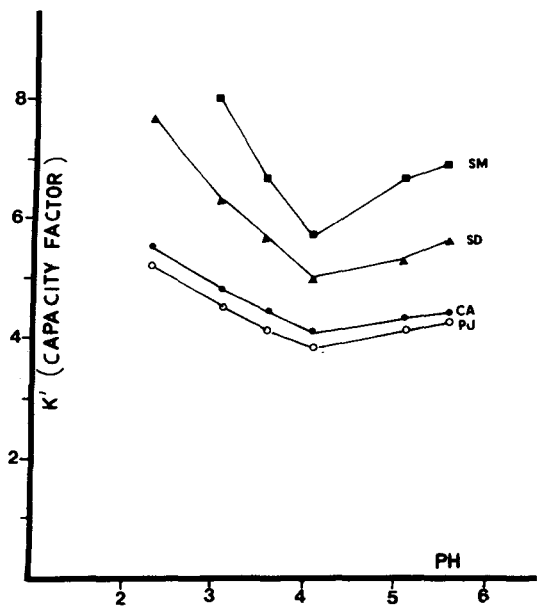

Fig. 2. Variations in capacity factor (k') versus pH with a Radialpak C 8 column.

TABLE I

RETENTION TIMES (MIN) OF PUTRESCINE, CADAVERINE, SPERMIDINE AND SPERMINE

Injection of a standard solution (100 pmoles per 20 μl) on ten different days using a Radialpak C 8 column. The internal standard has a retention time of 9.95 ± 0.18 min. $n = 10$.

	Putrescine	Cadaverine	Spermidine	Spermine
Mean	9.25	9.56	12.08	14.26
S.D.	±0.06	±0.15	±0.17	±0.19
C.V. (%)	0.64	1.65	1.40	1.33

TABLE II

PERCENTAGE RECOVERY OF STANDARD POLYAMINES ADDED TO SERUM AND URINE

The values represent the difference between the levels of polyamines in the biological fluid with and without load (each result is the mean of two assays).

Polyamine	Quantity (pmoles) of polyamine added to 2 ml of urine			Mean recovery (%)
	40	80	160	
Putrescine	36	78	162	96
Cadaverine	39	84	182	101
Spermidine	42	79	158	101
Spermine	30	62	145	81
	Quantity (pmoles) polyamine added to 2 ml of serum			
	40	80	160	
Putrescine	42	83	149	100
Cadaverine	25	77	168	88
Spermidine	25	71	154	82.5
Spermine	20	72	125	72.6

standard polyamine solution. A linear response is obtained for each polyamine for concentrations of 10—250 pmoles injected. This range corresponds to the values that were found in normal and pathological blood samples.

Chromatographic reproducibility is good because a standard solution containing 100 pmoles of each polyamine injected on ten different days ($n = 10$) gives for putrescine, cadaverine, spermidine and spermine, respectively, a coefficient of variation of 2, 3, 4 and 7%.

The limit of detection, which is defined as the minimum quantity of injected polyamine which gives a signal ratio to the background noise superior to 2, is 10 pmoles for spermine and 5 pmoles for the other polyamines. After purifica-

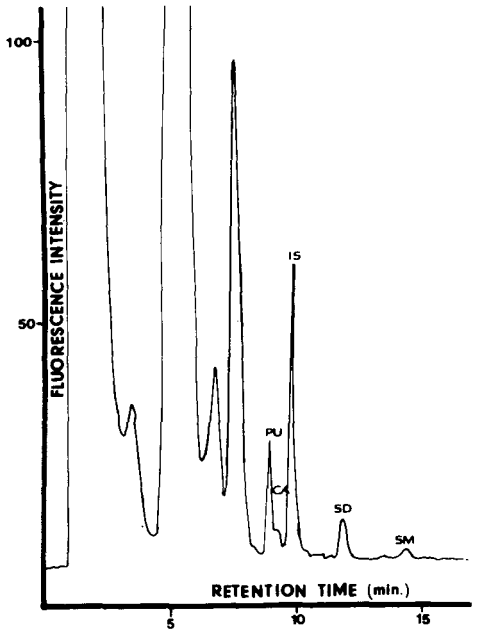

Fig. 3. Chromatogram of the free polyamines obtained from normal human plasma. Experimental conditions are defined in Materials and methods.

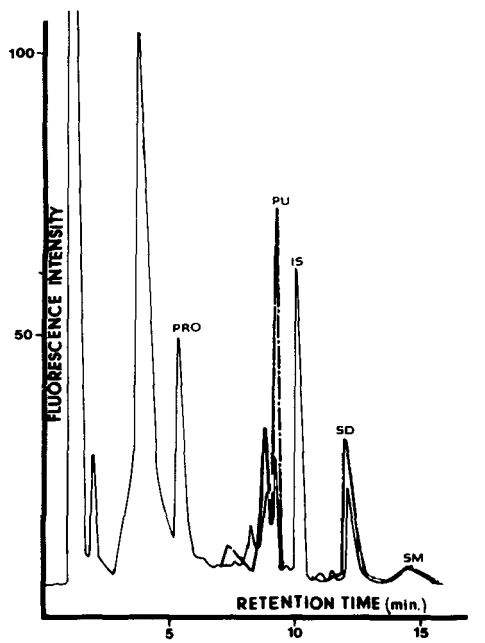

Fig. 4. Chromatogram of the free (——) and total (— · —) polyamines obtained from a 24-h urine sample (normal subject).

TABLE III

FREE POLYAMINE LEVELS FOUND IN NORMAL HUMAN SERUM, AND FREE AND TOTAL POLYAMINE LEVELS FOUND IN NORMAL HUMAN URINE

	Concentration in serum (nmoles/l)			
	Putrescine	Cadaverine	Spermidine	Spermine
Mean	155	7.72	45	17
S.D.	±122	±15	±35	±16
Range	42—388	0—45	19—104	0—52
(n = 11)				
	Urinary concentration (mmoles/mg creatinine)			
	Putrescine	Cadaverine	Spermidine	Spermine
Free polyamines				
Mean	1.49	0.015	0.87	0.46
S.D.	±0.56	±0.04	±0.71	±1
Range	0.83—2.43	0—0.12	0.22—2.06	0—2.91
(n = 12)				
Total polyamines				
Mean	4.86	0.54	3.31	0.99
S.D.	±0.88	±1.05	±2.37	±1.35
Range	3.31—5.81	0—2.64	0.83—6.97	0—3.62
(n = 12)				

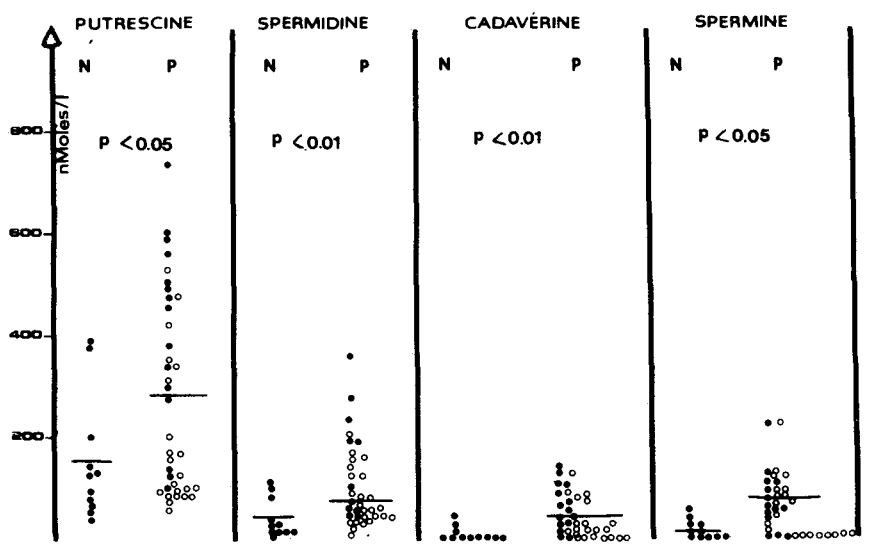

Fig. 5. Pre-treatment serum polyamine levels in cancer patients (P). (•), Breast cancers; (○), neck and head cancers. Comparison with normal subjects (N).

tion on Sep-Pak and extraction of the dansylated derivatives, the percentage recovery is 81—100% for the polyamines added to urine and 72—100% for the polyamines added to serum (Table II).

This technique has been applied to the urine and serum of normal subjects (Figs. 3 and 4, and Table III).

TABLE IV

SERUM POLYAMINE LEVELS IN PATIENTS WITH BREAST OR HEAD AND NECK CANCERS

The differences between the two groups were not significant.

	Polyamine concentration (nmoles/l)			
	Putrescine	Cadaverine	Spermidine	Spermine
Breast cancers (n = 15)				
Mean	412	59.8	133	106
S.D.	±193	±48	±105	±160
Head and neck cancers (n = 22)				
Mean	176	32	75	52
S.D.	±144	±36	±22	±59

TABLE V

VARIATION IN POLYAMINE* LEVELS ON DAYS 8 AND 30 IN PATIENTS RECEIVING OR NOT RECEIVING DFMO WITH TREATMENT

The results are expressed as percentages compared to day 1.

	With DFMO				Without DFMO			
	Pu	Ca	Sd	Sm	Pu	Ca	Sd	Sm
Day 8	−36	−27	−81	−89	+54	+19	+5	+10
	−30		−22		+42	−8	+140	+141
	−57	−45	+50	−100	+11	+400	+59	+400
	+110	+462	+22	+109	+128	0	−31	0
	−40	0	−69	−44	+35	+69	+108	+9
	+307	+400	−22	−13	+13	+166	+690	0
	−59	−13	−2	0	+22	0	+24	0
	−59	−100	−150		+17	−11	−37	+11
Day 30	−79	−100	−89	−100	−16	−58	−54	−89
	−39	0	0		−66	−100	−16	−100
	−74	−100	+14	−24	−49	0	−35	
	−86		−41	−38	−73	−95	−39	
	−17	−22	−16	0	−25	−23	+61	+158
	−79	−60	−12.5	−100	−91	−61	−16	−100
	−15	−100	−69	0	−38	−25	−51	−100
	−8	0	+400	+400				

*Pu = putrescine, Ca = cadaverine, Sd = spermidine, Sm = spermine.

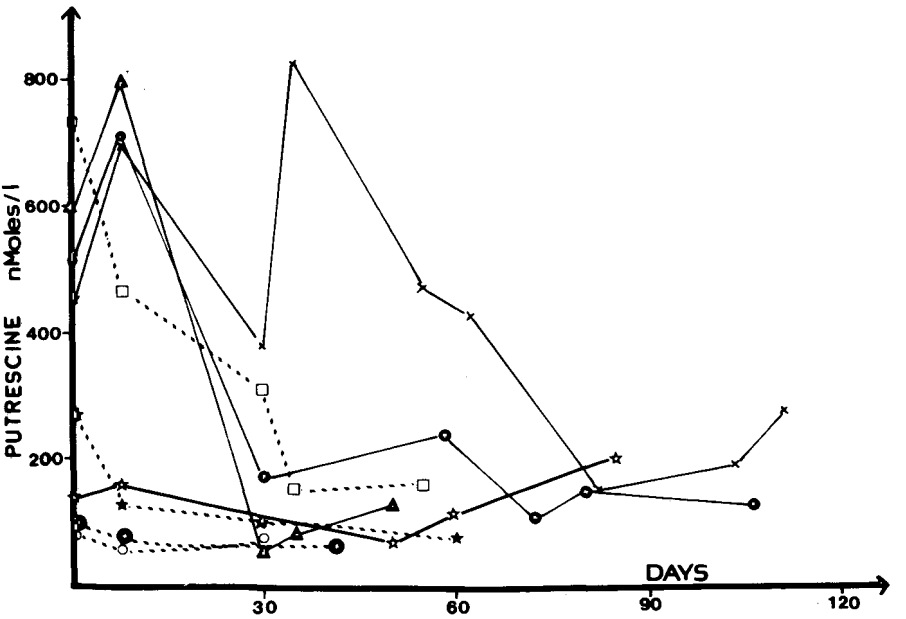

Fig. 6. Serum putrescine levels in eight cancer patients during induction treatment. (——), Without DFMO; (•••), with DFMO. Patients in which treatment brings about no remission (○, ★, ☆).

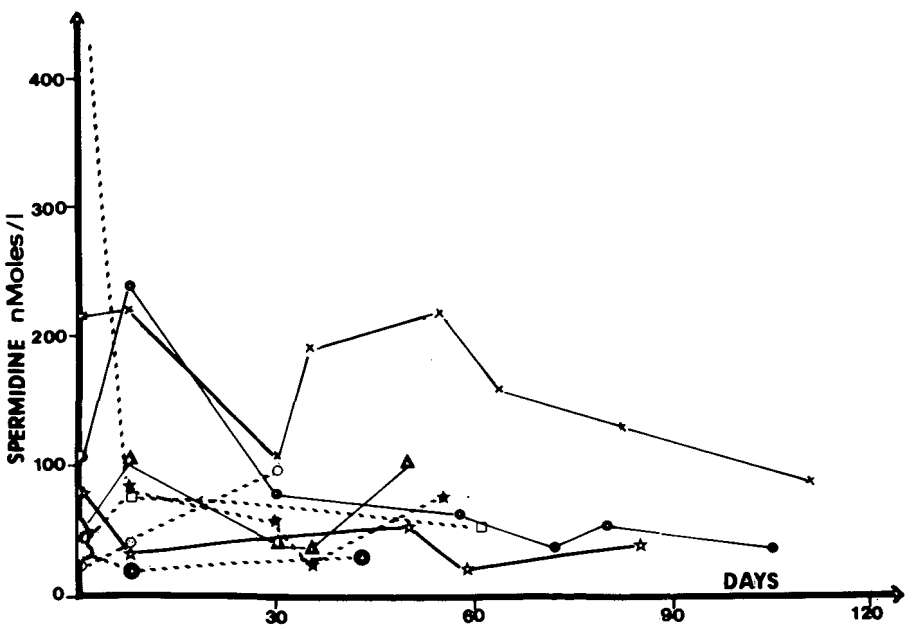

Fig. 7. Serum spermidine levels in eight cancer patients during induction treatment. (——), Without DFMO; (•••), with DFMO. Patients in which treatment brings about no remission (○, ★, ☆).

The results showed that levels in cancer patients before treatment were significantly higher than those in normal subjects (Fig. 5). Although, one must note, an important overlap exists between these two groups. The average values found in the breast cancer patients were higher for each polyamine than those found in patients with neck and head cancers (Table IV); however, the difference between these two groups was not significant. Table V shows polyamine variations, days 8 and 13, in patients who were treated or were not treated with DFMO. On day 8, patients treated with DFMO have, in general, a lower level of polyamines when compared to the first day. On the other hand, patients who were not treated with the inhibitor presented an increasing polyamine level.

On day 30 the polyamines are lowered in both cases, and in general the four polyamines vary in the same direction. Figs. 6 and 7 show the evolution of putrescine and spermidine in some patients during several months. The two polyamines evolve in a comparable manner.

Patients who have a low level of putrescine prior to any treatment and show no modification in their putrescine level during the course of treatment, are patients in which treatment brings about no remission. These patients have also a low spermidine level. For this second polyamine there is more overlap between patients presenting a remission and those in which the disease evolves.

DISCUSSION

In the different methods of polyamine assay using HPLC already discussed, classic columns were used, i.e. Bondapak C_{18} [15] and LiChrosorb RP-18 [11]. We chose a radial compression module because its small cartridge size permits an increase in flow-rate without an increase in pressure. Thus the equilibration time of the column, as well as the lapse time for the assay are shortened; therefore there is a gain of time in comparison to classic C_{18} columns. With daily use the Radialpak C 8 column has an effective lifetime of at least six months; however retention time varies from one column to another and each column must be standardized.

Stationary phases which are more polar such as CN [13] have also been recommended by certain authors. However, with the mobile phase which we used (TEAP—methanol) a CN Radialpak column does not efficiently separate the dansylated polyamine derivatives while a Radialpak C 8 column gives a good resolution in less than 15 min.

After having tested the mobile phases that are habitually used (distilled water—methanol; acetic acid—methanol; Pic B7 (Waters)—methanol) with the Radialpak C 8 column and having no success, we chose a technique using ion-pairing TEAP—methanol, ion-pairing having previously been applied to peptides [16]. In effect, phosphate ions at acid pH form a system of ion-pairing with the cationic polyamines. This diminishes the hydrophobic dansylated derivatives' retention time and thus assures a good separation. In addition, it is possible that the triethylamine recovers the free polar groups of the stationary phase, therefore giving better reproducibility.

Orthophthalaldehyde (OPA) has also often been retained in the formation of fluorescent polyamine derivatives [17]. The results using a Radialpak C 8

column are more reproducible with Dns-Cl than with OPA. In addition, the dansylated derivatives are stable, preparation is faster and they can be stored in ethyl acetate in darkness at 4°C for up to a week. The glass tubes used for this step and for hydrolysis must be siliconized because the polyamines are easily adsorbed onto glass.

The values we found for both the blood and urine of normal subjects in good health are comparable to those found by other authors [6, 18, 19]. However, some authors seemed to have found higher urinary polyamine values [20].

This polyamine assay technique was applied to blood from cancer patients. Most studies on polyamine variations in cancer patients were on urinary polyamines, in particular the study of putrescine and spermidine [6, 21, 22]. Our study on blood polyamines has shown that, as found with the urinary polyamines in cancer patients, blood polyamine levels are significantly elevated. We have also determined that this elevation was sustained by all four polyamines, confirming that cadaverine is also synthesized in man.

In our experience, blood polyamine levels of putrescine are more strongly and regularly elevated than those of the other polyamines. This fact was not demonstrated by the results of Bakowski et al. [19] because their polyamine assay technique shows interference at the level of putrescine. It seems, however, that the type of cancer has an influence on the level of blood polyamines found. Analogous results have been shown with urinary polyamine levels [23].

During treatment of cancer using chemotherapy, different authors have observed that urinary polyamines show a peak in the 24—48 h following onset of treatment [24]. We have ascertained that 8 days after the onset of chemotherapy, the plasma level of the four polyamines assayed remains elevated in the blood while the level of blood polyamines in patients treated with DFMO falls rapidly. This diminution generally concerns all four polyamines. However, this rapid drop has no prognostic significance in that there was no more remission in patients who were treated with DFMO than in those patients who received chemotherapy alone. We have also determined that those patients with low blood levels of putrescine and spermidine prior to any treatment and during treatment, are, contrary to any results found in the urine level [21], patients who do not respond to treatment. This difference can in part be explained by the fact that in the blood we measured the free polyamines, whereas in urine it is the total polyamines that are measured and are found to be elevated. It seems that in these patients there could be a modification in the conjugation of these polyamines [25] or in their clearance.

These results are not in accordance with the hypothesis of Russell who supposes that putrescine might reflect recruitment of tumor growth and spermidine reflect tumor cell death. Also one must certainly consider other difficult accessible parameters such as the speed of polyamine renewal, their cellular compartmentalization and the cell cycle [26].

In conclusion, the proposed assay for putrescine, cadaverine, spermidine and spermine permits the measurement of these cations in serum. Blood is often easier to obtain than 24-h urine samples, whose collection often poses problems. The results obtained show that the levels of these compounds are elevated in cancer patients and that these elevations concern all four cations.

It should be noted, however, that the elevations are variable from one type of cancer to another and that chemotherapy involves an increase of all the serum polyamines which lasts longer than the increase observed in the urine. DFMO, an ornithine decarboxylase inhibitor, masks the elevation of polyamines in subjects undergoing chemotherapy without, it seems, modifying the underlying evolution of their disease.

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Journal of Chromatography, 277 (1983) 101—114
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1792

ASSAY PROCEDURES FOR THE DETERMINATION OF BIOGENIC AMINES AND THEIR METABOLITES IN RAT HYPOTHALAMUS USING ION-PAIRING REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received February 15th, 1983; revised manuscript received May 16th, 1983)

SUMMARY

Procedures are described for the simultaneous determination, by high-performance liquid chromatography of adrenaline, noradrenaline, dopamine and 5-hydroxytryptamine using laurylsulphate as pairing ion and for the separate simultaneous determination of vanillylmandelic acid, dihydroxyphenylacetic acid, 3-methoxy-4-hydroxyphenylethylene glycol, 5-hydroxyindoleacetic acid and homovanillic acid using tetraethylammonium as pairing ion. Sample preparation consists of protein precipitation only and octadecylsilane coated silica is used for both sets of determinations as is electrochemical detection.

The chromatographic basis of each separation is discussed in the light of modern ideas of ion exchange and desolvation to enable the method to be modified chromatographically if other compounds are to be resolved or the assay method modified. The quantitative aspects of the methods are detailed and applied to amine and metabolite levels in rat hypothalamus. The values determined together with their sample variation and estimated limits of detection are quoted.

INTRODUCTION

The determination of adrenaline (A), noradrenaline (NA), dopamine (DA) and 5-hydroxytryptamine (5HT) in brain and other biological matrices has been the subject of several reports using pretreatment stages involving alumina adsorption to isolate the amines from predominantly acidic metabolites [1—4]. In these cases, chromatographic retention and separation have been achieved using aqueous methanol eluents with or without the addition of hydrophobic pairing ion, usually octylsulphate. The only separation and simultaneous quantitaton of the recognised metabolites of the above compounds vanillyl-

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mandelic acid (VMA), dihydroxyphenylacetic acid (DOPAC), 3-methoxy-4-hydroxyphenylethyleneglycol (MHPG), 5-hydroxyindoleacetic acid (5HIAA) and homovanillic acid (HVA) have been achieved by gradient elution in buffered aqueous methanol after extraction [5].

While numerous other reports have appeared in the literature describing methods for the determination of particular metabolites [6, 7] or amines [8] or unique combinations of amine and metabolites [9—14], these are open to criticism on the grounds that resolution is often inadequate for complete specificity of determination [15, 16].

More seriously, in many reported methods other amines and/or metabolites which have not been removed by any pretreatment step are not accounted for in the chromatographic separation. Such criticism is particularly relevant in view of recent methods where cerebrospinal fluid or brain tissue homogenate has been injected directly onto the chromatographic column subsequent only to protein precipitation [17, 18]. Such samples must necessarily contain all amines and metabolites and any specific estimation by chromatography must at least justify why the non-quantitated species do not interfere. Certain reports do in fact indicate doubts concerning the complete specificity of chromatography [10, 19].

The present work attempts to overcome these shortcomings and describes two isocratic separations, one suitable for the determination of the above amines in presence of the metabolites, the other for the determination of acidic and neutral metabolites in the presence of the parent amines. The biological matrix used is rat hypothalamus subjected to protein precipitation pretreatment only. Preliminary chromatographic retention data are obtained using ultraviolet detection and quantitation at the biological level is by electrochemical detection (ED). The compounds listed are chosen since they are currently believed to be the main biogenic amines and metabolites involved in central neurotransmission [20, 21].

EXPERIMENTAL

Equipment

Chromatographic measurements were carried out using a Waters Assoc. M 6000 A pump and M 440 fixed-wavelength (254 nm) or Bioanalytical Systems LC4 detectors. Columns were 200 × 4.6 mm slurry packed using 5-μm ODS Hypersil (Shandon Laboratories). The electrochemical detector was fitted with a polished glassy carbon electrode and operated at an applied potential of 0.70 V relative to a silver/silver chloride reference electrode. The column to detector cell fittings were modified to reduce dead volume.

Chemicals

DOPAC, NA (bitartrate), 5HIAA, HVA and VMA were obtained from Sigma. 5HT (sulphate) and DA (hydrochloride) were obtained from BDH and A (hydrogen tartrate) from Koch-Light. MHPG (piperazine salt) was obtained from Aldrich as were the pairing ions tetraethylammonium (TEA) and tetrabutylammonium (TBA) as the bromides. Laurylsulphate (SLS) as the sodium salt was obtained from Fisons. Acetonitrile was obtained from Rathburn

Chemicals and water was purified using a Milli Q system. All other chemicals used were of AnalaR or equivalent grade.

Sample preparation

Mature male rats of the Sprague—Dawley strain from our own breeding colonies were killed by cervical dislocation. The hypothalami were removed and placed in a vessel in an ice bath within 2 min of death. The tissues were weighed and homogenised by hand using a 1-ml glass Potter homogeniser within 1 h of removal from the animal. Approximately 0.1-g tissue samples were homogenised in 500 μl of 0.4 *M* perchloric acid. After centrifuging the homogenate at 3000 *g* for 30 min the supernatant (20 or 50 μl) was injected via a Rheodyne 7125 valve.

THEORETICAL BASIS OF SEPARATIONS

Previous high-performance liquid chromatographic (HPLC) determinations of these amine metabolites have been carried out in buffer solutions at pH values low enough to suppress ionisation of the predominantly acid metabolites. Retention in these cases is on the basis of varying hydrophobicity of essentially neutral solutes. In such cases the amines, being fully protonated, should elute rapidly. In some investigations pH is manipulated so as to achieve retention of certain of the amines as well as metabolites [15]. The corresponding methodology cannot be applied to the determination of bases alone since at pH values high enough to deprotonate the bases completely, catecholamines are chemically unstable [22] and in addition, the bonded hydrocarbon stationary phase is degraded [23]. The strategy in such cases has been to include a hydrophobic pairing ion such as octylsulphonate in a low pH buffer in order to retain bases by ion pairing and also to retain acid and neutral metabolites in the same system by ion suppression [24]. This latter procedure can result in crowded chromatograms with resolutions often quite inadequate for quantitation. Such methods are characterised by the use of very low solvent pairing ion concentrations.

Investigations on the mechanism of ion-pairing chromatography have shown that the dependence of capacity factors of solutes on solvent pairing ion concentration is complex [25] and that very often such a dependence goes through a maximum. Such behaviour has been explained on the basis of an ion-exchange—desolvation mechanism [26, 27]. Retention of a solute on a C-18 coated surface containing adsorbed pairing ion of charge opposite to that of the solute is described as occurring due to electrostatic binding coupled with desolvation of the neutralised solute on the available C-18 surface; i.e. on surface unoccupied by adsorbed pairing ion. Thus, if the adsorbed pairing-ion concentration is high due either to a high solvent concentration or to the use of a very strongly adsorbed pairing ion, retention of both charged and neutral species will actually be reduced due to the unavailability of free C-18 surface for desolvation.

The mixtures of basic biogenic amines and their predominantly acidic metabolites constitute an ideal system to which the above ion exchange—desolvation ideas may be applied for the purpose of obtaining improved

specificity in the assay of these compounds. At low pH the amines may be retained and resolved by suitable choice of anionic pairing ion at an appropriate concentration while the retention of acid and neutral metabolites may be minimized. Similarly at pH 7 the acidic compounds being appreciably ionised can be retained by use of a suitable cationic pairing ion while minimising the retention of the bases whether or not they are completely uncharged.

In principle, therefore, it should be possible to optimise the effects of increasing and decreasing retention of the different classes of solutes by choice and concentration of added pairing ion to achieve the required separations.

RESULTS AND DISCUSSION

Separation of amines

Fig. 1 shows the effect of SLS concentration in the solvent on the capacity factor (k') for the nine solutes studied. The amines show the predicted maxima

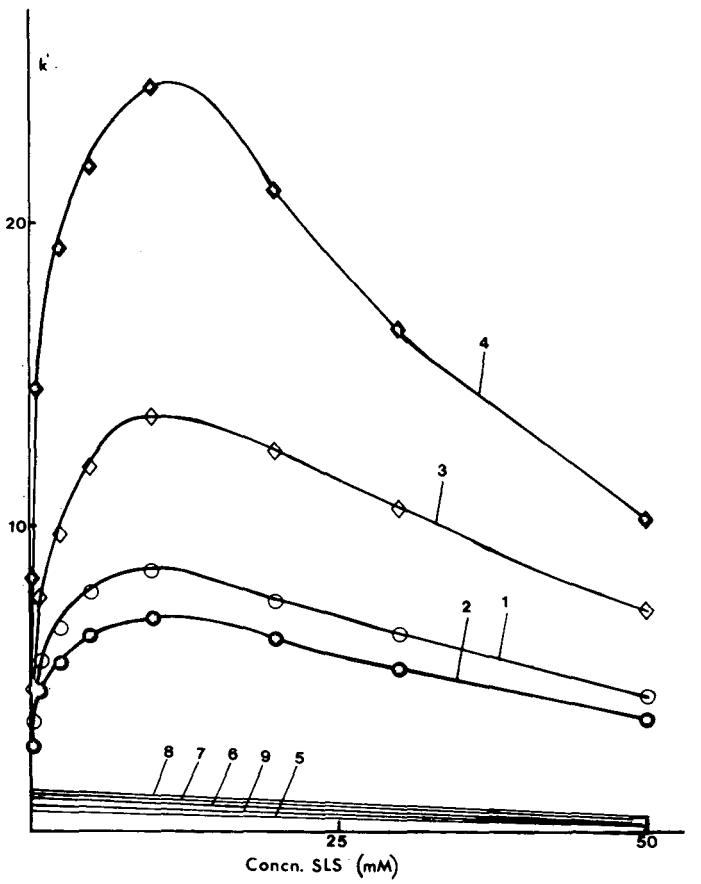

Fig. 1. Variation of capacity factor k' with SLS concentration for the nine amines and metabolites studied. Column 200 × 4.6 mm ODS Hypersil (5 μm); solvent 10 mM disodium hydrogen phosphate, pH 2 buffer containing 25% acetonitrile and 0.5 mM EDTA. Compound identification: 1 = A; 2 = NA; 3 = DA; 4 = 5HT; 5 = VMA; 6 = DOPAC; 7 = MHPG; 8 = 5HIAA; 9 = HVA.

while the metabolites show very low retentions which are further reduced by increasing SLS concentration. This pairing ion was chosen because of its ready availability and cheapness. Although in purely aqueous solvent it will produce very large capacity factors for bases [27], this is overcome by the inclusion of organic modifier which, in turn, will aid the rapid elution of neutralised acidic metabolites and endogenous materials from biological samples. Fig. 1 shows that pairing-ion concentration will dictate the degree of resolution obtained. While low concentrations (3—5 m*M*) can provide adequate resolution higher concentrations (10—50 m*M*) provide a less critical system with respect to pairing-ion concentration, avoid the problem of long equilibration times [28] and further reduce the retention of neutral components.

Representative separations of the major amines in a protein-precipitated homogenate are shown in Fig. 2a. Fig. 2b shows the separation of a standard mixture of amines together with their metabolites while Fig. 2c showing the separation of a spiked homogenate extract verifies the identities of the peaks shown. Comparison of Fig. 2a and b indicates the absence of both endogenous compounds and metabolite interference in the determination of amines using this system. The critical resolution was found to be the A/NA pair and acetonitrile afforded better selectivity than methanol which produced unreasonably long retention times for 5HT to achieve complete A/NA separation.

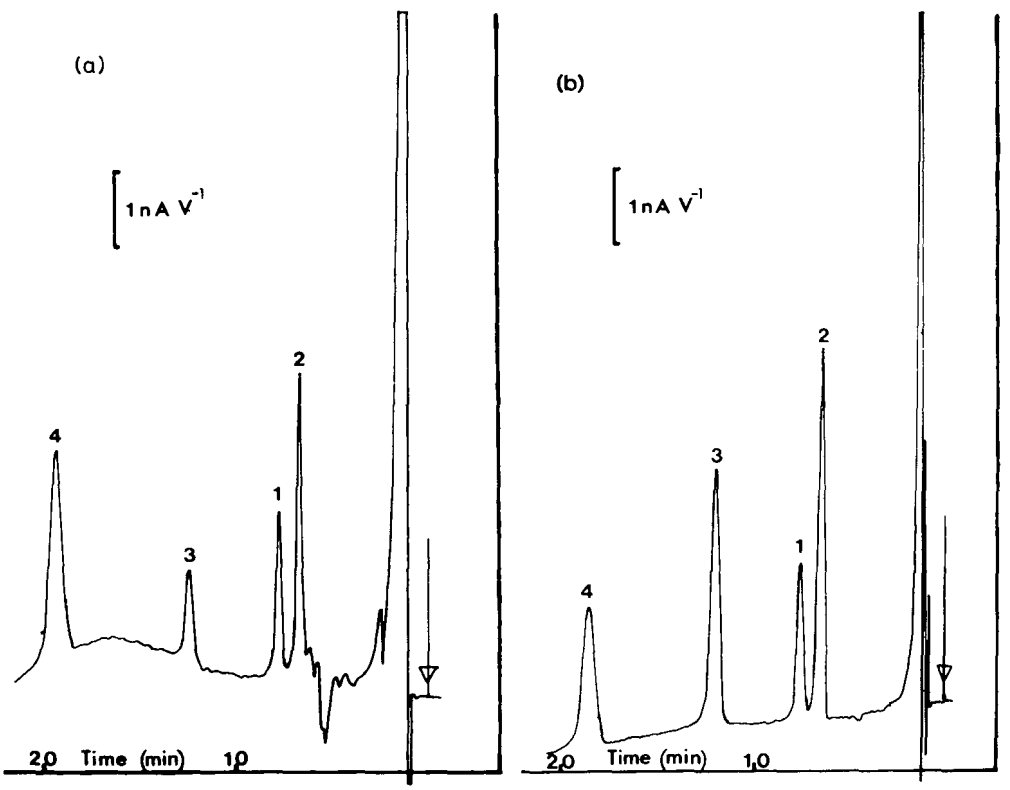

Fig. 2.

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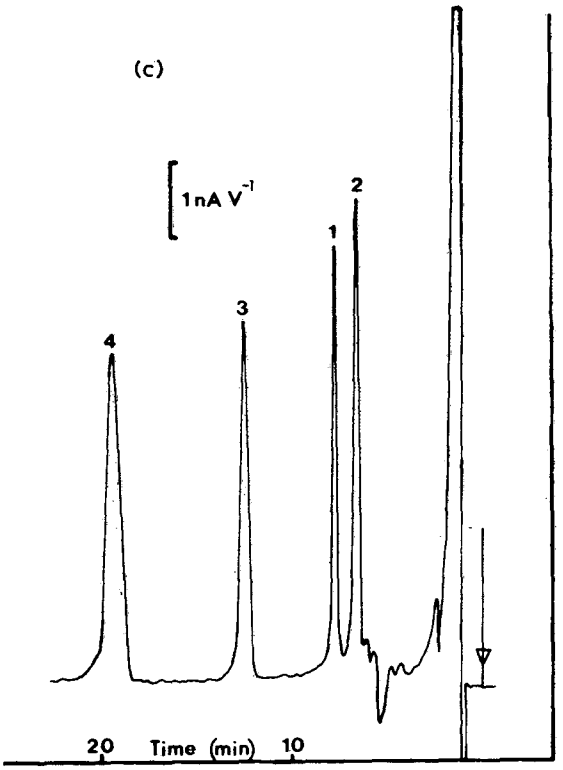


Fig. 2. Representative chromatograms showing separation of the major amines. (a) Supernatant from protein-precipitated tissue homogenate; (b) all amines and metabolites in aqueous solution; (c) tissue homogenate spiked with added metabolites. Conditions and compound identification as in Fig. 1 with SLS concentration 20 m*M*.

Separation of acid and neutral metabolites

To achieve this separation and concomitant minimal retention of amines it was found that the choice of pairing ion was critical. Very hydrophobic cationic pairing ions such as tetrabutylammonium or cetyltrimethylammonium while providing adequate resolution of the acidic species, reduced the retention of the neutral MHPG metabolite too rapidly with increasing pairing-ion concentration. The metabolite because of its highly hydroxylated structure and correspondingly low hydrophobicity was, in fact, less retained on most systems than the basic 5HT at pH 7.

Tetraethylammonium pairing ion proved to be the most suitable choice. Fig. 3 shows the variation of capacity factor, k', with TEA concentration for all solutes studied. At low TEA concentrations the neutral MHPG is highly retained as is 5HT while retention of the acidic metabolites is inadequate for reliable determination in biological matrices. Since TEA is a relatively weakly adsorbed pairing ion [26], as the TEA concentration is increased the retention of MHPG is only gradually reduced. The accumulation of charge on the C18 surface, however, decreases the retention of the 5HT markedly while having the expected ion pairing retention effect on the acidic anions. In this system the optimal concentration of TEA used was considered to be 30 m*M* and at this

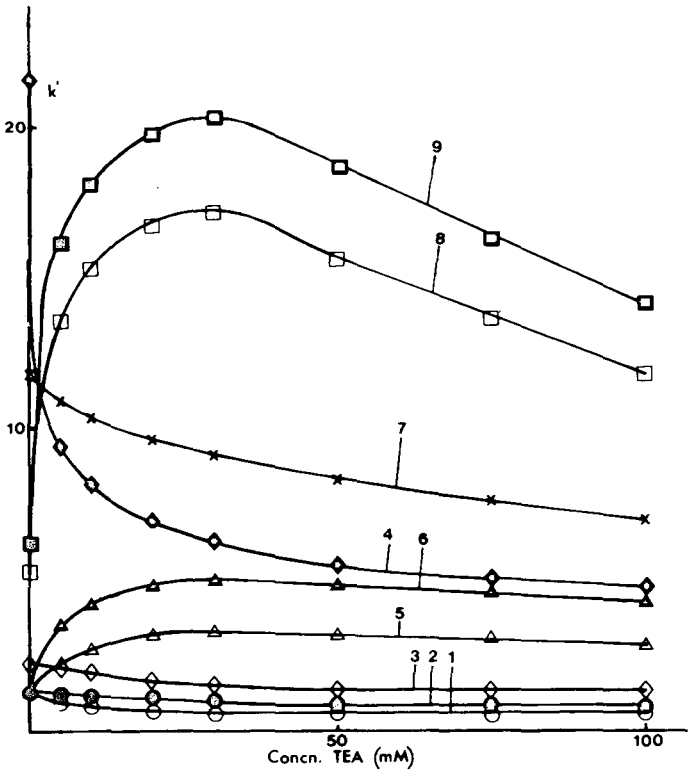
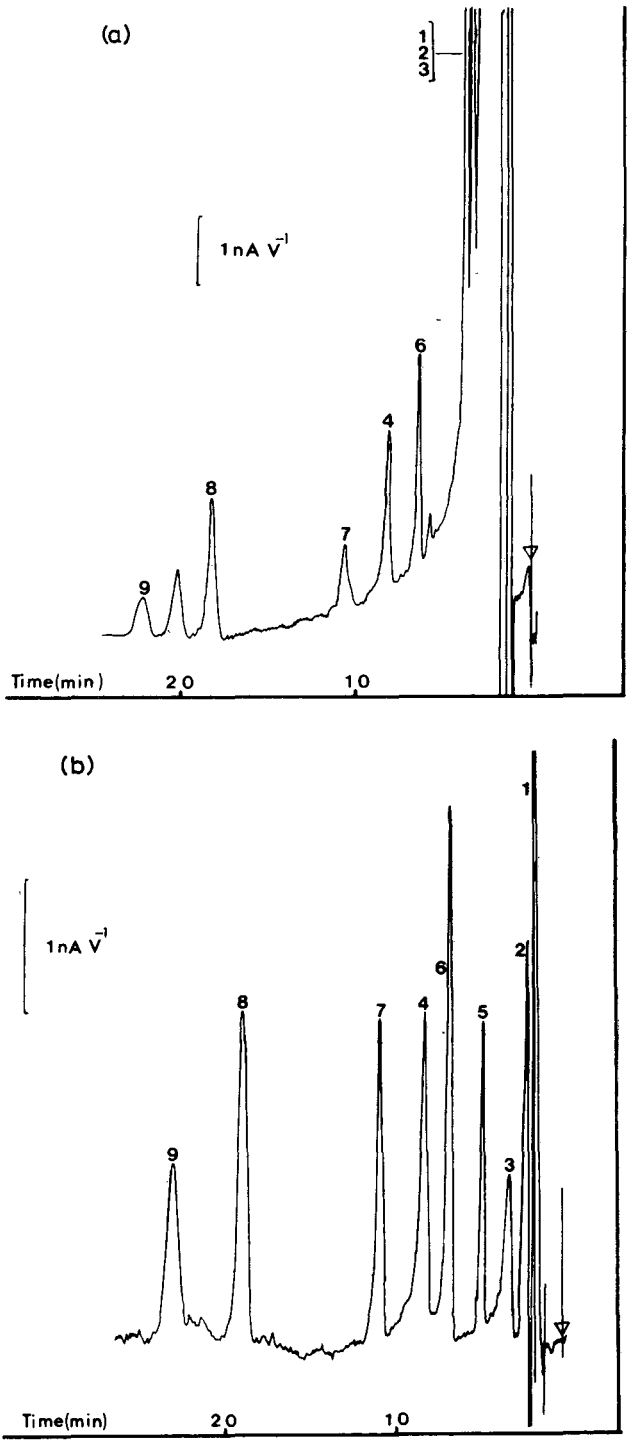


Fig. 3. Variation of the capacity factor k' with TEA concentration for the nine solutes studied. Column 200 × 4.6 mm ODS Hypersil (5 μm); solvent 10 mM disodium hydrogenphosphate, pH 7 buffer 0.5 mM in EDTA. Compound identification as in Fig. 1.

concentration NA, A and DA were rapidly eluted while 5HT in spite of its predominantly basic character was retained but resolved from the metabolite species. It was not possible to include organic modifier in this system for the purpose of reducing the peak due to endogenous materials.

Fig. 4a shows a representative chromatogram of tissue extract run on this solvent system. The signal due to endogenous material is much larger than in the case of the amine system but resolution is adequate. Fig. 4b is a chromatogram of a standard mixture of amines and metabolites and verifies that the amines other than 5HT are eluted with the residual endogenous compounds. Fig. 4a indicates an absence of VMA in the sample and this was found generally to be the case for other hypothalamus samples measured. Fig. 4c, a chromatogram of tissue spiked with all compounds, confirms the identities of the various peaks and the absence of VMA in the sample. In this solvent system few unidentified peaks were evident at the sensitivity required for the major metabolites. In Fig. 4a and c only one unidentified peak is noted between 5HIAA and HVA while in other brain samples assayed an additional peak was, on occasion, recorded after HVA. Resolution was adequate in all cases for quantitation of the specified metabolites.

It is emphasised that the two solvent systems described are not unique and


(a)
1
2
3
1nA V⁻¹
6
4
8
7
9
Time(min)
20
10
(b)
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Time(min)
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Fig. 4.

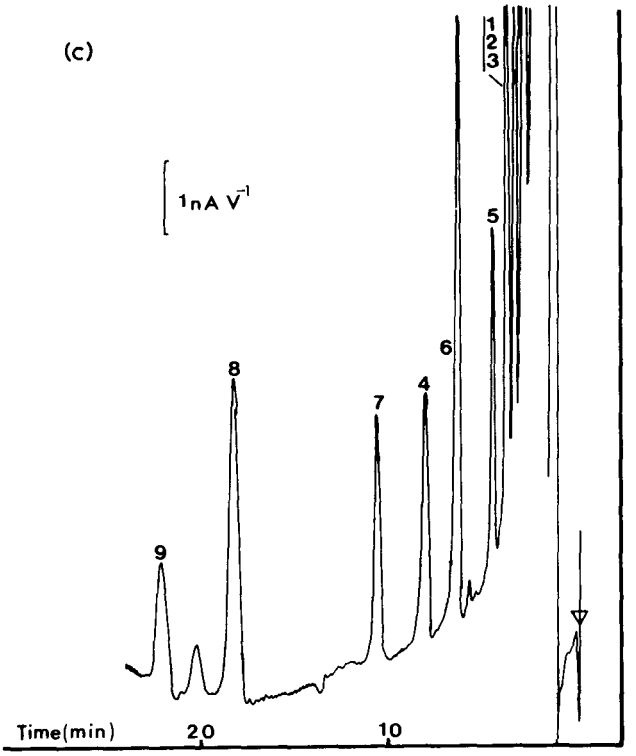

Fig. 4. Representative chromatograms showing separation of the amine metabolites and 5HT. (a) Supernatant from protein-precipitated tissue homogenate; (b) all amines and metabolites in aqueous solution; (c) tissue supernatant spiked with added metabolites. Conditions as in Fig. 3 with TEA concentration 30 m*M*. Compound identification as given in Fig. 1.

have been chosen as optimum for the particular purpose of determining the specified compounds. For other investigations it would be possible to alter retention times and order of elution by choice of different pairing ions and concentrations bearing in mind the dual retention characteristics of ion exchange and hydrophobicity.

Quantitation

For both amines and metabolites the standard addition method was applied to a pooled tissue sample to verify the linearity of the chromatographic response. Fig. 5a and b demonstrate the resultant plots, and confirm the linearity of response and verify the specificity of the solute peaks. The constants of the regression lines are listed in Table I. Concentrations of the endogenous catecholamines and metabolites are not quoted since different pooled samples were employed but the slopes of the regression lines indicate the relative sensitivities of the method for the different solutes. It is also seen from Table I that good agreement is obtained between experimental intercepts in Fig. 5a and b and those obtained by regression. The somewhat lower sensitivities apparent compared with other workers using similar detection systems [29] may be a consequence of the lower applied voltage and/or the

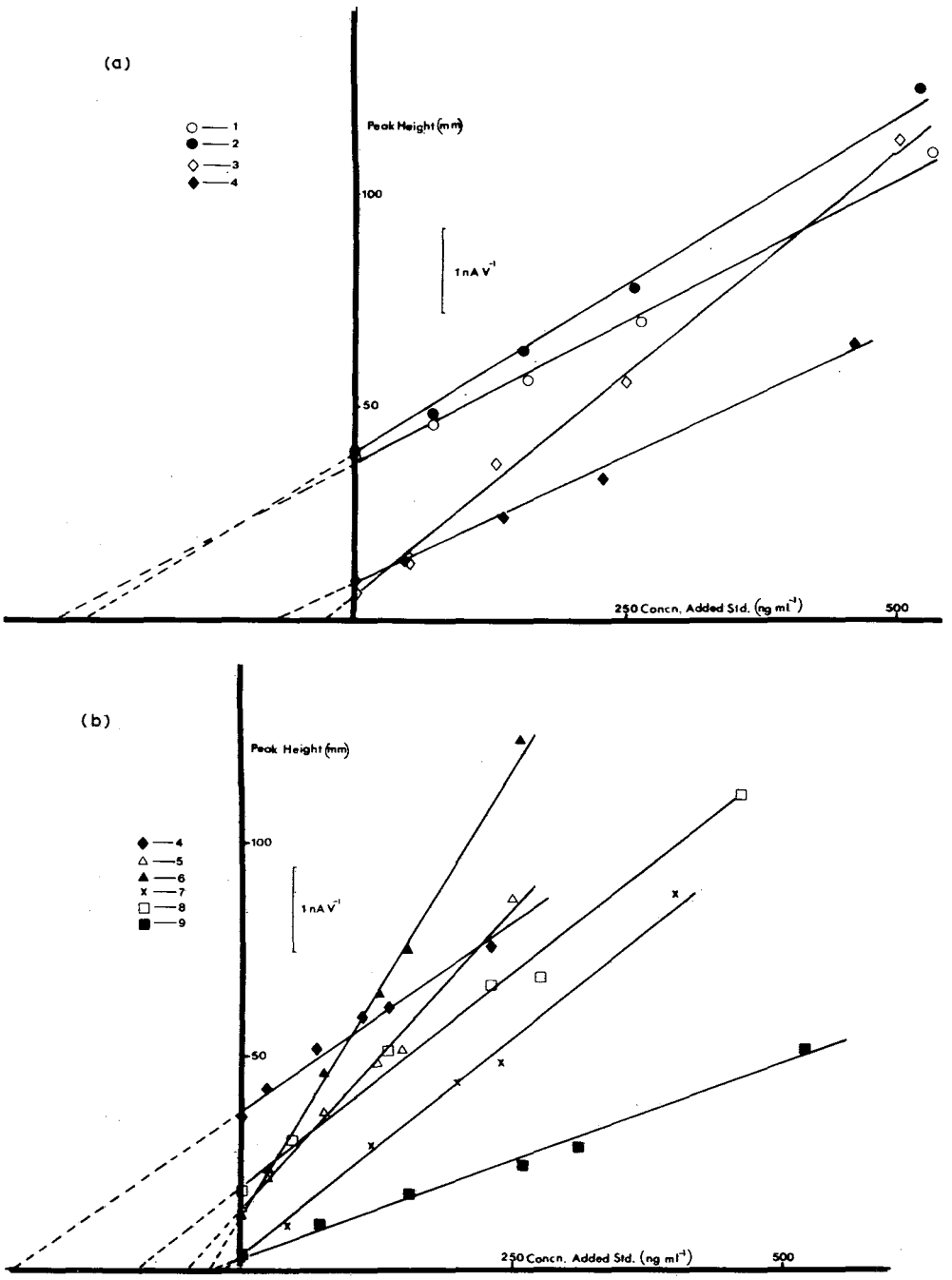

Fig. 5. Plots showing the variation of peak heights on addition of added standards to pooled protein-precipitated tissue homogenate. (a) Amines added, results from SLS system; (b) metabolites added, results from TEA system. Compound identification as in Fig. 1.

TABLE I

CONSTANTS OF THE REGRESSION LINES OBTAINED BY STANDARD ADDITION OF AMINES AND METABOLITES TO TISSUE SAMPLES

Compound	Constants of regression line					
	Slope	S.D. of slope	Intercept (regression)	Intercept (experimental)	S.D. of intercept	Correlation coefficient, r^2
A	0.125	$3.79 \cdot 10^{-3}$	37.3	38.4	1.04	0.998
NA	0.154	$3.32 \cdot 10^{-3}$	39.2	39.8	0.898	0.999
DA	0.205	$4.49 \cdot 10^{-3}$	5.02	6.3	1.16	0.999
VMA	0.282	$1.49 \cdot 10^{-3}$	13.7	14	1.99	0.994
DOPAC	0.433	$1.14 \cdot 10^{-3}$	11.4	12.4	1.54	0.998
MHPG	0.212	$8.19 \cdot 10^{-3}$	2.02	3.2	1.74	0.997
5HIAA	0.194	$1.13 \cdot 10^{-3}$	19.7	17.8	2.77	0.993
HVA	0.089	$5.43 \cdot 10^{-3}$	3.12	2.8	1.50	0.992
5HT bases	0.120	$6.11 \cdot 10^{-3}$	7.91	9.0	1.46	0.996
5HT acids	0.167	$9.58 \cdot 10^{-3}$	38.1	35.8	1.17	0.993

chromatographic solvent used. Table II shows results obtained on individual hypothalamus samples obtained by direct comparison of sample peaks produced with those of a set of aqueous standards. The 5HT values shown represent the means of the two values determined on the different solvent systems and the MHPG values refer to the unconjugated glycol. Also included in Table II are literature values for the concentrations of these compounds in rat hypothalamus.

The values obtained in the present study are in general agreement with those reported in the literature. None of the reported investigations, however, gave data for all of the compounds used in the present study. The apparent differences between present and literature values are probably a reflection of differences in technique. The present method involves minimal sample preparation with consequent minimal opportunity for losses. This may explain why noradrenaline and 5HT levels are similar to or greater than the highest levels previously reported. In the case of 5HT preliminary studies with aqueous standards showed its chemical instability so the high value in the present study may also be a reflection of the speed of sample preparation. Although HVA was reported in the literature it was not detected in the hypothalamic homogenates used for quantitation. It was, however, present in the homogenate used to establish retention times as indicated in Fig. 4a and c. The tissue for these experiments was isolated by different workers and it is likely therefore that the presence or absence of HVA may depend on minor variations in the size of the region isolated as hypothalamus. The limits of detection for the solutes by the present method determined in aqueous solution are also included in Table II. The chromatographic precision of the method (R.S.D. = 2.0%, n = 10) was considerably below the biological variation shown in Table II.

TABLE II

RESULTS OF AMINE AND METABOLITE DETERMINATIONS ON SIX TISSUE SAMPLES BY THE PRESENT METHOD TOGETHER WITH LITERATURE VALUES FOR CERTAIN COMPOUNDS IN THE MALE RAT HYPOTHALAMUS

Investigation method	Concentration ng/g wet tissue (R.S.D.)								
	A	NA	DA	5HT	VMA	DOPAC	MHPG	5HIAA	HVA
This investigation	78.3	3066	502	1812	20	100	97	409	25
Rat hypothalamus (n=6)	(51)	(27)	(12)	(17)		(17)	(14)	(10)	
HPLC—ED									
Limit of detection (ng/ml)	33	26	21	26	6.6	9.4	8.5	11	25
HPLC—ED [8]	—	808	150	—	—	—	—	—	—
HPLC—ED [30]	—	2780	596	1098	—	—	—	—	—
Gas chromatography—	—	2418	569	956	—	—	—	565	95
mass spectrometry	—	1740	488	1089	—	160	92	560	—
[31]	—	1707	359	741	—	—	—	414	116
	—	2327	465	931	—	—	—	483	111
HPLC—ED [32]	64	2080	410	—	—	46	—	—	35
	58								
	66								

CONCLUSION

The reported methods rely on minimum pretreatment of tissue samples with consequently least opportunity for sample loss. The chromatographic systems represent an advance on current literature systems since they provide improved resolution over all but gradient elution methods for all the accepted metabolites or amines and they also accommodate the presence of all of these compounds. The disadvantage of the two-solvent column system is believed to be more than compensated by the completeness of metabolite detection, the high capacity factors and improved resolution. The apparently lower sensitivities achieved are adequate for quantitation with the possible exception of VMA but could be improved by modification to column geometry and injection volume if required. The identities of the unknown compounds will be the subject of a continuing investigation as will determination of amine turnover rates for which purpose the assay methods described were designed.

ACKNOWLEDGEMENTS

The receipt of a Medical Research Council special equipment grant is gratefully acknowledged and P.F.C. gratefully acknowledges receipt of a Scottish Education Department research studentship.

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Journal of Chromatography, 277 (1983) 115—123
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1776

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH AMPEROMETRIC DETECTION OF PLASMA L-3,4-DIHYDROXYPHENYLALANINE IN PARKINSONIAN PATIENTS

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(First received January 26th, 1983; revised manuscript received May 6th, 1983)

SUMMARY

An assay for the quantitative estimation of L-3,4-dihydroxyphenylalanine in human plasma has been developed, using α-methylnoradrenaline as internal standard and ion-pair reversed-phase (C_8), isocratic high-performance liquid chromatography with amperometric detection. A citrate—phosphate buffer, pH 3.1, containing 2 m*M* EDTA disodium salt, 6.5 m*M* 1-octanesulphonic acid and 14% v/v methanol, provided good separation of the analytes from each other and from the carbidopa present in the plasma of Sinemet-treated Parkinsonian patients. The sensitivity, selectivity and precision of the method were found to be sufficient to enable its routine use for pharmacodynamic studies.

INTRODUCTION

L-3,4-Dihydroxyphenylalanine (L-dopa) a catechol α-amino acid, is widely used in the treatment of Parkinson's disease [1—4], usually in combination with a peripheral dopa decarboxylase inhibitor such as L-α-hydrazino-3,4-dihydroxy-L-α-methylbenzenepropanoic acid (carbidopa) [5] (Fig. 1).

Following the introduction of L-dopa for Parkinson's disease an extensive literature of fluorimetric analyses appeared [6—12]. However, the patient compliance and the considerable individual variation in therapeutic dosage, have

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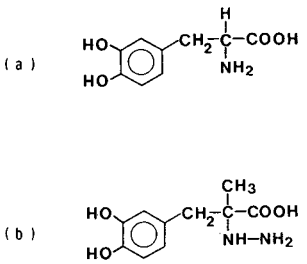

Fig. 1. Molecular structures of (a) L-dopa and (b) carbidopa.

prevented the widespread routine monitoring of L-dopa in plasma or urine; and as a consequence there has been comparatively little use of modern methods of analysis.

Radioenzymatic assays for L-dopa have been described [13, 14], but these tend to be expensive, time-consuming and unsuitable for small numbers of samples. Furthermore, there is the possibility of crossover interference from carbidopa in Sinemet-treated patients. High-performance liquid chromatography (HPLC) methods utilising ultraviolet detection [15, 16] or fluorescence detection [17] have the sensitivity to measure L-dopa in urine and pharmaceutical preparations; but the reliable measurement of plasma levels has awaited the development of a highly sensitive detector. The amperometric (electrochemical) detector introduced by Kissinger and co-workers [18, 19] has picomole sensitivity, is compatable with most reversed-phase HPLC systems; and now that problems with electroactive contaminants and electrical noise have been overcome, HPLC with amperometric detection is frequently the method of choice for catecholamines and related compounds [20]. Riggin et al. [21] described an HPLC method for measuring serum L-dopa and dopamine, using a bonded phase cation-exchange column and amperometric detection. More recently Freed and Asmus [22] reported a reversed-phase HPLC method suitable for plasma L-dopa, which gave improved resolution, but which was subject to undefined interfering peaks. Contrary to the experience of these workers we have found that reversed-phase ion-pairing HPLC systems based on citrate—phosphate (McIllvaine) buffers are ideally suited to separation and amperometric detection of catecholamines and related compounds [23, 24]. The advantages of citrate buffers have been appreciated by others [25, 26] but their acceptance has awaited the description of the mechanism of action, as recently given by Krstulović et al. [27].

This present paper deals with the development of an HPLC—amperometric method to measure L-dopa in the plasma of Parkinsonian patients treated with Sinemet, as part of a study of the effects of L-dopa on cerebral blood flow and metabolism, as assessed by positron emission (ECAT) scanning. The clinical results of this study will be published separately.

EXPERIMENTAL

Apparatus

Chromatography was performed using a Model 100A solvent delivery pump and a Model 210 sample injection valve (Altex Scientific, Berkeley, CA,

U.S.A.). The separations were achieved with a 15 cm × 4.6 mm I.D. Ultrasphere octyl (5 μm particle size) column (Altex), and detection was accomplished using a TL-4 glassy carbon electrode held at +0.72 V vs. Ag/AgCl reference electrode.

The electrode was controlled by an LC-4 amperometric detector (Bio-Analytical Systems, West Lafayette, IN, U.S.A.). Typical amperometric detector sensitivity settings for plasma L-dopa were 200 nA full scale on filter C. An aluminium Faraday cage enclosed the column, electrodes and amperometric detector and this was earthed to the Model RE. 541.20 Servoscribe chart recorder (Smiths Industries, London, U.K.).

Reagents

The ion-pairing agent 1-octane sulphonic acid was obtained from Fisons (Loughborough, U.K.). AnalaR grade methanol from May and Baker (Dagenham, U.K.) was found adequate. The standard L-dopa was obtained from Sigma (Poole, U.K.) and α-methylnoradrenaline was a gift from Hoechst Pharmaceuticals (Hounslow, U.K.). All other chemicals were of analytical grade and obtained from BDH Chemicals (Enfield, U.K.). The alumina (Brockmann grade 1, active neutral) was washed and activated as described previously [23].

Chromatography

Mobile phases consisted of citrate—phosphate buffer (pH 3.1) containing 6.5 m*M* 1-octanesulphonic acid and 14% methanol as organic modifier. The disodium salt of EDTA was added to a final concentration of 2 m*M*. Prior to use the mobile phase was filtered through Gf/f glass microfibre paper (Whatman Lab. Sales, Maidstone, U.K.) and helium degassed (BOC Special Gases, London, U.K.). All separations were carried out at ambient temperature.

Once set up, the system was run continuously to ensure stability and sensitivity. During sample injections the flow-rate was usually 1.2 ml/min and this was reduced to 0.1 ml/min when the system was not in use.

Dosing

Sinemet (Merck Sharp & Dohme, Hoddesdon, U.K.) was obtained from the hospital pharmacy as Sinemet 110 (10 mg carbidopa with 100 mg L-dopa) or Sinemet 275 (25 mg carbidopa with 250 mg L-dopa). Combinations of these two formulations were used in patient dosing.

Plasma samples

A cannula was inserted into a radial artery prior to the scanning procedure and blood was taken into lithium heparin tubes. These were immediately centrifuged (2050 *g*, 5 min, room temperature) and the separated plasma frozen and stored at −70°C prior to the analysis. The protocol outlined below (Fig. 2), generated four samples per patient, from scans during acute and chronic L-dopa treatment. The protocol was approved by the Research Ethics Committee of the Royal Postgraduate Medical School and Hammersmith Hospital and all patients gave their informed consent.

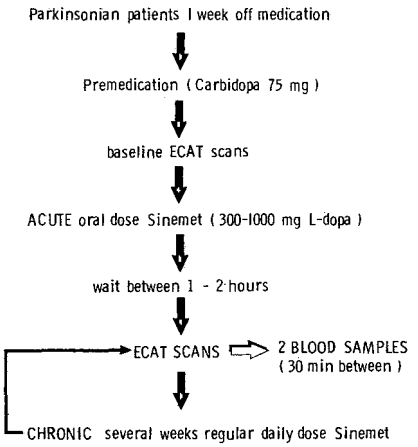

Fig. 2. Protocol for Parkinsonian patient study.

Quantitation

A stock standard of 10 μg/ml of L-dopa was prepared in 0.1 *M* hydrochloric acid and stored at 4° C. Spiked standards were prepared in drug-free plasma on the day of the assay, typically a range from 0—2000 ng/ml was employed. An internal standard of α-methylnoradrenaline was chosen to allow for variable recovery of L-dopa. Quantitation was thus achieved by comparison of the peak height ratio of L-dopa to α-methylnoradrenaline. A calibration curve of at least six spiked plasmas was run with every batch of samples.

Extraction

Conical polystyrene tubes were placed in an ice bath and the following reagents added: 60 mg activated alumina, 1 ml of 1 m*M* hydrochloric acid and 0.1 m*M* EDTA disodium salt, 100 μl of 10 μg/ml α-methylnoradrenaline and 1 ml of distilled water. Plasma samples were thawed, briefly centrifuged at 4° C to remove any fibrin aggregates and 1-ml portions were added to the tubes, followed by 1 ml of 3 *M* Tris—HCl buffer, pH 8.6. The tubes were mixed on a Spiramix rotary mixer (Denley, Bilinghurst, U.K.) for 15—20 min, then placed in ice and the fines of alumina allowed to settle. The supernatant was aspirated at the sink and the alumina washed three times with distilled water (previously adjusted to pH 7.0). In the final aspiration care was taken to remove all of the wash water, prior to elution with 200 μl of 0.1 *M* orthophosphoric acid — achieved by 2 min on the Spiramix mixer.

All tubes were centrifuged briefly at 2020 *g* at 4° C, finally removing the supernatant into small conical tubes (Eppendorf, 1.5 ml). Portions of the supernatant (usually 50 μl) were injected into the HPLC system, which was typically set at a sensitivity of 200 nA full scale in order to measure plasma L-dopa in the concentration range 100—4000 ng/ml. Storage of the separated eluent at −20° C had no deleterious effect on L-dopa levels for periods of up to five days.

RESULTS AND DISCUSSION

Optimization of pH

The pH was an important parameter to evaluate in the development of this

assay, since the pH of the mobile phase determines the concentration of the ionic forms of L-dopa and hence the degree of ion-pairing with citrate and 1-octanesulphonic acid [27]. Changing the pH from 6.0 to 3.1 increased the retention time of L-dopa from 1.0 to 2.5 min and increased the optimal applied potential from +0.50 V to +0.72 V vs. Ag/AgCl reference electrode.

Resolution

Using the HPLC–amperometric assay at a sensitiviity of 200 nA full scale, L-dopa and the internal standard, α-methylnoradrenaline, were resolved from other plasma constituents. This is illustrated with the chromatograms of extracted plasma samples from Sinemet-treated patients (Fig. 3).

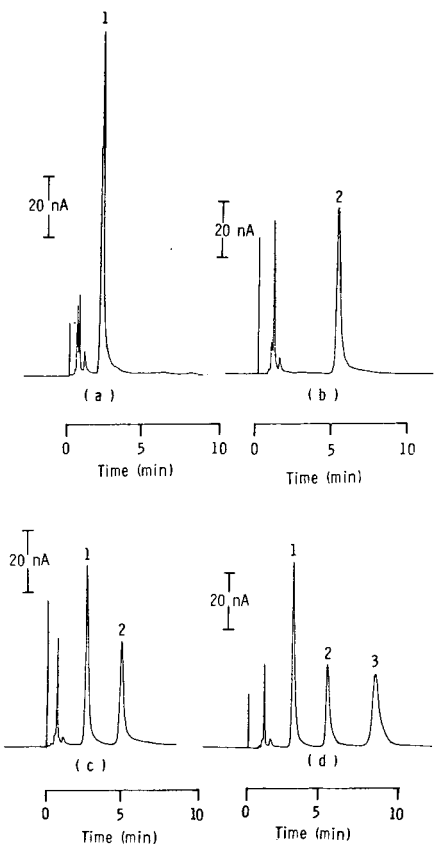

Fig. 3. Representative HPLC chromatograms of human plasma samples run through the assay. (a) Plasma from Sinemet-treated Parkinsonian patient without internal standard added, (b) drug-free plasma with internal standard added, (c) plasma from Sinemet-treated Parkinsonian patient with internal standard added (L-dopa = 1.60 μg/ml), (d) plasma from Sinemet-treated Parkinsonian patient with internal standard added and spiked with carbidopa (20 μg/ml). Chromatographic conditions: column, Ultrasphere octyl (5 μm particle size); eluent, 32 m*M* citric acid, 14 m*M* Na_2HPO_4, 2 m*M* Na_2EDTA, 6.5 m*M* 1-octanesulphonic acid, 14% methanol, pH 3.1; flow-rate, 1.2 ml/min; temperature, ambient; amperometric detector, +0.72 V vs. Ag/AgCl, 200 nA full scale, filter C. Peaks: 1 = L-dopa, 2 = α-methylnoradrenaline, 3 = carbidopa.

TABLE I

RETENTION TIMES FOR CATECHOL COMPOUNDS

For conditions, see text.

Compound	Retention time (min)
DOPAC	1.8
L-Dopa	2.5
α-Methylnoradrenaline	5.1
3-O-Methyl-dopa	5.5
α-Methyl-dopa	6.0
Carbidopa	6.8
Dopamine	8.4

Blank plasma chromatograms showed no interference which corresponded to the retention time of L-dopa or α-methylnoradrenaline. The reversed-phase ion-pairing HPLC system provided a good separation of L-dopa from carbidopa and other catechol compounds, with the compounds eluting in order of decreasing polarity (Table I). Since 3-O-methyl-dopa is a major metabolite of L-dopa [28] the possibility of its interference with plasma L-dopa measurements was investigated. In plasma samples from Sinemet-treated patients no 3-3-O-methyl-dopa could be detected after the alumina extraction, thus ruling out its interference in our samples.

Calibration

Fig. 4 shows a typical mean standard curve ($n = 6$) in plasma over a concentration range of 50–2000 ng/ml L-dopa, where peak height ratio was plotted as a function of L-dopa concentration. Linearity in standard curves of L-dopa was established over an extended range of up to 5 μg/ml in drug-free human plasma.

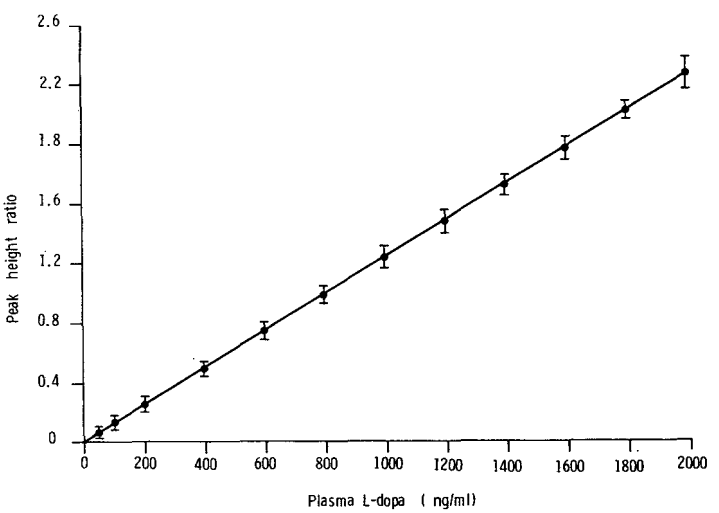

Fig. 4. Mean standard curve for L-dopa in plasma. Each point is the mean ± S.D. of six determinations.

Recovery

The absolute analytical recovery of L-dopa and α-methylnoradrenaline from human plasma was estimated by comparing the peak heights obtained from the injection of known quantities of the analytes with peak heights obtained from the injection of extracts of plasma samples spiked with the analytes. This gave values of 72% for L-dopa and 70% for α-methylnoradrenaline (n = 6).

Limit of detection

Measurement of L-dopa in the therapeutic range (and above) in 1-ml plasma samples was possible at a sensitivity of 200 nA full scale at +0.72 V vs. Ag/AgCl reference electrode on the amperometric detector. At this setting the absolute limit of detection was 25 ng/ml at a signal-to-noise ratio of 2. Since the therapeutic range is in the region of 0.10—3.0 μg/ml this is clearly satisfactory. A more sensitive assay could be achieved by increasing the current amplification on the detector, but this might lead to interferences e.g. from 3-O-methyl-dopa and so would need to be investigated.

During the course of our work on plasma L-dopa a more sensitive approach was described [29] utilising an HPLC system similar to that previously reported by us for catecholamines [24]. However, the elution order reported in this paper differs significantly from our own, in that the acidic metabolite dihydroxyphenylacetic acid (DOPAC) has the shortest retention in our system but an intermediate one in theirs. Furthermore in treated Parkinsonian patients, L-dopa is present in arterial plasma at μg/ml concentrations while DOPAC is in the ng/ml range. Thus for our application a sensitivity of 200 nA full scale on the amperometric detector enabled us to measure L-dopa without any interferences from DOPAC or 3-O-methyl-dopa.

Precision

Intra-assay. Replicate analysis of a pooled plasma sample containing L-dopa in the therapeutic range gave a coefficient of variation of 1.8% (n = 15, $\overline{X}$ = 720.3 ng/ml, S.D. = 13.1565).

Inter-assay. Analysis of a pooled plasma sample containing L-dopa in the therapeutic range over a 1-month period (the pool was stored in aliquots at —70°C between assays, once thawed the aliquots were not reused) yielded a coefficient of variation of 4.3% (n = 10, $\overline{X}$ = 693.5 ng/ml, S.D. = 29.7256).

Practical application

Using the method suggested, the plasma concentrations of L-dopa were monitored in Parkinsonian patients who had received Sinemet orally either following one week of drug abstinence, as an acute dose (range 300—1000 mg L-dopa, n = 8, $\overline{X}$ = 588 mg, S.D. = 219); or, following several weeks of daily optimal dosing, as a chronic dose (range 400—800 mg L-dopa, n = 8, $\overline{X}$ = 631 mg, S.D. = 150). The method described in this paper took advantage of an ECAT scanning study, which had been designed for other purposes; e.g. a positive clinical response had to be ensured after administering L-dopa.

This accounts for the differences in the doses given to the patients. The aim was to provide a single oral dose of L-dopa in the acute experiment equal to the total daily dose to which the patients were accustomed prior to the study.

TABLE II

PLASMA L-DOPA LEVELS IN SINEMET-TREATED PARKINSONIAN PATIENTS, DURING ECAT SCANNING (μg/ml)

Mean time interval A to B = 36 min, all values are the mean of two determinations.

Patient No.	Dyskinesis score	Actute treatment		Chronic treatment	
		A	B	A	B
1	+	3.19	2.83	0.75	0.59
2	—	2.20	2.05	0.50	0.51
3	—	2.70	2.20	0.18	0.26
4	—	2.80	2.28	1.16	0.94
5	++	3.55	3.25	0.48	0.48
6	+++	4.00	3.58	0.86	0.56
7	+	3.08	2.25	1.70	0.96

Samples were taken during ECAT scanning as indicated in Fig. 2, some preliminary results are shown in Table II. The determination of L-dopa in these patients was thought to be of value since previous workers have shown that clinical response can be related to plasma L-dopa concentration [30, 31]. Some evidence for this was obtained, as patients experiencing extra-pyramidal side effects following their acute dose tended to have higher levels of plasma L-dopa, viz. patients 5 and 6. The chromatograms from Sinemet-treated patients showed small carbidopa peaks, but no peaks corresponding to the retention time of DOPAC, 3-O-methyl-dopa or dopamine.

CONCLUSIONS

We have shown that citrate—phosphate buffers containing methanol and sulphonic acid ion-pairing agents give a good resolution of L-dopa and catecholamines on a reversed-phase (C_8) column. In contrast to Freed and Asmus [22] we found little column deterioration or aging with our system, and no interfering peaks in plasma samples. Any slight deterioration in column performance was usually due to proteinaceous material deposited on the head of the column — this was readily removed by use of a small spatula, followed by topping up with fresh packing material. With continuous pumping of mobile phase, resurfacing of the glassy carbon electrode was only occasionally necessary and the start-up time was a matter of minutes. The proposed method for the HPLC—amperometric assay of L-dopa in human plasma, would therefore seem to be suitable for the study of the effects of L-dopa (given as Sinemet) on the cerebral blood flow and metabolism of patients with Parkinson's disease and for therapeutic monitoring.

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Journal of Chromatography, 277 (1983) 125—136
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1789

ELECTROCHEMICAL ACTIVITY OF *o*-PHTHALALDEHYDE—MERCAPTOETHANOL DERIVATIVES OF AMINO ACIDS

APPLICATION TO HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF AMINO ACIDS IN PLASMA AND OTHER BIOLOGICAL MATERIALS

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(First received February 14th, 1983; revised manuscript received April 11th, 1983)

SUMMARY

o-Phthalaldehyde—mercaptoethanol (OPA) reagent is used to increase the sensitivity of post-column detection of amino acids after chromatographic separation. OPA-amino acids themselves chromatograph well on reversed-phase high-performance liquid chromatographic (HPLC) columns and can be determined by fluorimetric detection, enabling OPA to be used as a pre-column derivatisation reagent. In this paper electrochemical detection (in the 0.4—0.7 V range) of OPA-amino acids following reversed-phase HPLC is described. Using fluorimetric and electrochemical detection in series, confirmation of the identities of the amino acids can be obtained in a single chromatographic run. Particular amino acids (e.g. basic amino acids) are active at the lower potentials, so that they can be selectively detected if required. Other amino acids and peptides whose OPA derivatives have little or no fluorescent activity are electroactive, permitting their detection by HPLC of their OPA derivatives.

Application of this methodology to divers biological samples is illustrated. OPA derivatisation of amines is an example of a reaction in which the product is electroactive at a lower potential than the reactants. This type of reaction is likely to be particularly useful in extending the applications of electrochemical detection in HPLC.

INTRODUCTION

The sensitivity of traditional ion-exchange procedures for amino acid determination has been enhanced by the use of fluorogenic derivatisation reagents. Of these, *o*-phthalaldehyde—mercaptoethanol (OPA) reagent is notable because of its cheapness and its convenience due to its lack of intrinsic fluorescence,

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and its stability in water [1—3]. Polar amino acids are poorly retained on reversed-phase high-performance liquid chromatographic (HPLC) columns, but Lindroth and Mopper [4] showed that OPA derivatives of amino acids are well retained and resolved on reversed-phase columns. These authors and subsequently others [5, 6] described the determination of amino acids in biological materials, following pre-column derivatization with OPA, using reversed-phase HPLC with fluorescence detection.

Electrochemical detection (ED) provides a sensitive and specific method for compounds possessing intrinsic electrochemical activity. We [7, 8] and others [9, 10] have previously discussed the advantages of series fluorescence and electrochemical detection. Most amino acids are not intrinsically electrochemically active within the currently useful potential range, with the exception of tryptophan, tyrosine and cysteine [11]. The structure of OPA-amino acids derivatives contains an isoindole grouping [4]. In view of the electrochemical activity of indoles (such as tryptophan) we set out to determine whether OPA derivatives of the amino acids could be detected electrochemically. In this paper we show that they can be, and demonstrate the HPLC analysis of amino acids in various materials, using fluorimetric and electrochemical detection in series.

MATERIALS AND METHODS

Chromatography

A 15 cm × 4.6 cm column, packed with Hypersil ODS 5 μm, a guard column type A (both from Chrompack, London, U.K.) and phosphate buffers with methanol as organic modifier were used. For isocratic elution an Altex 110A pump was used with 0.1 *M* sodium phosphate buffer (pH 7.0)—AnalaR grade methanol (50:50) as eluent. For gradient elution two Kontron 410 pumps controlled by a Kontron 200 programmer were used. The eluent was a mixture of 0.05 *M* sodium phosphate buffer (pH 5.5)—AnalaR grade methanol in the proportions 80:20 for solvent A and 20:80 for solvent B. Samples were introduced using a 7125 Rheodyne injection valve with a 20-μl loop. Fluorescence (Kratos Fluoromat 950, excitation: 365 nm band pass filter, emission: 470 nm cut-off filter) and electrochemical (Bioanalytical Systems TL5 and LC-4) detectors were used in series. Chromatographic equipment was supplied by Anachem. (Luton, U.K.) (Altex, Bioanalytical Systems), Konton (St Albans, U.K.) and Kratos-Schoeffel (Manchester, U.K.).

Analytical procedure

OPA reagent was made up in the proportions described by Lindroth and Mopper [4] by dissolving 27 mg of *o*-phthalaldehyde in 500 μl absolute ethanol, and adding 5 ml of 0.1 *M* sodium tetraborate (in place of 0.4 *M* boric acid brought to pH 9.5 with sodium hydroxide [4]) followed by 20 μl of mercaptoethanol. The reagent was kept overnight before use, and 10 μl mercaptoethanol added if required (each 1—2 weeks) to maintain maximal yield.

Plasma (50 μl) was deproteinised with 4 volumes of AnalaR grade methanol by thorough mixing, standing for 10 min at 4°C and centrifugation for 5 min

at 8000 *g*. Water (50 μl), or mixtures of appropriate standard amino acid solutions (typically 100 nmol/ml final strength) were carried through the same procedure. One volume of supernatant was reacted with 4 volumes of OPA reagent at room temperature. At a timed 2-min interval after mixing the reactants, a 20-μl aliquot was injected into the HPLC system. Other biological materials were also studied at appropriate dilutions and with suitable standards. Cerebral spinal fluid (CSF) was diluted up to 5 times with methanol, and reacted as above. Brain was homogenised in 10 volumes of methanol and the supernatant reacted as above. However, further dilution of up to 10-fold was needed for the more abundant amino acids. Brain perfusates, in contrast, were reacted directly with an equal volume of OPA reagent. Gastric juice required up to a 5-fold dilution in addition to that of the standard procedure for plasma.

Standard amino acids, *o*-phthalaldehyde, mercaptoethanol, tryptophyl dipeptides and tryp-met-asp-pheNH_2 were obtained from Sigma (Poole, U.K.) and 3-hydroxykynurenine from Koch Light (Slough, U.K.). Bulk reagents were obtained from BDH (Poole, U.K.).

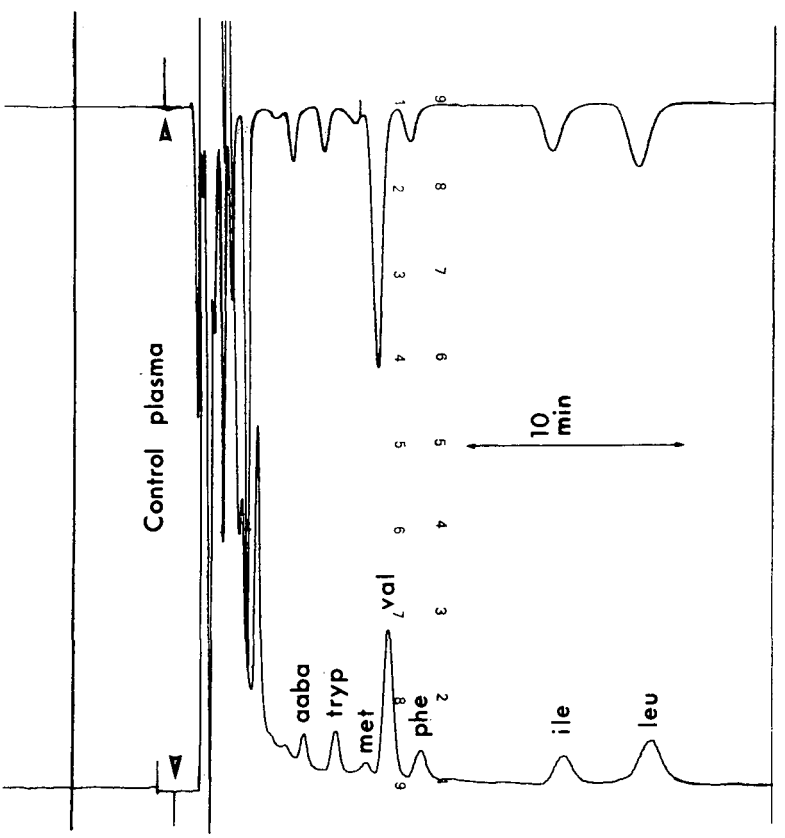

Fig. 1. Isocratic elution of large neutral amino acids in human plasma. Injection of 20 μl: equivalent to 1 μl plasma. Lower: ED trace at 1.0 V, f.s.d. = 100 nA; upper: fluorimetric trace (fluoromonitor 50 mV, × 10 setting). (Pen offset 24 sec in all chromatograms.)

RESULTS AND DISCUSSION

Using the isocratic conditions described, good resolution of a group of slower running (i.e. more hydrophobic) OPA-amino acid derivatives was achieved in about 20 min. Those detected in plasma were α-amino butyric acid (aaba), tryptophan (tryp), methionine (met), valine (val), phenylalanine (phe), isoleucine (ile) and leucine (leu) (Fig. 1). Recoveries of standards added to plasma were 95—100%. Each peak on the fluorescence detector was accompanied by an electrochemical peak with the working electrode set at 1.0 V (since the half-wave potential was expected to be in the range of the indole group). This group of amino acids in plasma is of interest, since it includes all but one (tyrosine, tyr) of a group of large neutral amino acids which compete with one another for uptake by the brain, affecting the rate of amino neurotransmitter synthesis [12].

For resolution of the full range of amino acids we utilised gradient elution (Fig. 2). Due to the higher methanol concentrations reached the molarity of the phosphate buffer was reduced by half. We were particularly concerned to resolve histidine (his) from glutamine (glu) for a particular application, and to resolve γ-aminobutyric acid (gaba) from alanine (ala), for use in brain studies. This was achieved by reducing the pH to 5.5 and utilising a gradient of 0 to 10% B over 10 min, then 10 to 85% B over 30 min, followed by 85 to 0% B in 5 min and 10 min re-equilibration. Resolution of asparagine (asp) from glutamate (glu) and tryptophan from methionine are incomplete. Variation of

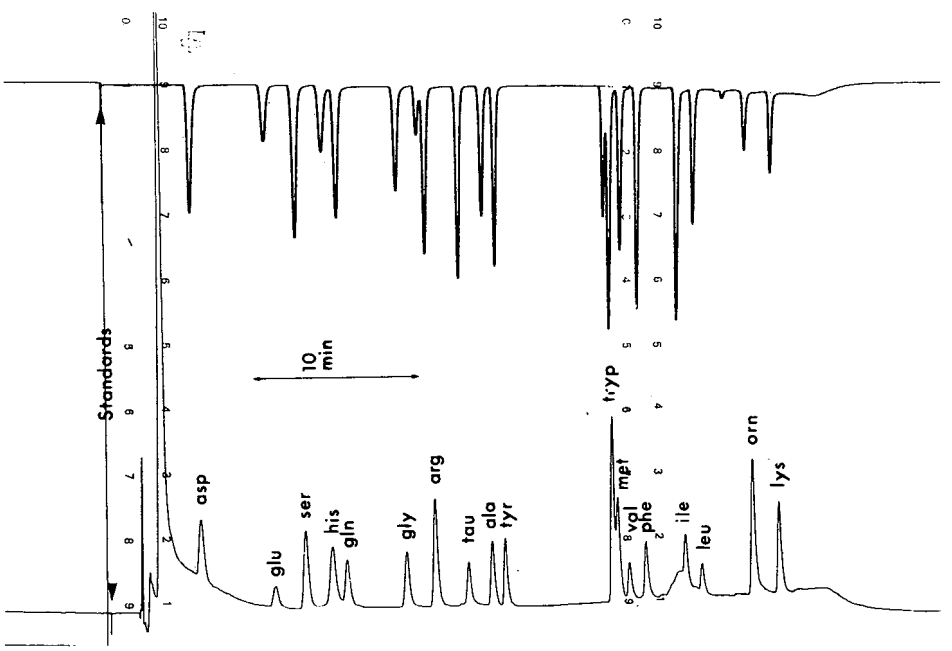

Fig. 2. Gradient elution of amino acid standard mixture 400 pmol each on column. Lower: ED trace at 0.5 V, f.s.d. = 50 nA; first peak is due to reagent (see text); upper: fluorimetric trace (Fluoromat 10 mV, × 1 setting).

the buffer species and its molarity, and of the pH and of the gradient profile permits optimisation for particular applications.

It is often stated that gradient elution is incompatible with ED. We have previously reported [13] that this is not the case for step gradient elution, provided that precautions are taken to minimise metal ion contamination of the eluent. The same holds for continuous gradient elution, the principal modification required being the substitution of sintered glass for metal frits on the solvent inlet lines.

The chromatogram of standard amino acids in Fig. 2 shows that with gradient elution also each fluorescence peak is accompanied by an electrochemical peak. Variation of the operating potential of the electrochemical detector revealed that the isoindolic OPA derivatives are active at quite low operating potentials (in this case 0.5 V) in contrast to the indoles and phenols which require a potential of 0.8—1.0 V. At this lower potential, baseline disturbance due to the gradient is small, as is the unretained peak. The ED trace from a blank also shows fewer interferences than the fluorimetric detector trace.

Since the fluorescent and electrochemical peak heights do not co-vary across different amino acids, the ratio of fluorescent to electrochemical activity at a

TABLE I

RATIOS OF FLUORESCENT TO ELECTROCHEMICAL ACTIVITY FOR AMINO ACIDS IN STANDARD MIXTURE AND IN HUMAN PLASMA

Amino acid	FL/$EC_{0.5}$ ratio*	
	Standard	Plasma
Isoleucine	4.47	5.43
Leucine	4.00	4.17
Valine	3.54	3.40
Phenylalanine	3.50	3.83
Taurine	3.45	3.88
(α-Aminobutyric)		2.70
Tyrosine	2.51	2.75
(Asparagine)		2.44
Glutamine	2.53	2.24
Glutamate	2.36	2.42
Aspartate	2.00	
(Threonine)		2.00
Methionine	2.00	
Alanine	1.92	1.78
Glycine	1.85	1.81
Serine	1.83	1.85
Arginine	1.45	1.50
Histidine	1.08	1.15
Lysine	0.82	0.80
Tryptophan	0.69	0.67
Ornithine	0.42	0.48

*FL/$EC_{0.5}$ ratio is the ratio of peak height on the fluorimetric detector at 10 mV × 1 to that on the electrochemical detector at 0.5 V, f.s.d. = 50 nA (settings of Figs. 2 and 3).

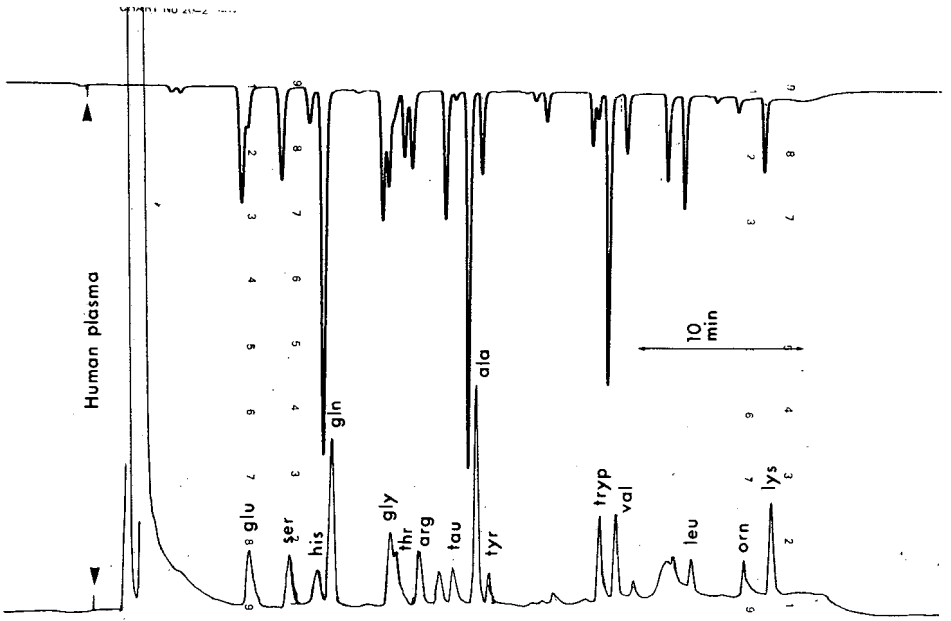

Fig. 3. Gradient elution of amino acids in human plasma. Conditions as Fig. 2.

given potential can be used to characterise the different amino acids. Table I shows the good agreement of the ratio obtained for standard amino acids, and for the amino acids in a human plasma sample (Fig. 3; the relatively poor agreement for isoleucine is probably due to interference on the ED trace).

If the operating potential is varied on successive runs, the electrochemical/fluorescence ratio for each amino acid increases with the potential, as expected. However, different amino acids show different profiles of activity (equivalent to different spectra). Thus at 0.4 V (Fig. 4A; same standard mixture as Fig. 3, ED sensitivity increased 2.5-fold) the basic amino acids retain a greater proportion of their electrochemical activity than the other amino acids. Conversely, differences between the amino acids are reduced at 0.6 V (Fig. 4B). If the operating potential is systematically varied from 0.4 to 0.7 V then profiles such as those shown in Fig. 5 are obtained.

OPA-amino acid derivatives show a less steeply increasing electrochemical activity with working potential (Fig. 5) than do simpler compounds, (e.g. catechols, indoles) presumably due to the interaction of multiple oxidisable groups in each derivative. The curves for the different amino acids fall into groups related to the chemical structure of the amino acid residue. The groups into which the profiles fell (of which space permits only representative examples in Fig. 5) include, in approximately ascending order of electrochemical/fluorescence ratio: 1, large neutral (val, leu, ile, phe) and taurine; 2, acidic (glu, asp); 3, small neutral (gly, ala, ser, thr); 4, basic (arg, his, lys, tryp, orn); 5, atypical: some amino acids show a steeper profile (tyr, his).

These observations imply that the amino acid residues, even those that are not intrinsically electroactive, will affect the electrochemical profile of the S-

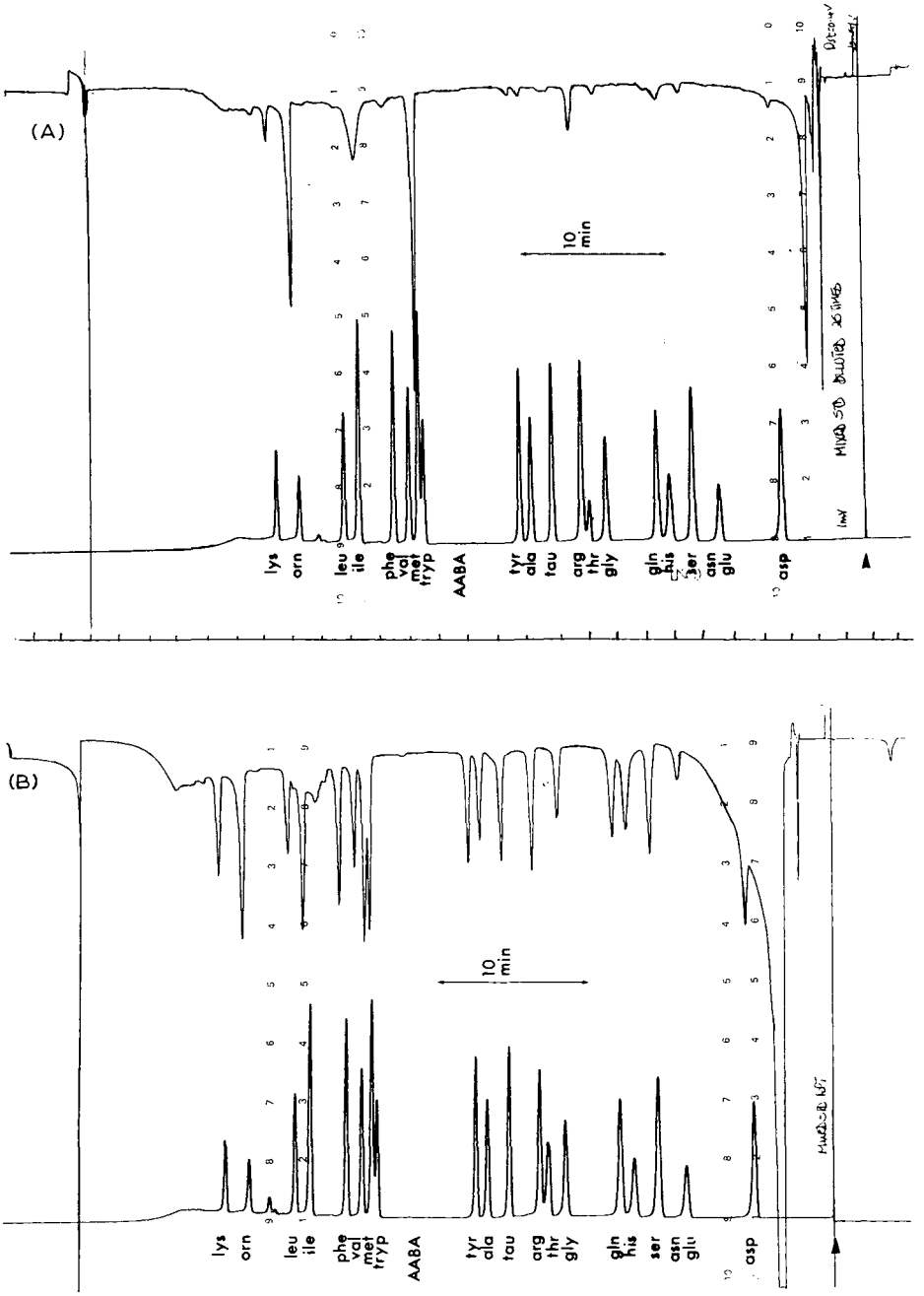

Fig. 4. Variation of electrochemical detector potential. Standard amino acid mixture, and lower (fluorimetric) trace exactly as Fig. 2; upper (ED) trace (A) at 0.4 V, f.s.d. = 20 nA; (B) at 0.6 V, f.s.d. = 50 nA. (N.B.: time zero at right.)

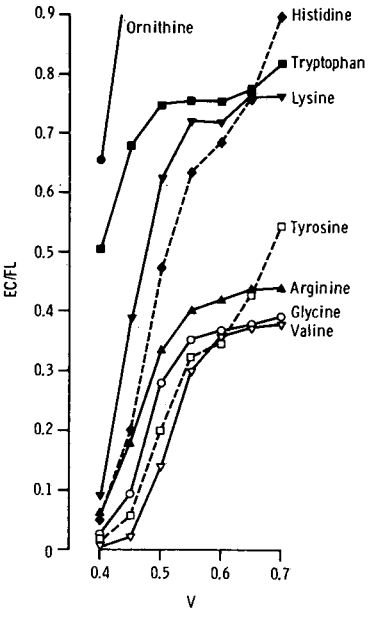

Fig. 5. Variation of electrochemical activity with working electrode potential. Vertical axis: peak height ratio of ED response (corrected to 50 nA f.s.d.) to fluorimetric response (at 10 mV × 1) for equimolar amounts of the amino acids stated.

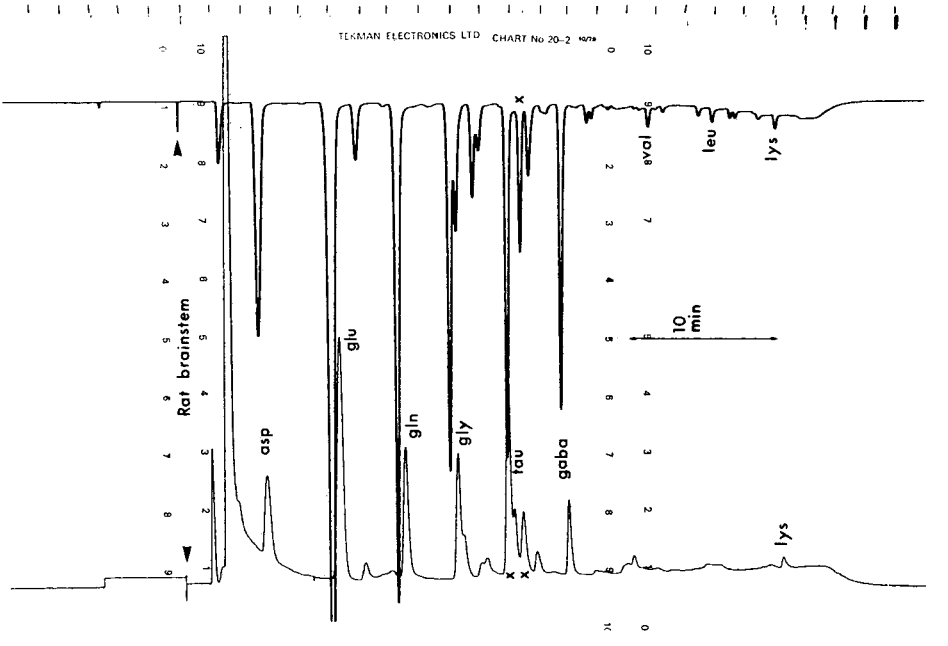

Fig. 6. Amino acids in rat brainstem. Conditions as Fig. 2, but fluorimetric detector, × 0.5 setting.

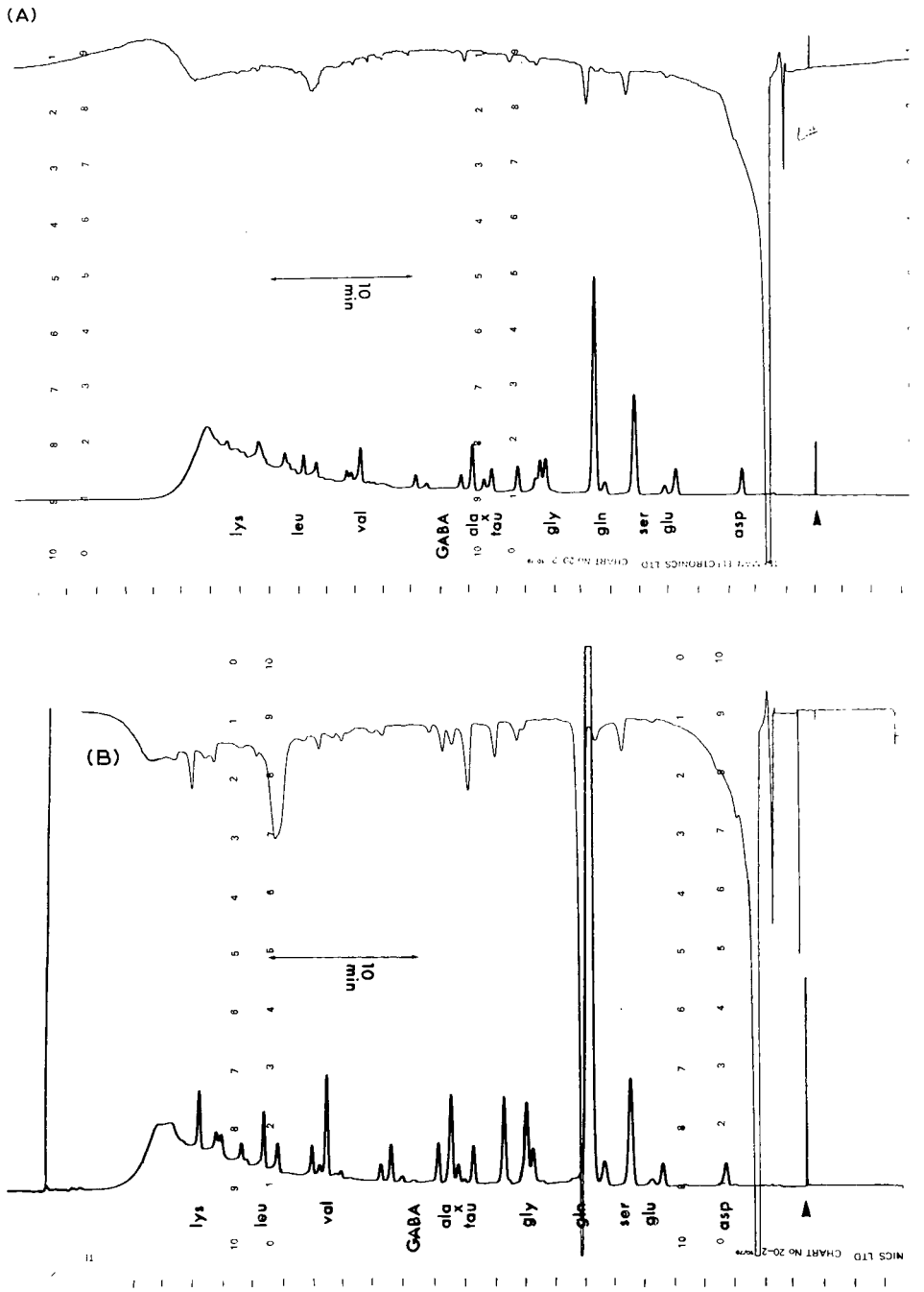

Fig. 7. (A) Amino acids in push—pull perfusate of rat hippocampus. Conditions: upper (ED) trace at 0.5 V, f.s.d. = 20 nA; lower fluorimetric trace 10 mV × 0.2 setting. (B) Amino acids in human lumbar CSF. Conditions: as A but ED trace (upper); f.s.d. = 50 nA.

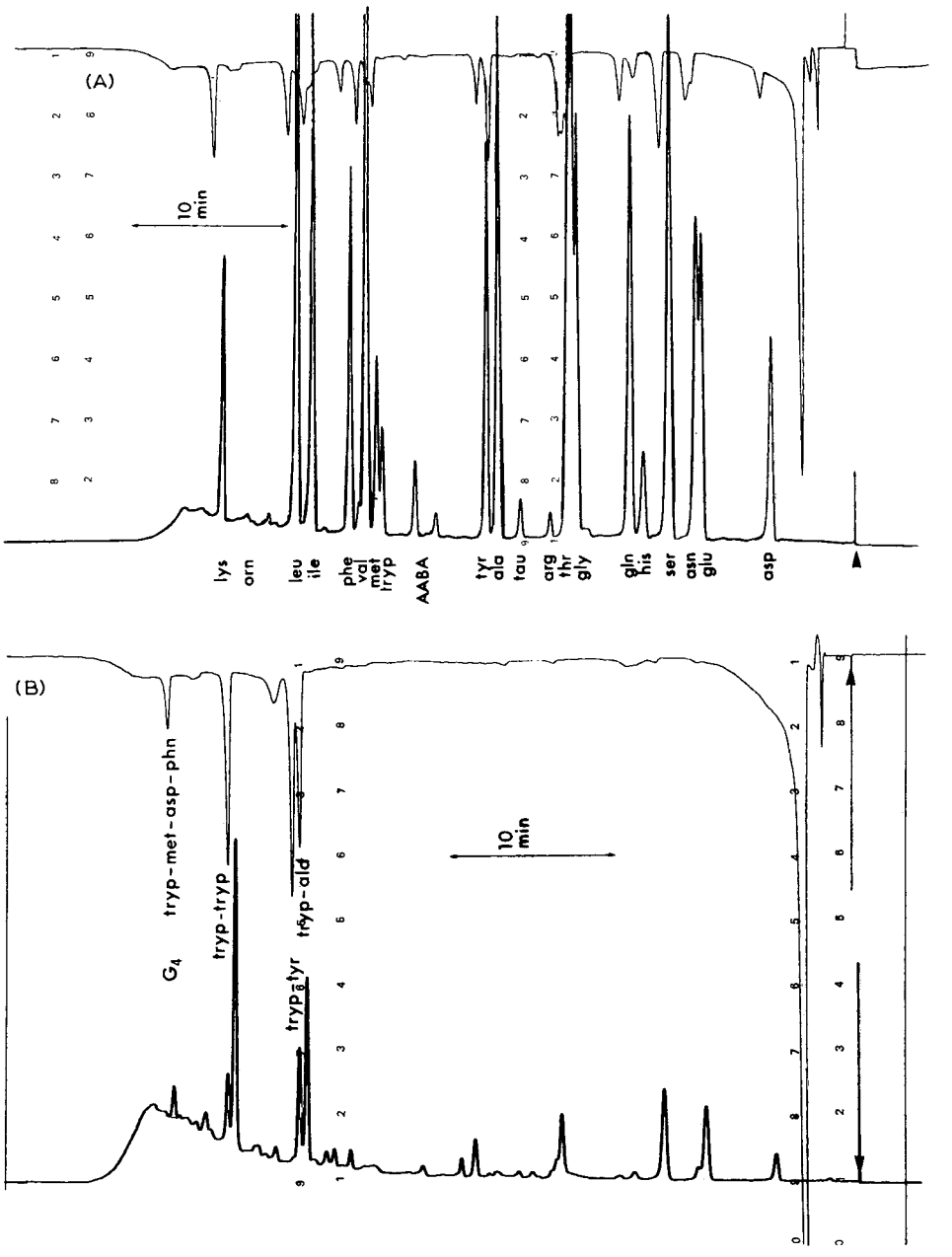

Fig. 8. (A) Amino acids in human gastric juice. Conditions: as Fig. 7B, but fluorimetric trace (lower) at 10 mV × 0.5 setting. (B) Separation of tryptophyl di- and tetra-peptide standards. Conditions as in Fig. 7B, but fluorimetric trace (lower) at 10 mV × 0.1 setting.

substituted isoindole which is common to all the derivatives. From inspection of Fig. 5 it can be anticipated that setting the potential at 0.5 V gives the most even spread of fluorescence to electrochemical ratio across the different amino acids. This indeed was the case in our hands, and is the basis of the selection of the $FI/EC_{0.5}$ ratio (see legend to Table I) in compiling Table I.

Figs. 6—8A demonstrate the application of the method to a variety of biological samples; rat brain (tissue extract), rat brain push—pull perfusate [14], human CSF and human gastric juice. The brain amino acid pool is dominated by aspartate, glutamate, glutamine, glycine, taurine and gaba. Since these are all among the faster running amino acid derivatives, an accelerated gradient could be used for rapid determination of brain amino acids. In this way the method can be modified to suit particular requirements. Electrochemical together with fluorimetric detection of OPA-amino acids offers improved versatility and specificity in the HPLC of the amino acids commonly found in biological materials.

OPA derivatives of a number of amino acids, including cysteine, kynurenine and 3-hydroxykynurenine show little or no fluorescence at the wavelengths usually employed for fluorimetric detection. These amino acids do however form derivatives, since we are able to detect them electrochemically (results not shown). OPA derivatives of the N-terminal amino acid of small peptides are also formed, but show marked fluorescence quenching compared to single amino acids. Fig. 8B shows the separation of standards of tryp-ala, tryp-tyr, tryp-tryp and C-terminal tetrapeptide common to gastrin and CCK, tryp-met-asp-phe-NH_2 (G_4). Variation of the working potential gave the results in Fig. 9, showing that while the fluorescent yield of the OPA derivative falls by some 32-fold in going from tryptophan to G_4, the electrochemical yield falls by 16-fold at 0.4 V and only 3-fold at 0.55—0.60 V. Expressed another way, ED at settings which give approximately equally sensitivity to fluorimetric detection for tryptophan, gives 3 to 8 times more sensitivity than fluorimetric detection for the dipeptides and 8 to 12 times more sensitivity than fluorimetric detection for G_4.

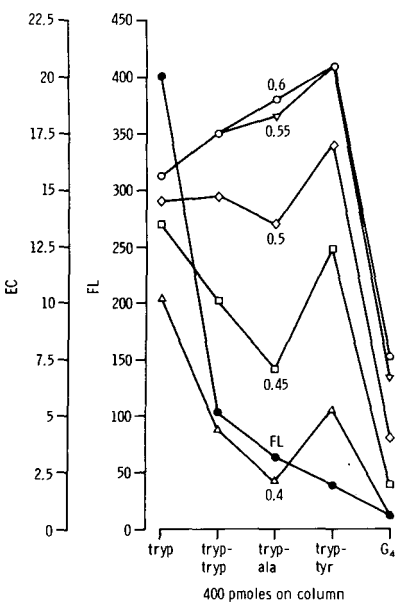

Fig. 9. Electrochemical and fluorescent activity with increasing peptide length. ED: peak height in nA at stated potential; fluorimetric detection: peak height in mm (200 mm = 1 mV).

Thus, in addition to its application to the HPLC determination of the usual amino acids, ED offers considerable promise for the determination of OPA derivatives of other amino acids and small peptides following HPLC separation. In addition to its specific application to the amino acid/peptide field, it is anticipated that the general technique, of which this is an example, of making derivatives that are electrochemically active in a range markedly lower than that of the parent species and of the derivatisation reagents will enable the scope of ED to be considerably enlarged.

ACKNOWLEDGEMENTS

We thank Peter Lindroth and Björn Josefsson (University of Göteborg, Sweden) for helpful correspondence and suggestions. The rat hippocampus push–pull perfusate samples were studied in collaboration with Annette Dolphin (NIMR, Mill Hill). Clinical samples were obtained under protocols approved by the Northwick Park–CRC ethical committee, or during routine patient care. Phillip Davies was an Applied Biology student, on placement from South Glamorgan Institute of Higher Education, Llandaff, Cardiff.

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Journal of Chromatography, 277 (1983) 137—144
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1764

RAPID, ISOCRATIC SEPARATION OF PURINE NUCLEOTIDES USING STRONG ANION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received November 30th, 1982; revised manuscript received April 18th, 1983)

SUMMARY

A method is presented for the rapid, isocratic separation of purine nucleotides using strong anion-exchange high-performance liquid chromatography at ambient temperature. The last peak of interest guanosine 5'-triphosphate (GTP) is eluted within 30 min and immediate reinjection is possible. All adenine and guanine nucleotides can be assayed with a single injection without the use of a gradient for elution. The procedure is particularly useful for the assay of NTP:AMP phosphotransferase reactions and/or the determination of changes in size of cellular purine nucleotide pools and computation of energy charges. An Altex Ultrasil AX prepacked column was used, and virtually identical results were obtained under similar conditions with the Whatman Partisil-10 SAX column. The eluting solution was 200 m*M* potassium phosphate, pH 6.85.

INTRODUCTION

The degree of energy charge loss from cells, and in particular the size of the purine nucleotide pool, is of importance in determining the recoverability of cells from periods of ischemia. Since AMP may be lost from cells irreversibly as adenosine through the action of 5'-nucleotidase, two enzymes that favor the formation of AMP during ischemia are of interest. These are adenylate kinase and guanosine 5'-triphosphate (GTP):AMP phosphotransferase, which catalyze the following freely reversible reactions:

*Some of the studies reported here are in partial fulfillment of the Ph.D. dissertation requirement.

0378-4347/83/$03.00

Adenylate kinase: 2 ADP ⇌ ATP + AMP
GTP:AMP phosphotransferase: ADP + GDP ⇌ GTP + AMP

Because of interest in the activities of these enzymes as well as determination of whole-cell energy charge under various conditions, a quick, accurate, and reproducible method for separating and quantitatively measuring purine nucleotides is required. Thin-layer chromatography was slow, with poor reproducibility, because of low concentration of reaction products. High-performance liquid chromatography (HPLC) seemed the method of choice, but the more universal reversed-phase ion-pairing technique failed to produce acceptable resolution of compounds of interest, probably due to interactions with the Tris buffer and other reaction components of the enzyme–substrate system. However, strong anion-exchange columns seemed to have none of the problems associated with multiple interactions between solvent, ion pairs, and reversed-phase support.

Other investigators have used the HPLC technique to determine the adenylate energy charge and/or specific nucleotide content of cells [1—10], or to assay various kinase reactions [10—12]. These preparations often require considerable sample cleanup for prolonged column life and all have used gradients to separate the mono-, di-, and triphosphate nucleotides, which produces a rising baseline with increasing ionic strength, and requires column re-equilibration before reinjection. By optimizing the eluent with respect to ionic strength, we have developed a method for the rapid, isocratic separations of adenine and guanine nucleotides with minimal sample cleanup. Elution of the slowest nucleotide, GTP, is complete within 30 min and immediate reinjection is possible. Only AMP and GMP have a resolution of less than 1 (0.93), which was within discrimination limits of the desk-top computing integrator.

METHODS

HPLC analysis of nucleotides

The HPLC assembly was a Tracor 900 series consisting of a Model 995 isochromatographic pump with a Model 970 variable-wavelength detector (Tracor Instruments), and a Rheodyne Model 7125 continuous-flow loop injector. The ultraviolet–visible detector was interfaced with a strip chart recorder and a Spectra-Physics System I computing integrator. The column was a prepacked, strong anion-exchange column (25 cm × 4.6 mm I.D.), the Altex Ultrasil AX with 10-μm diameter silica (Beckman Instruments). It was found that the Partisil-10 SAX (Whatman), of same dimensions and particle size, could be substituted with virtually identical results. A pre-column (25 cm × 4.6 mm I.D.) and a guard column (6.5 cm × 2.0 mm I.D.), both packed with 10—18 μm silica, were also used.

The eluent was 200 m*M* potassium phosphate buffer, pH 6.85, which was passed through a Millipore 0.45-μm filter and degassed prior to use. Flow conditions were typically 2.1 ml/min at ambient temperature with 186-bar back pressure of which about 48 bar was attributed to the precolumn. The column was stored under 100% ethanol when not in use, and equilibrated with about 40 ml buffer before use. Nucleotides were identified on the basis of

retention times (t_R) and detected by absorbance at 260 nm.

Concentrations of compounds of interest were computed by the external standard method utilizing standard predetermined time functions. The integrator was simultaneously calibrated for all six purine nucleotides by injection of an aqueous mixture containing the six standards. All standards were prepared from nucleotides of the highest purity available from Sigma, and as aqueous solutions of the sodium salt forms.

Enzyme assays

All enzyme assays were conducted in snap-cap polypropylene vials at 37°C. Reaction mixtures contained 4 m*M* $MgCl_2$, 1 m*M* $MnCl_2$, 50 m*M* Tris—HCl (pH 7.9), 1 m*M* nucleotide substrates, and an aliquot of enzyme preparation in a total volume of 100 µl. The substrates were either ATP plus AMP or ADP only for the adenylate kinase reaction, and either AMP plus GTP or ADP plus GDP for the GTP:AMP phosphotransferase reaction. Reactions were stopped by addition of 100 µl of 95% ethanol and after mixing were chilled to 0°C until analyzed.

Determination of purine nucleotide content and energy charge of isolated heart cells

Adult-rat cells were isolated by collagenase perfusion as described by Altschuld et al. [13]. The isolated cells were suspended in a calcium-free Krebs—Ringer phosphate buffer containing Na^+ (150 m*M*), K^+ (6 m*M*), Cl^- (126 m*M*), $MgSO_4$ (1.2 m*M*), phosphate (17.2 m*M*), taurine (7.5 mg/ml), creatine (3.35 mg/ml), 10 µl/ml vitamin mix (Gibco 100X), 10 µl/ml essential amino acid mix (Gibco 100X), 10 µl/ml non-essential amino acid mix (Gibco 100X), 0.68 m*M* glutamine, and bovine serum albumin (20 mg/ml), at pH 7.2. Cell suspensions (5 mg cell protein per ml) were incubated at 37°C, with and without glucose (11 m*M*), in a metabolic water bath under an atmosphere of oxygen or nitrogen. After 45 min, the cells from a 1-ml sample were centrifuged through 350 µl of bromododecane into 100 µl of 2 *N* perchloric acid, according to McCune and Harris [14]. The resulting acid extracts were neutralized with 50 µl of 0.2 *M* triethanolamine containing 2 *M* K_2CO_3 and centrifuged. A 50-µl sample aliquot was used for nucleotide analysis by HPLC.

RESULTS AND DISCUSSION

Fig. 1 illustrates the total resolution of all purine nucleotides at 2.5 nmoles each, except for AMP and GMP, which were near totally resolved. Table I lists these resolution (R) values, defined as $R = 2\,(t_2 - t_1)/(w_2 + w_1)$. AMP and GMP were eluted as slightly fused peaks and the resolution value of 0.93 rises or falls when the load was decreased or increased, respectively. A representative quantity of 2.5 nmoles was chosen as a midrange value that would be expected in nucleotide analyses. For other nucleotides, the resolution remained complete to 50 nmoles.

Table I also shows t_R values, theoretical plates per meter (N), and capacity

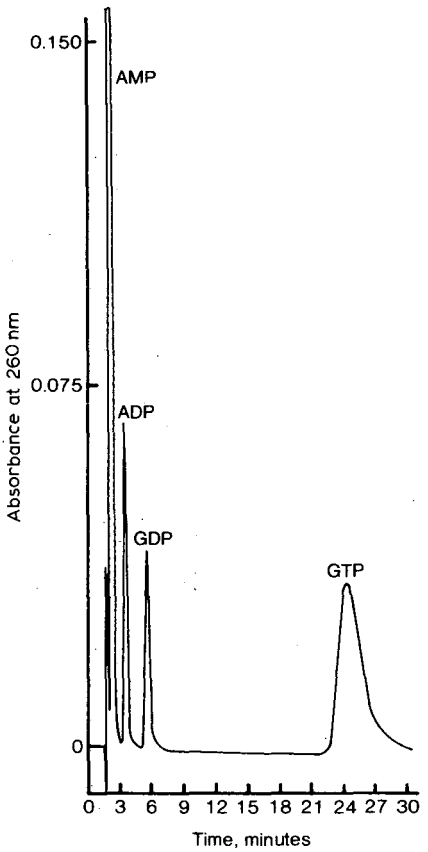

Fig. 1. Separation of purine nucleotide standards, 2.5 nmoles of each. For chromatographic conditions see Methods.

factor (k') values for all nucleotides. The number of plates per meter were calculated by:

$$N = 5.5\left[\frac{t_R}{w_{1/2}}\right]^2 \cdot \frac{1000 \text{ mm/m}}{250 \text{ mm column}}$$

where $w_{1/2}$ is the peak width at one-half of total peak height. The capacity factor was defined as $k' = (t_m - t_R)/t_m$, where t_m = 60 sec = one-half column volume/flow-rate. AMP and GMP had k' values at or above the recommended minimum of 1.00 and by fine-tuning the run parameters, the integrator had little difficulty computing individual peak areas. GTP has a rather high k' value but no other nucleotide elutes close enough to interfere with its resolution.

In order to determine linearity and reproducibility of the method, injections were made in triplicate. The average standard deviation was found to be 6.52% for adenine and guanine nucleotides in the range of 0.25 to 50 nmoles. The linear-regression equations indicated the correlation coefficient value for each nucleotide to be 0.99. These high values confirm the linearity of the detector response despite the wide range involved.

TABLE I

PURINE NUCLEOTIDE CHROMATOGRAPHIC SEPARATION DATA BASED UPON 2.5 NMOLES INJECTED FOR EACH NUCLEOTIDE

For chromatographic conditions see *HPLC analysis of nucleotides*. Equations used for plates per meter (N), capacity factor (k'), and resolution (R) values are described in Results and Discussion.

Nucleotide	t_R (sec)	N	k'	R
AMP	120	3912	1.00	
				0.93
GMP	142	3804	1.37	
				2.16
ADP	208	3372	2.47	
				3.08
GDP	342	3808	4.70	
				4.93
ATP	740	2832	11.5	
				4.42
GTP	1505	3140	24.1	

Figs. 2 and 3 illustrate the application of this HPLC method to the separation of nucleotides following GTP:AMP phosphotransferase and adenylate kinase reactions, respectively. Fig. 4 depicts the usefulness of the method for determining the size of purine nucleotide pools in isolated myocardial cells following incubation under aerobic conditions in the presence and absence of glucose. As expected, cells incubated in the presence of glucose (Fig. 4B) had a higher ATP content compared to cells that were incubated in its absence (Fig. 4A). The amount of GMP and GTP of cells under these conditions was too low to be detected. It appeared that the first peak (perhaps nucleosides) of both parts A and B to Fig. 4 might interfere with the quantitation of AMP. Though resolution was incomplete, refinement of integrator run parameters provided a trapezoidal baseline correction which maintained linearity of the peak area—concentration response.

The results in Table II show the effects on adenine nucleotide levels in heart cells subjected to anaerobic conditions. For comparison purposes, the effect of glucose is also shown. Although the amounts of adenine nucleotides generally declined when heart cells were incubated in an atmosphere of nitrogen, the decrease was much larger when glucose was unavailable. This was true regardless of whether the incubation was carried out under nitrogen or oxygen. The total nucleotide concentrations reported in Table II at time zero (before starting incubation), and after incubation without glucose (both aerobic and anaerobic), compare very well to previously reported values [13].

In summary, the HPLC procedure described here has many attractive features.

(a) Interference from enzyme reaction components does not occur in nucleotide determinations as shown by the chromatographic constants and analytical reproducibility.

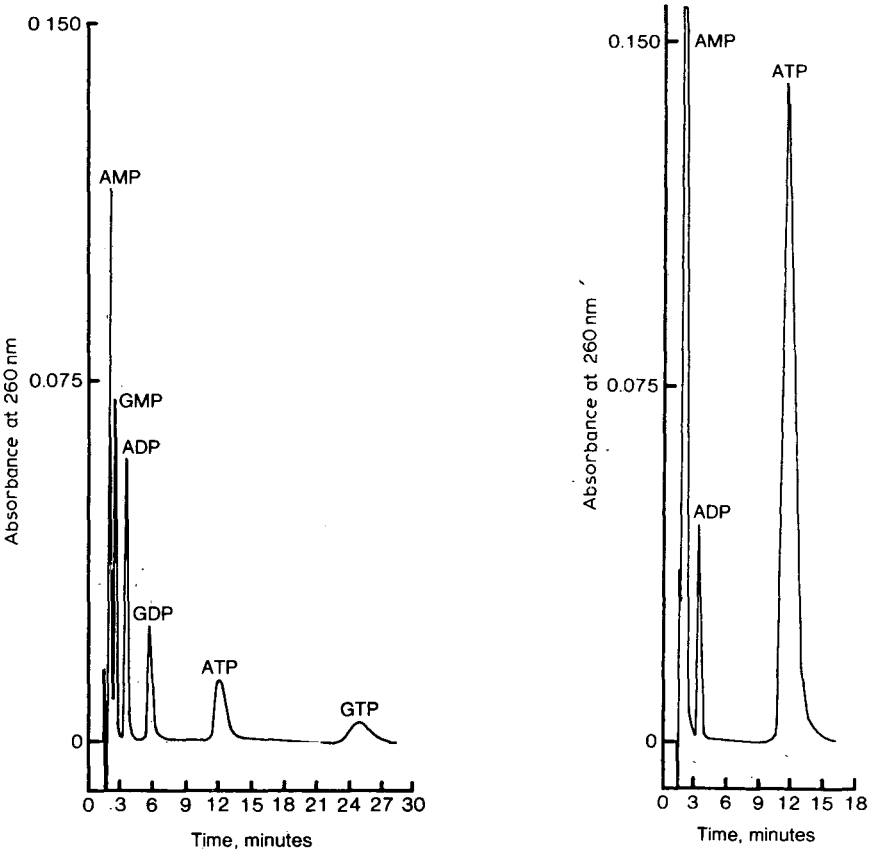

Fig. 2. Separation of reactants (AMP and GTP) from products (ADP and GDP) for a GTP:AMP phosphotransferase reaction, from a 50-μl injection of the sample directly without cleanup, as described in Methods.

Fig. 3. Separation of reactants (AMP and ATP) from product (ADP) for an adenylate kinase reaction, from a 50-μl injection of the sample without cleanup, as described in Methods.

(b) A simple eluting buffer at a pH value below 7.0 and nominal pressure assures reasonable column life and does not require any partial purification of the sample to be analyzed.

(c) The use of ambient room temperature does not require elevated temperatures that would contribute to solvent outgassing over extended periods of operation.

(d) The nucleotides of interest in these experiments are readily separated by isocratic elution employing a buffer of optimal ionic strength and pH. Analyses are rapid and time functions more constant than might be expected with a gradient.

(e) The utility of anion-exchange HPLC nucleotide analyses for heart cell extracts, standards, and enzyme-catalyzed reaction mixtures are shown by identical chromatographic constants.

TABLE II

EFFECT OF GLUCOSE UPON ADENINE NUCLEOTIDE POOLS OF ISOLATED MYOCARDIAL CELLS

Nucleotides were extracted into perchloric acid, which was then neutralized and a 50-μl aliquot used for HPLC analysis. Details of procedures provided in Methods. Time zero is a control value for nucleotides extracted from cells prior to incubation.

Nucleotide	Nucleotide concentration as nmoles per mg cell protein				
	Time zero	Incubated 45 min with glucose		Incubated 45 min without glucose	
		Aerobic	Anaerobic	Aerobic	Anaerobic
AMP	0.86	0.77	0.82	0.19	0.10
ADP	1.52	1.95	1.68	1.44	1.28
ATP	3.35	3.89	3.19	1.66	1.17
Total	5.73	6.60	5.68	3.29	2.55

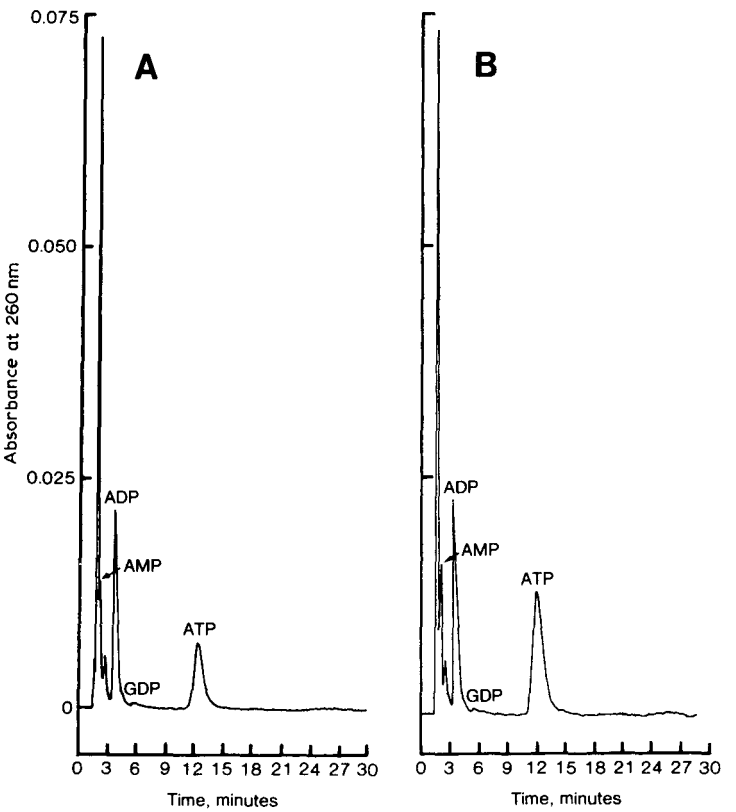

Fig. 4. Separation of purine nucleotides from 50-μl injections of neutralized perchloric acid extract of isolated myocardial cells incubated aerobically for 45 min. Part A represents incubation without glucose, and part B with glucose (11 m*M*) as described in Methods.

ACKNOWLEDGEMENTS

We are grateful to Dr. Gerald Brierley and his research group at Ohio State University for their assistance with studies on isolated heart cells.

These studies were supported in part by grants from the Miami Valley Chapter of the American Heart Association and the Biomedical Sciences Ph.D. Program at Wright State University.

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Journal of Chromatography, 277 (1983) 145—156
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1807

QUANTITATIVE ANALYSIS OF TOTAL THIAMINE IN HUMAN BLOOD, MILK AND CEREBROSPINAL FLUID BY REVERSED-PHASE ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received March 28th, 1983; revised manuscript received May 26th, 1983)

SUMMARY

Whole blood hemolysed by freezing, serum, cerebrospinal fluid, and milk of human origin were deproteinized by perchloric acid. Thiamine pyrophosphate and thiamine monophosphate were hydrolysed to thiamine by acid phosphatase. Chromatography was performed on C_{18}-coated silica using an *n*-octanesulfonate containing mobile phase methanol—aqueous citrate buffer pH 4.0 (45:55, v/v). In a post-column reaction $K_3Fe(CN)_6$ is used to oxidise thiamine to thiochrome, which is detected by fluorometry. Two ml blood is needed. The minimum detectable amount is 60 femtomol of thiamine. The intra-assay coefficient of variation (C.V.) is 2.3% and the inter-assay C.V. is 3.9%. The recovery of added thiamine pyrophosphate to blood samples was 98.7%. The reference range was found to be 88—157 nmol/l whole blood. Examples of the analysis of cerebrospinal fluid, serum and milk are given.

INTRODUCTION

The wide activity of thiamine (vitamin B_1) in carbohydrate metabolism is well known. Its main biologically active form, thiamine pyrophosphate (ThDP), is involved in the decarboxylation of α-keto acids and in the hexose-monophosphate shunt. Clinical aspects of thiamine deficiency have been extensively described (refs. 1—3, for example), such as in Wernicke's disease and in Korsakoff's psychosis. Alcohol abuse is accompanied by thiamine deficiency. Recently, attention was drawn to the occurrence of very high thiamine levels in postmortem serum of victims of sudden infant death syndrome [4], whereas the thiamine content of their erythrocytes, the erythrocyte transketolase activity and its stimulation by ThDP showed no abnormalities [4, 5].

0378-4347/83/$03.00

For diagnostic purposes, the direct quantification of total thiamine in whole blood, serum or erythrocytes is now advised. Determination of the ThDP-dependent transketolase activity may be useful to obtain extra information or whenever the direct analysis of total thiamine is not possible. Vitamin B_1 analyses have been performed by microbiological assays or by manual fluorometric methods (refs. 6—8, for example).

Recently the use of high-performance liquid chromatography (HPLC) for thiamine analysis in food, pharmaceutics, blood, etc., has been described (refs. 9—15, for example).

Most publications about thiamine analyses by HPLC deal with artificial mixtures, vitamin products or foods containing rather large amounts of thiamine. Only the method presented by Schrijver et al. [10] and the very similar but limited study of Kimura et al. [11] cope with the specific needs of a very low concentration (20—200 nmol/l) of thiamine and the complexity of the sample matrix of whole blood. Both groups converted the various thiamine phosphate esters present in blood to thiamine by enzymatic hydrolysis. Adsorption chromatography was performed using an aqueous mobile phase and either a silica [10] or a polyeter resin [11] stationary phase. Post-column derivatization and fluorometric detection of thiochrome completed the analysis. Especially the rapid deterioration of the silica column in thiamine analysis using adsorption chromatography [12] initiated our study.

We present an improved HPLC method of analysis of total thiamine in biological samples. Compared with the very few publications on this subject, our method is optimized with respect to sensitivity, time of analysis, chromatographic performance, sample treatment and costs. Reference values for total thiamine in blood are discussed.

MATERIALS

Acid phosphatase solution

Lyophilized acid phosphatase (EC 3.1.3.2) from potatoes (type II) was obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.; cat. no. P 3752), activity 0.4 units/mg solid. A solution of 10 mg enzyme per ml physiological saline was prepared just prior to use.

Taka-diastase solution

A solution of 200 mg Taka-diastase (Serva, Heidelberg, F.R.G.; cat. no. 35740) per ml of physiological saline was prepared.

Thiamine and ThDP solutions

Thiamine chloride hydrochloride (Merck, Darmstadt, F.R.G.; cat. no. 8181) and ThDP chloride (Boehringer, Mannheim, F.R.G.; cat. no. 133051) stock solutions contained approximately 30 μmol/l thiamine and 20 μmol/l ThDP, respectively, in 0.1 mol/l HCl. These solutions were stable in the dark at 4°C and the concentrations were calculated every month from their absorbance at 248 nm, as proposed by Penttinen [16]. However, after drying our thiamine as prescribed by the USP [17], we found a slightly higher value for the molar absorptivity (13,570 l/mol cm) than Penttinen. We used our own finding for

the determination of the stock concentrations. Two-hundred-fold dilutions with physiological saline of these stock solutions were used as working solutions for the chromatographic analysis.

Internal standard solution

This was an aqueous solution containing approx. 15 μmol/l salicylamide (2-hydroxybenzamide, Sigma Chemical Company; cat. no. S 07050), 0.6 mol/l sodium hydroxide and 1.8 mol/l sodium acetate.

Mobile phase

Methanol–aqueous sodium citrate buffer pH 4.0, 0.05 mol/l (45:55, v/v) was used. Sodium 1-octanesulfonate (Eastman Kodak, Rochester, NY, U.S.A.; cat. no. 10265) was added in a concentration 10 mmol/l of mixture. Degassing of the mobile phase was performed by ultrasonication under vacuum for 10 min. Flushing with a stream of helium gas did not improve the fluorescence intensity obtained during analysis and was considered unnecessary.

Oxidizing reagent

This was an aqueous solution of 2.5 mmol/l $K_3Fe(CN)_6$ and 3.0 mol/l NaOH. No degassing was necessary. The solution was stable for at least one month stored at room temperature and protected from light.

Reagents

HPLC grade methanol was obtained from Rathburn Chemicals (Walkerburn, U.K.). Reagent grade water was delivered by our Milli-Q deionization unit (Millipore, Bedford, MA, U.S.A.). Perchloric acid solutions 3.0 and 1.2 mol/l were obtained by dilution of 70% perchloric acid (Merck; cat. no 519). Reagents not specified were of analytical grade.

METHODS

Sample preparation

Whole human blood. Venous blood samples were collected in evacuated tubes containing either sodium heparinate or potassium ethylenediaminetetraacetate as anticoagulant. Blood samples were stored at −20° C. After thawing and homogenizing the sample, 2 ml were mixed vigorously with 2 ml of 1.2 mol/l cold perchloric acid and kept at 0° C for 15 min. Thereafter this mixture was centrifuged for 20 min at 2000 *g* and 4° C.

One milliliter of clear supernatant was mixed with 0.5 ml of internal standard solution. Finally 0.1 ml of acid phosphatase solution was added. Enzymatic hydrolysis occurred during overnight incubation at 37° C. Final pH is about 5.2. Samples treated this way were either directly analysed or stored at −20° C. Keeping prepared samples for 15 h at room temperature did not noticeably reduce their thiamine content.

Human serum or cerebrospinal fluid (CSF). Samples were stored at −20° C. After thawing and homogenizing, a 2-ml sample was mixed vigorously with 0.5 ml of 3.0 mol/l cold perchloric acid. The further procedure is as described for whole blood.

Human milk or manufactured baby milk. Samples were stored at −20°C and prepared just like whole blood. Centrifugation leads to a floating layer of fat and a clear solution, which is used for analysis.

Chromatographic analysis

The mobile phase was pumped at 1.2 ml/min by a Model 6000A pump (Waters Assoc., Milford, MA, U.S.A.). A 20-μl sample was injected by a Rheodyne valve (Model 7125, Rheodyne, Cotati, CA, U.S.A.). A μBondapak C_{18} analytical column (10-μm particles, column 30 cm × 3.9 mm, Waters Assoc.) and a corresponding guard column were used.

The post-column reactor consisted of a stainless-steel zero-dead-volume tee-piece (bore 0.25 mm, Valco Instruments, Houston, TX, U.S.A.) and a Teflon capillary (0.75 mm I.D., 1.8 mm O.D., 90 cm long) wound to a spiral tubular reactor 6.5 mm O.D. The Teflon coil was shielded for light. Column and post-column reactor were at ambient temperature (21—25°C).

A peristaltic pump delivered the oxidizing reagent at 0.3 ml/min; its pressure pulses were damped by a 1-m piece of silicon tubing between this pump and the tee-piece.

Detection was performed by a spectrofluorometer Model SFM-23LC (Kontron, Zürich, Switzerland) equipped with a 20-μl flow-through cell thermostatted at 25°C. The excitation was set at 367 nm, the emission was detected at 435 nm. The resulting signal was displayed on a Kipp BD 40 recorder (Kipp & Zonen, Delft, The Netherlands). Peak areas and retention times were calculated by a Minigrator computing integrator (Spectra Physics, Mountain View, CA, U.S.A.). A complete run took less than 7 min.

The total thiamine concentrations were calculated by the well-known internal standard method, using salicylamide as internal standard. The efficiency of the enzymatic hydrolysis during the sample preparation was checked for each batch of analyses by processing and analysing the working ThDP solution. A sample of a stock of frozen donor-blood was included in each batch of analyses for external quality-control purposes.

RESULTS AND DISCUSSION

Chromatographic experiences

The stability of thiamine is bad in alkaline solutions and fairly low in neutral aqueous solutions. However, in acid solutions it is very stable. Therefore, and because the pK_{a_1} is 4.8 [18], we selected the pH range 3.5—6.0 as most suitable for an ion-pair reversed-phase retention mechanism. Phosphate was often used as mobile phase constituent in HPLC analysis of thiamine (e.g. refs. 10—14). For our purpose its pK_a values are inappropriate. Moreover, the low solubility in water—methanol or water—ethanol of the Na_3PO_4 formed post column, leads to clogging of the detector. Citrate was found to be very useful as a buffer constituent.

A number of experiments were performed in which the mobile phase composition was optimized. A chromatogram obtained from the analysis of a test mixture is shown in Fig. 1. The separation of ThDP and ThMP (thiamine monophosphate) was even better at lower ionic strength or lower pH of the citrate

buffer. However, such change added one or two extra minutes to the total analysis time, which was considered a waste of time for the analysis of thiamine. We prefer hydrolysis of the ThDP and ThMP to thiamine because of its convenience for quantitation and because the fluorescence of the thiochrome phosphate esters differ from each other [16]. Besides, the analysis of a test mixture containing 0.1 mol/l HCl showed a slow spontaneous dissociation of ThDP to thiamine.

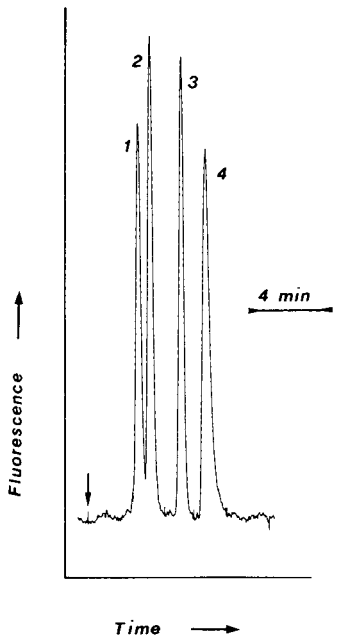

Fig. 1. Chromatogram obtained from a test mixture containing 61 nmol/l ThDP, 64 nmol/l ThMP, 7 μmol/l salicylamide and 82 nmol/l thiamine. For convenience the peaks are named after their parent compounds in the sample injected. Components elute in the order: 1, ThDP (k' = 0.3); 2, ThMP (k' = 0.6) 3, salicylamide (k' = 1.4) and 4, thiamine (k' = 1.9).

However, to demonstrate the suitability of our method to quantify also directly the ThDP content of whole blood, Fig. 2 shows chromatograms of samples before enzymatic hydrolysis (being the faster analysis) and after hydrolysis (being the more accurate analysis). The column was rinsed with methanol—water (70:30, v/v) after the analysis of each batch of samples. Comparing the performance of a new column with a column used four days a week for five months, we found a 24% decrease in the number of theoretical plates and a 2% shift in capacity factor, k', for thiamine. The pump pressure needed was increased from 110 to 160 bar upon aging of the column. Our analysis time (less than 7 min) is much shorter than that reported previously [10, 11]. Hence, our chromatographic system is economical as well as very stable.

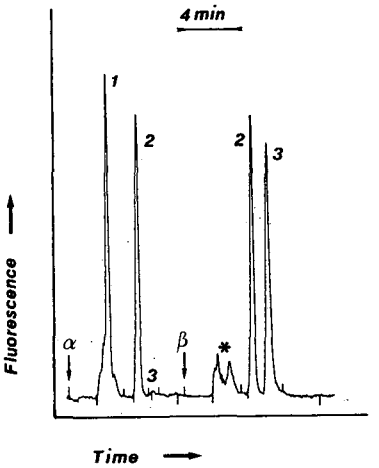

Fig. 2. Chromatograms obtained from a whole-blood sample containing 111 nmol/l total thiamine. The ThDP peak in sample α (without enzymatic hydrolysis) disappears and shows up as a thiamine peak in sample β (including enzymatic hydrolysis). 1, ThDP; 2, salicylamide; 3, thiamine. The asterisk (*) indicates unknown sample components mainly originating from the blood sample. (The new Kontron spectrofluorometer SFM 23B was used for this figure.)

Detection optimalization

The 45% (v/v) methanol in the mobile phase not only serves as a solvent modifier. Its use enhances the fluorescence intensity of thiochrome nearly three-fold and thus improves the signal-to-noise ratio. The observed enhancement of thiochrome fluorescence by the presence of different concentrations of either methanol or ethanol in the solution corresponds closely to Penttinen's results [16].

We tested the suitability of water—methanol (50:50, v/v) and water—ethanol (50:50, v/v) as solvent for the oxidizing reagent. No significant further increase of thiochrome fluorescence was noticed. Besides, methanol but especially ethanol, decreased the $K_3Fe(CN)_6$ stability in the reagent. Therefore, water became the solvent of choice for the oxidizing reagent and methanol was selected as constituent of the mobile phase. Wagner and Folkers [19] discussed the oxidation of thiamine. Important for the formation of thiochrome are the stabilization of a reaction intermediate by methanol and a pH of 11 or even higher. In our post-column reaction the pH is about 13 under the conditions given. The concentration of $K_3Fe(CN)_6$ does influence the thiochrome formation [7], as confirmed by own experiments. By varying both NaOH (1.0 to 3.75 mol/l) and $K_3Fe(CN)_6$ (0.5 to 8.0 mmol/l) concentrations the composition of the oxidizing reagent was optimized for the flow-rates employed.

Rapid mixing experiments using quartz cuvettes showed that thiochrome formation was complete (at least 95% converted) in about 15 sec under conditions equal to our post-column reaction. Obviously, an increased residence time in our post-column reactor leads to peak broadening. We selected a 90-cm length as an optimum for our Teflon capillary, corresponding to a 16-sec residence time.

Sample preparation experiences

Two different sample preparation procedures were tested; the TCA—diastase method as described by Schrijver et al. [10] using Taka-diastase, thiamine and ThDP solutions described under Materials; and a second method, as described in Methods, which will be called the $HClO_4$—phosphatase method. Deproteinization of the sample by trichloroacetic acid (TCA) and hydrolysis of the thiamine esters by Taka-diastase or Clarase have been used by several authors (e.g. refs. 6, 7, 10, 11). In view of several drawbacks that have been reported with TCA [6, 7] we tested denaturation with perchloric acid and found it very useful.

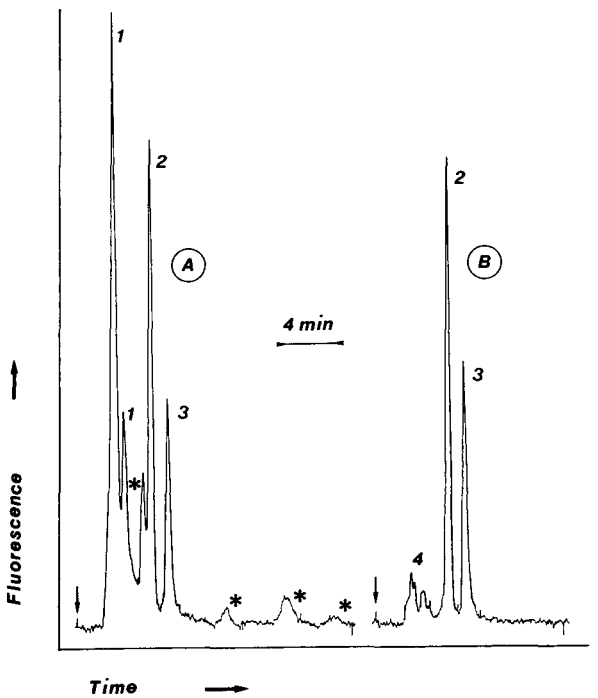

Fig. 3. Chromatograms demonstrating the differences between the TCA—diastase (A) and the $HClO_4$—phosphatase (B) treatments of the same whole-blood sample. Total thiamine contents corresponding to Fig. 3A and 3B were 107 and 126 nmol/l, respectively. 1, unknown sample components mainly from Taka-diastase; 2, salicylamide; 3, thiamine; 4, unknown sample components originating from the blood sample plus the acid phosphatase. Unknown impurities in Fig. 3A are indicated by asterisks (*).

The chromatogram presented in Fig. 3A was obtained from a whole-blood sample stored at −20° C and subjected to the TCA—diastase method. For blood samples stored at 4° C, the occurrence of fluorescent sample components eluting between the bulk of components near $k' = 0$ and the thiamine peak ($k' = 1.9$) was noticed more often. Fig. 3B shows the result of the $HClO_4$—phosphatase procedure for the same sample. The absence of the bulk of components (Fig. 3B) derived from a crude enzyme mixture allows an easy check of the completeness of ThDP and ThMP hydrolysis in the sample

analysed. In one experiment up to 3000 nmol/l ThDP were present in whole-blood samples fortified by ThDP addition. After $HClO_4$—phosphatase treatment no ThDP or ThMP could be found by chromatographic analysis. Reduction of the number and quantity of sample components makes the data handling easier and facilitates the insertion of an internal standard in the chromatogram.

The Taka-diastase preparation used contained a small amount of total thiamine of itself (in the order of 100 ng/g), whereas no total thiamine could be detected in the acid phosphatase.

The thiamine content of samples obtained by TCA—diastase treatment decreased upon freezing and thawing. The thiamine content of the sample in Fig. 3A was 119 nmol/l initially. The $HClO_4$-phosphatase method is clearly superior to the TCA—diastase method.

An example of analyses of whole-blood samples accompanied by a thiamine standard and a blank, is given in Fig. 4. Obviously, the chromatographic part of the assay can be easily automated.

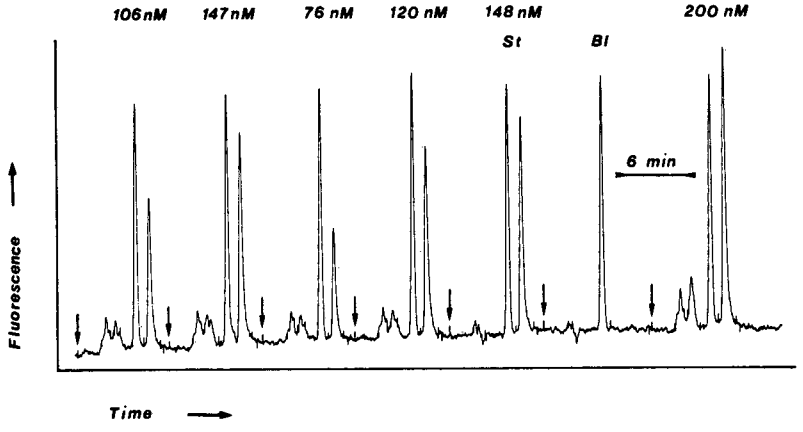

Fig. 4. Chromatograms showing five blood samples together with a thiamine standard (St) and a blank (Bl). Concentrations given are expressed in n*M* total thiamine in the original sample. The times of injection are indicated by arrows.

Thiamine or its esters gradually decomposed during storage of whole blood at 4°C. Five different samples of whole blood were divided and stored separately at 4°C and —20°C. A loss of 12—24% in total thiamine content was found for the 4°C samples and no loss for the frozen samples, after eleven days of storage. Myint and Houser [7] found no significant change in thiamine content upon storage at —20°C of serum and whole blood for eleven and two months, respectively. Hemolysing a blood sample by freezing—thawing increases its handling convenience and reduces the chance for sampling errors caused by sample inhomogeneity. It should be remembered that the total thiamine concentration in erythrocytes and leucocytes is one and two orders of magnitude higher, respectively, than in serum [6, 10].

Two final remarks must be made with regard to the $HClO_4$—phosphatase procedure. Salicylamide could not be included in the perchloric acid solution;

it became partially bound to the mass of denatured blood cells and proteins. No loss of salicylamide occurred when it was dissolved in the acetate solution.

Peak broadening or even splitting up the thiamine peak was noticed occasionally for samples having a pH below 5. If necessary, the pH of incubated samples or standards was adjusted to pH 5.2 by addition of 50 μl of 3 mol/l NaOH.

Precision, recovery and linearity

Donor blood containing disodium citrate as anticoagulant was frozen and thawed. After homogenization it was divided into portions and stored at −20°C in plastic tubes. Samples of such donor blood stocks were used for testing the precision, recovery and linearity of the method.

The intra-assay coefficient of variation, calculated from the analysis of ten samples from the same donor blood stock, was found to be 2.3% at a mean concentration of 74.3 nmol/l. The inter-assay coefficient of variation was found to be 3.9%, based on the analysis of ten donor blood samples in ten batches of analysis.

Because no absolute method was available for comparison, the accuracy of the method was investigated from recovery experiments. Weighed amounts of a 9.1 μmol/l ThDP solution in 0.1 mol/l HCl were mixed with known volumes of approximately 2 ml of donor blood containing 91 nmol/l total thiamine. A perfect linear relationship was found up to 400 nmol/l. Plotting the results of chromatographic analysis as a function of the expected thiamine concentration and using linear regression, we found $f(X) = 1.240 + 0.987X$ for $n = 9$; $r = 0.997$. Hence, the recovery was 98.7% of the ThDP added. At thiamine concentrations of 400–2000 nmol/l, a slight deviation of linearity was observed.

Based on the band width of the noise and considering a signal-to-noise ratio of 3 as the detection limit, the sample injected should contain at least 3 nmol/l thiamine, corresponding to an absolute amount of 60 fmol thiamine injected.

Reference values of total thiamine in blood

Whole-blood samples from 529 patients attending our hospital during two years were analysed elsewhere by the method of Schrijver et al. [10]. Analysing the distribution of the total thiamine content of these samples by a Bhattacharya plot as suggested by Naus et al. [20], we found a mean of 113 nmol/l and 95% confidence limits of 81–144 nmol/l. Taking into account that these samples had been stored at 4°C for up to one week before they were sent away for analysis, we conclude that these 95% limits are equal to the 95–155 nmol/l reference values published [10].

Next, we determined the total thiamine content in whole blood of 56 healthy volunteers (26 males and 30 females) by our own method. By means of the distribution-free method of Rümke and Bezemer [21] and setting the limits of percentiles at 2.5 and 97.5%, a reference range of 88–157 nmol/l was calculated with a reliability of 95%. The mean total thiamine content of our population was 117 nmol/l. The mean values for the 26 men and 30 women were 130 and 105 nmol/l, respectively. Fig. 5 shows the frequency histogram for our 56 volunteers. We believe that its skewness is partly caused by the different hematocrit values for males and females.

Table I summarizes some data on the total thiamine content of human whole blood gathered from the literature. A very good agreement exists between the HPLC analyses themselves and in comparison to the groups of older manual methods.

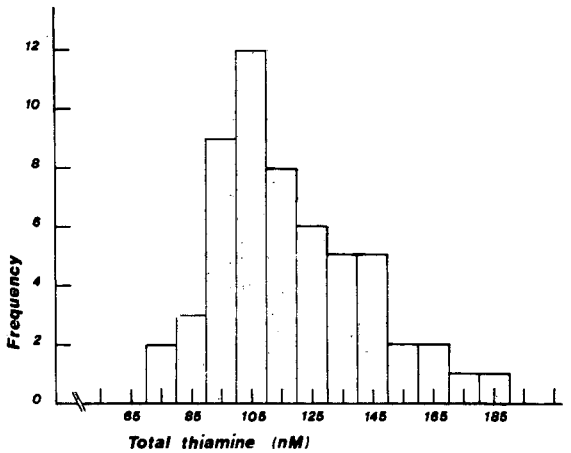

Fig. 5. Frequency histogram of the concentration of total thiamine (n*M*) in whole blood from 56 volunteers.

TABLE I

TOTAL THIAMINE CONCENTRATION IN HUMAN WHOLE BLOOD

Authors	Method	No. of samples	Range found (nmol/l)	Mean (nmol/l)
Burch et al. [6]	Manual—fluorometry	6	128—154	139
Myint and Houser [7]	Manual—fluorometry	44	34—142	87
Schrijver et al. [10]	HPLC—fluorometry	98	70—185	115
Kimura et al. [11]	HPLC—fluorometry	20	not given	137
Wielders and Mink (this study)	HPLC—fluorometry	56	71—185	117

Analysis of serum, CSF and milk

Chromatograms obtained from the assay of serum, CSF and milk are presented in Fig. 6. To our knowledge no HPLC method of thiamine analysis in CSF and (human) milk has been published before. The results obtained for a small number of samples of serum, CSF and milk are presented in Table II, together with data gathered from the literature. Our findings are very similar to those presented by authors using non-HPLC methods. Probably HPLC would be more accurate and less time-consuming than manual or microbiological methods.

Davis et al. [4] suggested a possible relationship between sudden infant death syndrome and the high thiamine content of manufactured baby milk in comparison to breast feeding. Therefore, three different brands of manufactured baby milk were analysed and found to contain 1520, 1450 and 2000 nmol/l total thiamine. The large discrepancy found between natural human milk and

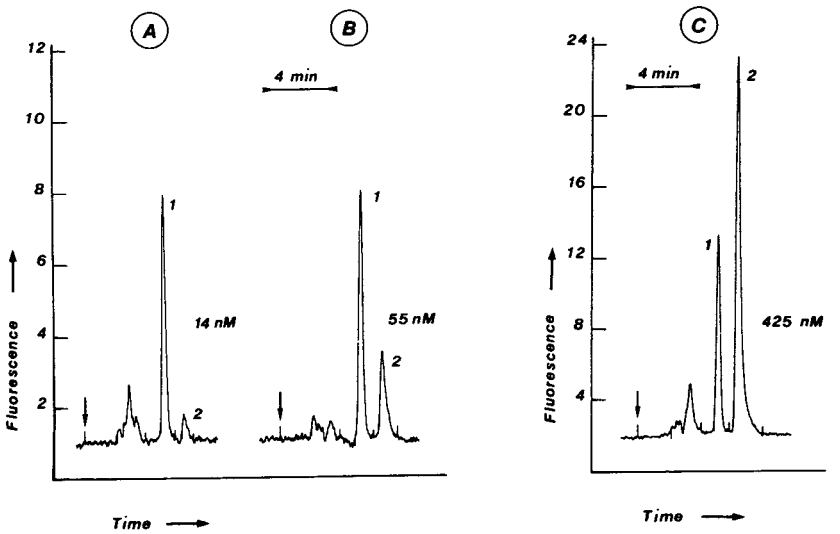

Fig. 6. Examples of typical chromatograms obtained from samples of human serum (A), human CSF (B) and human milk (C). Concentrations are given in nmol/l total thiamine in the original sample. Fluorescence intensity is given in mV recorder response. The salicylamide concentration in Fig. 6C was larger than in Fig. 6A and 6B. 1, salicylamide; 2, thiamine.

TABLE II

TOTAL THIAMINE CONCENTRATION IN HUMAN SERUM/PLASMA, CSF AND MILK

Thiamine or ThMP contents given in μg/l were converted to nmol/l.

Authors	Method	Material	No. of samples	Range found (nmol/l)	Mean (nmol/l)
Davis et al. [4]	Microbiological	Milk	9	163—1100	528
Burch et al. [6]	Manual—fluorometry	Plasma	6	15—30	24
Myint and Houser [7]	Manual—fluorometry	Serum	44	Trace—61	30
Rindi et al. [22]	Manual—fluorometry	CSF	20	not given	56
Wielders and Mink (this study)	HPLC—fluorometry	Milk	4	336—425	378
Wielders and Mink (this study)	HPLC—fluorometry	Serum	4	5—28	14
Wielders and Mink (this study)	HPLC—fluorometry	CSF	4	16—74	49

manufactured baby milk as far as the total thiamine content is concerned confirms Davis' analytical results. Our method will be useful for further study of this subject.

CONCLUSIONS

An optimalized and economical HPLC analysis for the quantification of total thiamine in biological samples at the nmol/l level is presented. The precision is high because of the selection of a purified enzyme preparation, the use of $HClO_4$ instead of TCA for protein precipitation, the insertion of an internal standard, and the fine chromatographic separations achieved. Recently,

Warnock [23] published an HPLC method for ThDP determination in erythrocytes using pre-column derivatization. His method was not able to measure free thiamine in a sample derived from erythrocytes, while ThDP is not stable at neutral pH. Together with Schrijver et al. [10] and Kimura [11], we prefer enzymatic hydrolysis of thiamine esters and on-line post-column derivatization to thiochrome. Our reference values confirm the only published data so far for HPLC analysis of thiamine in human blood [10]. Our method will be a valuable tool for monitoring thiamine content in CSF, serum and milk too.

ACKNOWLEDGEMENTS

We thank Mr. L.M.L. Habets for his technical assistance and his valuable contributions to the practical aspects of this study. The secretarial aspects were handled by Mrs. C.H.W.W. Wielders-Stassen.

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Journal of Chromatography, 277 (1983) 157—164
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1790

DETERMINATION OF ISOSORBIDE AS A METABOLITE OF ISOSORBIDE DINITRATE IN HUMAN URINE BY CAPILLARY GAS CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION

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(First received February 14th, 1983; revised manuscript received April 13th, 1983)

SUMMARY

A method for the determination of isosorbide as a metabolite of isosorbide dinitrate at concentrations down to 200 ng/ml in human urine is described. After addition of a known amount of isomannide as internal standard to 50 μl of urine, both compounds are extracted at basic pH into chloroform—isopropanol (4:1, v/v), which is then evaporated to dryness. They are then derivatized with heptafluorobutyric anhydride, and isosorbide is quantitated by capillary gas chromatography with electron-capture detection. A conjugate of isosorbide is determined in urine after enzymatic hydrolysis.

INTRODUCTION

Isosorbide dinitrate (ISDN) is a vasodilative organic nitrate. Very low plasma levels are reached after oral administration to man, and ISDN is detectable below its limit of quantitation in the 12—24 h urine only [1].

Several authors have reported that the major urinary metabolite of ISDN is the glucuronide of isosorbide. Rosseel and Bogaert [2] studied the urinary excretion of ISDN in rats and found (without quantitation) isosorbide before and after incubation of the urine for 20 h with β-glucuronidase—arylsulphatase. Sisenwine and Ruelius [3] measured plasma concentrations and urinary excretions of ISDN and its metabolites in the dog after administration of either an oral or intravenous dose of [^{14}C]ISDN; isosorbide and its polar conjugate in urine were measured after thin-layer chromatography and determination of radioactivity. Down et al. [4] studied the biotransformation of ISDN after oral administration of 5 mg of [^{14}C]ISDN and showed that nearly 50% of the drug was transformed to an isosorbide conjugate; concentrations of isosorbide were measured in urine after thin-layer chromatography and determination of radioactivity.

This present paper describes a procedure which permits the quantitative

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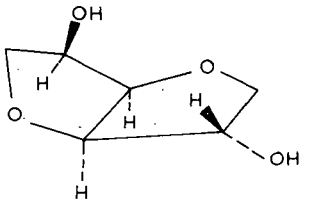
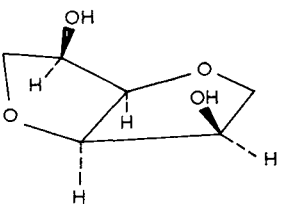


ISOSORBIDE



ISOMANNIDE

gas chromatographic determination of free isosorbide in human urine using isomannide as internal standard.

Conjugated isosorbide may also be assayed in urine after enzymatic hydrolysis. The limit of quantitation is 200 ng isosorbide per ml of urine.

EXPERIMENTAL

Chemicals and reagents

Isosorbide and isomannide were synthesized in our laboratories according to the method of Hockett and Fletcher [5] from D-sorbitol and D-mannitol, respectively. The solvents and reagents used are all of analytical grade: chloroform (Uvasol 2447; Merck, Darmstadt, F.R.G.), *n*-heptane (Merck, Uvasol 4366), isopropanol (Prolabo 20837; Rhône-Poulenc, Paris, France). Pyridine (Fluka 82702; Fluka, Buchs, Switzerland) was distilled at 115—116°C with potassium hydroxide pellets and stored over the same reagent. Heptafluorobutyric anhydride was purchased from Ventron (Ref. 1300-3; PCR, Karlsruhe, F.R.G.) and stored in 1-ml glass ampoules. Potassium carbonate (Merck 4928)—potassium hydrogen carbonate (Merck 4854) (2:3, w/w) was used as solid buffer. The extraction solvent is chloroform—isopropanol (4:1, v/v). Potassium dihydrogen phosphate (Merck 4873) was used as a saturated solution. The pH 5 buffer is prepared with 14.8 ml of 0.2 *M* acetic acid solution and 35.2 ml of 0.2 *M* sodium acetate solution, which are diluted up to 100 ml with water. The enzyme solution (β-glucuronidase—arylsulphatase) (Calbiochem, Los Angeles, CA, U.S.A.; B grade, 6.66 IU/ml β-glucuronidase, 3.41 IU/ml arylsulphatase) is diluted to one-tenth concentration with pH 5 buffer.

Calibration solutions

The methanolic solutions of isosorbide contain 10—250 ng per 25 μl. The methanolic solution of internal standard contains 250 ng of isomannide per 25 μl.

Equipment

A Hewlett-Packard Model 5880 A gas chromatograph equipped with computing integrator, electron-capture detector and automatic sampler is used. The column is a 12 m × 0.2 mm fused-silica capillary column coated with dimethylsilicone (OV-101) (Hewlett-Packard No. 19091-60010). Splitless injection is used with a 72-sec splitless period. The column is flushed with the carrier gas (argon—methane, 90:10) at a flow-rate of 2 ml/min; the septum purge is 5 ml/min and the auxiliary gas flow to the detector 30 ml/min. The injector temperature is 250°C and the detector is set at 300°C. The column

is at 80°C initially for 0.3 min and the temperature is then increased by 5°C/min up to 120°C; to wash out urine residues, it is held at 120°C for 1 min, then raised rapidly by 30°C/min up to 200°C for 15 min.

Enzymatic hydrolysis of isosorbide conjugates in urine

Twenty-five microliters of the internal standard solution are measured into a 10-ml glass tube, to which 50 μl of urine, 200 μl of pH 5 acetate buffer and 2.5 μl of β-glucuronidase—arylsulphatase mixture are added. This preparation is then kept for 24 h at 37°C and fresh enzyme (2.5 μl) is added four times during the incubation. The extraction is then performed as described below for free isosorbide.

Extraction of free isosorbide from urine

A 25-μl aliquot of the internal standard solution is measured into a glass tube, into which 50 μl of urine, 1 ml of distilled water and around 300 mg of potassium carbonate—potassium hydrogen carbonate (2:3, w/w) are then introduced. The stoppered tube is shaken on a Vortex mixer for 1 min, then 5 ml of chloroform—isopropanol (4:1, v/v) are added. The tube is restoppered and shaken mechanically for 15 min at 300 rpm with an Infors shaker, then centrifuged for 3 min at 2500 *g*. An aliquot of the organic phase is transferred to another tube and dried under a nitrogen stream at room temperature. Evaporation must be done very carefully and stopped just as the tube reaches dryness.

Derivatization and chromatography

To the dry residue are added 1 ml of heptane containing 0.5% pyridine and 10 μl of heptafluorobutyric anhydride. The medium is thoroughly mixed (Vortex mixer) for 20 sec and allowed to stand for 10 min at room temperature. Then 1 ml of potassium dihydrogen phosphate saturated solution is added, and the tube is shaken for 30 sec and centrifuged. The aqueous phase is frozen by immersing the tube in a methanol bath containing dry ice. An aliquot of heptane is transferred into a 250-μl conical glass flask (Hewlett-Packard 5080-8779) and a 1-μl portion is injected into the gas chromatograph using the splitless injection technique.

Calibration curves

Calibration samples are prepared by introducing 25 μl of a suitable methanolic isosorbide solution (10—250 ng) into 10-ml glass centrifuge tubes containing 50 μl of urine and a constant amount of internal standard (250 ng). The calibration curve is obtained from the ln—ln plot of the peak-area ratios versus the urine concentrations. The equation is calculated by the least-squares method. A calibration curve is prepared every day.

Human study

A healthy male subject, who had been advised to take no drugs during the fourteen days preceding the experiment and none besides ISDN throughout the duration of the study, received 5 mg of ISDN as one tablet of Isoket®. Urine was collected at the following time intervals: 0—4, 4—8, 8—12,

12—24, 24—48, 48—72, 72—96 and 96—104 h. The volume of each sample was measured and an aliquot was stored at −20°C.

RESULTS AND DISCUSSION

Reaction time

The derivatization was applied to isosorbide and isomannide, and the reaction time was varied from 5 to 60 min. Derivative formation was evaluated by peak areas; derivatization was almost immediate and the yield of isosorbide and isomannide was the same over the time range of 5—60 min. For convenience and safety, the suggested derivatization time is 10 min.

Capillary gas chromatography

The quantitative isosorbide determination by conventional packed column gas—liquid chromatography was difficult because of interfering peaks from the urine. The capillary column used here provides a suitable means of analysing isosorbide with the required efficiency.

Splitless injection

Splitless injection (solvent effect) is the appropriate technique for analysing samples containing trace-level components. To obtain a good solvent effect, the oven temperature must be low enough that the solvent does not leave the head of the column too quickly. This generally means that the oven should be 10—30°C below the boiling point of the solvent. In this technique, the sample components which are less volatile than the solvent (heptane) are reconcentrated at the beginning of the column during a 72-sec splitless period.

Urine hydrolysis of conjugated isosorbide

Preliminary experiments were carried out to determine the best conditions for the hydrolysis of the isosorbide conjugates in human urine. Stability tests showed that isosorbide and isomannide are stable for 24 h in human urine at 37°C. Samples of the same urine fraction, containing the conjugate of isosorbide, from a volunteer treated with ISDN were subjected to enzymatic hydrolysis. Enzymatic hydrolysis was studied in aliquots of this urine incubated with enzyme (β-glucuronidase—arylsulphatase) and acetate buffer (pH 5) for 2, 4, 6, 8, 22 and 24 h at 37°C; fresh enzyme (2.5 μl) was added four times during incubation. A maximum yield of isosorbide was obtained after incubation for 22 h.

Extraction

Isosorbide and isomannide are not extracted into organic solvents such as toluene or ethyl acetate. Mixed systems employing a non-polar solvent in combination with a highly polar solvent have, however, been used (chloroform containing isopropanol). Better isosorbide and isomannide extraction was obtained after saturation of urine samples with a basic solid buffer (potassium carbonate—potassium hydrogen carbonate, 2:3). The formation of emulsions with the extraction solvent was avoided by use of a solid buffer.

Linearity and reproducibility of calibration curves

Straight lines were obtained for calibration curves in the concentration range of 200—5000 ng/ml, and the method was validated over this concentration range (Table I). The day-to-day reproducibility of the standard curves was shown in three consecutive experiments carried out on separate days. On each occasion, the peak area ratio of isosorbide versus the internal standard (mean of duplicate analysis) plotted against five concentrations of isosorbide gave straight lines. A least-squares ln—ln regression line was generated from the fifteen data points of the three standard curves (Table I). It corresponds to the regression equation $\ln Y = 1.23276 \ln X - 10.7024$. A test of day-to-day reproducibility was made by expressing each data point as a percentage of the value read off in the ln—ln line for the corresponding concentration (Table I). The distribution of these normalized (concentration-independent) data had an overall average (± C.V.) of 100.9 ± 8.9%, demonstrating a good reproducibility between experiments. However, a variability between the curves is sometimes noted which is the reason why the calibration curve was generated daily.

TABLE I

LINEARITY AND REPRODUCIBILITY OF CALIBRATION CURVES

Concentration added to plasma (ng/ml)	Peak area ratio				$100 \times \frac{E}{C}$ (%)
	Experimental (E)			Calculated from ln—ln regression line (C)	
	Day 1	Day 2	Day 3		
200	0.015	0.018	0.012	0.015	100.0 120.0 80.0
500	0.051	0.051	0.044	0.048	106.3 106.3 91.7
1000	0.120	0.119	0.114	0.112	107.1 106.3 101.8
2000	0.275	0.279	0.260	0.264	104.2 105.7 98.5
5000	0.773	0.798	0.765	0.816	94.7 97.8 93.8
Average ± C.V. (%)					100.9 8.9

Precision and recovery

The repeatability of the method was checked by determining six urine samples spiked with several different concentrations. Table II shows the good reproducibility obtained with concentrations down to 200 ng/ml isosorbide. This concentration (200 ng/ml) may be taken as the limit of quantitation of the method.

TABLE II

PRECISION AND RECOVERY IN THE DETERMINATION OF ISOSORBIDE IN SPIKED HUMAN URINE

Amount added (ng/ml)	Mean amount found (ng/ml) (n = 6)	Precision (coefficient of variation)	Recovery (%)
200	196	4.6	98.2
500	500	2.7	100.0
1000	1010	4.2	101.0
2000	1946	4.1	97.3
5000	4730	0.6	94.6
Mean ± C.V. (%)			98.2 2.5

Interference from urine and the precursors of isosorbide

Fig. 1 shows the chromatograms of an extract of human urine (50 μl) and of the same extract spiked with 10 ng of isosorbide (200 ng/ml) and 250 ng of internal standard (isomannide). Isosorbide and the internal standard

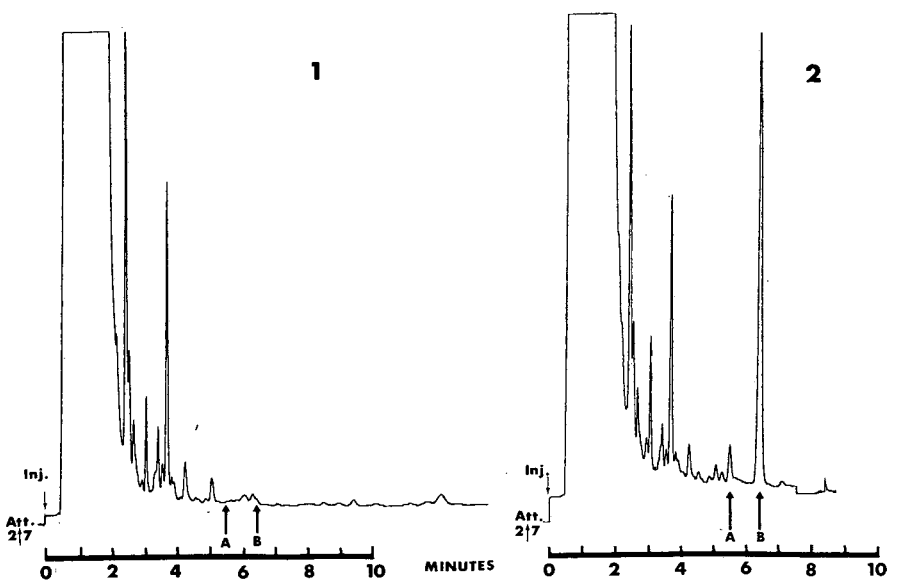


Fig. 1. Chromatograms of (1) human urine blank (50 μl urine); and (2) the same urine spiked with 10 ng of isosorbide (200 ng/ml) (A), and 250 ng of internal standard (B).

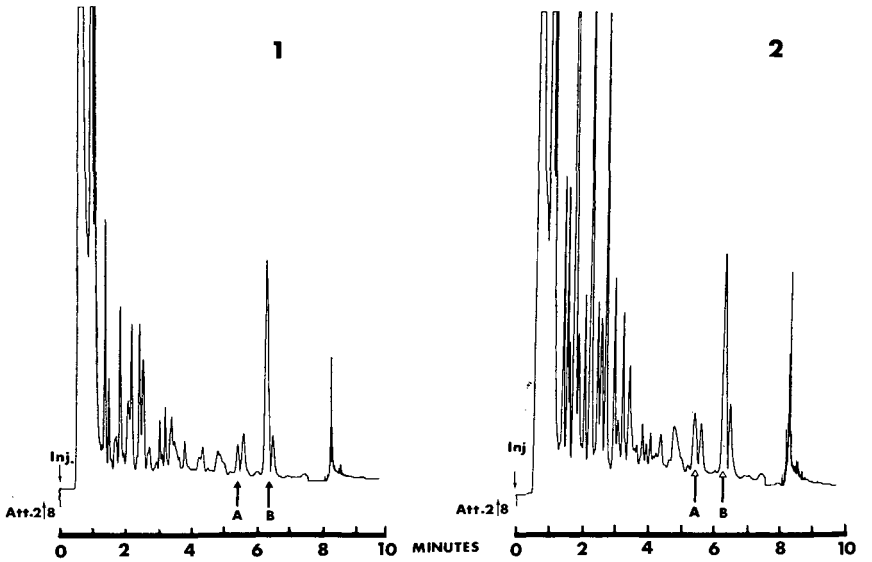


Fig. 2. Chromatograms of a urine aliquot (50 μl) collected between 4 and 8 h from a healthy subject treated with 5 mg of ISDN, (1) containing 696 ng/ml free isosorbide (A), and (2) containing 1296 ng/ml total isosorbide (free + conjugated, A) after enzymatic hydrolysis, both with 250 ng of internal standard (B).

are well separated from the normal components of the urine extract. Fig. 2 shows chromatograms of a urine aliquot (50 μl) before and after enzymatic hydrolysis; this urine was collected between 4 and 8 h from a healthy subject treated with 5 mg of ISDN. Isosorbide dinitrate and the two isosorbide

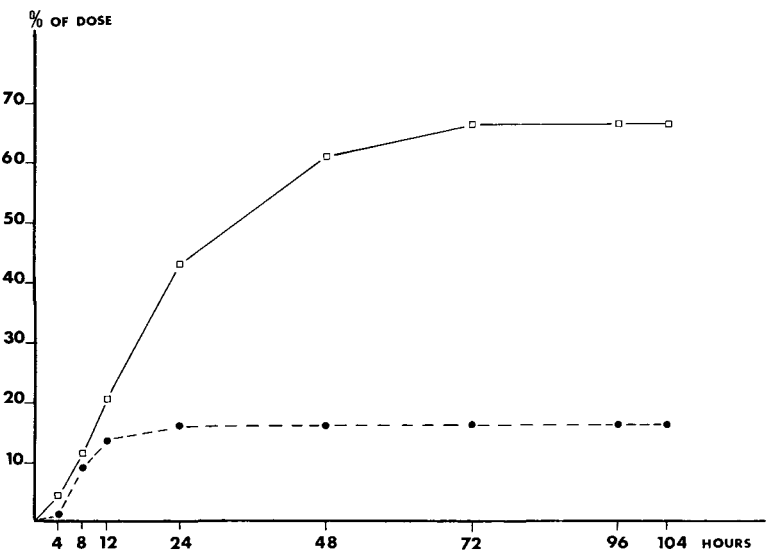


Fig. 3. Cumulative urinary excretions of free and conjugated isosorbide in one healthy subject after oral administration of 5 mg of ISDN as one Isoket tablet. (•), Conjugated isosorbide; (□), free isosorbide.

mononitrate metabolites (2-ISMN and 5-ISMN) were injected under the same conditions as isosorbide. These compounds were not recorded.

Application

The present method was used to determine the urinary excretion of the metabolite of ISDN after oral administration to one healthy subject. The corresponding plasma concentrations of the parent drug (ISDN) have also been determined [1]. Fig. 3 shows the curves of urinary free and conjugated isosorbide excretion in the subject given 5 mg of ISDN as one Isoket tablet. In the 0—104 h urine, 66.2% of the administered dose was recovered as free isosorbide and 15.8% as conjugated isosorbide.

CONCLUSION

The proposed gas chromatographic technique permits the quantitative determination of isosorbide in human urine at concentrations down to 200 ng/ml. This method can be applied to the determination of free and conjugated isosorbide as a metabolite of ISDN in urine.

ACKNOWLEDGEMENTS

The authors thank J. Godbillon, J. Richard and E. Urien (Biopharmaceutical Research Center, Ciba-Geigy, Rueil, France) for the synthesis of isosorbide and isomannide, and Dr. J. Moppert (Pharmacological Chemistry, Ciba-Geigy, Basle, Switzerland) for performing the clinical experiment.

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Journal of Chromatography, 277 (1983) 165—172
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1761

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF PLASMA ALLANTOIN

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(First received July 2nd, 1982; revised version received May 13th, 1983)

SUMMARY

A new method has been devised for the determination of nanomole levels of allantoin in human plasma. Allantoin was converted into xanthylallantoin, which was chromatographed on a reversed-phase silica gel using a mixture of acetonitrile—water (27:73) as mobile phase. The eluted compound was measured using an ultraviolet detector. The detection limit of the assay for plasma was about 100 ng/ml. This method was applied successfully to the determination of allantoin in human plasma after oral administration of 100 mg of aldioxa.

INTRODUCTION

Aldioxa (aluminium dihydroxyallantoinate), an anti-ulcer drug, is absorbed after hydrolysis to allantoin (I) in the digestive organs [1]. The determination of allantoin at the nanomole level is necessary in human bioavailability studies of aldioxa.

Several reports [2, 3] have been published on determination based on the color reaction of the aldehyde group of glyoxalic acid formed by the hydrolysis of allantoin. Some authors [4, 5] investigated the utilization of the color reaction between 4-dimethylaminobenzaldehyde and allantoin. Recently, Yuki et al. [6] reported colorimetric measurements of the condensation product

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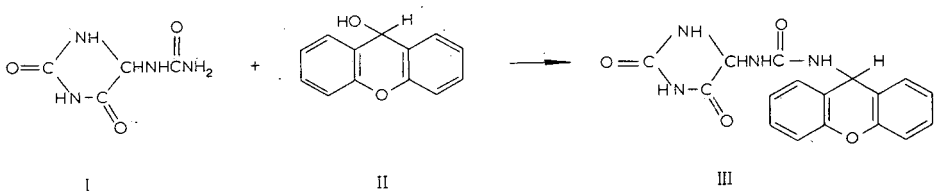


I II III

of allantoin with diacetylmonoximethiosemicarbazide. However, the quantitative analysis of allantoin at nanomole levels in blood has been lacking.

A new method has been devised for the determination of nanomole amounts of allantoin in human plasma. Allantoin was converted with xanthydrol (II) into xanthylallantoin (III), and the derivatized allantoin analyzed on reversed-phase silica gel by high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Materials

The standard solution of allantoin (Wako Pure Chemical Industries, Osaka, Japan) was freshly prepared in double-distilled water before analysis. Xanthylallantoin was prepared according to the method of Stewart [7]. The elemental analysis of the compound (as monohydrate) provided satisfactory results. Acetonitrile of UV grade was used. All other solvents and reagents were analytical reagents and used without further purification.

Apparatus

Chromatography was performed on a Shimadzu liquid chromatograph Model 3A, equipped with a variable-wavelength UV detector (Model SPD 2A, Shimadzu, Kyoto, Japan). Injection of samples was automatically performed with an autosampler (Atto, Tokyo, Japan), equipped with a sample bath kept at −1°C by circulation of chilled water. A step gradient of two mobile phases was carried out using a Shimadzu SGR-1A with a time programmer. A 2-μm line filter was inserted between the column and injector port. Plasma samples were homogenized in test tubes (7.5 × 1.0 cm) using a glass bar (0.6 cm diameter) attached to a motor. Evaporation of solvents was carried out with Sarvant Speed concentrator.

Chromatographic conditions

Separation was obtained with a Zorbax ODS column (15 × 0.46 cm; particle size, 5—6 μm; Du Pont, Hitchin, U.K.); the column eluent was monitored at 241 nm at a sensitivity of 0.04 a.u.f.s. A mobile phase of 27% acetonitrile in water was used for analysis of the derivatized allantoin, of which the retention time was 9 min. At 3 min after the appearance of the peak of this compound, the mobile phase was changed to 70% methanol in water for 18 min in order to sweep out interfering substances tightly adsorbed on the column. The mobile phase was again changed to the aqueous acetonitrile, and then the next sample injected after 27 min. The flow-rate was 1.0 ml/min at 43°C.

Extraction and clean-up

A human plasma sample (1.0 ml), which was obtained from fresh blood collected in a heparinized Vacutainer tube, was mixed with methanol (1.5 ml) and allowed to stand overnight in the refrigerator to precipitate most of the plasma proteins. The suspension was centrifuged and the supernant set aside for future use. The precipitate was homogenized with methanol (1.0 ml) to extract the remaining allantoin. After centrifugation, the precipitate containing traces of allantoin was again extracted by homogenizing with methanol (1.0 ml) in the same way. The three methanolic supernatants were combined and evaporated to dryness. The resultant residue was homogenized with methanol (2.5 ml), and the supernatant obtained by centrifugation was evaporated to dryness. Water (1.0 ml) was added to the residue and the resulting solution was washed with three 1.0-ml portions of ether–ethyl acetate (1:1, v/v). The defatted solution was passed through a Sep-Pak C_{18} cartridge (Waters Associates) and the resin washed with water (0.5 ml). The resin was regenerated by washing with methanol (3.0 ml). The combined solution of the eluate and washings was evaporated to dryness, and the resulting residue submitted to derivatization followed by analysis.

Derivatization

To the above residue was added 50% acetic acid in water (0.7 ml), and the solution was cooled to 0°C in an ice-bath. A solution (0.2 ml) of xanthydrol (25 mg) in dimethylformamide (DMF, 1.0 ml) was added. The mixture was allowed to stand at 0°C for 1 h, and was then evaporated to dryness. The resulting residue was suspended in a solution (100 μl) of 50% DMF in water, and diluted with mobile phase (1.0 ml of the aqueous acetonitrile). The diluted suspension was allowed to stand in the refrigerator, and then centrifuged at 8000 *g* for 1 min. An aliquot (0.5 ml) of the suspension was placed in the sample bottle of the autosampler, and an aliquot (50 μl) was injected into the liquid chromatography column.

Quantitative determination

The concentration of allantoin in plasma was directly calculated from a calibration curve. The curve (external standard) of peak height of the derivatized allantoin was obtained by analysis of blank plasma (1.0 ml) to which known quantities (0.5—3.0 μg) of allantoin were added. The curves were prepared for each day of analysis to establish the linearity and reproducibility of the HPLC system.

Drug administration

Five healthy volunteers (23—46 years old, 53—78 kg) were administered a single dose of 200 mg of Bestass (Nisshin Pharmaceutical Co., Tokyo, Japan) which included 100 mg of aldioxa. The drug was administered after overnight fasting, and blood samples were collected in heparinized tubes at 0, 0.5, 1, 2, 3, 5, 8, and 24 h after administration. The volunteers took no food or water until after the collection of the 3-h sample; then their diet was no longer restricted. The samples for the determination of endogenous allantoin were taken in the same way without administration of the drug. The samples were stored at −20°C, and analyzed within three days.

RESULTS AND DISCUSSION

Pre-treatment and derivatization

The plasma sample required a pre-treatment consisting of deproteinization by methanol, elimination of lipid by an organic solvent, and elimination of interfering compounds by a reversed-phase cartridge. Without this pre-treatment an abnormal increase in column pressure was observed, resulting in a shortened column life. The cartridge allowed the passage of allantoin with no adsorption, and could be regenerated by washing with methanol.

The need for repeated extraction by methanol could not be avoided, since a single extraction resulted in a low recovery (about 50%) of allantoin. Although another organic solvent for deproteinization, acetonitrile [8], was used instead of methanol, the recovery was not greatly improved. In addition, extraction from dried plasma (obtained by evaporating plasma under reduced pressure) with methanol was tried. A poor recovery was obtained in this case as well. These facts indicate that plasma has a marked tendency to adsorb allantoin and the allantoin resists desorption from the protein.

Xanthylation of allantoin was carried out by reaction with xanthydrol in 50% acetic acid, using the method previously reported [7]. Since the reagent was used in large excess (about 2000-fold in this case), the reaction mixture contained a large quantity of unreacted reagent with xanthone and xanthene being produced by disproportionation of the reagent [9]. These interfering substances were almost eliminated before injection onto the analytical column. The reaction mixture was evaporated to dryness and the resulting residue suspended in a small volume of DMF (100 μl) that was enough to dissolve the xanthylallantoin. Upon dilution with the aqueous acetonitrile mobile phase, most of the interfering compounds were reprecipitated and were removed by centrifugation.

Use of more than 100 μl of DMF in the above treatment led to the incomplete HPLC separation of xanthylallantoin due to tailing of the DMF. The interfering substances that are reprecipitated upon dilution with mobile phase, should be removed after standing overnight in the refrigerator. Otherwise, gradual precipitation occurs in the sample bottle (cooled at $-1°C$) of the autosampler. The precipitate might clog up the needle which sucks the sample solution from the bottle, leading to inaccurate sampling. This multi-step clean-up procedure enabled the analysis of over 300 samples without any abnormal increase in the column pressure and without any deterioration of resolution on the reversed-phase column.

Chromatography

As shown in the chromatogram of a plasma sample spiked with allantoin (Fig. 1c), a symmetrical peak and good separation could be obtained for xanthylallantoin. When plasma samples were analyzed with the single mobile phase of 27% acetonitrile, several peaks continued for more than 60 min after the appearance of the compound. These prolonged peaks interfered with the next injection of sample. This effect could be eliminated by switching to another mobile phase, i.e. 70% methanol. The interfering substances were quickly swept from the column as several peaks appeared after the change of

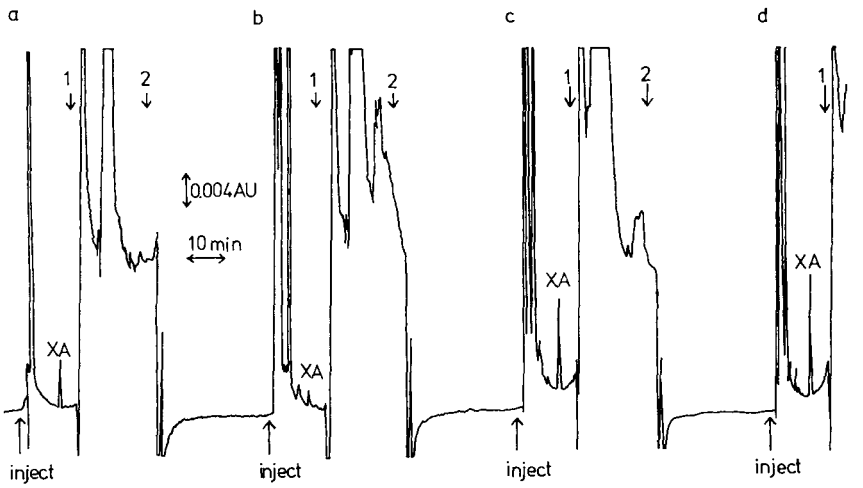


Fig. 1. Continuous analysis of derivatized allantoin. The following samples (1.0 ml) were derivatized to xanthylallantoin (XA) and analyzed under conditions described in Experimental: (a) standard allantoin (1 μmol) in water; (b) blank plasma; (c) plasma spiked with standard allantoin (2 μg); (d) volunteer plasma 1 h after administration of aldioxa. The arrows 1 and 2 indicate the flow start of mobile phases 27% acetonitrile in water and 70% methanol in water, respectively.

the mobile phase (Fig. 1). The switch was performed with a step gradient device connected to the autosampler with a time programmer. One sample could be automatically analyzed in 57 min. A typical successive chromatogram is shown in Fig. 1.

Recovery and precision

Allantoin was added to plasma and then analyzed by the method described above. At the same time, an aqueous allantoin solution was derivatized without the pre-treatment described for plasma. Fifty micrograms of the sample were injected and peak heights of xanthylallantoin measured. The overall recovery of allantoin from plasma was calculated by comparing these peak heights; the results are listed in Table I. The coefficient of variation for identical plasma

TABLE I

RECOVERY ON EXTRACTION AND DERIVATIZATION OF ALLANTOIN

Allantoin added to 1 ml of plasma (μg)	n*	Recovery (% ± S.D.)	Coefficient of variation (%)
0.5	5	98.4 ± 6.0	6.1
1.0	5	104.8 ± 4.6	4.4
1.5	4	93.4 ± 4.9	5.2
2.0	5	96.2 ± 5.4	5.6
3.0	4	103.2 ± 4.7	4.6

*n = number of determinations.

samples varied from 4% to 6%. The precision of the method was evaluated by repeated analysis of plasma with added standard allantoin. The detection limit of the assay was about 100 ng/ml of plasma.

Stability of xanthylallantoin

It was found that the peak height of xanthylallantoin decreased with the passage of the time when the reaction mixture after the derivatization was allowed to stand even at temperatures as low as 0°C. The stability was examined using standard xanthylallantoin (Fig. 2). Acetic acid gradually decomposed the compound even at 0°C, producing an unidentified compound with a retention time of 11.5 min (Fig. 3). However, no decomposition in DMF, which has a high solubility for xanthylallantoin among several solvents tested, occurred at 0°C, although moderate decomposition occurred at ambient temperature. Therefore acetic acid, a solvent for derivatization, was removed by evaporation after derivatization, and the residual xanthylallantoin was dissolved in the mobile phase containing DMF. The resulting solution was cooled at 0°C in an autosampler bottle until it was injected into the analytical column.

Human studies

A small peak in the chromatogram of blank plasma (Fig. 1b) appears at the retention time of xanthylallantoin. The retention time did not differ from that of xanthylallantoin when the analyses were carried out using several mobile phases consisting of acetonitrile and water in several proportions, indicating that the peak is due to endogenous allantoin in the plasma. The endogenous concentration was measured in two volunteers. As seen in Fig. 4, a fluctuation during a day was observed within the range 0.7—1.2 μg/ml of plasma. Archibald [10] and Stahl et al. [11] measured the concentration in individuals using a colorimetric method, and reported the different ranges of 3—6 and 6—11 μg/ml

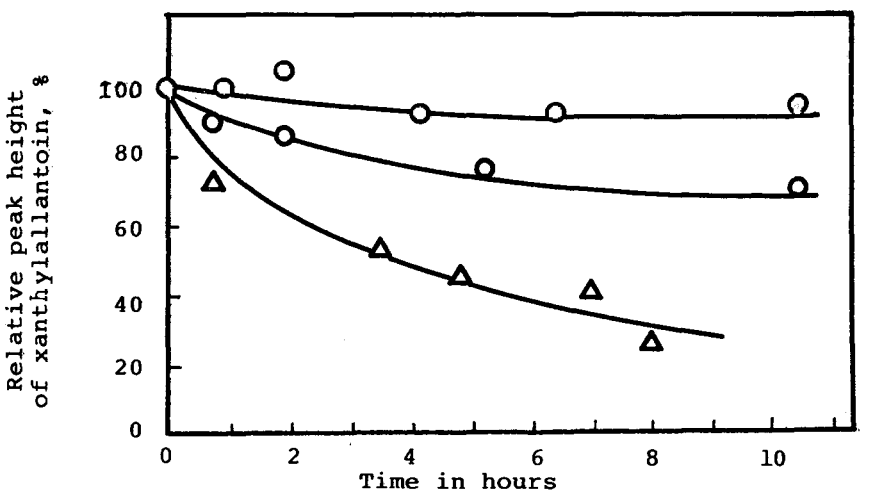
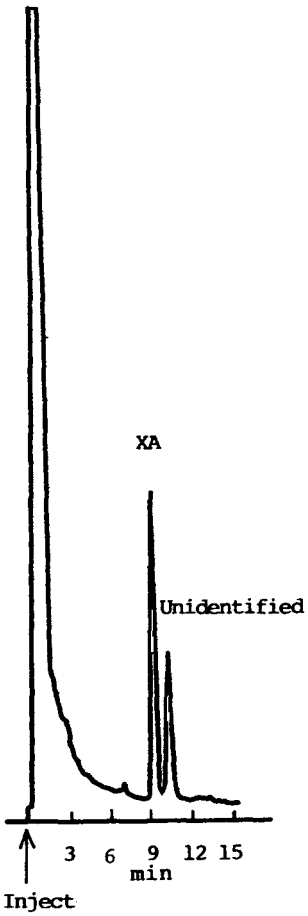


Fig. 2. Stability of xanthylallantoin. Xanthylallantoin (5 μg) in the acetonitrile mobile phase (1.0 ml) containing 7% DMF (•) or 7% acetic acid (○) was allowed to stand at 0°C, and at room temperature in DMF (△).



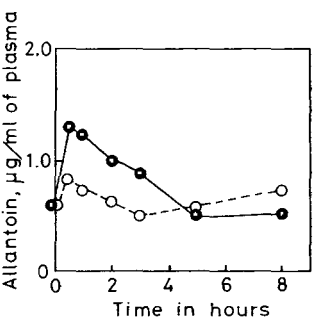


Fig. 3. Production of an unidentified compound from xanthylallantoin. Xanthylallantoin (5 μg) in the acetonitrile mobile phase (1.0 ml) containing 7% DMF was allowed to stand for 24 h at room temperature, and then analyzed.

Fig. 4. Fluctuation of endogenous allantoin in plasma.

of plasma and serum, respectively. These values are much higher than our data. Such a difference may be caused by the different methods used for the determination or by the nature of the specimens from the volunteers.

Allantoin concentration after the administration of aldioxa preparation was measured. The concentration of exogenous allantoin was conveniently calculated by subtracting the allantoin concentration before administration from that after administration, and was then plotted as a function of time (Fig. 5). Allantoin reached its maximal plasma level 1—2 h after administration, and then slowly fell to the pre-administration level 24 h after administration.

This assay with a fully automated system has proved to be reproducible, sensitive, and selective for the determination of allantoin in human plasma at the therapeutic level, and has been used continuously over a year. The sample size required in this procedure makes it suitable for performing pharmacokinetic studies which require multiple biological samples.

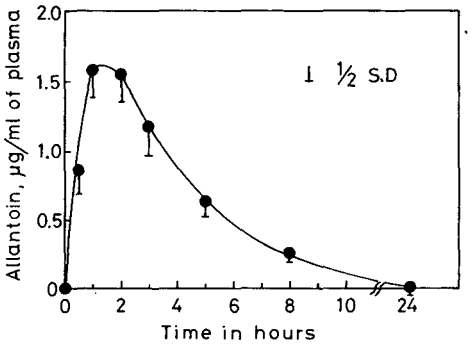


Fig. 5. Plasma concentration of exogenous allantoin after oral administration of 100 mg of aldioxa.

This effective liquid-chromatographic analysis of allantoin modified with xanthydrol indicates that this reagent can be used as a UV-labeling reagent for other drugs having an amide group.

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Journal of Chromatography, 277 (1983) 173—182
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1811

RAPID AND SENSITIVE METHOD FOR THE MICROASSAY OF NITROSOBENZENE PLUS PHENYLHYDROXYLAMINE IN BLOOD

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(First received January 12th, 1983; revised manuscript received June 3rd, 1983)

SUMMARY

An assay method has been developed for the determination of the combined concentration of nitrosobenzene plus phenylhydroxylamine (as nitrosobenzene) in small volumes of blood. The initial step in the procedure consisted of the simultaneous oxidation of phenylhydroxylamine to nitrosobenzene and of ferrous hemoglobin to methemoglobin by ferricyanide. Nitrosobenzene in the ferricyanide-treated blood samples was then extracted into ethyl acetate, and separated and quantitated by reversed-phase high-performance liquid chromatography with electrochemical detection. The sensitivity limit for nitrosobenzene in blood was in the pmol/ml concentration range, less than 100 μl of blood was required for assay, and the procedure was convenient for routine multisample use. In comparison with previous assays, this method was more sensitive, had a lower coefficient of variation, and required 25—40 fold smaller blood sample volumes. The method was combined with the orbital sinus bleeding technique in order to follow the nitrosobenzene time course in vivo using small serial blood samples from rats treated with intraperitoneal injections of phenylhydroxylamine or aniline.

INTRODUCTION

A variety of aromatic amine and nitro compounds, including drugs such as dapsone and chloramphenicol, industrial chemicals such as aniline, and environmental chemicals such as 4-aminobiphenyl and 2-napthylamine, undergo in vivo conversion to N-hydroxy and nitroso derivatives during their metabolic clearance. These metabolic intermediates have been suggested to be mediators of the toxic effects of the parent compounds, which include methemoglobinemia, blood dyscrasias, mutagenicity, and carcinogenicity [1—5]. In most cases, only a small percentage of a dose of the parent compound is converted to N-hydroxy and nitroso metabolites, and these compounds are usually relatively unstable in biological systems. Accordingly, detection and quantita-

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tion of these important metabolites has been difficult because: (1) their concentrations in blood and tissues are low, requiring detection methods with high sensitivity, and (2) loss of the unstable compounds is likely to occur during extraction and separation procedures.

An additional factor complicating the in vivo quantitation of aromatic N-hydroxy and nitroso compounds has been demonstrated in studies of the formation of phenylhydroxylamine and nitrosobenzene during aniline clearance in vivo. These experiments showed that phenylhydroxylamine and nitrosobenzene are interconverted in blood via an enzyme-mediated redox cycle [3]. Thus, in order to evaluate the extent of aniline N-oxidation in vivo, it is necessary to measure the sum of the phenylhydroxylamine and nitrosobenzene concentrations in blood. The assay procedure used in the above experiments continues to be perhaps the most widely used method for quantitation of the sum of nitrosobenzene plus phenylhydroxylamine in blood [6]. It is a colorimetric method that is sensitive in the nanomolar range, but requires relatively large volumes of blood (2—3 ml) and has a high sample-to-sample variability. In addition, several extraction and derivatization steps are required, making the method inconvenient for routine assay of large numbers of samples.

Recently, sensitive techniques utilizing high-performance liquid chromatography (HPLC) and electrochemical detection have been introduced to determine nitrosobenzene or phenylhydroxylamine [7—10]. These methods, designed to measure either nitrosobenzene or phenylhydroxylamine alone in aqueous solutions or in tissue homogenates, have not been adapted for the quantitation of aniline N-oxidation products in blood. This communication presents a simple and rapid procedure for the combined assay of nanomolar concentrations of the major aniline N-oxidation products (phenylhydroxylamine plus nitrosobenzene) in small volumes of blood. The procedure is significantly more sensitive than the previous colorimetric assay, allowing the blood sample volumes required for routine analysis to be reduced to less than 100 μl. Loss of phenylhydroxylamine, which is unstable in aqueous solution [8], is avoided by conversion of phenylhydroxylamine to nitrosobenzene prior to extraction and chromatography; interference by biological materials is avoided by a single extraction step and HPLC separation; and quantitation of nitrosobenzene is accomplished by electrochemical reduction as the column effluent passes across a glassy carbon electrode. This method is suitable for routine analysis of multiple small blood samples serially obtained from laboratory rodents.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing 200—250 g were obtained from Camm Research (Wayne, NJ, U.S.A.) and maintained in hanging stainless-steel cages on standard lab chow (Wayne Lab Blox, Allied Mills, Chicago, IL, U.S.A.) and water ad lib.

Chemicals

Phenylhydroxylamine was prepared by zinc-catalyzed reduction of nitro-

benzene in the presence of aqueous ammonium chloride [11] and recrystallized three times from benzene—hexane. Nitrosobenzene (Aldrich, Milwaukee, WI, U.S.A.) was purified by vacuum sublimation. Aniline · HCl (Aldrich) was dissolved in water and purified by extraction into ethyl acetate at neutral pH, precipitation with hydrogen chloride gas, and recrystallization two times from ethanol—ethyl acetate. These compounds were stored in the dark at −20°C under nitrogen. Other chemicals were reagent grade (Aldrich) and were used without further purification. Solvents were distilled-in-glass grade (Burdick & Jackson Labs., Muskegon, MI, U.S.A.).

HPLC system

Chromatography was performed on a system consisting of a Model 6000A solvent delivery system (Waters Assoc., Milford, MA, U.S.A.), a Rheodyne No. 7125 injector port, a Whatman guard column, a Partisil PXS 5/25 ODS column (Whatman, Clifton, NJ, U.S.A.), and an LC-3 amperometric detector with an electrochemical cell containing a glassy carbon working electrode (Bioanalytical Systems, West Lafayette, IN, U.S.A.). A working electrode potential of −200 mV versus a Ag/AgCl reference electrode was used throughout, and the system was operated at a flow-rate of 1.2 ml/min.

Analytical procedure

Standard solutions of nitrosobenzene and phenylhydroxylamine in aqueous 1% methanol were prepared by addition of the compounds, dissolved in 25 μl methanol, to 2.5-ml aliquots of water. Standard solutions of nitrosobenzene in rat blood were prepared using blood obtained by cardiac puncture and divided into 2.5-ml aliquots. Nitrosobenzene was added to the blood samples in 25 μl methanol to yield the desired final concentrations. The blood samples were then mixed by gentle swirling for 1 min and 75-μl aliquots were assayed for nitrosobenzene.

For in vivo experiments, groups of rats received intraperitoneal injections of phenylhydroxylamine dissolved in 5% Tween 80 in 0.9% saline, or aniline · HCl dissolved in 0.9% saline. Serial blood samples were subsequently obtained from the orbital sinus [12, 13] of each animal in 75-μl heparinized capillary tubes and assayed for nitrosobenzene as described below.

The 75-μl blood samples were immediately hemolyzed in 175 μl of 0.005 *M* phosphate buffer, pH 7.0. Fifty μl of 20% potassium ferricyanide in water and 50 μl of 10% potassium cyanide in water were added with brief mixing, followed by 350 μl water-saturated ethyl acetate. The samples were extracted by rapid mixing with a vortex mixer for 2 min, then briefly centrifuged and stored over ice for up to 1 h.

Samples of the organic phases were injected into the HPLC system using a 20-μl sample loop attached to the injector port. Nitrosobenzene in the sample was quantitated by comparing the height of the nitrosobenzene peak to a standard curve of nitrosobenzene peak height versus concentration in rat blood.

RESULTS

HPLC of standard solutions of nitrosobenzene in ethyl acetate yielded a

single peak (retention volume = 8.3 ml) by electrochemical detection in the reducing mode. The response of the detector was linear over a nitrosobenzene concentration range of 20—400 pmol per 20 μl, which in this assay corresponds to a concentration range of 5—100 nmol/ml in the aqueous solutions prior to dilution and extraction. When aqueous solutions of nitrosobenzene (100 nmol/ml) were diluted with phosphate buffer, treated with ferricyanide and cyanide, and extracted with ethyl acetate, as described in Methods, over 90%

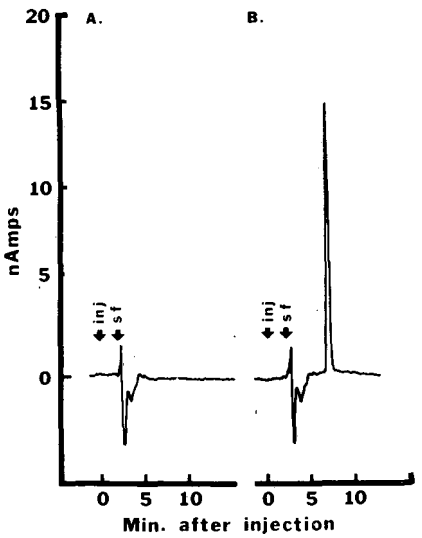

Fig. 1. Electrochemical detection of nitrosobenzene after HPLC of ethyl acetate extracts of rat blood. (A) Chromatogram of 20 μl of the ethyl acetate extract of control blood; (B) chromatogram of 20 μl of the ethyl acetate extract of blood containing 75 nmol/ml nitrosobenzene. inj = injection of the sample into the chromatographic system, sf = solvent front.

TABLE I

DETECTION AND QUANTITATION OF PHENYLHYDROXYLAMINE + NITROSOBENZENE IN ETHYL ACETATE EXTRACTS OF RAT BLOOD

Extraction efficiency of nitrosobenzene from blood*	63 ± 8.1%
Retention volume	8.3 ± 0.04 ml
Sensitivity limit**	0.5 nmol/ml
Coefficient of variation*** (at 5 nmol/ml)	6% (n = 5)
Standard curve linear regression parameters	
Y-intercept	0.15 nA
Slope	0.192 nA/(nmol/ml)
Correlation coefficient	0.996

*Mean recovery of nitrosobenzene at concentrations of 5, 25, 50, 75, and 100 nmol/ml blood.

**The sensitivity limit is taken as the concentration at which the nitrosobenzene peak height is twice the background noise level. This concentration was determined by a linear extrapolation of the standard curve.

***A single nitrosobenzene-spiked blood sample was divided into five aliquots which were extracted and assayed separately. The coefficient of variation was calculated as (S.D.)/(mean nitrosobenzene peak height) for the five aliquots.

of the nitrosobenzene was recovered in the organic phase. When aqueous solutions of phenylhydroxylamine (100 nmol/ml) were similarly treated, over 90% of the phenylhydroxylamine was also recovered in the organic phase as nitrosobenzene. In samples of rat blood spiked with nitrosobenzene (100 nmol/ml), 60% of the added nitrosobenzene was recovered after hemolysis in phosphate buffer, ferricyanide/cyanide treatment, and ethyl acetate extraction. Nitrosobenzene recovery was 8—10% lower in samples not treated with cyanide. An equivalent fraction of phenylhydroxylamine (100 nmol/ml) was recovered as nitrosobenzene from spiked rat blood samples after a similar extraction procedure. Stability experiments indicated that no loss of nitrosobenzene occurred from the organic phase after extraction from blood when the samples were maintained over ice for up to 4 h.

Fig. 1 shows typical chromatograms of ethyl acetate extracts of control and nitrosobenzene-spiked rat blood samples. The only major peak after the solvent front was that of nitrosobenzene, and no endogenous compounds were detected by electrochemical reduction in the vicinity of the nitrosobenzene peak. Total analysis time was approximately 8 min.

Peak height over the concentration range of 0—100 nmol/ml in rat blood was linearly related to concentration; regression analysis of the data yielded a correlation coefficient of 0.996. The limit of sensitivity (signal-to-noise ratio =

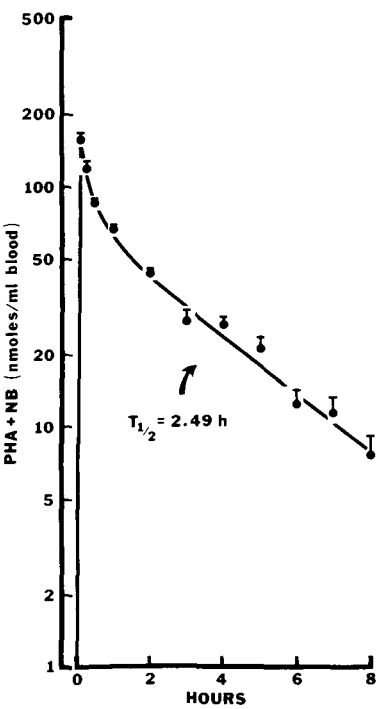

Fig. 2. Semi-logarithmic plot of phenylhydroxylamine + nitrosobenzene concentration in blood after intraperitoneal injection of phenylhydroxylamine (200 μmol/kg). Points and error bars shown are means ± S.D. (n = 4). The half-life of the terminal phase was determined from a regression line fitted to the final seven data points. PHA = phenylhydroxylamine, NB = nitrosobenzene.

2) was 500 pmol/ml blood and the coefficient of variation at 5 nmol/ml was 6%. Extraction efficiency was relatively constant over the range of 5—100 nmol/ml at 63 ± 8.1% (S.D.). These data are presented in Table I.

The use of the assay method in vivo is illustrated by the quantitation of phenylhydroxylamine + nitrosobenzene in serial blood samples from rats after intraperitoneal injection of phenylhydroxylamine or aniline · HCl. Standard solutions of nitrosobenzene were routinely assayed before and after each group of experimental samples, and indicated excellent stability of electrode sensitivity for nitrosobenzene. Fig. 2 shows the blood concentration time course of phenylhydroxylamine + nitrosobenzene after phenylhydroxylamine (200 μmol/kg) administration. Absorption was rapid, with the initial blood sample at 5 min showing the highest concentration of phenylhydroxylamine + nitrosobenzene (155 nmol/ml blood). Two phases of elimination then followed: a relatively rapid phase occurring over the first hour after administration, and a slower terminal phase which was linear on the semi-log plot with a half-life of 2.49 h.

The time course of phenylhydroxylamine + nitrosobenzene in rat blood after aniline · HCl (1.5 mmol/kg, intraperitoneally) was also determined (Fig. 3). Phenylhydroxylamine + nitrosobenzene formation was rapid, with the initial level of 57 nmol/ml at 10 min being the maximum observed. The clearance of phenylhydroxylamine + nitrosobenzene displayed complex kinetics, with a linear initial phase (half-life = 3.52 h) and a terminal phase of more rapid elimination.

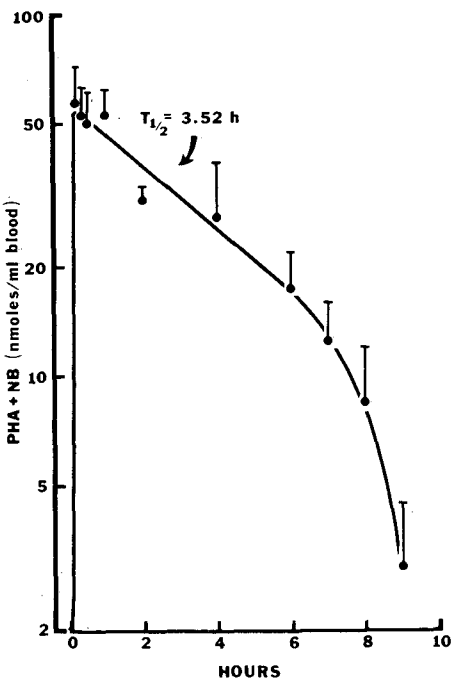

Fig. 3. Semi-logarithmic plot of phenylhydroxylamine + nitrosobenzene concentration in blood after intraperitoneal injection of aniline (1.5 mmol/kg). Points and error bars shown are means ± S.D. (n = 4). The half-life of the initial phase was determined from a regression line fitted to the first seven data points. PHA = phenylhydroxylamine, NB = nitrosobenzene.

DISCUSSION

This paper presents a simple and rapid method for quantitation of the combined amount of phenylhydroxylamine and nitrosobenzene present in small volume blood samples. Like the method of Herr and Kiese [6], the initial step is the addition of ferricyanide to the hemolyzed blood sample. This treatment oxidizes both phenylhydroxylamine to nitrosobenzene and hemoglobin to methemoglobin. Nitrosobenzene is more stable in blood than phenylhydroxylamine [11] and is more easily extracted from aqueous solution. Oxidation of hemoglobin to methemoglobin improves the extraction efficiency of nitrosobenzene from blood by eliminating ferrous heme as a binding site for nitrosobenzene [14]. The addition of cyanide to block methemoglobin by the formation of cyanomethemoglobin results in an additional small increase in the amount of nitrosobenzene extracted, presumably by preventing interaction between nitrosobenzene and ferriheme. Recovery of nitrosobenzene from ferricyanide-treated aqueous solutions of either nitrosobenzene or phenylhydroxylamine was greater than 90%, indicating that ferricyanide quantitatively oxidized phenylhydroxylamine to nitrosobenzene, but did not further oxidize nitrosobenzene to nitrobenzene.

Nitrosobenzene in the oxidized blood samples was extracted into ethyl acetate and assayed directly, after HPLC, by electrochemical reduction at a glassy carbon electrode. Electrochemical detection is a highly sensitive method which has been used with a variety of oxidizable and reducible substances in aqueous solution. When used with HPLC, electrochemical detection provides additional specificity beyond that usually associated with HPLC separations [15]. This is because electrode potentials may be selected which oxidize or reduce compounds of interest, but which do not cause electrochemical reactions in compounds eluting near compounds of interest. With this detection method, an interfering compound not only must co-chromatograph with the compound of interest, it must also show similar electrochemical characteristics. In the present assay, no other easily reducible compounds were found near the nitrosobenzene peak in chromatograms of ethyl acetate extracts of rat blood. Thus background noise from endogenous compounds was very low, which increased the sensitivity of the assay. In addition, a peak with the appropriate retention time could be identified as nitrosobenzene with a high degree of confidence.

The extraction efficiency of ethyl acetate for nitrosobenzene in equal volumes of aqueous solution was greater than 90%, whereas the extraction efficiency against equal volumes of blood was reduced to approximately 63%. This reduction in extraction efficiency was comparable to the 60% extraction efficiency obtained by Herr and Kiese [6] using carbon tetrachloride as the organic phase. Incomplete extraction may result from entrapment of nitrosobenzene in the membrane lipids and denatured protein of the erythrocytes. Although a rapid elimination of nitrosobenzene within the first minute after addition to blood could have resulted in the loss of some of the compound, this is unlikely because no further loss of nitrosobenzene was found when samples were extracted 3 min, rather than 1 min, after nitrosobenzene addition. Recent studies by Eyer and Lierheimer [16] also indicate that

nitrosobenzene is relatively stable in erythrocyte suspensions, with a half-life of approximately 50 min. Although in the present studies the extraction of nitrosobenzene from blood was not complete, extraction efficiency showed excellent reproducibility and was constant over the concentration range of the assay. Therefore, correction for incomplete extraction was straightforward.

The performance of the assay in vivo was demonstrated by determining the blood time course of phenylhydroxylamine + nitrosobenzene in rats after intraperitoneal injection of phenylhydroxylamine. By utilizing the orbital sinus blood sampling technique to collect serial 75-μl blood samples for assay, blood levels of phenylhydroxylamine + nitrosobenzene could be followed over time in individual animals. Phenylhydroxylamine was absorbed rapidly after intraperitoneal injection with maximal blood levels of phenylhydroxylamine + nitrosobenzene occurring within 5 min. The elimination time course showed two phases occurring during the 8 h following administration. Elimination according to an apparent two-compartmental model obviously could result from several mechanisms; however, this finding is consistent with recent data obtained in erythrocyte suspensions indicating the presence of several non-heme reversible intracellular binding sites for nitrosobenzene [16].

As indicated in the Introduction, cyclic interconversion of phenylhydroxylamine and nitrosobenzene within erythrocytes concomitant with the oxidation of hemoglobin to methemoglobin was demonstrated in the 1950's [17—19]. From these and other data, Kiese [3] postulated that phenylhydroxylamine-induced methemoglobin formation is mediated by a cyclic redox system including phenylhydroxylamine, nitrosobenzene, oxyhemoglobin, and methemoglobin. In this system, the blood levels of phenylhydroxylamine and nitrosobenzene would be governed by the position of the redox equilibrium as well as by the total amount of N-oxidized compound present. Since the relative concentrations of the hydroxylamine and nitroso forms are not known under varying experimental conditions, appropriate evaluation of the total amount of methemoglobin-forming compound present in blood must include quantitation of both phenylhydroxylamine and nitrosobenzene. An advantage of the assay procedure reported here for these types of studies is that it allows rapid and convenient measurement in a single procedure of the total amount of putative toxic compounds present (that is, both the hydroxylamine and nitroso forms).

Kiese [20] also demonstrated the presence of phenylhydroxylamine + nitrosobenzene in the blood of a dog during methemoglobinemia induced by aniline given intravenously. Subsequent studies showed that intravenous infusion of low levels of phenylhydroxylamine into a dog was associated with extensive methemoglobinemia [21]. From these results, Kiese [21] proposed that phenylhydroxylamine was a toxic metabolite derived from aniline responsible for the methemoglobinemia observed after aniline in vivo.

Data presented here show that phenylhydroxylamine + nitrosobenzene was also detectable in rat blood in significant levels after intraperitoneal injections of aniline · HCl. Maximal levels are reached within 10 min after injection. Elimination over the subsequent 7 h approximates first-order kinetics, and the half-life of this elimination phase is longer than that seen after phenylhydroxyl-

amine treatment. This lengthened half-life may reflect the elimination rate of the parent compound, aniline [22].

The terminal phase of phenylhydroxylamine + nitrosobenzene elimination after aniline treatment appears to deviate from linearity. This deviation was not seen at equivalent blood levels of phenylhydroxylamine + nitrosobenzene following intraperitoneal injections of phenylhydroxylamine. Several possible explanations exist for the observed elimination pattern. A concentration-dependent change in the metabolic disposition of aniline when blood levels of aniline are low could lead to a relative decrease in the proportion of aniline undergoing N-oxidation with respect to other clearance pathways during the terminal phase of elimination. Other alternatives include non-linear elimination kinetics of aniline, with phenylhydroxylamine + nitrosobenzene levels reflecting the kinetics of the parent compound; or an increase in the activity or availability of elimination pathways in the erythrocyte for nitrosobenzene and/or phenylhydroxylamine as the level of these compounds, or of methemoglobin, in the erythrocytes declines.

These preliminary results indicate the usefulness of the method described here for the investigation of the formation and elimination kinetics of phenylhydroxylamine + nitrosobenzene. The combined amount of these two putative toxic intermediates was measured in blood samples 25—40-fold smaller than required by previous methods, with no loss in sensitivity, and a decreased error. This assay, when combined with appropriate blood sampling technique, will greatly decrease the difficulty of studying the kinetics and toxicology of aniline N-oxidation in vivo by permitting blood time course data to be obtained from individual small laboratory animals. In addition, it is anticipated that this method may be easily modified for analysis of other aromatic hydroxylamine and/or nitroso compounds in biological systems.

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Journal of Chromatography, 277 (1983) 183—189
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1781

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF DIPYRONE AND ITS ACTIVE METABOLITE IN BIOLOGICAL FLUIDS

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(First received February 8th, 1983; revised manuscript received May 6th, 1983)

SUMMARY

New high-performance liquid chromatography methods were developed to measure dipyrone and its active metabolite 4-monomethylaminoantipyrine (MAA) in biological fluids. While no detectable level of the unchanged dipyrone was found in plasma of four subjects taking 1000-mg oral doses of the drug, values ranging from 1.25 to 14.99 μg/ml of MAA were observed. Twenty-four hour urinary excretion of MAA varied from 9.56 to 21.21 mg. Concentrations as low as 0.25 μg/ml of MAA can be measured with acceptable coefficients of variation. The method, therefore, is suitable for microquantification of MAA in biological fluid which enables convenient and rapid assessment of the drug disposition in body.

INTRODUCTION

Dipyrone (metamizol, sulpyrine), sodium N-(1,5-demethyl-3-oxo-2-phenyl-pyrazolin-4-yl)-N-methylaminomethanesulfonate, is an effective analgesic and antipyretic agent. Although administration of this drug is associated with severe agranulocytosis [1, 2] and prolongation of bleeding time [3, 4], it is being widely used in many countries in daily doses of as high as 4 g [4]. The analgesic potency of dipyrone seems to be largely due to its rapid biotransformation to its active metabolite, 4-monomethylaminoantipyrine (MAA) [5]. Limited information is available as to the pharmacokinetics of this analgesic [6]. To study the time-course of dipyrone in the body a spectrophotometric method is used [6]. Furthermore, a 0.09—0.70 mg/g range of dipyrone and its major metabolite has been measured in rat tissue hemogenate using a high-performance liquid chromatography (HPLC) method [7].

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0378-4347/83/$03.00

The purpose of this paper is to report a highly sensitive and specific HPLC method suitable for micro determination of MAA in plasma and urine following administration of single doses of dipyrone to human subjects.

EXPERIMENTAL

Apparatus

The HPLC system consisted of a Model 224 U instrument (Waters Assoc., Milford, MA, U.S.A.) equipped with a dual-channel fixed-wavelength (254 and 280 nm) ultraviolet detector.

For determination of dipyrone a column, 61 cm × 2 mm I.D. with 35—50 μm particle size anion-exchanger packing (Bondapak AX/Corasil, Waters Assoc.), was used. Mobile phase was phosphate buffer, pH 5.6—methanol (85:15) which was pumped at a flow-rate of 0.5 ml/min and initial pressure of 0.7 MPa.

A reversed-phase column, 30 cm × 3.9 mm I.D. with 10-μm particle size packing (μBondapak C_{18}, Waters Assoc.), was used to quantify MAA. The mobile phase was water—methanol—acetic acid (75:20:5) which was pumped at a constant flow-rate of 1 ml/min and an initial pressure of 3.5 MPa.

Chemicals

All organic solvents were of analytical grade. Water was distilled and deionized. Mobile phasss were filtered through 0.45-μm pore size filters (Millipore, Bedford, MA, U.S.A.) before utilization. Antipyrine (Aldrich, Milwaukee, WI, U.S.A.) and sodium salicylate (E. Merck, Darmstadt, F.R.G.) were used as internal standards (IS) in MAA and dipyrone assays, respectively. Powder of dipyrone was a gift from Hoechst Laboratories of Iran.

The metabolite (MAA) was prepared by hydrolysis of dipyrone at 50°C, extraction with chloroform in alkaline environment, separation by means of thick-layer chromatography on silica gel using ethyl acetate—methanol—chloroform (20:20:60) as solvent, re-extraction with chloroform and then crystallization in isopropyl alcohol and diethyl ether. The chemical structure of the metabolite was confirmed using nuclear magnetic resonance (NMR) spectroscopy in accordance with Yoshioka et al. [8, 9] and mass spectroscopy. Melting point of MAA was 170—172°C under the hot-stage condition.

Sample preparation

Dipyrone in plasma. To create two separate layers and direct dipyrone into an alcoholic layer, excessive amount of potassium carbonate powder (600—1000 mg) was added to tubes containing 1—2 ml plasma and 1 ml IS solution (40 μg sodium salicylate in methanol). Tubes were shaken for 1 min and centrifuged. Methanol layers were separated and evaporated under flow of nitrogen and reconstituted in 0.5 ml mobile phase and aliquots of 25—50 μl were injected into the chromatograph.

For the purpose of quantification on three occasions, 100 μl of freshly prepared solutions of dipyrone in water containing 0, 1, 10, 20, 40, 100, 200 and 400 μg of the drug were added to 0.9 ml blank plasma and extracted using the mentioned method. These standard samples correspond to plasma dipyrone

concentrations of 0 to 40 μg/ml. Peak height (dipyrone at 254 nm, IS at 280 nm) ratio method was used and the relationship between the observed ratios and corresponding dipyrone concentrations was examined.

The percent intact dipyrone in tablets was also measured using the Bondapak AX/Corasil column. Twenty tablets of 500 mg dipyrone were weighed, ground to powder and quantities equal to 5 mg of drug were dissolved in 100 ml water. To three 1-ml volumes of this solution was added 1 ml solution of IS containing 40 μg sodium salicylate. Ten μl of the final preparations were injected into the instrument and their dipyrone contents were quantified by examining the observed peak height ratios against those of solutions of equal strenth prepared from pure powder.

MAA in plasma and urine. To 1 ml of plasma or urine samples were added 0.5 ml IS solution (40 μg antipyrine), 0.5 ml of 0.1 *N* sodium hydroxide and 10 ml benzene. After vigorous shaking for 20 min, the benzene layer was separated, transferred into 15-ml centrifuge tubes, evaporated under flow of nitrogen and the residual was dissolved in 0.25 ml methanol. Aliquots of 25 μl were injected into the chromatograph.

To quantify MAA in plasma, four series of standard solutions were prepared by adding 0, 0.25, 0.50, 1, 2, 5, 10, 20 and 40 μg MAA to 1 ml blank plasma and extracting according to the above mentioned method. Standard solutions for determination of MAA in urine contained 0, 10, 20, 50, 100 and 200 μg of the latter in 1 ml urine. Four series of such solutions were examined. Peak height ratios of MAA (at 254 nm) and IS (at 280 nm) were measured and a standard curve was prepared by plotting ratios versus amount of MAA. Statistical parameters were computed using a programmable calculator (Model 41C, Hewlett-Packard, Corvallis, OR, U.S.A.).

Analysis of both dipyrone and MAA in biological samples was always accompanied by preparation of freshly prepared standard solutions.

Recovery of MAA

The efficiency of the MAA extraction method was examined by preparing two series of solutions similar to those of standard solutions but MAA (in methanol) was added to the separated benzene layer after the extraction.

Subjects

Four healthy male subjects volunteered for this experiment. They ranged in age and weight between 20—30 years and 54—65 kg, respectively. Two tablets of 500 mg dipyrone (Novalgin, Hoechst, Iran) were ingested with a glass of water after an overnight fast and at least 1.5 h before breakfast. Venous blood samples were taken from forearms by heparinized disposable syringes at 0, 0.5, 1, 2, 4, 6 and 8 h post-dosing and plasma portions were separated. Following administration, total urine outputs at 0, 1.5, 3, 5, 7, 11, 12.5, 14 and 24 h were collected. Plasma and urine samples were kept frozen until the time of analysis. For determination of intact dipyrone, however, fresh plasma samples up to 4 h post-dosing were analyzed immediately after the collection.

Data treatment

The AUTOAN [10] and NONLIN [11] programs were used to estimate the

best-fit line through the data points and calculate the half-life ($t_{½}$) of the terminal phases of MAA curves.

RESULTS AND DISCUSSION

Fig. 1 depicts peaks representing intact dipyrone and IS added to blank plasma. The drug and IS appeared 6.3 and 11.2 min after injection into the HPLC system. No interfering peaks were observed. An excellent linear relationship (correlation coefficient of 0.996) was found between peak height ratios and the amount of added dipyrone. The best-fit line through the points was described by $y = 0.0429x - 0.0021$. The observed coefficients of variation (C.V.) ranged from 2 to 7% except for solutions containing 0.1 μg dipyrone per ml of plasma which showed a C.V. of 30%. The minimum quantifiable concentration (MQC) was, therefore, set at 1 μg/ml. Analysis of a set of standard solutions after 12 h referigeration showed no significant reduction in response. This indicates the stability of dipyrone under the utilized condition at least for 12 h.

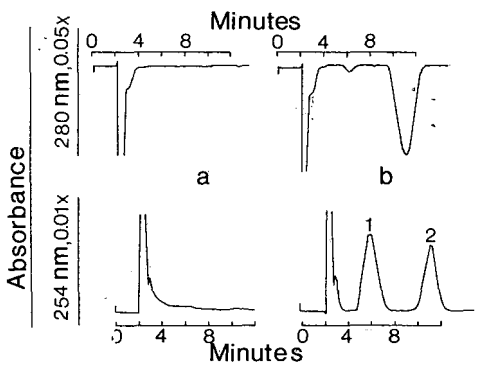
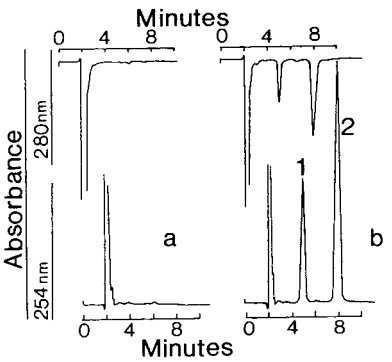


Fig. 1. Chromatograms of (a) blank plasma (b) dipyrone spiked plasma (4 μg/ml). Peaks: 1 = dipyrone, 2 = sodium salicylate.

A chromatogram of a plasma sample taken from subjects during the first 4 h after post-administration of 1 g dipyrone showed no peak with specifications of dipyrone. This observation indicates that the concentration of dipyrone in plasma of subjects taking 1 g dipyrone is substantially less than our MQC of 1 μg/ml.

Chromatograms of plasma and urine samples extracted with benzene and analyzed using a reversed-phase column are shown in Figs. 2 and 3, respectively. Peaks representing MAA and antipyrine were observed 5.2 and 8.0 min after injection into the chromatograph, respectively. Blank plasma and urine contained no interfering compounds. The selectivity of the assay was assured by routine examination of UV absorbance ratios at 280 over 254 nm for MAA (0.33) and antipyrine (0.31). An excellent linear relationship was found between the peak height ratios (MAA at 254/IS at 280) and the amount of MAA in samples. For the pooled data, the best-fit lines through the observed points were described by $y = 0.0161x - 0.0018$ and $y = 0.0200x - 0.0278$ for plasma and urine solutions with correlation coefficients of 0.998 and 0.999,



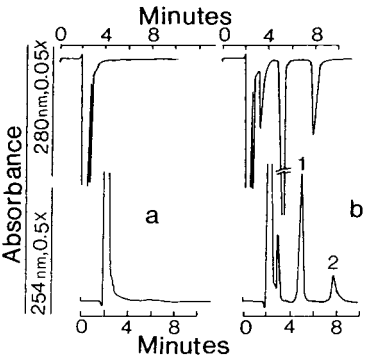


Fig. 2. Chromatograms of (a) blank plasma and (b) plasma of subject 1, 1 h after administration of 1000 mg dipyrone. Peaks: 1 = MAA, 2 = antipyrine. a.u.f.s., 0.05.

Fig. 3. Chromatograms of (a) blank urine and (b) urine of subject 1, 1.5 h following administration of 1000 mg dipyrone. Peaks: 1 = MAA, 2 = antipyrine.

respectively. Coefficient of variation varied from 2 to 9% within the examined ranges except for plasma samples containing 0.25 μg MAA, which was 11%. Averages of 78.1 and 86.1% with standard deviations of 3.8 and 4.7 were found to be extractable from plasma and urine, respectively.

After administration of dipyrone an extra peak was consistently present in urine. The latter which appeared 3.3 min after injection of the urine extracts into the instrument (Fig. 3) was, presumably, another metabolite of dipyrone. No attempt was made to identify the structure of this peak.

Table I depicts plasma MAA concentrations and the elimination half-lives ($t_{1/2}$) in four subjects following administration of single 1000-mg doses of dipyrone. Maximum plasma MAA concentration (C_{max}) varied from 7.52 to 14.99 μg/ml and attained (T_{max}) within the first 2 h post-dosing. At 8–9 h after administration, the concentration was between 1.65 and 2.82 μg/ml. The sensitivity of the method was found adequate to follow plasma and urine concentrations of MAA following administration of a single 1000-mg oral dose of dipyrone since concentrations as low as 0.25 μg/ml were quantifiable with acceptable C.V. values. However, the observed MQC can be further increased by utilization of larger plasma volumes.

Disposition kinetics of MAA conferred the characteristics of a one-compart-

TABLE I

PLASMA MAA CONCENTRATIONS (μg/ml) AND ELIMINATION HALF-LIVES ($t_{1/2}$) AFTER ORAL ADMINISTRATION OF 1000 mg DIPORONE TO HEALTHY SUBJECTS

Subject No.	Time (h)							$t_{1/2}$ (h)
	0.5	1	2	4	6	8	9	
1	9.85	14.99	12.98	6.04	2.86	1.57	—	1.87
2	8.52	14.74	13.67	8.74	6.29	2.82	—	2.22
3	5.91	9.45	9.27	6.86	3.44	1.90	—	2.21
4	1.14	4.88	7.52	4.02	2.54	1.65	1.25	2.46

TABLE II

CUMULATIVE URINARY MAA EXCRETION (mg) AND HALF-LIVES ($t_{½}$) OF EXCRETION-RATE PLOTS FOLLOWING ORAL ADMINISTRATION OF 1000 mg DIPYRONE TO HEALTHY SUBJECTS

Subject No.	Time (h)								$t_{½}$ (h)
	1.5	3	5	7	11	12.5	14	24	
1	4.43	9.94	15.07	16.67	17.74	17.83	17.95	17.99	1.60
2	5.81	12.64	14.01	17.21	19.75	20.29	21.16	21.21	2.44
3	—	4.77	8.67	11.33	—	14.50	—	15.28	2.96
4	—	2.65	4.01	6.05	6.83	—	8.78	9.56	—

ment model with a first-order input kinetic. The observed $t_{½}$ of MAA disappearance from plasma ranged from 1.87 to 2.46 h with a mean and standard deviation (S.D.) of 2.19 h and 0.23, respectively. The observed MAA plasma concentration—time curves seem to agree with those reported earlier [6] using a spectrophotometric assay. These authors reported a mean C_{max} value of approximately 8 ± 4 (S.D.) μg/ml. The amount of MAA excreted in urine and $t_{½}$ values of excretion-rate plots as a result of a 1000-mg dose of dipyrone are shown in Table II. A total of 9.56—21.21 mg of the metabolite was recovered in urine during the 24-h collection period. Excretion rate—time plots had comparable $t_{½}$ values to those of plasma MAA concentration—time curves (1.60—2.96 h). Due to a substantial fluctuation in the urinary excretion of MAA in subject 4, the $t_{½}$ value of the excretion rate—time plot was not considered.

Upon administration, dipyrone is rapidly, and to a great extent, biotransformed to its major metabolite, MAA [6] which seems to be more effective than the parent compound [7]. In our laboratory, using an anion exchange system, we were able to quantify dipyrone in concentrations as low as 1 μg/ml when added to blank plasma. Following oral administration of 1000 mg dipyrone, however, we failed to detect even traces of the unchanged drug in plasma. This phenomenon is not likely to be caused by a lack of dipyrone chemical stability. The administered tablets contained 102—109% of the claimed dipyrone content. Furthermore, it has been found by others that chemical degradation of dipyrone is minimal in methanol [7]. In this work, we extracted dipyrone into methanol by adding excessive sodium carbonate to saturate the aqueous layer. The absence of significant changes in the peak height ratios of dipyrone-spiked plasma solutions (standards) after 12 h refrigeration supports this suggestion. It, therefore, seems reasonable to suggest that the absence of intact dipyrone in plasma of subjects taking this drug may be due to its rapid and extensive metabolism to MAA upon the first-pass through the gut and/or liver. This metabolic characteristic of dipyrone is similar to that of acetylsalicylic acid which upon administration is rapidly biotransformed to salicylic acid [12].

This method is superior to that of Tamura et al. [7] in sensitivity and peak resolution. Their method was applied to measure dipyrone and its metabolites in muscle in 0.09—0.70 mg/g range and failed to achieve a complete separation

of the resultant peaks. Dipyrone is administered orally, intravenously or intramuscularly as single doses of 500—2500 mg to relieve pain. The method outlined here seems to be quite suitable to follow the time course of its major metabolite, MAA, in human urine and plasma.

ACKNOWLEDGEMENTS

Supported by grant 400-3-57/2, Ministry of Culture and Advanced Education, Iran. Assistance of Mr. Abas Yaser-Afra is gratefully acknowledged.

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Journal of Chromatography, 277 (1983) 191—199
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1793

DETERMINATION OF CANRENONE, THE MAJOR METABOLITE OF SPIRONOLACTONE, IN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received February 23rd, 1983; revised manuscript received May 20th, 1983)

SUMMARY

An assay procedure for measuring plasma and urine levels of canrenone is described. The drug is extracted with *n*-hexane—toluene (1:1, v/v) after adding spirorenone as internal standard, and is then separated from plasma constituents and metabolites by high-performance liquid chromatography followed by UV detection at 285 nm. The limit of detection is less than 5 ng/ml. Interference with a series of spironolactone and canrenone metabolites was not observed. Plasma levels and renal excretion of canrenone after oral administration of 200 mg of spironolactone and intravenous injection of 200 mg of potassium canrenoate to a healthy male volunteer were measured.

INTRODUCTION

Spironolactone (7α-acetylthio-17-hydroxy-3-oxo-17α-pregn-4-ene-21-carboxylic acid-γ-lactone) is a synthetic steroid which is widely used in the treatment of oedema, cirrhosis of the liver and hypertension [1]. Its mechanism of action is competitive antagonism of aldosterone [2]. Spironolactone is metabolized in man to a variety of compounds which can be subdivided into two classes [3—5]. In the first group, the sulphur atom of the parent drug is retained in the molecule. The second group is formed by dethioacetylation of spironolactone and subsequent biotransformation steps. The most important compound in this second class is canrenone (Fig. 1). It is pharmacologically active and accounts for the major part of the activity of spironolactone, at least after multiple oral administrations [6]. Potassium canrenoate is used as intravenous formulation due to its higher water solubility in comparison to canrenone and spironolactone. In vivo, potassium canrenoate is rapidly converted to canrenone in an equilibrium reaction [7].

0378-4347/83/$03.00

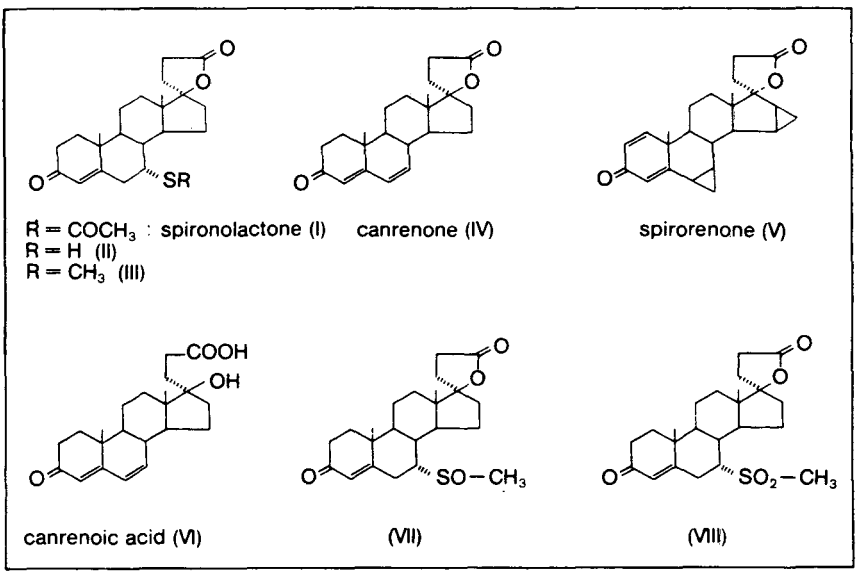

Fig. 1. Structures of spironolactone and some of its metabolites and of the internal standard, spirorenone.

Canrenone is assayed in biological fluids mostly by a fluorimetric method, first described by Gochman and Gantt [8], and modified by Sadee et al. [9] and by Karim et al. [10]. Neubert and Koch automated this procedure [11]. However, this method lacks specificity, which was concluded from a comparison of plasma levels and pharmacologic effects following administration of either spironolactone or potassium canrenoate [12], and therefore other investigators have looked for different methods with higher reliability.

The most selective method at present seems to be a high-performance liquid chromatographic (HPLC) assay described by Neurath and Ambrosius [13]. However, in this procedure normal-phase columns are used which cannot be handled as easily as reversed-phase columns. Furthermore, an internal standard eliminating extraction errors was not used by these authors.

Therefore, the aim of the present study was to establish an assay procedure for canrenone using reversed-phase chromatography, with spirorenone (Fig. 1) as internal standard, and to apply this method to the determination of canrenone levels in the plasma and urine of a healthy male volunteer.

EXPERIMENTAL DESIGN

Subject and medication

A healthy male volunteer (33 years old, 63 kg body weight) was given 200 mg of potassium canrenoate (Sincomen®-pro injectione) intravenously. Blood samples were taken 5, 10, 15, 20, 30, 45, 60, 90 min and 2, 4, 6, 8, 12 and 24 h after drug administration. The samples were immediately centrifuged and the plasma stored at −20°C until analysis. One week later the same test

subject received an oral dose of 200 mg of spironolactone (Sincomen-100). Sampling times were 15, 30, 45, 60, 90 min and 2, 4, 6, 8, 12 and 24 h after drug intake.

Chemicals

Methanol, *n*-hexane and toluene were of analytical-reagent grade (Merck, Darmstadt, F.R.G.) and were used without further purification.

Extraction procedure

A 0.2-ml aliquot of plasma or urine was pipetted into an 8-ml stoppered test tube containing 300 ng of spirorenone (Fig. 1) as internal standard. The samples were extracted two times with 1 ml of toluene—*n*-hexane (1:1, v/v) by vortexing for 1 min and subsequent centrifugation at 1200 *g* for 5 min. The organic layers were combined and taken to dryness under a slight stream of nitrogen. The residue was taken up in 250 μl of the HPLC eluent and 200 μl were injected for analysis.

Alternatively, with low concentration samples, 1 ml of plasma or urine were used with varying amounts of internal standard. Extraction then was performed twice with 3 ml of toluene—*n*-hexane (1:1, v/v).

The extraction efficiencies were determined with 0.2-ml plasma and urine samples containing 31.25, 125 and 300 ng of canrenone. With 1-ml plasma samples recoveries were calculated by comparing two standard curves obtained by plotting peak heights of canrenone before and after extraction versus the amount of drug.

Chromatographic system

The HPLC system consisted of a solvent delivery pump (Waters, Königstein, F.R.G., type 6000 A) and a UV detector with variable wavelength (Schoeffel SF 770) set at 285 nm. The chromatographic columns used were either a Shandon ODS Hypersil (60 × 4.6 mm, 3 μm; Bischoff, Stuttgart, F.R.G.) or a Spherisorb ODS-2 (125 × 4.6 mm, 5 μm; Bischoff) with pre-columns (20 × 4.6 mm). Injection was accomplished with a Rheodyne RH 7120 system or alternatively with an automatic sampling device (WISP, Waters).

The mobile phase was methanol—water in the ratio 60 : 40 (v/v), using the 3-μm column and 65 : 35 (v/v) with the 5-μm column. The flow-rate was 1 ml/min in both cases. The whole system was operated at ambient temperature.

Standard curves were constructed with 0.2-ml plasma and urine samples containing 15.6, 31.25, 125, 250 and 500 ng of canrenone and 300 ng of spirorenone, and 1-ml samples with 10, 20, 40 and 60 ng of drug and 100 or 300 ng of internal standard. These samples were analyzed as described above. Peak areas were calculated from the peak height and the width at half height. Peak area ratios (canrenone/spirorenone) were plotted against the concentration of canrenone and the calibration curves were obtained by regression analysis.

RESULTS

Assay of plasma and urine

Recoveries from 0.2-ml plasma and urine samples were about 85% (Table I).

With 1-ml plasma samples 82% was found. The recovery of the internal standard was 81% as had been investigated earlier [14].

Chromatograms of plasma and urine extracts are shown in Figs. 2 and 3 using the longer column with 5-μm particles. With both columns the limit of detection was less than 5 ng/ml when extracting 1-ml samples. However, for routine analysis the short columns cannot be recommended, because they deteriorate fairly rapidly and lose their resolution.

TABLE I

EXTRACTION RECOVERIES

Recoveries (mean % ± S.D.) were determined by extracting 0.2 ml of plasma or urine (n = 7 each) spiked with different amounts of canrenone and comparing the peak areas measured to those of unextracted drug.

	Amount spiked (ng per 0.2 ml)		
	31.25	125	500
Plasma	85 ± 24	N.D.*	85 ± 7
Urine	88 ± 8	80 ± 6	87 ± 5

*Not determined.

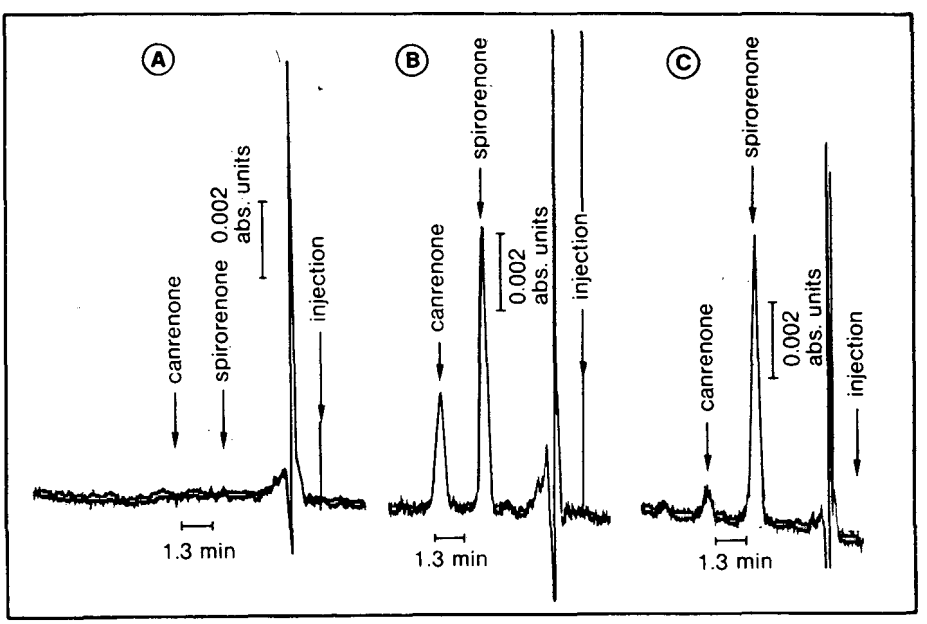

Fig. 2. HPLC chromatograms of 0.2-ml plasma samples obtained with a Spherisorb ODS-2 column (125 × 4.6 mm, 5 μm) before (A), and 4 h (B) and 12 h (C) after oral ingestion of 200 mg of spironolactone and addition of 300 ng of internal standard (B and C).

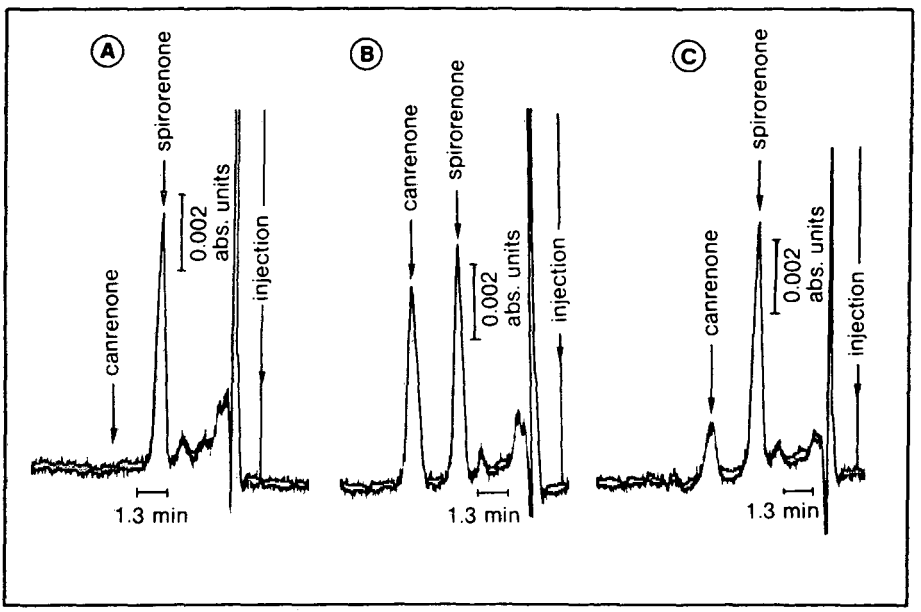

Fig. 3. HPLC chromatograms of 0.2-ml urine samples obtained with a Spherisorb ODS-2 column (125 × 4.6 mm, 5 μm) from blank urine (A), from a 0—2 h fraction (B) and from a 12—24 h fraction after adding 300 ng of internal standard.

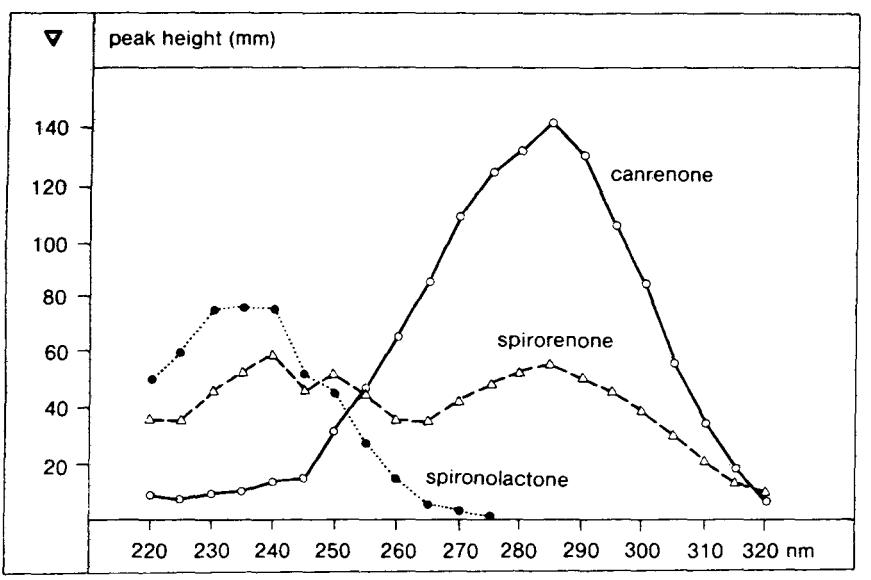

Fig. 4. UV spectra of canrenone, spironolactone and spirorenone obtained by measuring HPLC peak heights as a function of the wavelength of the UV detector.

The retention times of canrenone and of spirorenone were 7.8 and 5.1 min, respectively, with the 125-mm column. Spironolactone was eluted in this system after 5.8 min. However, with the UV detector set at 285 nm, spironolactone was not detected in the concentration range of interest. Sprionolactone has its UV maximum at 235 nm (Fig. 4), while canrenone and spirorenone exhibit a maximum at 285 nm.

This is also the case for those metabolites which are formed after dethioacetylation of the parent drug. The retention time of canrenoic acid, which is one metabolite of this group, was 1.1 min. Compounds of the second series of biodegradation products in which the sulphur atom is retained in the molecule include substances II, III, VII and VIII (Fig. 1). They were eluted after 6.8, 6.5, 2.2 and 2.1 min, respectively. Furthermore, at 285 nm no interference with canrenone or spirorenone was observed.

Calibration curves for the determination of unknown plasma and urine levels are listed in Table II. They were obtained by plotting peak height or peak area ratios of canrenone/spirorenone versus the concentration of the drug. Mean values for the slope and the intercept of the calibration lines obtained on five different days upon extraction of 0.2-ml plasma samples with 300 ng of internal standard and evaluation of peak areas are 0.114 ± 0.087 and 0.008 ± 0.001, respectively. Linearity of the calibration lines was positively tested up to 1500 ng/0.2 ml. The precision of the assay method at different concentration levels is given in Table III.

TABLE II

MATHEMATICAL EQUATIONS OF THE CALIBRATION CURVES FOR THE DETERMINATION OF UNKNOWN CANRENONE CONCENTRATIONS

The curves were obtained by spiking 0.2 ml and 1 ml of plasma or urine with various amounts of drug and 100 ng or 300 ng of internal standard.

Sample	Sample volume extracted (ml)	Amount of standard added (ng)	Column particle size (μm)	Calibration curve	Correlation coefficient
Plasma	0.2	300	5	$Y = 0.045 + 0.010X^{\star}$ ($n = 14$)	0.999
Plasma	1	100	3	$Y = 0.042 + 0.019X^{\star\star}$ ($n = 10$)	0.996
Plasma	1	300	3	$Y = 0.029 + 0.015X^{\star\star}$ ($n = 10$)	0.99
Urine	0.2	300	5	$Y = 0.020 + 0.009X^{\star}$ ($n = 6$)	0.099997

* Y = (peak area of canrenone)/(peak area of spirorenone), X = ng of canrenone.
** Y = (peak height of canrenone)/(peak height of spirorenone), X = ng of canrenone.

Plasma and urine levels

Plasma concentrations measured in the test subject after oral ingestion of 200 mg of spironolactone and intravenous injection of 200 mg of potassium canrenoate are illustrated in Fig. 5. Following oral administration the maxi-

mum concentration was 210 ng/ml at 4 h after ingestion. Intravenous administration resulted in a maximum plasma level of 3136 ng/ml, 20 min post administration.

Renal excretion of unchanged canrenone was less than 0.5% of the dose after both ways of administration (Fig. 6).

TABLE III

PRECISION OF THE ASSAY METHOD

Precision was obtained from consecutive determinations on the same day of canrenone in 0.2 or 1 ml of plasma and urine and calculations of peak area ratios.

Concentration (ng per 0.2 ml)	Plasma (%)	Urine (%)
31.25	6.1 (n = 7)	7.3 (n = 8)
125	4.1 (n = 7)	5.5 (n = 7)
500	2.2 (n = 7)	2.2 (n = 7)
20 (ng/ml)	N.D.*	4.3** (n = 10)

*Not determined.
**Peak height ratio.

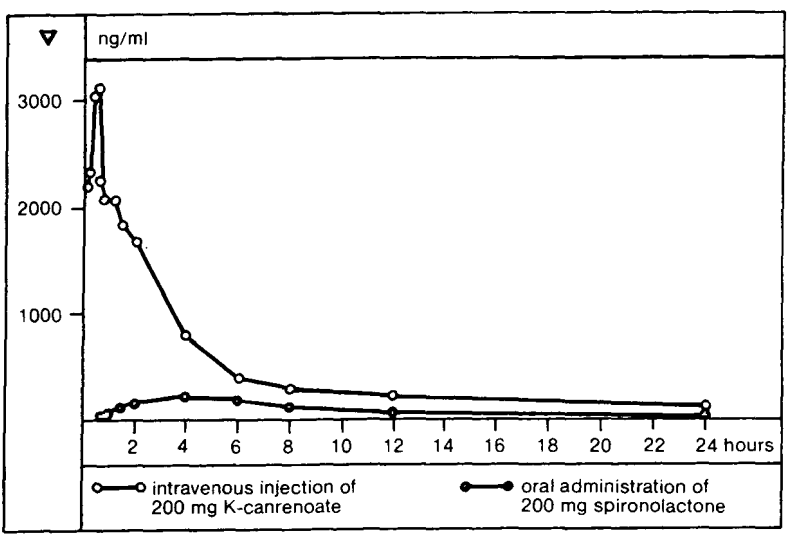

Fig. 5. Plasma level of canrenone in a healthy male volunteer who had received 200 mg of spironolactone orally and 200 mg of potassium canrenoate by intravenous injection.

DISCUSSION

Several methods have been described in the literature for the determination of canrenone in biological fluids. The most widely used procedure is the fluorimetric assay, indicating maximum levels of 400—500 ng/ml after oral

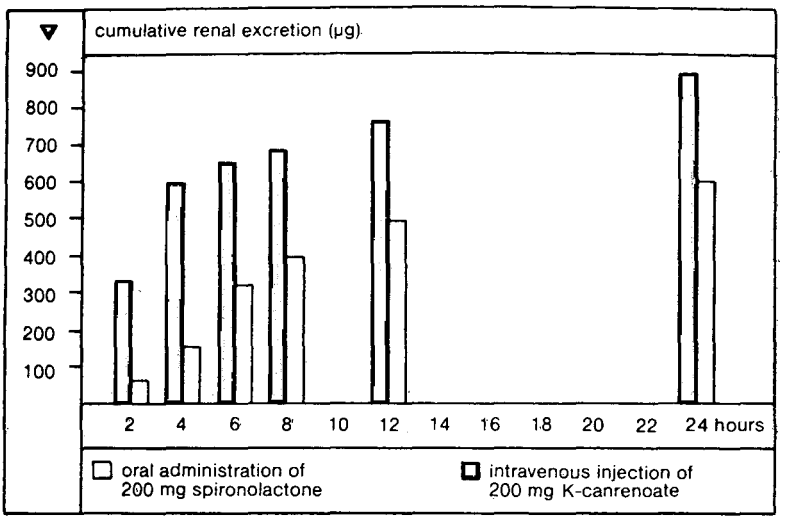

Fig. 6. Cumulative urinary excretion of canrenone in a male test subject after oral administration of 200 mg of spironolactone and intravenous injection of 200 mg of potassium canrenoate.

administration of 100 mg of spironolactone. The HPLC method described by Neurath and Ambrosius [13] yielded peak values of 45 ng/ml after the same dose. This is in the same order of magnitude as the results obtained in the present study considering the higher dose administered and the very small body weight of the test subject studied resulting in a diminished volume of distribution and, therefore, higher plasma levels.

The method of determining canrenone described here differs from the procedure by Neurath and Ambrosius by using *n*-hexane—toluene for extraction, by a reversed-phase column instead of normal-phase HPLC and by the addition of an internal standard.

The advantage of reversed-phase HPLC systems as compared to normal-phase chromatography usually lies in better suitability for routine analysis. Furthermore, an internal standard which is used in the present procedure compensates for losses during the extraction process. Thus the assay procedure described seems to be more suitable for the routine analysis of canrenone in plasma and urine samples. An application of this method in clinical pharmacokinetics will be reported later.

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Journal of Chromatography, 277 (1983) 201—208
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1762

METHODS FOR THE DETERMINATION OF LORAZEPAM AND CHLORDIAZEPOXIDE AND METABOLITES IN BRAIN TISSUE

A COMPARISON WITH PLASMA CONCENTRATIONS IN THE RAT

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(First received August 9th, 1982; revised manuscript received April 13th, 1983)

SUMMARY

Rapid and sensitive methods are described for determining lorazepam and for determining chlordiazepoxide and its metabolites in brain tissue of the rat. Lorazepam was determined by means of solvent extraction and electron-capture gas—liquid chromatography and concentrations as low as 5 ng/g tissue could be measured. High-performance liquid chromatography with UV detection was used to determine chlordiazepoxide and its metabolites and was sensitive to 0.1 μg/g tissue. The methods were used to investigate the brain and plasma pharmacokinetics of these compounds in animals that had been chronically treated with lorazepam or chlordiazepoxide. In both experiments brain and plasma levels of all compounds assayed were found to correlate highly.

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0378-4347/83/$03.00

INTRODUCTION

The plasma kinetics of many benzodiazepines have been extensively reported in both animals and man [1], although plasma levels of these compounds are not good predictors of their behavioural effects [2–6].

Although there are already many methods available for determining benzodiazepine concentrations in plasma [7–13], there is a need for routine methods for determining these compounds in brain tissue. Increased blood–brain permeability has been observed following electroconvulsive shock [14, 15], and similar pharmacokinetic changes may underlie other alterations to in vivo responses to drugs. Previous workers have investigated the brain pharmacokinetics of animals receiving either single low doses of [^{14}C]lorazepam or high doses of unlabelled compound [16]. Similarly, early studies on the metabolism of chlordiazepoxide used high doses of [^{14}C]chlordiazepoxide [17]. The purpose of this study was to develop rapid and sensitive methods for determining unlabelled lorazepam and for determining unlabelled chlordiazepoxide and its metabolites (Fig. 1) in brain tissue of the rat. In both studies plasma and brain kinetics of the drugs were to be compared in animals receiving once daily injections of the compounds for 5 or 10 days, using doses that are known to induce metabolising enzymes [6, 18]. Lorazepam has been determined in plasma using gas chromatography (GC) with either mass spectrometry [7], or electron-capture detection [13], and more recently using high-performance liquid chromatography (HPLC) [8]. Electron-capture GC was used in the present study since we have previously found this method to be reliable and sensitive for determinations of lorazepam in plasma and in urine [13].

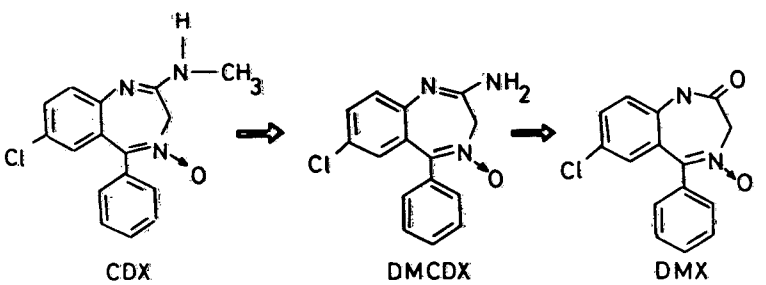

Fig. 1. The metabolic pathway of chlordiazepoxide in the rat, showing chlordiazepoxide (CDX) and its two principal metabolites, desmethylchlordiazepoxide (DMCDX) and demoxepam (DMX).

Methods for determining chlordiazepoxide and its metabolites in plasma have utilised reversed-phase HPLC [9–12], and this technique was therefore used in this study.

EXPERIMENTAL

Apparatus and chromatographic conditions

Gas chromatograph. The analytical instrument was a Model 5830A or 5840A Hewlett-Packard gas chromatograph equipped with a 15-mCi ^{63}Ni electron-

capture detector. The column was coiled glass, 183 cm × 2 mm I.D., packed with 3% OV-25 on Chromosorb W HP (80—100 mesh, Supelco, Bellefonte, PA, U.S.A.). The carrier gas was argon—methane (95:5) at a flow-rate of 30 ml/min. The operating temperatures were: injection port 310°C, column 255°C, and detector 310°C. The column was primed daily by an injection of 2—3 μl of an azolectin solution in benzene (1 mg/ml).

HPLC. A Waters Assoc. (Milford, MA, U.S.A.) high-performance liquid chromatograph consisting of a Model 6000A pump for delivery of mobile phase, a WISP Model 610B automatic sample processor and a 30 cm × 3.9 mm stainless steel C-18 μBondapak (10 μm) reversed-phase column was used. The chromatograph was linked to a Model 440 UV detector operated at 254 nm.

The mobile phase for the chromatography was prepared by mixing 450 ml methanol—acetonitrile (50:50), 550 ml water and 1 ml 1 *M* sodium acetate. The flow-rate was 2.0 ml/min. The organic and aqueous components of the mobile phase were filtered prior to use.

Drugs

Lorazepam (Wyeth, U.K.) was dissolved in a vehicle of 2% benzyl alcohol, 18% polyethylene glycol and 80% propylene glycol to concentrations of 2 and 4 mg/ml. This was then further diluted with water to give concentrations of 0.25 and 0.50 mg/ml. Chlordiazepoxide hydrochloride (Roche, U.K.) was dissolved in water to give a concentration equivalent to 25 mg/ml chlordiazepoxide free base. Injection volumes were 2.0 ml/kg.

Standards and solvents

Pure samples of lorazepam and oxazepam were kindly supplied by Wyeth (Radnor, PA, U.S.A.). Standard solutions were prepared by dissolving 10 mg of each compound in 2—3 ml absolute alcohol and diluting to 100 ml with distilled water. Working standards were prepared by appropriate dilution with distilled water.

Chlordiazepoxide (CDX) hydrochloride, demoxepam (DMX), desmethylchlordiazepoxide (DMCDX) and chlorodesmethyldiazepam (RO 5-3027) were all kindly donated by Dr. W.E. Scott of Hoffmann-La Roche (Nutley, NJ, U.S.A.). Standard solutions were prepared by dissolving these compounds in methanol. Solutions of CDX, DMX and RO 5-3027 are stable when stored in amber bottles at 4°C for up to four months but DMCDX is unstable and was prepared fresh for each determination.

Organic solvents were liquid-chromatography grade and were obtained from commercial sources.

Drug administration

Experiment 1. Twenty-one male hooded Lister rats (Olac. Bicester), weighing 350—400 g, received a daily intraperitoneal (i.p.) injection of 0.5 mg/kg lorazepam for 5 days. A further 21 animals received a similar 5-day treatment with 1.0 mg/kg lorazepam. On the 5th day groups of animals were killed at intervals of 30, 90 and 240 min after administration of the drug. Immediately after death blood samples were collected in heparinised tubes and the animals' brains were removed and stored at −20°C. Plasma was separated by centrifugation and also stored at −20°C.

Plasma concentrations of lorazepam were determined as described previously [13].

Experiment 2. Twenty-eight male hooded rats (350—400 g) received once daily i.p. injections of 50 mg/kg CDX for 10 days. On the 10th day groups of animals were killed at intervals of 30, 120, 240 and 360 min after the final injection. Plasma and brain samples were collected and stored as in Experiment 1.

Plasma concentrations of CDX and its metabolites DMCDX and DMX were determined as previously described [12].

Determination of lorazepam in brain

Oxazepam was used as the internal standard since it has pK_a values similar to those of lorazepam [19] and also undergoes the same thermal rearrangement on the chromatographic column [20]. A series of round-bottomed stoppered tubes was prepared containing 75 ng oxazepam (75 μl of 1.0 μg/ml stock solution). Each brain minus the cerebellum was bisected along the midline. Half-brains were weighed, homogenized in 3.5 ml 0.4 *M* perchloric acid and the homogenate transferred to one of the tubes. (Each half-brain weighed about 0.6 g.) The pH was adjusted to approx. 9 by addition of 2 *M* sodium carbonate solution; 4 ml benzene—dichloromethane (80:20) were added, the tubes vortexed for 1 min and then centrifuged at 400 *g* for 10 min.

The organic phase was transferred to a tube containing 1.5 ml 4 *M* hydrochloric acid. The tubes were vortexed, centrifuged and the organic phase discarded. The aqueous phase was washed with a further 4 ml of benzene—dichloromethane. After discarding the organic phase the pH of the aqueous phase was adjusted to approx. 9 by addition of 2 *M* sodium carbonate solution; 2.0 ml benzene—dichloromethane were added. After mixing and centrifugation, the organic phase was removed, evaporated to dryness at 40°C under conditions of mild vacuum and reconstituted with 175 μl toluene (containing 15% isoamyl alcohol); 6 μl were injected into the chromatograph.

Standard curve. A series of tubes containing 75 ng oxazepam and 12.5, 25, 50, 75, 100 or 150 ng lorazepam were prepared. Drug-free control brain samples were homogenized in 3.5 ml 0.4 *M* perchloric acid and added to each of the tubes. A standard curve was obtained by taking these tubes through the extraction process. Standards were assayed with each set of unknowns. Since the chromatographic peaks corresponding to both oxazepam and lorazepam are Gaussian, the standard curve was obtained by plotting lorazepam concentration against lorazepam to oxazepam peak height ratio.

Determination of chlordiazepoxide and metabolites in brain

A series of tubes containing 10 μg of the internal standard RO 5-3027 was prepared (0.1 ml of a solution containing 100 μg/ml evaporated to dryness). Brains were weighed, homogenized, centrifuged and made alkaline as described for the lorazepam determination. After addition of the sodium carbonate solution, 4 ml benzene—isoamyl alcohol (98.5:1.5) were added and the tubes vortexed for 1 min and centrifuged. The organic phase was transferred to a pointed centrifuge tube and evaporated to dryness under mildly reduced

pressure. The residue was reconstituted in 200 μl HPLC-grade methanol and transferred to an autoinjection tube; 25—30 μl were injected into the chromatograph.

Standard curve. A standard curve was obtained in a similar manner to Experiment 1. Standard tubes contained 10 μg RO 5-3027 and 0.1, 0.25, 0.5, 1.0, 2.0, 5.0 and 10.0 μg CDX, DMX and DMCDX. Standards were again assayed with each set of unknowns.

Statistics

Brain/plasma concentration ratios were analysed using analysis of variance with time (and dose in the lorazepam experiment) as the independent factor(s).

RESULTS AND DISCUSSION

Experiment 1

Under the described conditions, the retention times of oxazepam and lorazepam are 4.9 and 6.4 min, respectively (see Fig. 2). Plots of peak height ratio against lorazepam concentration were linear over the concentration range studied and yielded correlation coefficients of 0.99 or greater.

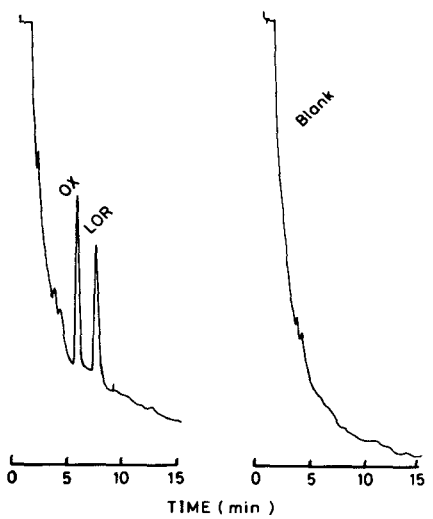

Fig. 2. A chromatogram of a drug-free control brain extract (right) and a sample to which 75 ng of oxazepam (OX) and 50 ng lorazepam (LOR) had been added (left).

Since the pK_a values of lorazepam are 1.3 and 11.5 and of oxazepam are 1.7 and 11.6 [19] it is important that the aqueous phase is not too basic before the extraction into benzene—dichloromethane. Concentrations of 5—10 ng/g tissue could be determined using this method and recoveries of 50% were obtained. Coefficients of variation for identical samples (n = 5) were 9.6% at 25 ng and 8.6% at 100 ng/g tissue.

The mean concentrations of lorazepam in brain tissue and in plasma, together with the brain/plasma concentration ratios are given in Table I. At

TABLE I

CONCENTRATIONS OF LORAZEPAM IN BRAIN AND IN PLASMA AS A FUNCTION OF TIME AFTER I.P. ADMINISTRATION OF 0.5 AND 1.0 mg/kg

Figures are means ± S.E.M.

	Time (min)	Brain concn. (ng/g)	Plasma concn. (ng/ml)	Brain/plasma
0.5 mg/kg lorazepam	30	189 ± 9	79 ± 3	2.39 ± 0.07
	90	94 ± 8	23 ± 2	4.26 ± 0.39
	240	16.1 ± 4.2	3.6 ± 1.0	4.53 ± 0.80
1.0 mg/kg lorazepam	30	306 ± 16	176 ± 16	1.78 ± 0.13
	90	163 ± 17	43 ± 3	3.75 ± 0.28
	240	26 ± 4	6.8 ± 0.7	3.75 ± 0.60

any given time after the administration of 0.5 or 1.0 mg/kg lorazepam the brain tissue/plasma (B/P) ratios showed little variation. At all time points the ratio was greater than 1.0 indicating a localization of lorazepam in brain tissue. The B/P ratio varied significantly with time [$F(2,36) = 12.9, p < 0.001$], reflecting an equilibration delay at 30 min after each dose. At this time there was also a

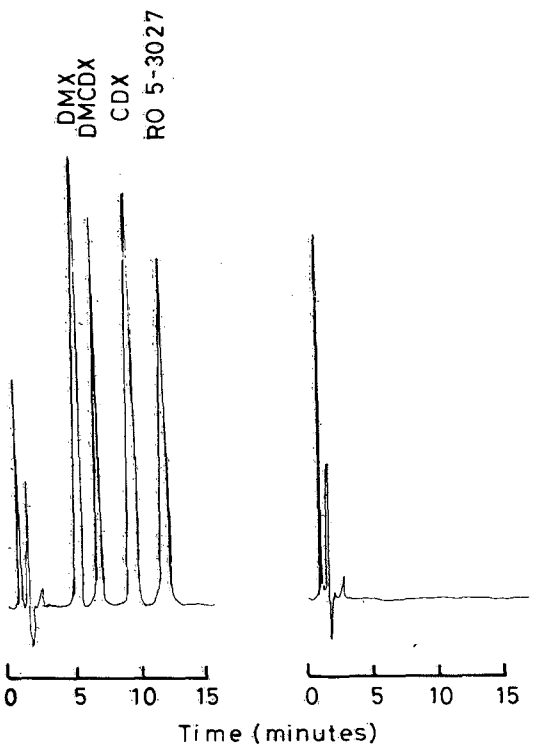

Fig. 3. A chromatogram of a drug-free control brain extract (right) and a sample to which chlordiazepoxide (CDX), desmethylchlordiazepoxide (DMCDX) and demoxepam (DMX), 5 μg of each, and the internal standard chlorodesmethyldiazepam (RO 5-3027), 10 μg have been added (left).

significant difference between the B/P ratios for the two doses ($p < 0.01$), but there were no significant differences between doses at the other times when samples were taken. For both doses plasma and brain half-lives were just less than 1 h.

There was a high correlation between the brain and plasma concentrations of lorazepam ($r = 0.88$, $p < 0.001$).

Experiment 2

The retention times for DMX, DMCDX, CDX and chlorodesmethyldiazepam are approximately 5.5, 7, 9.5 and 12 min, respectively (see Fig. 3). Plots of peak area or height ratio (relative to the internal standard) against concentration were linear for each compound up to concentrations of at least 10 μg/g brain tissue and gave correlation coefficients of greater than 0.99. Recoveries of 75—85% were obtained and concentrations as low as 0.1 μg/g brain tissue could

TABLE II

REPLICABILITY OF IDENTICAL SAMPLES

Brain concentration (μg/g)	Coefficient of variation* ($n = 6$)		
	Chlordiazepoxide	Desmethylchlordiazepoxide	Demoxepam
0.25	12.0	11.6	8.9
2.0	2.7	9.1	3.2

*Standard deviation divided by mean, in per cent.

TABLE III

CONCENTRATIONS OF CHLORDIAZEPOXIDE AND METABOLITES IN PLASMA AND IN BRAIN AS A FUNCTION OF TIME AFTER I.P. ADMINISTRATION OF 50 mg/kg

Figures are means ± S.E.M.

		Brain (μg/g)	Plasma (μg/ml)	Brain/plasma
30 Minutes	Chlordiazepoxide	25.3 ± 4.0	15.3 ± 1.7	1.62 ± 0.13
	Desmethylchlordiazepoxide	3.9 ± 0.5	1.6 ± 0.2	2.43 ± 0.13
	Demoxepam	0.5 ± 0.1	4.0 ± 0.3	0.13 ± 0.01
120 Minutes	Chlordiazepoxide	13.5 ± 2.9	8.2 ± 1.6	1.61 ± 0.05
	Desmethylchlordiazepoxide	6.5 ± 1.2	1.7 ± 0.4	4.23 ± 0.58
	Demoxepam	0.8 ± 0.1	5.2 ± 0.7	0.15 ± 0.03
240 Minutes	Chlordiazepoxide	6.2 ± 0.9	3.9 ± 0.6	1.68 ± 0.20
	Desmethylchlordiazepoxide	4.9 ± 0.6	1.2 ± 0.2	4.54 ± 0.60
	Demoxepam	0.6 ± 0.1	4.0 ± 0.4	0.16 ± 0.02
360 Minutes	Chlordiazepoxide	3.9 ± 1.1	2.4 ± 0.8	1.80 ± 0.22
	Desmethylchlordiazepoxide	4.5 ± 1.0	0.8 ± 0.2	5.91 ± 0.61
	Demoxepam	0.5 ± 0.2	3.3 ± 0.9	0.18 ± 0.02

be determined for all compounds. Coefficients of variation for identical samples are shown in Table II. The mean brain and plasma concentrations of CDX and its metabolites are given in Table III.

The B/P ratios for CDX and DMX showed little variation, although the ratio for DMCDX increased with time [$F(3,24) = 7.44$, $p < 0.002$]. There were significant correlations between plasma and brain concentrations of CDX, DMX and DMCDX ($r = 0.98$, 0.74 and 0.66, respectively, $p < 0.001$ for each compound).

Plots of log plasma concentration or log brain concentration against time for CDX were linear ($r > 0.9996$). Plasma and brain half-lives calculated from the gradients were 103 and 107 min, respectively.

The methods described for determining lorazepam and for determining CDX and its metabolites in brain tissue were rapid, clean and sensitive and will be useful to workers needing to follow the brain pharmacokinetics of benzodiazepines in the investigation of drug interactions.

ACKNOWLEDGEMENTS

R.G.L. is supported by a School of Pharmacy postgraduate award. S.E.F. is a Wellcome Trust senior lecturer. This work was supported in part by Grants MH-34223 and AM-32050 from the United States Public Health Service.

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Journal of Chromatography, 277 (1983) 209—215
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1770

ANALYSIS OF THE ANTIMALARIAL, MEFLOQUINE, IN BLOOD AND PLASMA USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received January 17th, 1983; revised manuscript received May 6th, 1983)

SUMMARY

An analytical method is described for the quantitation of mefloquine, a new antimalarial agent, in plasma and blood. A structurally similar quinolinemethanol compound, WR 184,806, is used as the internal standard. The method employs a three-step extraction procedure followed by reversed-phase high-performance liquid chromatography, and octanesulfonate is used as an ion-pairing reagent. Detection is achieved at 222 nm. The entire procedure is relatively simple and requires only 1 ml of sample. Good accuracy and precision are obtained over the wide concentration range tested.

INTRODUCTION

Mefloquine [(*R**,*S**)-(±)-α-2-piperidinyl-2,8-bis(trifluoromethyl)-4-quinolinemethanol] hydrochloride is a recently developed antimalarial agent. It is effective as a single oral dose against multidrug-resistant malaria in man [1, 2]. It also provides suppressive prophylaxis against *Plasmodium vivax* and *P. falciparum* infections in human volunteers [3, 4]. Several methods have been published on the analysis of mefloquine in blood and/or plasma, including thin-layer chromatographic (TLC) [5], high-performance liquid chromatographic (HPLC) [6], ion-selective electrode [7], gas—liquid chromatographic (GLC) [8], and selected-ion monitoring (SIM) [9] methods. All of these methods have some inherent drawbacks. Several of them require a large sample size (5 ml) [6—8]. Certain methods used a relatively uncommon technique such as

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0378-4347/83/$03.00

an ion-selective electrode to detect hydrophobic cations in aqueous solution [7], or relatively sophisticated and expensive instrumentation such as gas chromatography—mass spectrometry [9]. Some of the methods require derivatization [7—9] and some do not use internal standard [5, 7]. The TLC technique, in addition to lacking the resolving power of HPLC [10], is further hampered by its sensitivity to changes in ambient conditions, a limited concentration range and by plate-to-plate differences [5]. Furthermore, in the reported methods [5—9], the evaporation step is carried out in ethyl acetate, isopropyl acetate or ether. It has been our experience that evaporation in these solvents can cause major decomposition of mefloquine even when solvents of the highest commercial grade or redistilled solvents are used.

Therefore, a relatively simple, yet sensitive and selective, method was developed for the analysis of mefloquine in blood and plasma and is currently being employed in pharmacokinetic studies.

EXPERIMENTAL

Chemicals

Mefloquine hydrochloride [WR 142,490 HCl, (*R**,*S**)-(±)-α-2-piperidinyl-2, 8-bis(trifluoromethyl-4-quinolinemethanol) hydrochloride] and WR 184,806 H_3PO_4 {[DL-2,8-bis(trifluoromethyl)-4-[1-hydroxy-3-(N-*tert.*-butylamino)-propyl] quinoline phosphate} were obtained on contract from Ash Stevens (Detroit, MI, U.S.A.), and Starks Associates (Buffalo, NY, U.S.A.), respectively. ^{14}C-Labeled mefloquine ([^{14}C] methanol, 57.8 mCi/mmole) was obtained on contract from Research Triangle Institute (Research Triangle Park, NC, U.S.A.). Quinine, primaquine, chloroquine, sulfadoxine, pyrimethamine, WR 180,409, WR 194,965, WR 171,669, WR 177,602 and WR 160,972 were obtained from Walter Reed stock. Solvents were purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Commercially available ion-pairing reagent, low UV PIC B8, was purchased from Waters Assoc. (Milford, MA, U.S.A.). Other chemicals were of the best commercially available grade.

Extraction of mefloquine

To disposable glass centrifuge tubes with Teflon-lined screw caps, were added 1 ml of blood or plasma and 1 ml of deionized water. Contents were mixed by vortexing and appropriate amounts of mefloquine and WR 184,806 (the internal standard) were added. Contents of the tubes were mixed again by vortexing and 1 ml of 0.5 *M* dibasic sodium phosphate was added and samples were vortexed well to ensure homogeneity. All extractions were carried out by slow mixing for 15 min using a Roto-Torque rotator (Cole-Parmer Instrument Co., Chicago, IL, U.S.A.) and centrifugation for 10 min at about 2500 *g* and 4° C in a Sorvall refrigerated RC-3B centrifuge (Dupont, Newtown, CT, U.S.A.). Blood or plasma samples were extracted with 5 ml of ethyl acetate–hexane (3:2, v/v). The organic phase was extracted with 2 ml of methanol plus 3 ml of 0.5 *M* monobasic ammonium phosphate. To the methanolic aqueous phase were added 2 ml of 0.2 *M* perchloric acid and the resultant mixture was extracted with 6 ml of dichloromethane. The dichloromethane phase was transferred to clean tubes and evaporated to dryness under nitrogen at room

temperature. The residue was redissolved in 100 μl of mobile phase just prior to analysis.

High-performance liquid chromatographic analysis

The chromatographic system consisted of a Model 6000A pump (Waters Assoc.), a Model U6K injector (Waters Assoc.), a Model LC-85 variable-wavelength UV detector (Perkin-Elmer, Norwalk, CT, U.S.A.), a Model 385 strip chart recorder (Linear Instruments, Irvine, CA, U.S.A.) and a 30 cm × 3.9 mm (I.D.) 10-μm particle size μBondapak C_{18} column (Waters Assoc.). The column was at ambient temperature. The mobile phase consisted of 0.005 *M* low UV PIC B8 in methanol—water (70:30, v/v) and the flow-rate was 1.0 ml/min. Injections were made using 10—50 μl of the sample and the UV signal was monitored at 222 nm and 0.01 or 0.02 a.u.f.s.

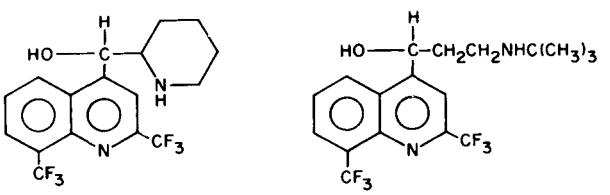
Quantitation

For each analysis, a standard curve was generated by adding known, varying amounts of mefloquine to outdated blank human blood or plasma (Walter Reed Army Medical Center Blood Bank, Washington, DC, U.S.A.) prior to extraction. Known and constant amounts (50 or 250 ng) of WR 184,806 (the internal standard) were also added to all samples prior to extraction. Standards (10—150 ng or 25—1500 ng) were selected to encompass the range of experimental values. Spiked samples were treated as unknowns to evaluate the accuracy and precision of the method. Quantitation was achieved using the peak height ratio of mefloquine to WR 184,806.

RESULTS AND DISCUSSION

The quinoline-derived antimalarials are used in the treatment of erythrocytic forms of the disease and it has been observed that the infection causes greater accumulation of these drugs in the red blood cells [11, 12]. The time-dependent concentration profile of antimalarials in both plasma and blood (and therefore also in red blood cells if hematocrit is known) may be of importance in understanding their action and optimizing the therapy. Therefore, it was deemed necessary to develop an assay which would allow analysis of mefloquine in both of these biological fluids.

In addition, quantitation of relatively low levels of mefloquine required combination of sensitive detection and relatively clean samples. Sufficient sensitivity was achieved at 222 nm, the apparent maximum absorbance for



I II

Fig. 1. Chemical structures of mefloquine (I) and WR 184,806 (II) (the internal standard).

mefloquine. A three-step extraction procedure was developed to provide clean samples compatible with high-sensitivity detection at 222 nm. Mefloquine and the internal standard are weak bases as can be seen from their chemical structures (Fig. 1) and the extraction procedure utilized this characteristic. These compounds were first extracted from biological matrix into ethyl acetate–hexane from which they were then extracted into methanolic acidic buffer. Perchloric acid was added to the methanolic buffer extract and the compounds were finally extracted as ion pairs into dichloromethane. The overall extraction efficiency was determined using 214 ng of [^{14}C] mefloquine and ranged between 60% and 70% for plasma and blood. Chromatographic separation was achieved by reversed-phase HPLC in the presence of octanesulfonate ion-pairing reagent. Representative chromatograms of extracts from blank plasma, spiked plasma and patient plasma and blank and spiked blood depicted in Figs. 2 and 3 demonstrate that sensitivity and clean sample criteria were met.

In order to cover a large (10—1500 ng/ml or ng/g) concentration range without sacrificing accuracy or precision, it was advisable to use two standard curves. The lower end standard curve was designed for 10—150 ng/ml or ng/g concentration range and the higher end standard curve for 25—1500 ng/ml or ng/g. Each standard curve consisted of at least eight points covering the anticipated assay range. Routinely, excellent linearity and a negligible Y-intercept were found. Using a least-squares linear regression analysis, representative equations of the line and the regression coefficients (r^2) for lower end and higher end standard curve were: $Y = 0.0132X + 0.0051$, $r^2 = 0.991$ and $Y = 0.00242X - 0.00531$, $r^2 = 0.999$, respectively.

The validation of the method was performed by using spiked plasma and blood. The results are summarized in Tables I–III and show good accuracy and precision in both plasma and blood.

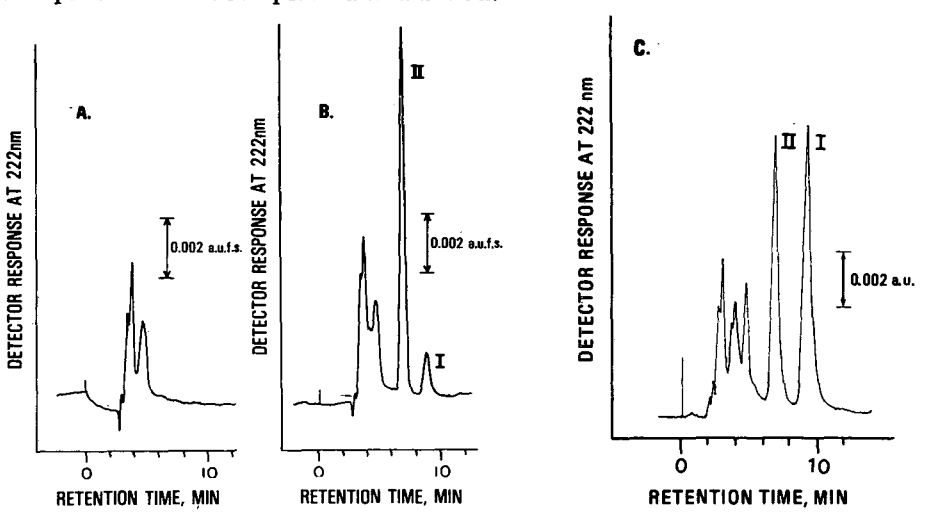


Fig. 2. High-performance liquid chromatogram of extracts from (A) blank human plasma, (B) human plasma spiked with 250 ng of WR 184,806 (II) and 50 ng of mefloquine (I), and (C) patient sample spiked with 250 ng of WR 184,806 (II) and containing 486 ng of mefloquine (I). In all instances, 25 μl of a total 100 μl were injected. For additional details, see the Experimental section.

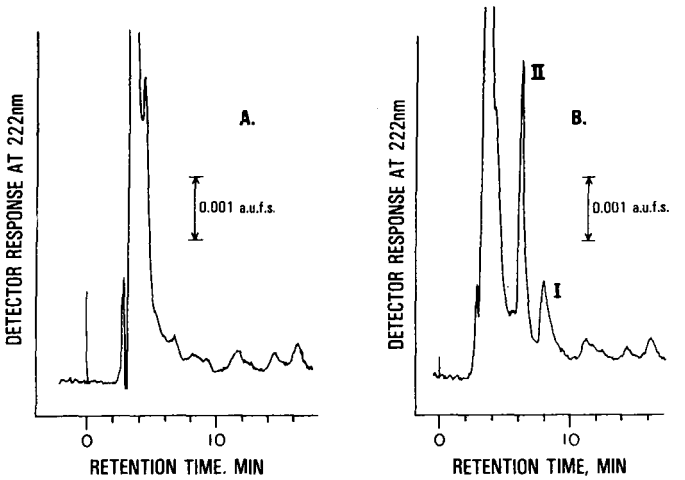


Fig. 3. High-performance liquid chromatogram of extracts from (A) blank human blood and (B) human blood spiked with 50 ng of WR 184,806 (II) and 15 ng of mefloquine (I). In both instances, 50 μl of a total 100 μl were injected. For additional details, see the Experimental section.

TABLE I

PRECISION AND ACCURACY DATA FOR MEFLOQUINE ANALYSIS IN PLASMA

Data represent a compilation of three separate experiments performed by the same investigator. Spiked unknowns were bracketed by a standard curve ranging from 25 to 1500 ng of mefloquine. For additional details, see the Experimental section.

Amount added (ng)	Amount measured (ng, mean ± S.D.)	C.V. (%)	*n*
50.0	49.2 ± 4.12	8.4	12
100.0	99.9 ± 2.16	2.2	4
300.0	303.2 ± 4.02	1.3	8
1400.0	1413.9 ± 19.5	1.4	9

TABLE II

PRECISION AND ACCURACY DATA FOR ANALYSIS OF HIGHER CONCENTRATIONS OF MEFLOQUINE IN BLOOD

Data represent a compilation of two separate experiments performed by the same investigator. Spiked unknowns were bracketed by a standard curve ranging from 25 to 1500 ng of mefloquine. For additional details, see the Experimental section.

Amount added (ng)	Amount measured (ng, mean ± S.D.)	C.V. (%)	*n*
50.0	49.6 ± 2.84	5.7	8
100.0	98.0 ± 5.73	5.8	8
1400.0	1442.5 ± 41.3	2.9	6

TABLE III

PRECISION AND ACCURACY DATA FOR ANALYSIS OF LOWER CONCENTRATIONS OF MEFLOQUINE IN BLOOD

Data represent a compilation of three separate experiments performed by the same investigator. Spiked unknowns were bracketed by a standard curve ranging from 10 to 150 ng of mefloquine. For additional details, see the Experimental section.

Amount added (ng)	Amount measured (ng, mean ± S.D.)	C.V. (%)	*n*
15.0	14.6 ± 1.92	13.2	12
30.0	29.4 ± 1.96	6.7	12
50.0	49.8 ± 1.49	3.0	12
90.0	92.9 ± 4.21	4.5	9

TABLE IV

RETENTION TIMES FOR MEFLOQUINE AND RELATED COMPOUNDS

Retention times were obtained under the HPLC conditions used for mefloquine analysis. For additional details, see the Experimental section. The elution time of the solvent front was 3.0 min.

Compound	Retention time (min)
Mefloquine	8.8
WR 184,806	6.8
WR 160,972	4.0
WR 177,602	7.1
Pyrimethamine	4.7
Sulfadoxine	3.4
Quinine	5.4
Primaquine	5.7
Chloroquine	5.9
WR 180,409	14.5
WR 194,965	18.8
WR 171,669	63.0

In order to check for possible interferences, chromatographic retention times for related compounds were also examined under these conditions and the data are presented in Table IV. Pyrimethamine, sulfadoxine, quinine, primaquine and chloroquine are other commonly used drugs for the treatment of malaria while WR 180,409 [DL-*threo*-α-(2-piperidyl)-2-trifluoromethyl-6-(4-trifluoromethylphenyl)-4-pyridinemethanol], WR 194,965 [(4-(*tert.*-butyl)-2-(*tert.*-butylaminomethyl)-6-(4-chlorophenyl)phenol], and WR 171,669 {halofantrine; [1-(1,3-dichloro-6-trifluoromethyl-9-phenanthryl)-3-di(*n*-butyl)aminopropanol]} are in investigational stages. WR 160,972 (2,8-trifluoromethylquinoline-4-carboxylic acid) is the major reported metabolite of mefloquine [13, 14] and WR 177,602 is a *threo* diastereoisomer of mefloquine. Based on the retention times, none of the compounds tested would be expected to interfere with mefloquine.

In separate additional experiments (data not shown), [^{14}C]mefloquine (542 ng) was added to fresh canine blood and samples were maintained for seven days at various temperatures. Using the analytical method described herein, essentially the same peak height ratio (mefloquine/WR 184,806) was found for freshly spiked samples and spiked samples which were kept for seven days at temperatures ranging from −20°C to +37°C. Furthermore, on the basis of chromatographic radioactivity data, no decomposition of mefloquine was observed during extraction or seven-day incubations.

In summary, the analytical method described here offers the sensitivity, selectivity and reliability necessary for accurate and precise determination of mefloquine in only 1 ml of blood or plasma. The method is relatively simple and is suitable for pharmacokinetic studies where it is being currently employed. Furthermore, the procedure is also easily adaptable for analysis of other quinoline antimalarials.

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Journal of Chromatography, 277 (1983) 217—227
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1777

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF AMEZINIUM IN HUMAN PLASMA

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(First received January 26th, 1983; revised manuscript received May 13th, 1983)

SUMMARY

A method with internal analogue standardization is described for the determination of 4-amino-6-methoxy-1-phenyl-pyridazinium methyl sulphate (ameziniummetilsulfate, Regulton®, LU 1631) in plasma. The method involves a cation-exchange filtration, a dequaternization reaction and subsequent determination by high-performance liquid chromatography. There is no interference by plasma components or amezinium metabolites found in plasma extracts. When using a 2-ml sample the lower limit of detection is 0.75 ng/ml; the lower limit of determination is about 2 ng/ml. Interassay coefficients of variation decrease from about 14% at the lowest concentration to about 5% at 20 ng/ml and above.

INTRODUCTION

Ameziniummetilsulfate* is a new antihypotensive agent [1] which differs from classic sympathomimetics by its completely novel structure and its unprecedented pattern of actions [2—5]. It has proved its value in the treatment of postural cardiovascular dysregulations in humans with a regimen of 10 mg b.i.d. [6—11]. The pharmacokinetic behaviour of amezinium has been described in detail for doses of 20 mg and above [12, 13] using a double-isotope derivatization technique with a lower level of determination of 3 ng/ml [14].

Since amezinium has been introduced into therapy a widespread interest in more specialized investigations has arisen in which determinations of amezinium concentrations in plasma play a salient role. Thus, the need has

*In this paper referred to as amezinium.

0378-4347/83/$03.00

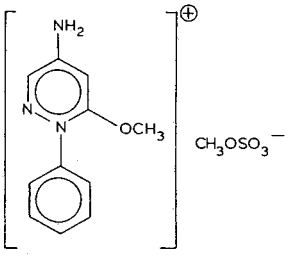
developed for a selective and sensitive method which can be practised in a well-equipped hospital laboratory or other. To this end, it should avoid the use of isotopes and highly sophisticated instrumentation and, rather, base itself on readily available techniques like high-performance liquid chromatography (HPLC).

This paper describes the development and evaluation of a selective determination method for amezinium in the lower nanogram range employing a cation-exchange filtration step prior to dequaternization by nucleophilic attack and subsequent separation and quantification by HPLC.

MATERIAL AND METHODS

Substances

Ameziniummetilsulfate. 4-Amino-6-methoxy-1-phenyl-pyridazinium methyl sulphate (ameziniummetilsulfate, LU 1631*) was first synthesized [1] in the Hauptlaboratorium of BASF Aktiengesellschaft. [methyl-^{14}C] Amezinium (specific activity: 336 MBq/mmole ≙ 9.08 mCi/mmole) was supplied by the Isotope Laboratory of BASF Aktiengesellschaft [1]. Being a pyridiazinium salt, amezinium is soluble and stable in water, methanol and ethanol; it decomposes, however, in alkaline media (pH > 8). The nature of the anion does not have any influence on the pharmacological action; it is only taken into account in so far as, in this study, all concentration data are based on the molecular weight 313 of the methyl sulphate, regardless of the chemical species actually handled.



Internal standard. 4-Amino-6-methoxy-1-*p*-tolyl-pyridazinium methyl sulphate (*p*-methyl-amezinium) was kindly supplied by Dr. Thyes, Hauptlaboratorium of BASF Aktiengesellschaft, who also provided in analytical grade quality 5-amino-2-phenyl-3(2H)-pyridazinone (LU 1724) and 5-amino-2-*p*-tolyl-3(2H)-pyridazinone (*p*-methyl-LU 1724), both products of the dequaternization procedure.

Other reagents. Cellulose phosphate (Whatman Grade P I; small ion capacity 3.7 mequiv./g dry weight) was used after a cycle of purification steps (washing with 1 *M* sodium chloride solution until supernatant is colourless: $\Delta E^{1\,cm}_{280-360\,nm} < 0.005$). The purified preparation can be stored without deterioration in the refrigerator at about 4° C for at least eight weeks.

All other reagents used were of the best commercially available quality.

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*Commercially available as Regulton®. Manufacturer: Nordmark-Werke, Uetersen, F.R.G.

Human plasma was supplied by the Transfusion Services of the Municipal Hospitals Mannheim and Ludwigshafen.

Preparation of spiked samples and determination of the nominal concentration

A standard solution of amezinium in human plasma is prepared from an aqueous stock solution (5 μg/ml) the exact concentration of which is determined spectrophotometrically (λ_{max} = 289 nm; ϵ = 15,100 cm^2 $mmol^{-1}$). From this standard solution containing 50.48 ng/ml the series of spiked samples with amezinium concentrations listed in Table VI is made by diluting with human plasma. The dilution steps are verified by weighing, the concentrations corrected according to the result of this weighing and taken as the basis for subsequent calculations as nominal concentrations. The human plasma samples thus prepared are divided into several series of aliquots of 2.0 ml and frozen at —25° C until analysis.

HPLC equipment

The equipment used comprises a Rheodyne sample injector, Type 7125, a high-pressure pump unit Waters Type 6000A, an HPLC column, Merck-Hibar® (250 × 4 mm), filled with LiChrosorb RP-8/7 μm (Merck), and a UV detector, Jasco-Uvidec-100 III, operated at 280 nm. The mobile phase is a mixture of acetonitrile—water (25:75, v/v) which is filtered (Teflon Fluorophore filter) and degassed. The flow-rate is 2 ml/min at about 20—28 MPa.

Analytical procedure

Ion-exchange columns of cellulose phosphate in 2-ml disposable syringes with the outlet closed with cotton wool are prepared (bed volume 0.9—1.1 ml) and washed twice with 2 ml of water. After addition of 44.84 ng of internal standard the plasma sample (0.1—2 ml; 2 ml throughout method evaluation) is applied to the column. The columns suspended in suitably sized tubes are centrifuged for about 10 min at 1000 *g*, washed with two consecutive 2-ml portions of water, and the unchanged amezinium and internal standard eluted with 1 ml of 1 *M* NaCl, centrifuging each time.

To this eluate 0.1 ml of 10 *M* NaOH is added and kept for 30 min at room temperature. Extraction of the resulting pyridazinones is performed by shaking with 4 ml of methylene chloride for 10 min. The organic layer is evaporated to dryness in a stream of nitrogen (temperature < 30° C). The residue is taken up in 100 μl of mobile phase and the maximum volume possible injected into the HPLC system.

Data recording and data reduction

The chromatogram is registered by means of a recorder and the region enclosing substance and internal standard is simultaneously digitized and stored on a magnetic tape cassette (A/D converter, Type BASF-TKL, with a cassette unit MFE 5450, Messrs. Fey, Munich, F.R.G.). The relevant chromatographic parameters (retention times, peak heights, and peak height ratios) are calculated by means of the GC evaluation program of Caesar and Klier [15] in a version adjusted to the requirements of an internal analogue standardization on the Honeywell-Bull Computer (BASF Data Processing Centre) via terminal.

Calculations

Amezinium concentration is calculated using peak height ratios according to the formula

$$c_A = \frac{m_A}{V} = \frac{k \cdot m_{St} \cdot Q}{V}$$

where c_A = amezinium concentration; m_A = the amount of amezinium in the assay; V = volume of sample; m_{St} = the amount of internal standard in the assay; Q = peak height ratio amezinium to internal standard; and k = proportionality factor between peak height ratio and mass ratio ($k = \frac{h_{St}}{h_A}$ for $m_{St} = m_A$).

The proportionality factor k is calculated for each series of analyses from three calibration samples of different concentrations done in duplicate. Possible systematic deviations in terms of an increase are to be judged as amezinium losses.

The data are summarized and tabulated, and various statistical parameters and tests based on the experimental data calculated using a Wang 2200.

To examine statistically the results to be discussed, the following calculations are carried out with confidence levels of 99%: nominal versus actual value comparisons for estimation of recoveries; testing of the regression coefficient for deviation from 1; testing of the ordinate intercept for deviation from 0 by t-test [16]; and linearity test of the calibration line by F-test [16]. Regression and correlation analysis are performed according to general instructions [17].

To estimate accuracy and precision of the method the mean values and their standard errors are determined for each concentration in all series of analyses. In the calculation of the quotients and differences (in order to determine accuracy) standard errors are calculated by error propagation.

RESULTS

Cation-exchange filtration step

One of the most important steps in the proposed method of determination is the use of phosphorylated cellulose as cation-exchanger onto which amezinium is adsorbed from plasma solutions. Owing to the hydrophilic nature of the polymer matrix, it can be desorbed under mild conditions; with 1 *M* sodium chloride solution recoveries of more than 90% are obtained if the elution volumes are equal to or greater than the bed volume of the exchanger. By this technique separation of amezinium and its metabolites, none of which carries a positive charge [18], can be accomplished readily. Furthermore, the greater part of interfering organic material from plasma is removed. In order to exploit these advantages fully, care has to be taken to minimize background from the reagents and especially from the exchanger material which is not of analytical grade. It had been shown that a simple although extensive washing procedure yields exchanger material suitable for analysis in the lower nanogram range provided that not more than 1 ml of exchanger is used for one determination, otherwise quantification is seriously hampered by background noise during HPLC. This poses some restrictions to the scope of this step which become

TABLE I

RECOVERY OF AMEZINIUM AFTER CATION-EXCHANGE SEPARATION

Amezinium concentration = 191.0 ng/ml plasma.

Plasma volume (ml)	Percolate (%)	Wash water (%)	Eluate	
			(%)	(ng)
1	<1	<1	91.4	174.6
2	10.4	15.1	70.5	269.3
3	32.8	15.6	50.1	287.1

evident from Table I, where extraction yields obtained with radioactive amezinium are summarized. The recovery in the eluate of amezinium from plasma is volume-dependent, ranging from 90% with 1 ml of plasma to 50% with 3 ml of plasma. This appears to be due to the limited although relatively high capacity of the exchanger material which is used up by exchangeable material from the plasma. Accordingly, the loss during this step is accounted for by those amounts of amezinium appearing in the percolate and the wash waters. Unfortunately, for reasons specified above, this cannot be circumvented by the use of more exchanger material. Therefore, with this limitation of exchanger volume no gain in sensitivity of the method can be achieved using 3 ml instead of 2 ml of plasma.

Dequaternization

Since during the first step involved in this method no marked concentration can be achieved which would be a necessary prerequisite for a further purification and quantification by HPLC, use was made of one prominent property of the amezinium molecule, its reaction with nucleophilic agents to yield the corresponding pyridiazinone which is easily extracted by organic solvents. From the nucleophiles tested we finally chose NaOH which, within a reasonable reaction time at room temperature, produced the pyridazinone in high yields with a minimal amount of interfering by-products, as can be seen

TABLE II

YIELDS OF THE DEQUATERNIZATION STEP (INCLUDING EXHAUSTIVE EXTRACTION WITH CH_2Cl_2)

Concentration = 242.4 ng of LU 1631 per ml.

Final NaOH concentration (*N*)	Reaction time (min)	Yield (%)
0.1	30	29.7
0.1	120	49.5
1.0	30	83.5
5.0	30	93.3

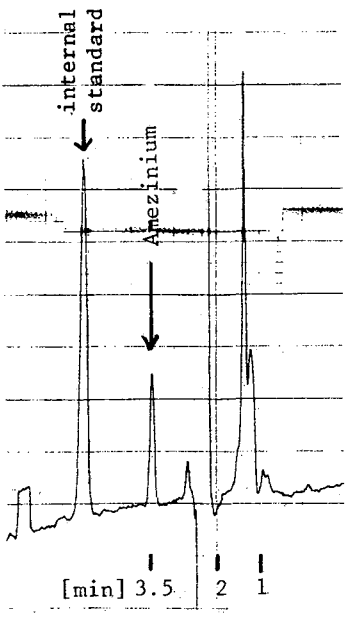

Fig. 1. Chromatogram of the dequaternization products of 10.56 ng of amezinium and of 44.84 ng of internal standard, isolated from 2 ml of plasma.

from Table II and Fig. 1. The chemical nature of the reaction product was verified by a comparison with the authentic compound with regard to retention times in HPLC, R_F values in thin-layer chromatography with different mobile phases, and UV spectrum.

Extraction of the pyridazinones

As shown in Table III, extraction from the alkaline sodium chloride solution can be accomplished by a series of organic solvents. For obvious reasons use of methylene chloride in a ratio of 4:1 is recommended to achieve a high recovery in a volatile organic phase. It will be shown later that methylene chloride in spite of its marked polarity will produce acceptable background levels even for the lower nanogram range aimed at. Therefore, no attempts to further purify the extract, for example by a cycle of back- and re-extraction, are necessary.

TABLE III

YIELD OF DEQUATERNIZATION PRODUCT FROM SALINE MEDIUM BY EXTRACTION WITH FOUR VOLUMES OF ORGANIC SOLVENT

Concentration = 242 ng of LU 1724 per ml.

Solvent	Extraction yield (%)
Cyclohexane	0
Diethyl ether	37.5
Ethyl acetate	64.9
Dichloromethane	81.8

Suitability of the chromatographic method

It can be seen from Fig. 1 that the chromatographic method adopted here is suited for perfect separation of the dequaternization products of amezinium and the internal standard during a 10-min chromatographic run. There is no interference by material originating from plasma or cellulose phosphate. The baseline remains satisfactory provided that not more than 1 ml of cellulose phosphate is used (Fig. 2a). In this case a background noise is observed simulating an apparent concentration of amezinium which will be quantified later. Furthermore, Fig. 2b—d clearly demonstrates by means of three representative examples with plasma of individuals treated with amezinium that no compounds show up with retention times being the same or similar to that of the dequarternized internal standard. Thus, all chromatographic requirements for the application of the method are met.

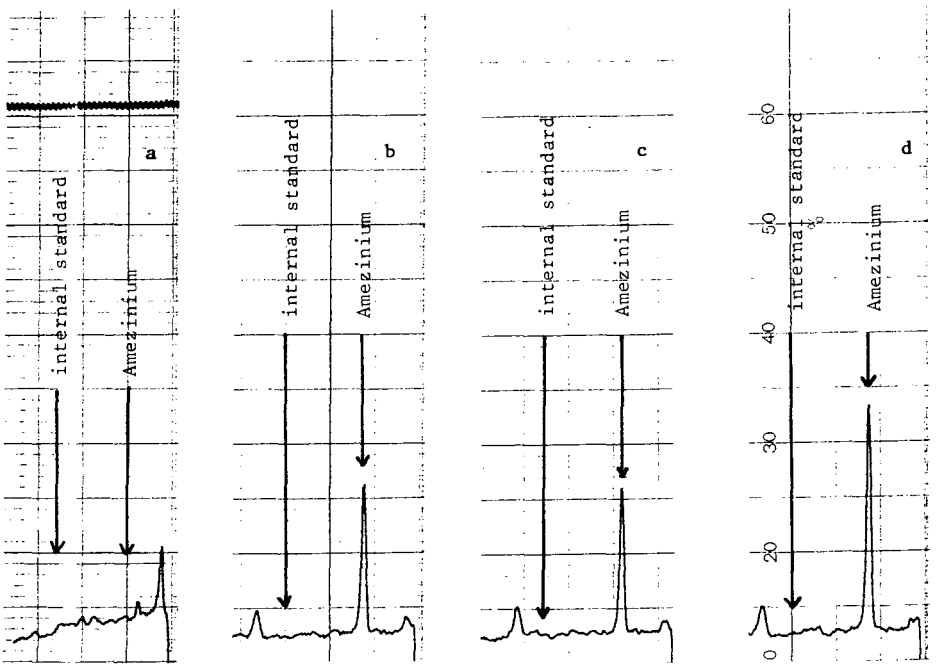

Fig. 2. Chromatograms obtained from (a) 2 ml of blank plasma, and (b—d) plasmas of three volunteers, after repetitive oral application of amezinium (10 mg b.i.d.), carried through the analytical procedure: (b) volunteer A, day 1, 8.00 p.m., (c) volunteer B, day 6, 8.00 p.m., (d) volunteer C, day 11, 8.00 a.m.

Suitability of the internal standard

In a concentration range of 2—20 ng per injection the dequaternization products of amezinium and the internal standard (mass ratio 1:1.093) show identical behaviour with respect to possible adsorption losses in chromatography and peak form in elution which can be seen from the constancies of peak height ratios: they average around 1.45 with a variation coefficient of 2.6%.

Even after subjecting amezinium and internal standard to the entire

TABLE IV

CONSTANCY OF PEAK HEIGHT RATIOS AFTER ENTIRE ANALYTICAL PROCEDURE

n = 5, 2-ml samples.

Mass ratio amezinium/ p-methyl-amezinium (ng/ng)	Peak height ratio	
	$\bar{x} \pm s_x$	C.V. (%)
100.96/348.57	0.4011 ± 0.0113	2.8
50.48/174.28	0.4143 ± 0.0169	4.1
10.09/ 34.86	0.4010 ± 0.0146	3.6

procedure, including the cation-exchange step, dequaternization, extraction of products, and chromatography, the peak height ratios for a mixture of amezinium and internal standard at low, intermediate and high concentrations remain constant, suggesting closely identical behaviour during analysis (Table IV).

Peak height ratios, indeed, are a suitable parameter for the measurement of amezinium concentration after internal standardization with its methyl analogue. The calibration line obtained is linear within the range of about 2.4—25 ng of LU 1724 per injection (F-test); its ordinate intercept of Q = +0.015 significantly differs from 0, but it can be neglected due to its low value.

The reproducibility of HPLC analysis is satisfactory showing variation coefficients of 2.1% at 2.4 ng/injection and 0.4% at 24 ng/injection, the correlation coefficient covering the entire range being $r \geqslant 0.999$).

Overall yield of entire procedure

If amezinium is subjected to the analytical procedure described above, about 45—55% of it finally arrives at the quantification step, a value to be regarded as satisfactory taking into account the numerous stages where losses can occur. It is in reasonable accordance with the value of about 48% which can be calculated from the yields of individual steps.

Evaluation of the method

Within a period of five weeks a number of plasma samples of different known amezinium concentrations were subjected in duplicates to the method described above. In Table V the results of these test series are listed versus the nominal concentrations.

Recovery. Table VI gives data on the recovery of added amezinium which represents a measure of the accuracy of the results if selectivity of the method is given.

Within the concentration range 1—20 ng/ml differences of —1% to 26% between actual and nominal concentrations are found with a surplus of 26% at the lowest concentration being significant. A look at the absolute differences reveals that this observation may be explained by a background of about 0.2 ng/ml which, of course, should turn up quite distinctly in blank plasma determinations which will be discussed later. Taking this background (0.23 ± 0.053 ng/ml) into account, recoveries listed in Table VI in parentheses can be esti-

TABLE V

RESULTS OF METHOD EVALUATION

The individual values represent the means of duplicate determinations.

Nominal concentration (ng/ml)	Series 1 (ng/ml)	Series 2 (ng/ml)	Series 3 (ng/ml)	Series 4 (ng/ml)	Series 5 (ng/ml)	Series 6 (ng/ml)	Series 7 (ng/ml)	Series 8 (ng/ml)	Series 9 (ng/ml)	Series 10 (ng/ml)
1.02	1.3577	1.4074	1.6643	0.9838	1.1367	1.2664	1.5381	1.2927	1.2810	0.9645
2.04	1.8373	2.7079	2.1404	2.3451	1.6333	2.1336	2.5051	2.2953	2.3225	2.1733
5.07	5.8465	5.7691	4.8627	5.5744	4.2761	5.4808	5.5163	5.2451	4.4850	4.9003
10.08	10.4940	10.3359	9.2317	10.8760	8.5279	11.8495	10.0574	10.1769	8.2407	9.9308
19.75	21.6532	19.6030	19.2135	21.7584	21.6997	20.3430	20.9790	20.3594	19.4870	19.9366

TABLE VI

EVALUATION OF AMEZINIUM METHOD: DATA ON ACCURACY AND PRECISION

Values in parentheses are corrected for background of blank plasma samples (0.229 ± 0.053 ng/ml; $\bar{x} \pm s_{\bar{x}}$).

n	Nominal concentration (ng/ml)	Actual concentration (ng/ml, $\bar{x} \pm s_{\bar{x}}$)	Precision		Accuracy	
			S.D. (ng/ml)	C.V. (%)	Actual/nominal (%)	Actual − nominal (ng/ml)
10	1.02	1.29 ± 0.07 (1.06 ± 0.08)	0.22	17.22	126.4 ± 6.9* (104.0 ± 8.6)	0.27 ± 0.07 (0.04 ± 0.09)
10	2.04	2.21 ± 0.10 (1.98 ± 0.11)	0.31	13.96	108.3 ± 4.8 (97.1 ± 5.4)	0.17 ± 0.10 (−0.05 ± 0.11)
10	5.07	5.20 ± 0.17 (4.97 ± 0.18)	0.54	10.41	102.5 ± 3.4 (98.0 ± 3.5)	0.13 ± 0.17 (−0.09 ± 0.18)
10	10.08	9.97 ± 0.34 (9.74 ± 0.34)	1.08	10.79	98.9 ± 3.4 (96.7 ± 3.4)	−0.10 ± 0.34 (−0.33 ± 0.34)
10	19.75	20.50 ± 0.31 (20.27 ± 0.31)	0.97	4.72	103.8 ± 1.5 (102.7 ± 1.6)	0.75 ± 0.31 (0.52 ± 0.31)

*Significantly different from 100%.

mated which now do not deviate significantly from the expected values, the absolute deviation ranging between −0.33 and +0.52 ng/ml. Using this experimentally verified correction, accurate results are obtained which is confirmed by a correlation analysis between nominal and corrected actual values (individual data) with a regression coefficient of $b = 1.029 \pm 0.0126$ ($r = 0.995$) which is not significantly different from 1 at the 99% level of confidence.

Precision. Data on the precision of the entire analytical procedure are given in Table VI. It can be seen from this table that the coefficients of variation (C.V.) range from about 5% at 20 ng/ml to 18% at 1 ng/ml. This inter-assay variation is expectedly greater than the intra-assay variation which was estimated to increase from C.V. ≈ 4% at 20 ng/ml to C.V. ≈ 17% at 1 ng/ml. It is obvious that the precision of the method is unsatisfactory at the lowest concentration of 1 ng/ml.

If, in routine practice, a coefficient of variation of 15% is still accepted for duplicate determinations (this corresponds to a ratio of 1 to 1.25 in the individual determinations) we have to expect that about 5% of the analyses need to be repeated.

Following such a procedure, which has proved its value in routine practice of a number of analytical methods, only minor variations are found for the data obtained here with respect to accuracy. The risk of a falsification of statements resulting from outliers not eliminated is, however, minimized.

If the standard deviations are regarded as a function of the observed mean values, a linear relation may be assumed in first approximation for the concentration range up to 10 ng/ml. Regression analysis gives values of 0.1 ± 0.03 ng/ml for the ordinate intercept (absolute error) and of 0.09 ± 0.005 for the slope corresponding to a weighted relative error of about 9%. This linear function can be used as a weighting function in any fitting procedures where homogeneous variances are required. For values above 5 ng/ml the commonly used weighting function $1/c_A^2$ can be adopted without serious distortions of error structure.

Limit of determination and detection. The lower limit of determination (defined as that concentration which can still be determined with coefficients of variation of about 10%) is about 5 ng/ml according to the data discussed above with the use of 2-ml plasma samples . If one is prepared to accept a somewhat lower reliability corresponding to coefficients of variation of up to 15%, concentrations of down to 2 ng/ml can be estimated.

To determine the lower limit of detection of unchanged amezinium, human blank plasma is subjected to the analytical method described in this paper. A mean value of 0.23 ± 0.17 ng/ml ($\bar{x} \pm s_x$) results from the values obtained. If the lower limit of detection is defined as three times the standard deviation above the mean blank value, it is estimated at 0.75 ng/ml.

Experiences in routine practice

In the meantime the method described has been applied to the determination of amezinium concentrations in human plasma after application of 10 mg per os. It demonstrated its suitability to follow the time course up to 32 h after administration. Without any modification it can be used to determine amezinium in human urine at 25 ng/ml, the lowest concentration encountered

after 1 mg intravenous administration, a coefficient of variation of < 3% was found.

ACKNOWLEDGEMENT

The authors wish to thank Mrs. Ursula Kripp for her skilful and knowledgeable assistance.

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Journal of Chromatography, 227 (1983) 229—237
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1804

DETERMINATION OF AMIODARONE AND ITS N-DEETHYLATED METABOLITE IN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(Received March 3rd, 1983)

SUMMARY

A high-performance liquid chromatographic (HPLC) method utilizing hexane extraction and a normal bonded phase column (NH_2-alkylamine) was developed to measure serum concentrations of amiodarone and its N-deethylated metabolite. A single analysis requires 8 min. The one-step extraction efficiencies of amiodarone and the internal standard are greater than 90%. The method is linear between 0.05 and 20.0 μg/ml. The average relative standard deviation of the slope of the standard curve is 4% and the single day coefficient of variation is 3.2%. The use of hexane extraction for sample cleanup and a bonded phase column for chromatography result in a sensitive and reproducible system well suited to laboratories monitoring serum concentrations of multiple drugs by HPLC. A preliminary study has shown the assay to be useful for the investigation of the pharmacokinetics of this agent.

INTRODUCTION

Amiodarone is an antiarrhythmic agent which has demonstrated remarkable efficacy and safety in the treatment of refractory ventricular and supraventricular arrhythmias [1—7]. Using conventional dosage regimens, there is generally a period of 1—2 weeks from the beginning of drug administration until arrhythmia suppression is noted. In patients whose arrhythmias are refractory to all other therapy, such a delay in the onset of a therapeutic effect is life-threatening.

Aggressive therapy with amiodarone intended to shorten or abolish this lag time between the start of therapy and arrhythmia control has been dis-

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couraged by the paucity of information concerning amiodarone's biodisposition. Without knowledge of the adsorption, distribution, metabolism and excretion of the drug and the relationship between serum concentration and therapeutic effect, a rational approach to the initiation of therapy has not been possible. Progress in these areas has been hampered by the lack of a rapid, simple and sensitive procedure for the determination of the concentration of amiodarone in biological fluids.

We report here a method for the determination of amiodarone and its N-deethylated metabolite [8] in serum utilizing high-performance liquid chromatography (HPLC). This method is rapid, clean and durable and therefore well suited to laboratories measuring several compounds by HPLC.

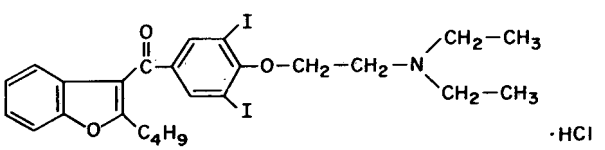
EXPERIMENTAL

Apparatus

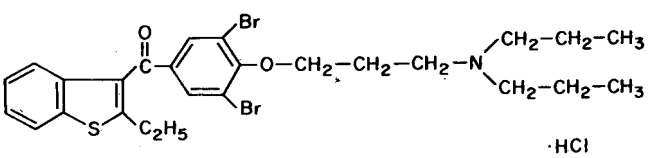
Analyses were performed using a Varian Model 5020 high-performance liquid chromatograph interfaced with a Varichrome variable-wavelength detector and a CDS 111L digital integrator (Varian Instruments, Palo Alto, CA, U.S.A.). Chromatography was performed using a 30 cm × 4 mm Varian NH_2-10-alkylamine column preceded by a 4 cm × 4 mm guard column packed with 40-μm silica particles (Vydac®, Varian Instruments). A heater block maintained the column at 30°C. The flow-rate was 1.5 ml/min which developed a pressure of 44 bars. The detector wavelength was set at 248 nm with an 8-nm bandwidth. Chromatograms were displayed on a strip chart recorder (Linear Instruments, Irvine, CA, U.S.A.).

Chemicals and reagents

The hexane, methylene chloride and methanol used as elution solvents were glass distilled (Burdick and Jackson Labs., Muskegon, MI, U.S.A.). All other solvents and reagents were ACS reagent grade. Amiodarone [2-butyl-3-(3,5-diiodo-4-β-diethylaminoethoxybenzoyl)benzofuran] and the internal standard



AMIODARONE



INTERNAL STANDARD

Fig. 1. Chemical structures of amiodarone and internal standard.

(IS) 2-ethyl-3-(3,5-dibromo-4-β-dipropylaminopropoxybenzoyl)benzothiophene (Fig. 1) were generously supplied by Labaz (Brussels, Belgium). Pooled serum from human volunteers receiving no medication was used in the preparation of standards.

Extraction procedure

A stock solution of IS, 100 μg/ml in methanol, was prepared and kept at 4°C to minimize evaporation. All solutions were handled with glass capillary micropipettes. For serum extraction 10 μl of IS stock solution was placed in the bottom of a 16 × 100 mm borosilicate tube. To each tube 0.2 ml of 3 *M* sodium acetate buffer (pH 4.8) and 0.5 ml serum were added. This was mixed briefly followed by the addition of 5 ml of hexane. Each tube was then vortexed vigorously for 2 min and centrifuged at 7000 *g* for 1 min. The upper hexane layer was removed with a pipette, placed in a 10-ml pear-shaped flask and evaporated to dryness under reduced pressure. The residue was redissolved in 65 μl of mobile phase with vigorous shaking. From this a sufficient quantity (30—40 μl) was recovered to load a 25-μl injector loop.

Mobile phase

The mobile phase consisted of methylene chloride—hexane—methanol—glacial acetic acid—0.117 *N* perchloric acid in methanol (50:39:6:1:1, v/v). This solution was made daily and placed in an ice water bath during use to prevent bubbles from forming in the pump inlet tubing. Column temperature was maintained at 30°C with a heating block.

Calculations

Peak areas of standards and unknowns were normalized by dividing the amiodarone (A) peak area by the IS peak area for each sample. For the pharmacokinetic study sample concentrations were calculated by comparison of their A:IS peak area ratios to that of a single standard serum containing a known amount of amiodarone. The full equation was:

$$C_u = \frac{A_u}{A_{IS}} \times \frac{A_{IS}}{A_{std}} \times C_{std}$$

where C_u and C_{std} are the concentrations of the unknown and the standard serum respectively and A_u, A_{IS} and A_{std} are the peak areas of the unknown, IS and standard serum respectively.

Kinetic parameters were calculated [9] with a non-linear regression computer program (NONLIN [10]) linked with a pharmacokinetic analysis program (AUTOAN [11]) as follows:

$AUC_{0\rightarrow\infty}$ = area under the serum concentration vs. time curve extrapolated to infinity (trapezoidal rule)

β = slope of the last linear phase of the log blood concentration vs. time plot

$t_{½\beta}$ = half-life of β-phase = ln 2/β

V_d = volume of distribution = $\frac{\text{Dose}}{AUC_{0\rightarrow\infty} \times \beta}$

$$Cl_{bl} = \text{blood clearance} = \frac{\text{Dose}}{AUC_{0\to\infty}}$$

RESULTS

Sample collection

Blood was collected from normal volunteers and from patients receiving amiodarone as part of their anti-arrhythmic therapy. In collecting these samples, it was discovered that the use of tubes containing a gelatin to separate serum from cells resulted in a significant decrease in the recovery of amiodarone from the sample. When blood from three individuals was divided between serum separator tubes (Corvac®, Sherwood Medical, St. Louis, MO, U.S.A.) and plain glass tubes without the serum separator, the recovery of drug in the former was only 67% of the latter (n=3). In all the studies described herein, only plain glass tubes were employed for sample collection.

Extraction

Methylene chloride, ethyl acetate, chloroform and hexane were investigated as extraction solvents. All but hexane were found to extract a number of serum components which interfered with the detection of amiodarone, its metabolite and/or IS at 248 nm. Hexane extracted no interfering peaks with absorbance at 248 nm. When the extraction solvent was buffered at pH 4.8, nearly quantitative recovery of amiodarone was observed. Amiodarone added to serum at final concentrations of 0.2, 2.0 and 20 μg/ml resulted in one-step extraction efficiencies of 90, 94 and 99%, respectively. These studies were repeated for the internal standard with similar results. No information concerning the recovery of metabolite could be derived owing to the small amount obtainable by the procedure described.

High-performance liquid chromatography of amiodarone

The use of the NH_2-10-alkylamine column permitted the separation of the IS, amiodarone and the N-deethylated metabolite of amiodarone without chromatographic interference. Typical chromatograms of amiodarone, the metabolite and the IS are shown in Fig. 2. The same column has been in use for more than one year, during which period there have been no changes in pressure requirements and no loss of resolution. Droplets of polar solvents from other users of the same chromatograph do not affect retention volumes or peak widths, but do release polar compounds from the column which temporarily interfere with the assay.

The heater block was employed to maintain the column at 30°C in order to avoid minor fluctuations in retention due to changes in ambient air temperature. Finally, a wavelength of 248 nm was chosen for monitoring the column effluent rather than 243 nm, the absorption maximum for amiodarone (unpublished data), because our preliminary studies revealed that methylene chloride, a major component of the elution solvent, absorbs significantly at 243 nm. The 248-nm wavelength resulted in a more favorable signal-to-noise ratio.

The elution solvent, composed of methylene chloride, hexane and methanol,

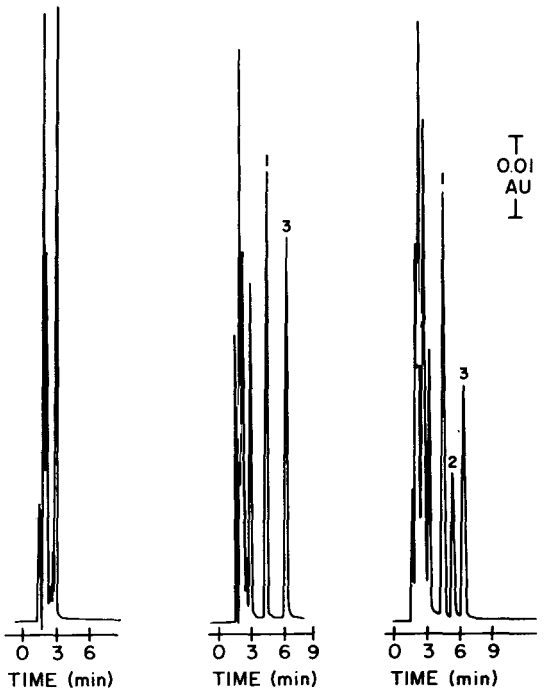


Fig. 2. HPLC of amiodarone and its metabolite. Serum was obtained from a patient prior to (left), during initial (center) and after chronic (right) therapy. Extraction and chromatography were performed as described under Experimental except no IS was added to the prior to therapy sample to demonstrate the absence of interfering peaks in the region of the IS. Peaks: 1 = internal standard, IS; 2 = metabolite; 3 = amiodarone.

was found to separate the compounds of interest from each other and from other serum peaks when acetic acid was added to this mixture to reduce peak tailing and perchloric acid was added as an ion-pairing agent.

Interference studies

Interference studies were performed as follows: A number of commonly used therapeutic agents were dissolved in elution solvent and injected onto the column. The results are shown in Table I. The agents with retention volumes which might have interfered with the assay (furosemide, propranolol, theophylline and caffeine), were then added to pooled human serum and subjected to the extraction and chromatographic procedures described. No peaks were found which would interfere with the determination of amiodarone, its N-deethylated metabolite or the IS.

The lack of endogenous or exogenous interference was confirmed by examining the sera of eight volunteers receiving no medication and multiple cardiac patients receiving a variety of cardiac and non-cardiac drugs including lidocaine, procainamide, quinidine sulfate, furosemide, propranolol, digoxin, hydrochlorothiazide, isosorbide dinitrate, thyroxin and diazepam. No interfering peak was encountered.

TABLE I

RELATIVE RETENTION OF COMMON THERAPEUTIC AGENTS AND AMIODARONE

Compounds were dissolved in HPLC elution solvent and 25 μl were injected onto the chromatographic column. V = Retention volume of compound; V_0 = retention volume of elution solvent.

Compound	Capacity factor (k') $\frac{V - V_0}{V_0}$	Separation factor (α) $\frac{k'_{\text{compound}}}{k'_{\text{amiodarone}}}$
Amiodarone	2.63	1.00
N-Deethylated amiodarone	2.05	0.78
Internal standard (IS)	1.59	0.60
Caffeine	1.84	0.70
Chlorpromazine	4.56	1.73
Digoxin	>10.00	>4.00
Disopyramide	>10.00	>4.00
Furosemide	2.21	0.84
Lidocaine	4.29	1.63
Phenobarbital	0.88	0.33
Phenytoin	0.96	0.37
Prednisolone	5.22	1.98
Prednisone	3.53	1.34
Procainamide	>10.00	>4.00
Propranolol	2.65	1.01
Quinidine	>10.00	>4.00
Theophylline	2.74	1.04

Methodologic verification

In order to determine the stability and sensitivity of the assay, amiodarone solutions at concentrations of 0.1, 0.2, 0.5, 1.0, 2.0, 5.0 and 10.0 μg/ml were prepared by adding known quantities of drug in methanol to pooled drug-free human serum. The final methanol concentration never exceeded 1% (v/v) in any sample. At least two of these standards plus a serum blank were run on each analysis day for a total of eleven days over 1.5 months. From these data, a standard curve was constructed each day by plotting the ratio of the amiodarone peak to the IS peak versus amiodarone concentration. The straight line obtained could be described by the equation: $y = 0.84x + 0.07$, where the average relative standard deviation for the slope was 4% and the average regression coefficient (r^2) had a value of 0.9997. The standard deviation of multiple determinations of a single sample performed on the same day was 3.2% (n=14). The assay was found to be linear over a sample concentration range of 0.05—20.0 μg/ml. Serum samples frozen at —20°C remained stable for at least seven months.

Clinical studies

We developed this assay for amiodarone in serum to investigate the pharmacology of this clinically important agent. To determine the utility of our assay for such work, a pilot pharmacokinetic study was performed.

The single-dose kinetics of amiodarone were studied in five healthy male volunteers ages 24—57 years. Amiodarone, 5 mg/kg, was administered as an

TABLE II

SINGLE DOSE PHARMACOKINETIC PARAMETERS IN HEALTHY VOLUNTEERS

Results of a pharmacokinetic study of five healthy male volunteers. A single dose of amiodarone, 5 mg/kg, was infused over 15 min and serum sampled at designated intervals for 24—48 h. β = Slope of the last linear phase of the log blood concentration vs. time plot; $t_{½\beta}$ = half-life of the β phase; V_d = volume of distribution per kg; AUC = area under the serum concentration vs. time plot; Cl_{bl} = blood clearance; $\bar{X}$ = mean; S.D. = standard deviation.

Subject No.	β (h^{-1})	$t_{½\beta}$ (h)	V_d (l/kg)	AUC (μg/ml h)	Cl_{bl} (ml/min kg)
1	0.066	10.46	5.40	14.00	5.94
2	0.046	14.94	4.90	22.26	3.76
3	0.058	11.95	10.80	8.00	10.44
4	0.080	8.66	5.95	10.50	7.94
5	0.066	10.57	4.76	15.93	5.24
$\bar{X}$	0.063	11.32	6.36	14.14	6.66
S.D.	0.012	2.34	2.52	5.50	2.59

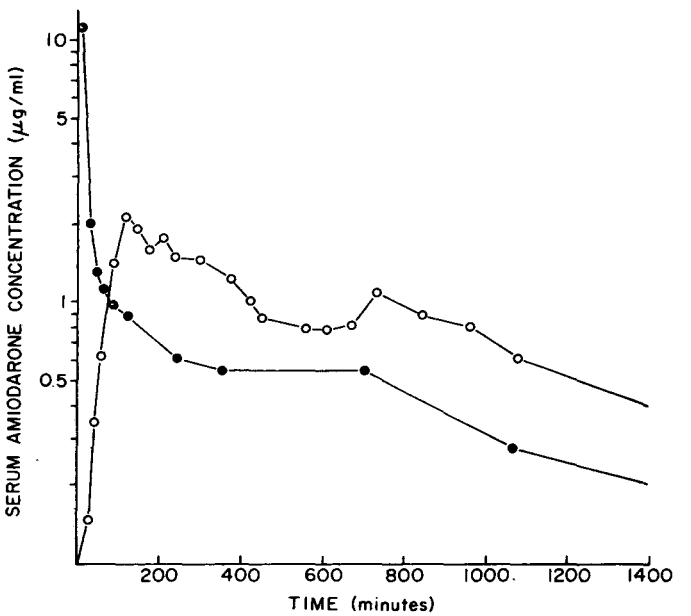


Fig. 3. Single dose intravenous and oral pharmacokinetic study in a normal volunteer. A 34-year-old white male received amiodarone, 5 mg/kg intravenously (•—•) and several months later was redosed with a single 1400-mg oral dose (○—○). Each point represents the average of duplicate determinations of each sample.

intravenous infusion over 15 min. During the next 24—48 h, 15—20 serum samples were obtained at designated intervals from a heparin lock placed in the arm opposite that used for drug infusion. The data fit a two-compartment open pharmacokinetic model [11] with an r value of 0.997. The results of this study are shown in Table II.

The pharmacokinetic evaluation was repeated several months later in one volunteer following a single oral dose of 1400 mg. The elimination half-life ($t_{½\beta}$) was within 1% of that observed after intravenous administration (Fig. 3). The bioavailability of the oral preparation (Cordarone®, Labaz) was 0.48.

DISCUSSION

The method we present here has been used in our laboratory for over 16 months and is rapid, accurate and stable.

Although other methods have been reported [12—16] they have certain disadvantages when compared to the method presented here. Riva et al. [12] and Cervelli et al. [13] reported methods using extraction steps which require more than 30 min. The method reported by Andreasen et al. [14] uses no internal standard. The internal standards used by Riva et al. [12] and Flanagan et al. [15] are chemically dissimilar from amiodarone. Finally, the method reported by Lesko et al. [16] utilizes a silica column which has the chromatographic stability and maintenance problems common among unbounded column materials.

The method reported above has an extraction step which takes only 2 min to complete, utilizes an internal standard very similar to amiodarone and employs a NH_2-alkylamine column. This bonded phase column has proved resistant to the effect of water and other polar solvents which are used in reversed-phase assays performed on the same chromatograph.

For these reasons we feel this method is a valuable alternative to those currently available and should facilitate the pharmacologic investigation of this important agent.

ACKNOWLEDGEMENTS

This work was supported in part by the Pediatric Pharmacology/Toxicology Center Grant from the trustees of Rainbow Babies and Childrens Hospital. Dr. Mostow is a Research Fellow of The American Heart Association, Northeast Ohio Affiliate Inc.

The authors would like to express their appreciation to Dr. Terrence Sheehan of Varian Instruments for his assistance in the development of this method.

The authors gratefully acknowledge Labaz for supplying the purified amiodarone and internal standard utilized in the development of this method.

After initial pre-treatment of a new column with sequential washes of methylene chloride, isopropanol, water, isopropanolol, methylene chloride and hexane, 48 h of continuous mobile phase flow at 0.5 ml/min were required to achieve the chromatographic conditions described.

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Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1802

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF BAMIFYLLINE AND ITS THREE METABOLITES IN HUMAN PLASMA

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(First received March 1st, 1983; revised manuscript received May 6th, 1983)

SUMMARY

The performance of a gradient elution normal-phase system and of two isocratic elution reversed-phase ion-pair systems was investigated for the high-performance liquid chromatographic separation of bamifylline and its three metabolites. Three packings and mobile phases were tested and the best separation was achieved using a reversed-phase ion-pair system with Hypersil ODS 3 μm as stationary phase. The effects of alkyl chain length and concentration of alkyl sulfonates, salt concentration, column temperature and competing amine on separation and peak tailing are discussed. The determination of bamifylline and metabolites in human plasma using the defined optimal chromatographic conditions is reported.

0378-4347/83/$03.00

INTRODUCTION

Bamifylline* is a xanthine derivative obtained by bisubstitution of theophylline. The drug is used in the treatment of asthma and reversible airway obstructions. The pharmacokinetic profile and metabolism of the drug have been studied in man using various methods [1—3]. Three metabolites of bamifylline were identified (Fig. 1): AC 85, AC 155 and AC 119, but AC 85 and AC 155 were not separated. This paper describes three different high-performance liquid chromatographic (HPLC) systems we investigated in order to achieve optimal separation of bamifylline and its three metabolites.

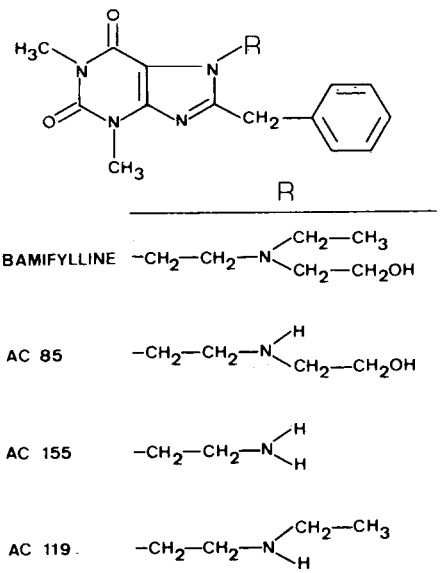

Fig. 1. Chemical structure of bamifylline and its metabolites.

Choice of the counter-ion size and concentration for the selected chromatographic procedure is discussed. The isocratic method developed here, using an internal standard, is found to be suitable for therapeutic determinations and pharmacokinetic investigations. Samples can be injected at 20-min intervals.

EXPERIMENTAL

Chemicals and reagents

Bamifylline and its metabolites AC 85, AC 155 and AC 119 were provided by Spret-Mauchant Laboratories (Spret-Mauchant, Gennevilliers, France). Standard aqueous solutions from 1.0 to 40 μg/ml of each compound were extemporaneously obtained from stock solutions (100 μg/ml) prepared in methanol—water (50:50, v/v) and stored at +4° C in brown glass flasks for up to

*Trademark: Trentadil.

one month. The internal standard, fenetylline, was supplied by Gerda Laboratories (Gerda, Tassin, France); a 25-μg/ml standard solution of fenetylline was prepared in methanol—water (50:50, v/v).

Acetonitrile for reversed-phase HPLC, potassium chloride and hydrochloric acid of "Titrisol" grade were respectively purchased from Prolabo (Prolabo, Paris, France) and from Merck (E. Merck, Darmstadt, F.R.G.). Pentane-, hexane-, heptane- and octanesulfonic acid sodium salts were provided by Fluka (Fluka, Buchs, Switzerland).

Chromatography

Different HPLC systems were studied using two different instrumentations:

(1) An isocratic system consisting of the following components: a Waters Model 6000 A pump (Waters, Paris, France); a Pye-Unicam spectrophotometer (Pye-Unicam, Paris, France) operated at 275 nm; a Rheodyne 7125 injection valve (Touzart et Matignon, Vitry, France) equipped with a 50-μl loop; the detector output was connected either to a Kontron W + W 610 recorder (W.W. Electronic Inc., Basel, Switzerland) or to a HP Model 3390 A integrator (Hewlett-Packard, Paris, France).

(2) A gradient system equipment consisting of a Model 1084 B Hewlett-Packard dual-head pump, equipped with a 79875 A variable-wavelength detector operated at 275 nm and a 79.850 B LC terminal.

Three different commercially available supports were used for the column packing: Nucleosil C18 (5 μm), Nucleosil NH_2 (5 μm) (Macherey-Nagel, Düren, F.R.G.) and Hypersil ODS (3 μm) (Shandon, Runcorn, U.K.). These chemically bonded silica gels were slurry packed in 15 cm × 4.6 mm I.D. stainless-steel columns with a Haskel pump (Touzart et Matignon, Paris, France), according to a method described by Coq et al. [4] using *n*-butanol as the slurry medium and methanol as the packing solvent.

Three distinct mobile phases were tested with different stationary bonded phases and mobile phases. Their characteristics are given in Table I.

Sample prepration and plasma extraction

Venous blood samples (5 ml) were collected into 10-ml Vacutainer green-stoppered tubes (Becton-Dickinson, Missisauga, Canada) and centrifuged at 900 *g*. When the determination was not carried out immediately, the plasma was frozen at −20° C in plastic tubes. In these conditions, no degradation of drugs was noted after two months' storage.

A 2-ml volume of plasma was added to 200 μl of 2 *N* HCl (pH of the final mixture = 2.6), 100 μl of 25 μg/ml internal standard solution and 7 ml of methylene chloride—ethyl ether mixture (4:7, v/v) in 10-ml Teflon-lined screw-capped glass tubes. Tubes were shaken for 10 min on a Laboral oscillating agitator (Prolabo, Paris, France) and then centrifuged at 900 *g* for 10 min. The upper (organic) layer was discarded, 100 μl of 5 *N* NaOH (final pH = 11.6) and 7 ml of methylene chloride—diethyl ether (4:7, v/v) were then added to the aqueous layer. Tubes were shaken for 10 min and spun at 900 *g* for 5 min. The upper organic phase was then collected in conical base glass tubes and evaporated to dryness under a very light nitrogen stream at

TABLE I

STATIONARY AND MOBILE PHASE CHARACTERISTICS FOR THE DIFFERENT CHROMATOGRAPHIC SYSTEMS

HPLC system	Stationary phase	Mobile phase			Flow-rate (ml/min)
Normal-phase chromatography [1]	Nucleosil NH_2, 5 μm (Macherey-Nagel)	Pump A Isooctane—methylene chloride (20:80, v/v) Pump B Isopropanol	Time (min) 0.0 3.0 6.0 10.0	% B 5.0 10.0 15.0 5.0	1.5
Ion-pair reversed-phase chromatography [2]	Nucleosil C18, 5 μm (Macherey-Nagel)	Aqueous phase (pH=2.0) KCl 0.2 *N* (14.9 g/l) =25 ml HCl 0.2 *N*=75 ml Distilled water=900ml Heptanesulfonate=1.5 g Acetonitrile		73% (V) 27% (V)	1.2
Ion-pair reversed-phase chromatography* [3]	Hypersil ODS, 3 μm (Shandon)	Aqueous phase (pH=3.0) Distilled water=1000 ml Heptanesulfonate=1.5 g KCl=3 g 1 *N* Acetic acid to pH=3.0 Acetonitrile		78% (V) 22% (V)	1.3

*This method must be considered as the definitive system.

35—40°C. The dry residue was redissolved in 200 μl of mobile phase and 50 μl were then injected into the chromatograph.

Calibration curves and calculation

Plasma samples were spiked with increasing amounts of bamifylline and each metabolite (final concentrations: 0.05, 0.1, 0.5, 1, 2 μg of each drug per ml of plasma) and 100 μl of 25 μg/ml internal standard solution. The samples were submitted to the extraction procedure described and standard curves were generated for each series of determinations by plotting peak height ratios (drug/internal standard) against known drug concentrations.

Plasma concentrations were interpolated from these standard curves. Accurate results in the range 0.01—2 μg/ml could also be alternatively obtained using a HP integrator; in this case, calibration was obtained from a 0.5 μg/ml standard solution.

RESULTS AND DISCUSSION

Chromatographic system development

Chromatograms obtained using the different HPLC systems previously

described are shown in Fig. 2. Poor selectivity was obtained using Nucleosil NH_2 (A_1) but separation was improved after impregnation of this packing with heptane sulfonate (A_2). Unfortunately, this system was unstable and resolution decreased quickly since no counter-ion could be pumped through the column using isooctane—methylene chloride—isopropanol as mobile phase. In spite of the good selectivity observed, this system was rejected due to the insolubility of heptanesulfonate in the mobile phase. Using the ion-pair reversed-phase system with Nucleosil C18 (5 μm) as stationary phase, metabolites AC 85 and AC 155 were not separated, whatever alkylsulfonate counter-ion size or percentage of organic phase modifier we used. Hypersil ODS (3 μm) appeared to be a suitable alternative: a correct selectivity was obtained, although a concomitant and conflicting increase in peak broadening was noted. We were unable to explain this increase in peak broadening. In one paper, Cooke and Olsen [5] related conclusions of two authors indicating that sufficiently small particles may involve finite kinetics of solute transfer between stationary and mobile phases [6], or thermal effects due to high frictional flow resistance [7] and so negate any advantage using such packing materials. Karger et al. [8]

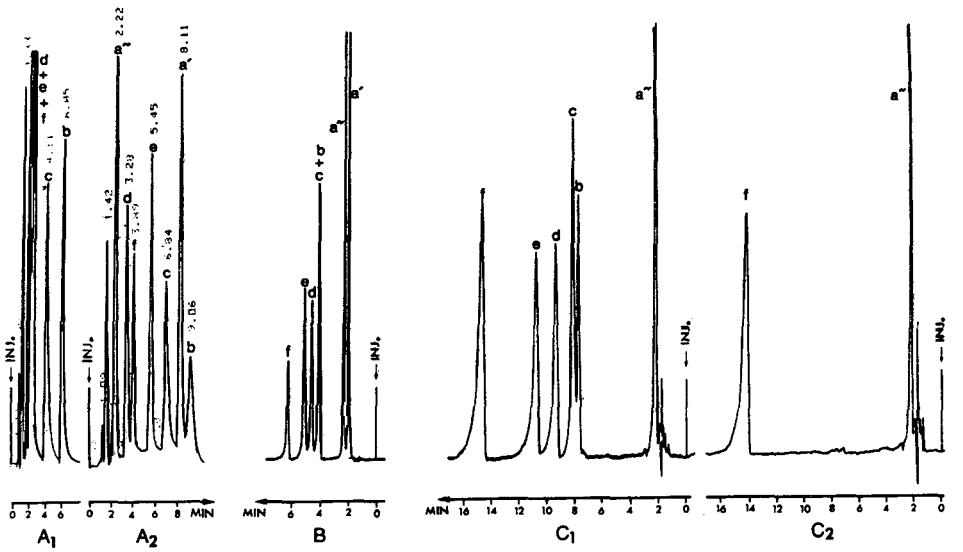

Fig. 2. (A) Chromatograms of a standard solution (in mobile phase) spiked with caffeine (a''), bamifylline (d), fenethylline (f), AC 119 (e), AC 155 (c) and AC 85 (b) at concentrations ranging from 5 to 10 μg/ml of each compound. These chromatograms were obtained before (A_1) and after (A_2) stationary phase was impregnated with counter-ion. Other chromatographic conditions are described in Table I [1]. UV wavelength = 275 nm. (B) Chromatogram of a standard solution (in mobile phase) spiked with theophylline (a'), caffeine (a''), AC 85 (b), AC 155 (c), bamifylline (d), AC 119 (e) and fenethylline (f) at 10 μg/ml for each compound. Chromatographic conditions are described in Table I [2]. UV wavelength = 275 nm. (C) Chromatogram (C_1) of a plasma extract spiked with AC 85 (b), AC 155 (c), bamifylline (d) and AC 119 (e), at 0.5 μg/ml for each compound and 1.25 μg/ml of fenetylline (f). This plasma contained caffeine (a'') at 3.2 μg/ml before spiking. Also chromatogram (C_2) of a blank plasma extract containing only fenetylline (f) at 1.25 μg/ml and caffeine (a'') at 3.2 μg/ml. In these two cases (spiked and blank plasma), chromatographic conditions are described in Table I [3]. UV wavelength = 275 nm.

reported that the most prevalent causes responsible for the poor peak shape observed when a secondary chemical equilibrium was used in the control of separation were competing side-reactions and slow kinetics in the chemical equilibrium steps. In this last case, band symmetry and efficiency can be improved by a change in temperature or in solvent conditions (ionic strength, etc.).

As will be seen later, the problem of peak tailing was partially resolved when potassium chloride (3 g/l) was added to the aqueous mobile phase.

The HPLC system 3 described in Table I allowed the separation of all the compounds of interest and was used for further studies.

Influence of the alkyl chain length and concentration of the pairing reagent on retention and selectivity of drug compounds

First of all the influence of counter-ion size on the retention of bamifylline and its metabolites was studied. Effects of pentane, hexane, heptane and octanesulfonate were successively investigated. As described in Fig. 3a, the capacity factor ($k' = (t_R - t_0)/t_0$) for a given mobile phase increased exponentially with the carbon number in the alkyl chain of the counter-ion. Investigation of selectivity factors (Fig. 3b) obtained from $\alpha = k'y/k'x$, where $k'y$ and $k'x$ are respectively the capacity factors of the y-th and x-th samples components (y being more retained than x), showed a relative improvement or constancy except in the case of α for bamifylline/AC 155, when increasing the alkyl chain length of the counter-ion. An hypothesis to explain this particular behaviour is that the accessibility of the ammonium group of bamifylline $-N\begin{matrix}\diagup CH_2-CH_3 \\ \diagdown CH_2-CH_2OH\end{matrix}$ is less than that of the other compounds. In agreement with the ion-pair mechanism proposed by Bidlingmeyer [9], i.e. the ion-pair model, it can be assumed that the formation of "ion-pair" is not complete between counter-ion and bamifylline. This difficulty to obtain an "ion-pair" may be especially important as the number of carbon atoms in the alkyl sulfonate chain length increases. Consequently, the retention of the ion-pair counter-ion—bamifylline increases less than that of the ion-pair counter-ion—AC 155.

Fig. 4. shows the effect of heptane sulfonate concentration in the mobile phase on capacity and selectivity factors. Concentrations from 0.1 to 2 g/l were successively used. According to the results achieved, a good compromise was obtained between retention time and selectivity factors of the different compounds by using a mobile phase containing 1.5 g/l heptanesulfonate. In these chromatographic conditions, AC 85 and AC 155 were separated with a resolution of less than 1, while the resolution between AC 155 and bamifylline, and between bamifylline and AC 119 was at least 1.0 and 1.2.

Influence of salt concentration, column temperature and competing amine on peak broadening and retention

When the KCl concentration in aqueous mobile phase was increased up to 3 g/l (Table II) to mask unreacted silanols, a decrease in capacity factors was

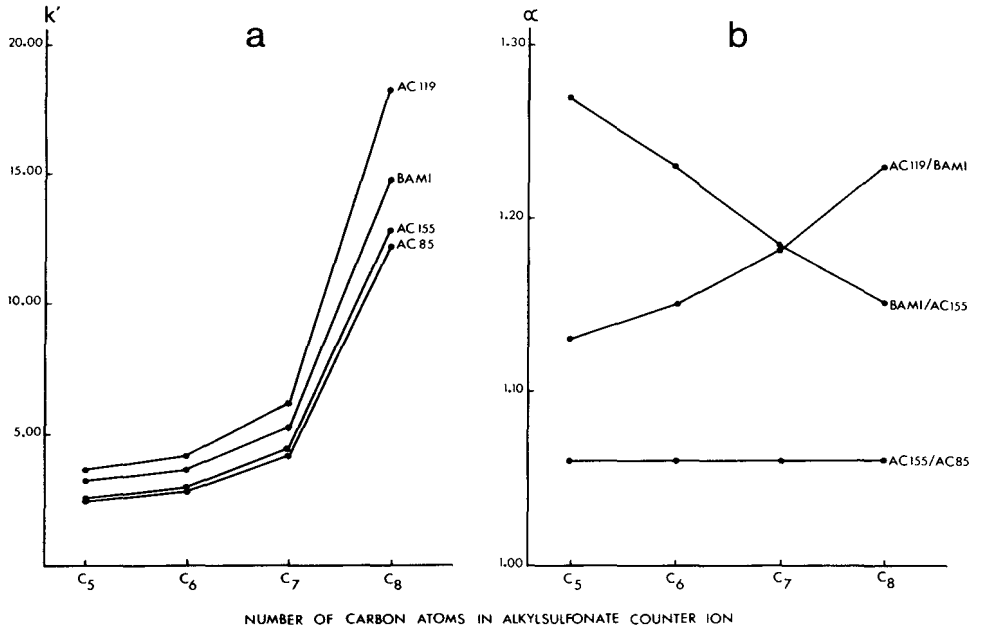

Fig. 3. Effect of counter-ion size on the k' values (a) and selectivity factor values α (b) for bamifylline and its metabolites. Column: Hypersil ODS, 3 μm (150 × 4.6 mm). Mobile phase: as described in Table I [3].

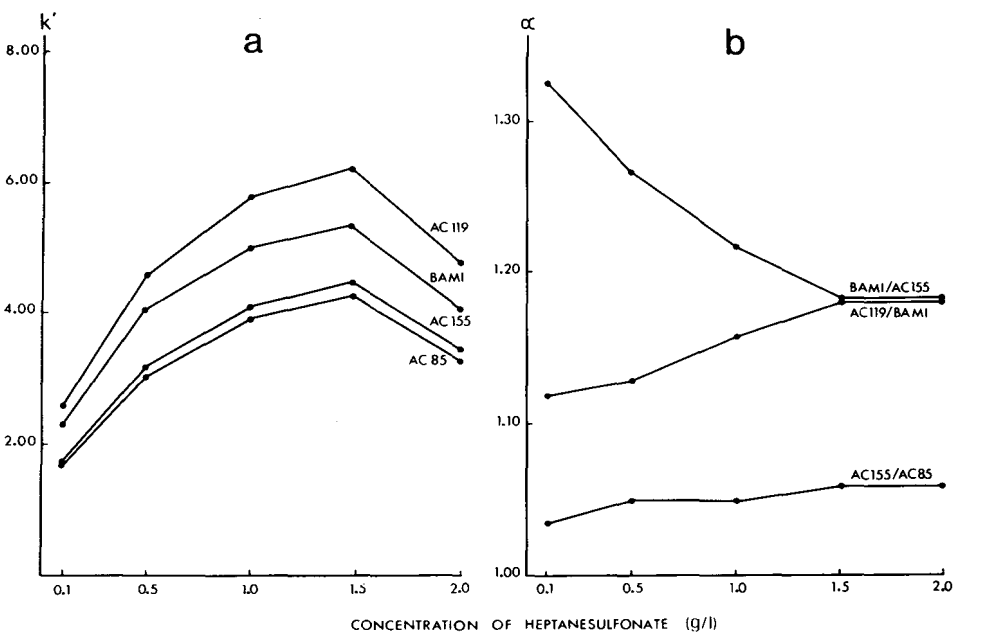

Fig. 4. Effect of heptanesulfonate concentration on the k' values (a) and selectivity factor values α (b) for bamifylline and its metabolites. Column: Hypersil ODS, 3 μm (150 × 4.6 mm). Mobile phase: as described in Table I [3].

TABLE II

EFFECT OF KCl OR Na_2SO_4 CONCENTRATION IN AQUEOUS MOBILE PHASE ON NUMBER OF THEORETICAL PLATES (N), CAPACITY FACTOR (k'), SELECTIVITY FACTOR (α) AND PEAK ASYMMETRY*

Chromatographic conditions (except for KCl or Na_2SO_4 concentrations) are described in Table I [3].

Salt conc. (g/l)	AC 85		AC 155		Bamifylline			AC 119			α		
	N	k'	N	k'	N	k'	Asym.	N	k'	Asym.	AC 155 / AC 85	Bamifylline / AC 155	AC 119 / Bamifylline
KCl													
0	2830	7.10	2800	7.70	790	12.0	2.7	540	14.0	0.9	1.08	1.56	1.17
1	11025	2.73	8940	2.89	3695	3.71	4.5	3500	4.33	5.3	1.06	1.28	1.17
2	12720	2.13	11025	2.27	6100	2.82	2.5	6080	3.33	2.75	1.07	1.24	1.18
3	14400	1.93	12280	2.04	7260	2.51	2.0	7250	2.98	2.5	1.06	1.23	1.19
4	8930	3.10	8200	3.28	6980	3.94	2.5	6900	4.63	4.3	1.05	1.20	1.17
Na_2SO_4													
3	6720	2.64	4520	2.82	1160	4.0	Tailing peak	1020	4.73	Tailing peak	1.07	1.42	1.18

*Measured at 10% of peak height.

observed, and peak asymmetry (measured at 10% of the peak height) was reduced but remained still higher than that obtained with Nucleosil C18 (5 μm), 2.0 versus 1.0, for bamifylline. Selectivity factors were unchanged or decreased (bamifylline/AC 155) and theoretical plates were enhanced. When KCl concentrations higher than 3 g/l were used these global improvements disappeared. Substitution of 3 g/l KCl by 3 g/l Na_2SO_4 in aqueous mobile phase did not give satisfactory results. It is common knowledge that the chloride ion may be detrimental to the HPLC instrument; however, as the use of chloride ion resulted in good chromatographic separations, and as no damage to the chromatographic apparatus occurred after a six-month work period, we suggest that the chloride ion may be used in the mobile phase. Nevertheless, we advice users to wash the HPLC instrument carefully, pumping through at least 50 ml of distilled water once a week.

The effect of temperature was studied after the column was set in a chromatograph oven at temperatures from 30 to 65°C. We noted that selectivity factors were enhanced between AC 155 and bamifylline (1.23 up to 1.41) but decreased between bamifylline and AC 119 (1.19 down to 1.13) and between AC 85 and AC 155 (1.06 down to 1.00).

Improvement in peak shape was observed when *n*-nonylamine was added to aqueous mobile phase (with or without 3 g/l KCl) at concentrations ranging from $1.3 \cdot 10^{-4}$ to $2.0 \cdot 10^{-3}$ *M*, but a simultaneous decrease of resolution appeared.

Both of these modifications (temperature or competing amine) were then discarded.

Linearity, sensitivity, specificity

Standard curves were obtained by measuring the peak height ratios (drug/internal standard) on chromatograms obtained from drug-free plasma spiked with bamifylline, its metabolites and fenetylline. Linear curves were observed when plotting peak height ratios versus concentration (0.01, 0.1, 0.2, 0.5, 1, 2 μg of each compound per ml). Each value was the mean of six measurements. The calibration curves for AC 85, AC 155, bamifylline and AC 119 could be respectively expressed by the following equations: $Y = 0.981X$ ($r = 1.00$), $Y = 1.01X$ ($r = 1.00$), $Y = 0.986X$ ($r = 1.00$) and $Y = 0.992X$ ($r = 1.00$). The detection limit (signal/background = 3) was 0.01 μg/ml for each compound. No interference with endogenous components (uric acid, creatinine) or with tested drugs (10 μg/ml each in plasma) such as clobutinol, almitrine, bromhexine, troleandomycin, doxycycline, furosemide, salbutamol, altizide, spironolactone, canrenoate potassium and eprazinone was found.

Reproducibility, recovery

Within-day reproducibility was determined by carrying out fourteen determinations of plasma spiked with 0.2, 0.5 and 1.0 μg/ml of bamifylline and each metabolite. Day-to-day reproducibility was obtained by carrying out every day and during ten days a determination from plasmas spiked with 0.5 μg/ml of each drug compound. Coefficients of variation (Table III) were respectively less than 8.2% and less than 6.3%.

TABLE III

WITHIN-DAY AND DAY-TO-DAY REPRODUCIBILITY OF THE METHOD

	Drug concentration (μg/ml)	Coefficient of variation (%)			
		AC 85	AC 155	Bamifylline	AC 119
Within-day (n = 14)	0.2	6.92	8.17	4.71	6.71
	0.5	5.27	5.14	4.17	5.61
	1.0	4.88	6.30	4.54	6.94
Day-to-day (n = 10)	0.5	6.22	4.97	4.13	6.30

A recovery study was carried out by adding a known amount of bamifylline and metabolites to a drug-free plasma sample at two concentrations (0.5 and 1.0 μg/ml). After extraction, the dry residue was dissolved in a mobile phase containing an internal standard amount equivalent to a 100% extraction yield. For each concentration, five extractions were performed; means of peak height ratios were computed and compared to the peak height ratio of an injected amount of drug compound and internal standard equivalent to a 100% extraction yield. As can be noted from Table IV, recovery percentages are greater for bamifylline than for its metabolites.

TABLE IV

RECOVERY DATA FOR ASSAY OF BAMIFYLLINE AND ITS METABOLITES

Plasma concentration (μg/ml)	n	Mean recovery (% ± S.D.)			
		AC 85	AC 155	Bamifylline	AC 119
0.5	5	62.6 ± 3.0	75.2 ± 3.4	94.2 ± 3.5	72.4 ± 3.6
1.0	5	61.6 ± 2.3	71.4 ± 3.9	89.6 ± 2.7	70.6 ± 2.1

Clinical applications

A typical chromatogram obtained, using the method previously described, from a subject treated for three months with bamifylline is illustrated in Fig. 5. The chromatographic separation of bamifylline and its metabolites was achieved in less than 15 min. The retention times (t_R) of AC 85, AC 155, bamifylline, AC 119 and internal standard were 6.8, 7.2, 8.3, 9.4 and 12.6 min, respectively. This chromatogram shows two unidentified peaks (unknown minor metabolites?) at t_R = 8.0 min (X) and t_R = 11.2 min (Y).

Preliminary results of plasma concentration of bamifylline and metabolites were obtained from ten subjects receiving orally 600 mg of Trentadil per day (2 × 300 mg) for at least one month. Mean plasma concentrations at the trough (just before the next-morning dose) and at nearly the peak level (60 min after

oral administration) were, respectively, 0.16 and 0.43 μg/ml for AC 85, 0.12 and 0.12 μg/ml for AC 155, 0.34 and 1.28 μg/ml for bamifylline, 0.21 and 0.28 μg/ml for AC 119.

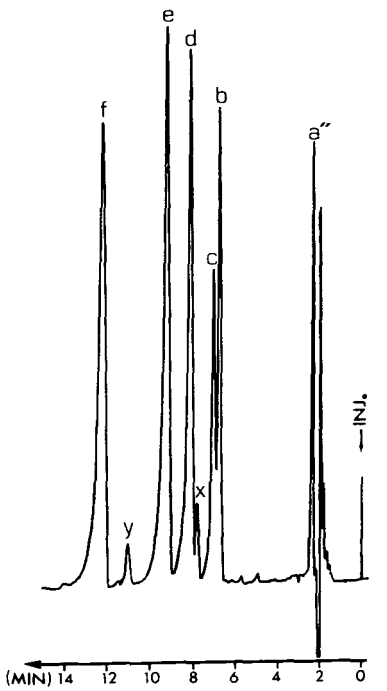

Fig. 5. Chromatogram of a plasma extract from a patient receiving a 600-mg oral dose of bamifylline (Trentadil) for three months and spiked with 1.25 μg/ml of fenetylline (internal standard). Chromatographic conditions are described in Table I [3]. a'' = caffeine, b = AC 85, c = AC 155, d = bamifylline, e = AC 119, f = fenetylline, x and y = unknown peaks (minor metabolites?).

ACKNOWLEDGEMENTS

This work was supported by a grant from INSERM (CRL no. 825 020). The technical assistance of J. Bonnaud is appreciated.

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Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1791

QUANTITATIVE HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHIC ANALYSIS OF AMPICILLIN IN HUMAN URINE

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(First received February 15th, 1983; revised manuscript received May 6th, 1983)

SUMMARY

A quantitative high-performance thin-layer chromatographic (HPTLC) method was developed for the analysis of ampicillin in urine. A dioxane—water—*n*-butanol—formic acid mobile phase system and HPTLC Silica gel 60 F254 as stationary phase were used. Quantitation was realized on a Zeiss PMQ 2 densitometer connected to a Varian A-25 recorder and an Apple II microcomputer or alternatively with an HP 9830A computer and HP 9862A plotter. A good linear range of detection (0.05—1.00 μg) at 480 nm was obtained. Standard statistical methods demonstrated good reproducibility (coefficient of variation is not greater than 3%). The method is appropriate for ampicillin quality control and pharmacokinetic studies.

INTRODUCTION

Ampicillin (D-(—)-α-aminobenzylpenicillin, anhydrous or as trihydrate or sodium salt), derived from 6-aminopenicillanic acid, is an antibiotic used therapeutically on a wide scale because of its low toxicity and its biological activity against a broad spectrum of Gram-positive and several Gram-negative pathogens. Its pharmacokinetic properties (relatively rapid systemic absorption, small extent of biotransformation and nearly complete elimination in the urine) ensure its high applicability in the treatment of systemic and urinary infections [1—3]. The assay of ampicillin currently consists of the following

0378-4347/83/$03.00

official and non-official procedures: microbiological agar diffusion [4], iodine titration [5], spectrophotometry [6, 7], non-aqueous acid and base titration [8], fluorometry [9], high-performance liquid chromatography [10—14], and thin-layer chromatography [15]. The microbiological assay proved to be slow and tedious and to lack precision and specificity. The chemical and spectrophotometric assay require available functional groups and as such are limited by the fact that they depend on reactions that may also occur with other components of the sample. High-performance liquid chromatography (HPLC), because of its effective separation, selective detection and appropriate sensitivity, reproducibility, accuracy and precision, offers the best potential for quantitating the intact drug and/or its degradation products or metabolites in stability, pharmacokinetic and quality control studies. Thin-layer chromatography and especially high-performance thin-layer chromatography (HPTLC) are becoming, due to the great evolution of microelectronics (densitometers connected to microcomputers) and appropriate equipment (chromatographic plates, spotting, developing and visualization equipment), methods equivalent to HPLC in terms of all the mentioned criteria and superior in terms of flexibility [16, 17].

Because none of the mentioned references provides a rapid and flexible analytical method applicable to pharmacokinetics, when a large number of blood and urine samples must be analysed, this study was undertaken to develop a selective and reliable method for ampicillin analysis in human urine using HPTLC. The second reason for the present work arises from the intention to ascertain the applicability of HPTLC among the other analytical methods used in pharmacokinetics.

EXPERIMENTAL

Apparatus

Investigations were carried out at various times with two different systems: (1) a Zeiss PMQ 2 densitometer (Opton, 7082 Oberkachen, F.R.G.) connected to an A-2 recorder (Varian, Palo Alto, CA, U.S.A.) and an Apple II microcomputer (Apple Computer Inc., CA, U.S.A.); and (2) a Zeiss PMQ 2 densitometer connected with an HP 9830 A calculator and an HP 9862 A plotter (Hewlett-Packard, CA, U.S.A.).

Chromatographic plates, spotting and developing equipment

The satisfactory plates were HPTLC plates of silica gel 60 F254 for nano-TLC, 0.25 mm thick, 10 × 10 cm (E. Merck, Darmstadt, F.R.G.). By means of Drummond microcaps (1 μl), 1-μl aliquots of the samples were spotted onto the chromatographic plates. The spots were dried and developed at room temperature with the solvent system (mobile phase) in a Camag twin-trough chamber, 20 × 10 cm, previously saturated with solvent vapours. Development was continued until the distance between the origin and the solvent front was 7 cm. The plates were air-dried, sprayed with ninhydrin solution and treated for 5 min at 110°C to visualize the spots. The R_F of ampicillin was 0.65.

Chemicals and reagents

Ampicillin trihydrate, 860 μg/mg activity, was obtained from the Institute for Pharmacy and Drug Quality Control, Ljubljana, Yugoslavia.

Ninhydrin reagent (0.3 g of ninhydrin, 100 ml of *n*-butanol and 3 ml of glacial acetic acid) was prepared according to the method of Stahl [18].

Ammonium sulphate, p.a., chloroform, p.a., benzalkonium chloride, p.a., dioxane, p.a. and formic acid, p.a., were also used.

Preparation of solutions and chromatographic plates

Solvent for standard solution. This was a mixture of dioxane—water (4:1) with formic acid to give pH 5.

Mobile phase. This was a mixture of dioxane—water—*n*-butanol—formic acid (70:15:15:1.25).

Standard solution. An amount of the working ampicillin standard equivalent to 290.697 mg was weighted on a Mettler H542 balance and dissolved in 250 ml of the solvent for standard solution. The solution was shaken and ultrasonicated until it became totally clear.

Chromatographic plates for the calibration curve and ampicillin extraction ratio from urine. Solutions with 0.1, 0.15, 0.20, 0.25, 0.35, 0.40, 0.50 and 0.86 μg/ml were prepared by diluting the standard solution, and spotted onto the chromatographic plates parallely without and with 1 μl of urine extract. As we wanted to obtain the ampicillin extraction ratio from urine, a known amount of ampicillin was added to urine which was further treated in the same manner as that with ampicillin after oral administration.

Chromatographic plates for the statistical evaluation of the method. Ten 1-μl aliquots containing 0.5 μg of ampicillin in dioxane—water mixture were spotted on plate 1; another ten aliquots containing 0.5 μg of ampicillin in urine extract were spotted on plate 2; plates 3 and 4 were arranged in the same way but contained 1.5 μg of ampicillin. Plates 1 and 2 were evaluated on the recorder without subtracting the base line and the line of the blank sample, while plates 3 and 4 were evaluated on the plotter after subtracting the base line and the line of the blank sample.

Conditions for quantitation

Densitograms were obtained using the densitometer in the remission mode [16, 17] at 480 nm, slit 3.5 mm wide and 0.1 mm high, table 200 mm/min *y* axis; *x* axis was adjusted manually, white background.

Protocol of in vivo pharmacokinetic study and preparation of urine samples

The pharmacokinetic study was carried out with three male volunteers (VI, VII, VIII). Each of them swallowed in the evening a capsule of Ampicillin® (Lek, Ljubljana) containing 500 mg of ampicillin trihydrate and urine was collected during the night (8 h). VIII repeated the trial three times. Next morning, urine volumes were measured and 10-ml aliquots were transferred into separating funnels together with 3 g of ammonium sulphate. Separately, we prepared 30 ml of chloroform with 500 mg of benzalkonium chloride. It was added in three aliquots to each separating funnel and ampicillin was extracted from the aqueous to the organic phase. The extracts were collected

in round-bottomed flasks and chloroform evaporated at 40°C to give a volume of less than 10 ml. The solutions were transferred into 10-ml volumetric flasks which were filled to volume with chloroform. Aliquots (1 μl) of the samples were spotted onto the chromatographic plates.

Assay and calculations

Ampicillin concentrations in urine samples were determined using statistically treated relation between ampicillin quantity on the chromatogram and area under the Gaussian curve (AUC), resulting from the densitometric measurements. Eqns. 1 and 2 were selected to obtain the calibration curve and ampicillin concentrations in the urine samples:

$$\mathrm{AUC} = A \cdot h \tag{1}$$

$$\mathrm{AUC} = (b \pm e_b) + (a \pm e_a) \cdot Q \tag{2}$$

where AUC = area under the Gaussian curve, A = width of the peak at half height, h = height of the peak from the base line, Q = ampicillin quantity in the spot, a and b = linear regression coefficients for the calibration curve — the slope of the calibration curve and intercept with AUC axis when Q is zero, and e_a and e_b are the errors of a and b. The data from the chromatograms were transferred to computer memory. The base line and the line of the blank sample were subtracted and the corrected Gaussian curve reflected the amount of ampicillin in the sample. The reproducibility of the method was tested using standard statistical parameters: arithmetic mean, variance, standard deviation, relative standard deviation (coefficient of variation), standard error, relative standard error and 95% confidence interval of the mean.

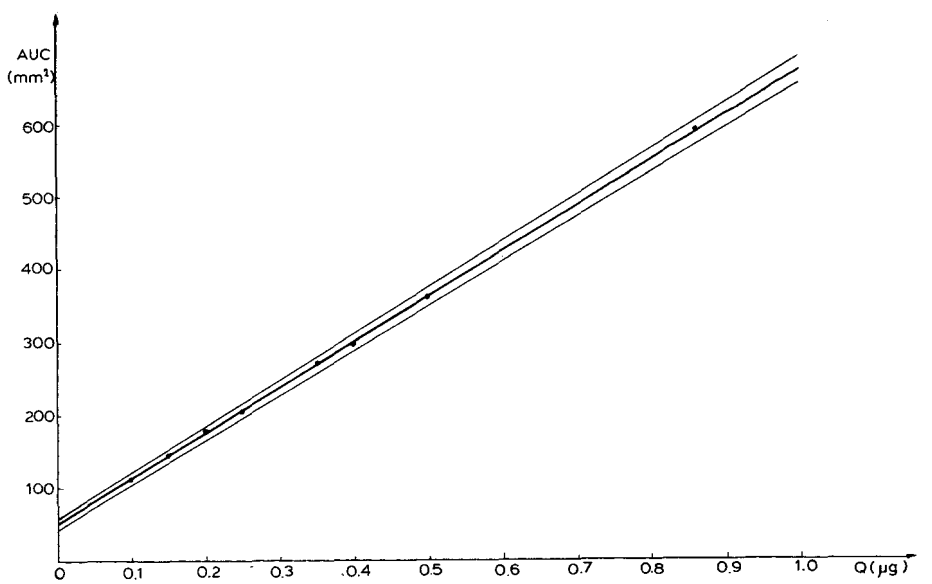


Fig. 1. Calibration curve for densitometric determination of ampicillin in water and urine. AUC = (51.21 ± 6.65) + (621.17 ± 8.06)Q. r = 0.9998, n = 16, P = 0.05, each point represents the mean of two measurements.

RESULTS

Ampicillin trihydrate was dissolved in dioxane—water using formic acid as pH moderator. The solution was easily spotted on the chromatographic plate, rapidly dried and after development gave spots of suitable quality. It is well known that ampicillin is unstable in aqueous solutions and the pH of its maximal stability coincides with the pH of its minimal solubility (pH 5) [19]. The mentioned fact did not represent the limitation, because our working concentrations at pH 5 were greater than the ampicillin intrinsic solubility (C_0 = 0.0223 M).

Calibration

Fig. 1 shows the calibration curve for densitometric determination of ampicillin in water and urine using eqns. 1 and 2. Base line and line of the blank sample which result from endogenous urine components were subtracted by computer. The extraction ratio, obtained using the calibration curve and benzalkonium chloride as phase-transfer catalyst, was 82.5%.

The results of the statistical evaluation are summarized in Table I.

TABLE I

STATISTICAL PARAMETERS OF AUC VALUES USED FOR DENSITOMETRIC DETERMINATION OF AMPICILLIN

	Plate 1	Plate 2	Plate 3	Plate 4
Arithmetic mean	378.4	384.7	1050.0	1136.4
Variance	219.5	299.4	902.8	1255.1
Standard deviation	14.8	17.3	30.0	35.4
Relative standard deviation (%) (coefficient of variation)	3.9	4.5	2.9	3.1
Standard error	4.7	5.4	9.5	11.2
Relative standard error (%)	1.2	1.4	0.9	1.0
95% Confidence interval of the mean	±10.6	±12.3	±21.5	±25.3

Quantitative analysis

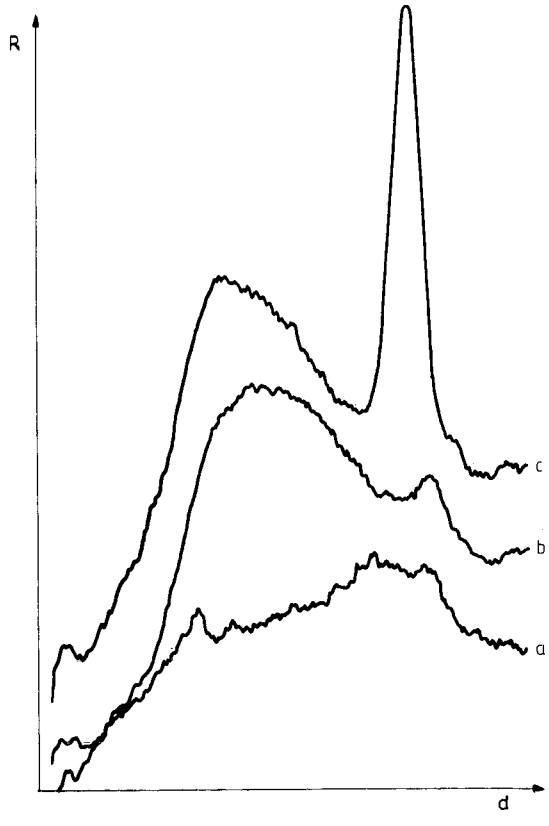
Figs. 2 and 3 illustrate use of the computer to subtract the base line and the line of the blank sample to give the corrected ampicillin curve.

Figs. 4 and 5 show curves for ampicillin in urine after extraction of known and unknown quantities of ampicillin.

Chromatographic parameters for the samples and their quantitative evaluation are given in Table II. Ampicillin quantities in spots are calculated considering the extraction ratio. Table III shows the calculation of concentrations of unmetabolized ampicillin in urine after oral administration of a 500-mg capsule.

DISCUSSION

The results obtained show that the HPTLC method for the determination of unchanged ampicillin can be used in in vitro as well as in in vivo ampicillin



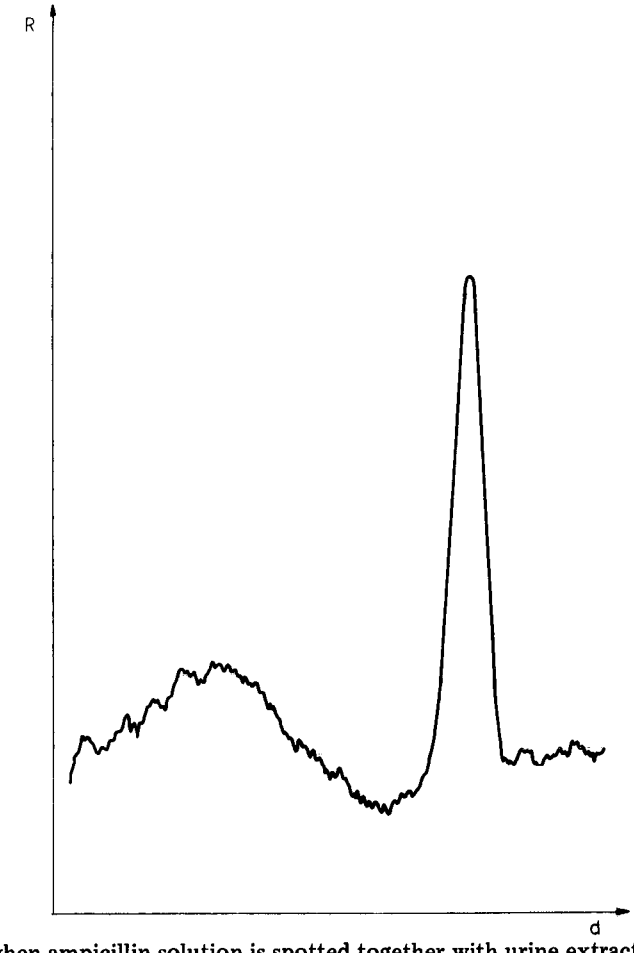


Fig. 2. Base line curve (a), black sample curve (b) and ampicillin curve (c) when ampicillin solution is spotted together with urine extract. R = percentage remission, d = distance in cm.

Fig. 3. Corrected ampicillin curve after subtracting base line and line of blank sample.

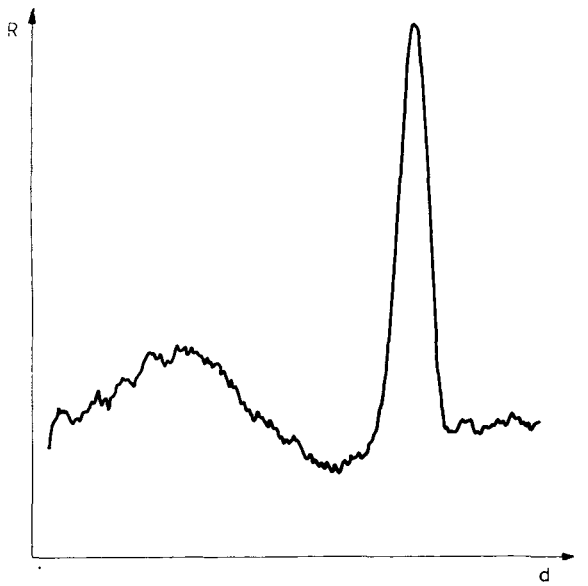


Fig. 4. Ampicillin curve after extraction from urine (original concentration = 0.86 μg/μl, concentration after extraction = 0.71 μg/μl).

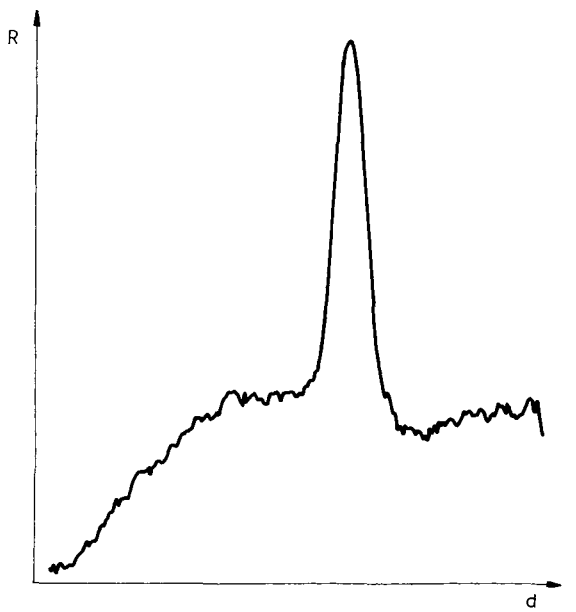


Fig. 5. Ampicillin curve after oral administration and sample extraction for VIII, third experiment.

studies. In drug quality control it could be the method of choice for several reasons: it is rapid, it enables the simultaneous measurement of a large number of samples under the same conditions, and it shows great reproducibility and

TABLE II

CHROMATOGRAPHIC PARAMETERS FOR AMPICILLIN DETERMINATION IN URINE SAMPLES

Sample	Peak height (cm)	Peak width at half height (mm)	AUC (mm^2)	Ampicillin quantity in spot (μg)
VI 1	5.2	7.0	364.0	0.606
VII 1	4.5	6.5	292.5	0.436
VIII 1	3.6	6.2	223.2	0.327
VIII 2	5.1	6.8	346.8	0.569
VIII 3	6.3	6.5	409.5	0.690
VIII 4	6.3	6.5	409.5	0.690

TABLE III

URINARY AMPICILLIN CONCENTRATIONS AND QUANTITIES, APPROPRIATE URINE VOLUMES, AND PERCENTAGE OF UNMETABOLIZED AMPICILLIN IN URINE AFTER ORAL ADMINISTRATION OF 500 mg OF AMPICILLIN

Sample	Urinary ampicillin concentration (mg/ml)	Urine volume (ml)	Quantity of urinary ampicillin (mg)	Percentage of unchanged ampicillin excreted
VI 1	0.606	300	181.8	42.27
VII 1	0.436	410	178.7	41.55
VIII 1	0.327	450	147.6	34.33
VIII 2	0.569	250	142.2	33.08
VIII 3	0.690	220	151.8	35.30
VIII 4	0.690	210	144.9	33.69

sensitivity compared to other methods, especially the official ones. The method is particularly suitable for pharmacokinetic studies. The criteria for an analytical method to be used in pharmacokinetics are fulfilled and the results obtained for urinary ampicillin concentrations coincide well with those in the literature [20].

Since the urinary ampicillin concentrations in the kinetic study (urine sample collection during appropriate time intervals) were higher than 0.1 mg/ml, the sensitivity of the method was not tested separately. However, the limit of quantitation per ml of urine can be given, i.e. 0.05 mg/ml. The proposed method for ampicillin, whose metabolites are therapeutically inactive and are present in very low concentrations in urine [12], proved to be very useful for ampicillin antimicrobial activity evaluation under in vivo conditions.

The proposed method demonstrates some advantages of HPTLC over other chromatographic methods: the simultaneous measurement of a large number of samples, a very flexible system for absorption and fluorescence measurements, scanning of spectra directly from densitograms, separate chromatographic and detection systems, impure samples do not interfere (clean-up is not needed), and very dilute solutions can be spotted repeatedly. Significant improvement was attained using the Apple microcomputer, which had control

of scanning on the densitometer, preserves the data on discs and defines the integrating conditions for AUC calculations. It computes, comparing with standard values, appropriate statistical parameters and prints out the results in full. Using the specific programs for subtracting the base line and the line of blank sample, better reproducibility and accuracy were obtained.

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Journal of Chromatography, 277 (1983) 261—272
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1768

THIN-LAYER CHROMATOGRAPHIC DETERMINATION OF PROCAINAMIDE AND N-ACETYLPROCAINAMIDE IN HUMAN SERUM AND URINE AT SINGLE-DOSE LEVELS

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(First received August 4th, 1982; revised manuscript received May 6th, 1983)

SUMMARY

Thin-layer chromatographic methods were applied for bioavailability studies of procainamide in serum and urine. Detection of the parent compound and the major metabolite was performed in the ultraviolet range at 275 nm. Using 100-μl samples, detection limits were 60 ng of procainamide—HCl per ml serum and 7 μg/ml urine, and 60 ng of N-acetylprocainamide—HCl per ml serum and 5 μg/ml urine. Advantages over previous methods are discussed. From serum and urine data of five volunteers, the bioavailability of procainamide from a 250-mg dragee preparation compared with an intravenous dose was verified. Pharmacokinetic data were computed using one-compartment open models. Results corresponded well with values previously published.

INTRODUCTION

Procainamide (Fig. 1) is an effective and widely used anti-arrhythmic drug [1]. Pharmacokinetic and metabolism data reported in the literature are primarily concerned with long-term therapy [1—9]. N-Acetylprocainamide, the potent [2] major metabolite, has to be regarded separately, since it has a comparatively lower clearance and a longer half-life than the parent drug. Two groups of human patients, related to their metabolite serum levels, should be discriminated, i.e. slow and fast acetylators. The latter are expected to have

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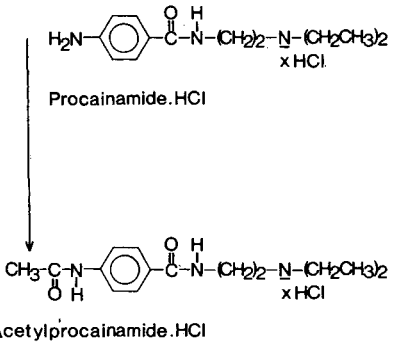


Fig. 1. Structure of procainamide · HCl and its metabolite.

a comparatively lower risk [4] of developing a syndrome resembling lupus erythematosus which may be induced by the parent drug.

For therapeutic monitoring in serum, detection limits of 1 μg/ml procainamide · HCl and 1 μg/ml N-acetylprocainamide · HCl, respectively, suffice. Assays published in the last decade have utilized gas chromatography (GC) [10—12], high-performance liquid chromatography (HPLC) [13—19], and thin-layer chromatography (TLC) [20—22]. In most cases, serum aliquots of 0.5—1 ml were used.

For single-dose pharmacokinetics, however, especially for bioavailability studies of various preparations, analytical methods for serum and urine are required which are practicable, selective and accurate. They should allow the simultaneous assay of both compounds at the 100 ng/ml serum and 10 μg/ml urine levels. Since large numbers of individual blood samples are usually needed, preferably small serum aliquots should be used for each analysis.

The present report describes sensitive TLC assays for procainamide and N-acetylprocainamide in serum and urine. In one simple extraction step, 100-μl samples are cleaned-up for chromatographic analysis.

EXPERIMENTAL

Reagents

The reagents used were 1 mmol/l carbonate buffer pH 10.9 AR, ethyl acetate AR, 1,4-dioxane AR, methanol AR, concentrated ammonia solution (25%) AR.

Equipment

A Zeiss KM3 chromatogram spectrometer with microoptics and a Servogor® 210 (Metrawatt) recorder were used. Separation was performed on silica gel HPTLC plates F 254 (no. 5642, E. Merck, Darmstadt, F.R.G.) in a Camag twin-trough HPTLC chamber 20 cm × 10 cm (no. 25254). For sample clean-up and spotting, a Vortex® mixer, a centrifuge, glass-stoppered tubes (ca. 8 ml), conical glass-stoppered tubes (ca. 8 ml) and a Desaga Autospotter® were used.

Serum procedure

In a glass-stoppered tube, 100 μl of serum were treated with 1 ml of buffer

pH 10.9. The serum was extracted with 5 ml of ethyl acetate for 30 sec on a Vortex mixer. The phases were separated by centrifugation (5 min), and 4 ml of the organic phase were transferred into a conical tube and evaporated to dryness at 40° C under a stream of nitrogen. The residue was dissolved in 100 µl of ethyl acetate.

Using the Desaga Autospotter*, 75 µl were transferred onto the HPTLC plate as a series of consecutive droplets of approx. 100 nl each. Since each drop evaporated before the next was applied, narrow spots were obtained which were suitable for HPTLC.

Urine procedure

In a glass-stoppered tube, 100 µl of urine were treated with 1 ml of buffer pH 10.9. The urine was extracted with 5 ml of ethyl acetate for 30 sec on the Vortex mixer. The phases were separated by centrifugation (5 min) and 75 µl were transferred onto the HPTLC plate.

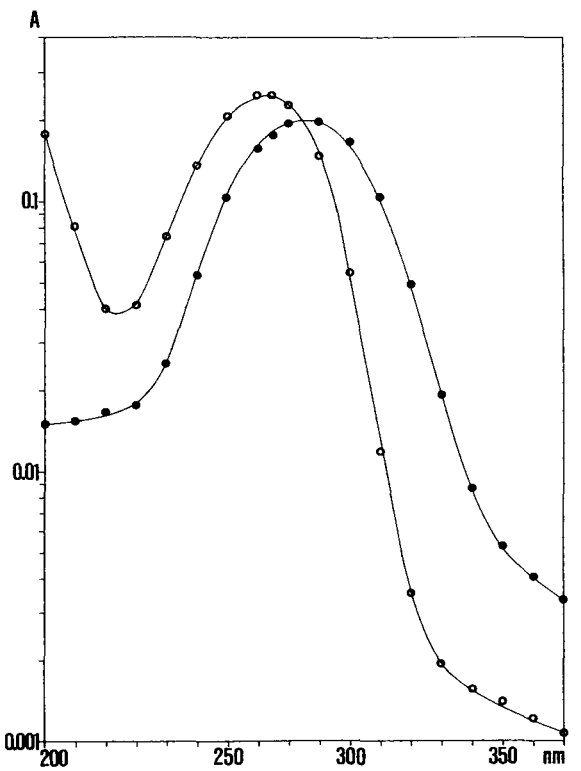


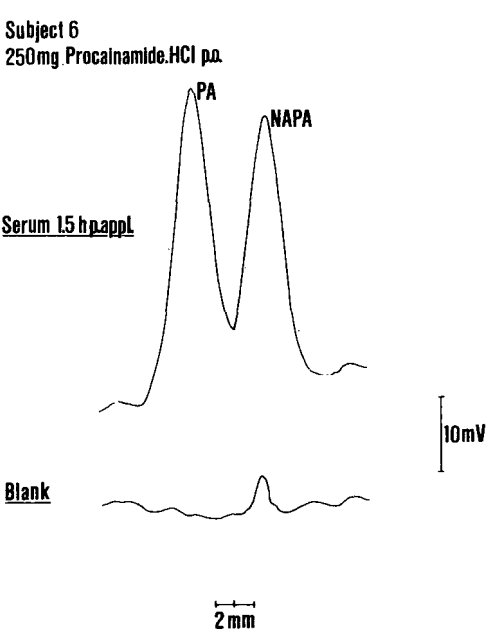
Fig. 2. In situ ultraviolet spectra of procainamide · HCl (•) and N-acetylprocainamide · HCl (○) on an HPTLC plate; 0.5 µg per spot.

*Modified version, Tygon® tubes of larger diameter [Technicon flow-rated, code 116-0549-09 (white)] and 60-cm long Hostaflon® tubes were used.

Chromatography

The twin-trough HPTLC developing chamber contained 10 ml of solvent in one compartment. The plate was developed with dioxane—concentrated ammonia (9:1) in the dark, without previous saturation, over a distance of 4 cm. R_F values were procainamide 0.55, N-acetylprocainamide 0.45.

Measurements were carried out in the direction of the solvent flow with an effective slit (microoptics) of 4.5 mm × 0.15 mm at a wavelength of 275 nm (Fig. 2), scanning speed 50 mm/min and paper speed 240 mm/min (Figs. 3 and 4).



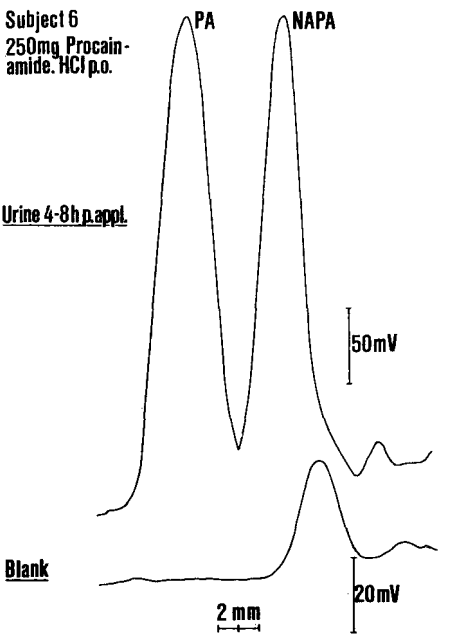


Fig. 3. Determination of procainamide · HCl (PA, 0.90 μg/ml) and N-acetylprocainamide · HCl (NAPA, 0.55 μg/ml) in serum, compared to a blank.

Fig. 4. Determination of procainamide · HCl (PA, 103 μg/ml) and N-acetylprocainamide · HCl (NAPA, 53 μg/ml) in urine, compared to a blank.

Quantitation

Peak height evaluation leads to a better precision and accuracy compared to peak area determinations, since these results depend much less on the quality of peak separation and on the baseline noise. Therefore, calibration functions were determined for each compound from the peak heights of the standards (Fig. 5).

These functions were non-linear. Deviation from linearity, however, could be expressed in terms of a parameter k_m according to Kufner and Schlegel [23]:

$$C = \frac{C_{max} \times E_{rel} \times k_m}{1 - E_{rel} + k_m}$$

where C = unknown concentration, C_{max} = maximum calibration standard,

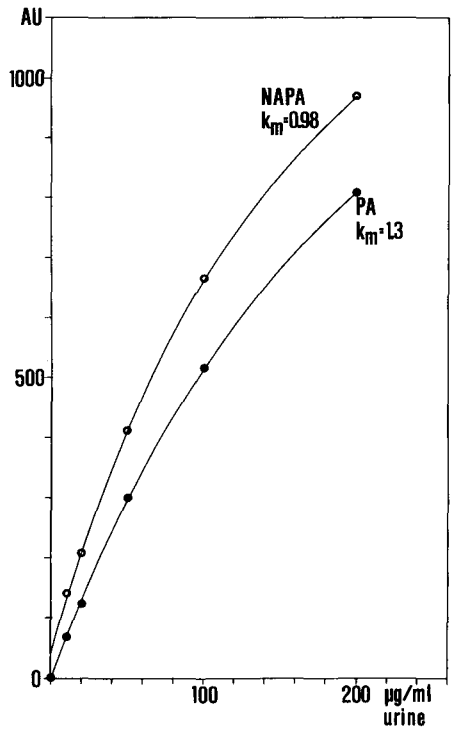


Fig. 5. Non-linear calibration graphs for procainamide · HCl (PA, •) and N-acetylprocainamide · HCl (NAPA, ○).

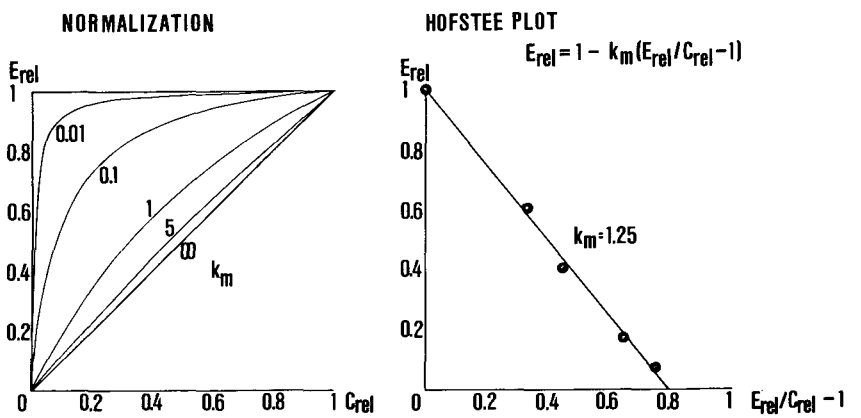


Fig. 6. Determination of k_m, parameter of non-linearity, from a Hofstee plot.

E_{rel} = peak height/maximum peak height, and k_m = parameter of non-linearity. The latter was obtained from a Hofstee plot following normalization of peak heights and concentrations (Fig. 6).

RESULTS

Serum

The compounds were added to blank serum in five concentrations over the range 0.1—2.0 μg/ml serum. Each sample was split into six portions of 0.1 ml, so that six equal series were formed. Each series was then analyzed in turn so that a total of six independent analytical results were available for each concentration.

Quality criteria of an analytical method are selectivity, accuracy, reproducibility and sensitivity [24, 25]. The corresponding parameters were derived from the analytical results given in Table I (except inter-assay reproducibility).

TABLE I

RECOVERY, PRECISION AND ACCURACY OF PROCAINAMIDE AND N-ACETYLPROCAINAMIDE IN SERUM

n = 6 determinations, concentration in μg/ml.

Procainamide · HCl			N-Acetylprocainamide · HCl		
Added	Found (mean ± S.D.)	Accuracy	Added	Found (mean ± S.D.)	Accuracy
2.00	2.01 ± 0.02	—0.01	2.00	2.00 ± 0.00	0.00
1.00	1.02 ± 0.08	—0.02	1.00	1.00 ± 0.07	0.00
0.50	0.45 ± 0.03	+0.05	0.50	0.49 ± 0.04	+0.01
0.20	0.21 ± 0.02	—0.01	0.20	0.21 ± 0.02	—0.01
0.10	0.10 ± 0.01	±0.00	0.10	0.09 ± 0.02	+0.01
Blank	0		Blank	0	

Selectivity. The serum blank does not contain any peak which could interfere with the assay (Fig. 3).

Accuracy. This was expressed by the deviation (bias) of the mean value of the results from the theoretical value: accuracy = $C_{added} - \bar{C}_{found}$. In each case the average accuracy was <0.01 μg/ml serum. Regression coefficients were greater than 0.998.

Precision (intra-assay reproducibility). This was defined in terms of standard deviations of the results obtained from each sample. It was constant in the concentration range considered: procainamide · HCl, S.D. = 0.03 ± 0.03 μg/ml serum; N-acetylprocainamide · HCl, S.D. = 0.03 ± 0.03 μg/ml serum.

The limit of detection was 60 ng/ml serum for both compounds. It was calculated as precision × 2 [26].

Inter-assay reproducibility. This was tested in two spiked control sera. From values obtained between September and November 1979, the following degrees of inter-assay reproducibility were determined (μg/ml, mean ± S.D.): For control 1, procainamide · HCl = 2.07 ± 0.22, and N-acetylprocainamide · HCl = 1.95 ± 0.12. For control 2, procainamide · HCl = 0.54 ± 0.13, and N-acetylprocainamide · HCl = 0.56 ± 0.08.

Urine

The compounds were added to blank urine in five concentrations over the range 10—200 μg/ml urine. Each sample was split into six portions of 0.1 ml, so that six equal series were formed. Each series was than analyzed in turn so that a total of six independent analytical results were available for each concentration.

Quality criteria of the urine method were defined by the corresponding parameters calculated from the analytical results given in Table II (except inter-assay reproducibility).

TABLE II

RECOVERY, PRECISION AND ACCURACY OF PROCAINAMIDE AND N-ACETYLPROCAINAMIDE IN URINE

n = 6 determinations, concentration in μg/ml.

Procainamide · HCl			N-Acetylprocainamide · HCl		
Added	Found (mean ± S.D.)	Accuracy	Added	Found (mean ± S.D.)	Accuracy
200	200 ± 0	0	200	201 ± 2	−1
100	101 ± 9	−1	100	99 ± 5	+1
50	49 ± 6	+1	50	48 ± 2	+2
20	21 ± 3	−1	20	23 ± 2	−3
10	10 ± 2	0	10	11 ± 1	−1
Blank	0		Blank	0	

Selectivity. The assay is practically free from blanks for both substances; a small blank as observed near the metabolite R_F was in the range of the detection limit (Fig. 4).

Accuracy. For each substance, the average accuracy was <0.3 μg/ml urine. Regression coefficients were greater than 0.998.

Precision (intra-assay reproducibility). This was constant in the concentration range considered. For procainamide · HCl, S.D. = 3.3 ± 3.6 μg/ml urine; for N-acetylprocainamide · HCl, S.D. = 2.4 ± 1.5 μg/ml urine.

The limit of detection (DL), taken as precision × 2, was 7 μg/ml urine for procainamide · HCl and 5 μg/ml urine for N-acetylprocainamide · HCl.

Inter-assay reproducibility. This was tested in two spiked control urines. From values obtained between September and November 1979, the following degrees of inter-assay reproducibility were determined (μg/ml, mean ± S.D.): For control 1, procainamide · HCl = 196 ± 9, N-acetylprocainamide · HCl = 194 ± 12. For control 2, procainamide · HCl = 50 ± 9, N-acetylprocainamide · HCl = 50 ± 7.

Pharmacokinetics

In a cross-over study, procainamide · HCl was administered intravenously and orally in doses of 250 mg to six healthy male volunteers (age 25—31 years, height 165—189 cm, weight 56—74 kg). Blood was sampled and urine collected in fractions up to 24 h post administration.

Using TLC, serum levels were found in the ranges 0.05—2 μg/ml for procainamide · HCl and 0.05—0.7 μg/ml for N-acetylprocainamide · HCl. Urine levels were found in the ranges 5—600* μg/ml for procainamide · HCl and 5—90 μg/ml for N-acetylprocainamide · HCl.

Pharmacokinetic profiles were calculated based on one-compartment open models. This is demonstrated by the serum kinetic results of subject 2 following intravenous and oral doses (Figs. 7 and 8) and by mean cumulative excretion values of five** volunteers (Fig. 9).

Following intravenous dosage, the initial serum concentration of 1.7 ± 0.3 μg/ml procainamide · HCl corresponded to a volume of distribution of 144 ± 20 l and a distribution coefficient of 2.1 ± 0.2 l/kg. The drug was eliminated with a half-life of 2.4 ± 0.4 h. The area under the curve was 6.0 ± 0.9 μg h/ml procainamide · HCl. The total clearance was 711 ± 106 ml/min.

N-Acetylprocainamide serum levels rose after a lag time of 10 ± 14 min with a half-life of 4 ± 0 min. Maximum levels of 0.4 ± 0.1 μg/ml N-acetylprocainamide · HCl were reached 0.7 ± 0.2 h post administration. The metabolite was eliminated with a half-life of 8 ± 2 h. The area under the curve was 4.3 ± 1.0 μg h/ml N-acetylprocainamide · HCl.

Following oral treatment, the drug was absorbed after a lag time of 13 ± 10 min with a half-life of 9 ± 6 min. Maximum serum levels of 1.2 ± 0.2 μg/ml were reached 0.8 ± 0.3 h post administration. The elimination half-life of 2.5 ± 0.3 h and the area under the curve of 4.8 ± 1.0 μg h/ml corresponded well with the data after intravenous dosage (see Tables III and IV). Metabolite serum levels rose after a lag time of 17 ± 10 min with a half-life of 16 ± 10 min.

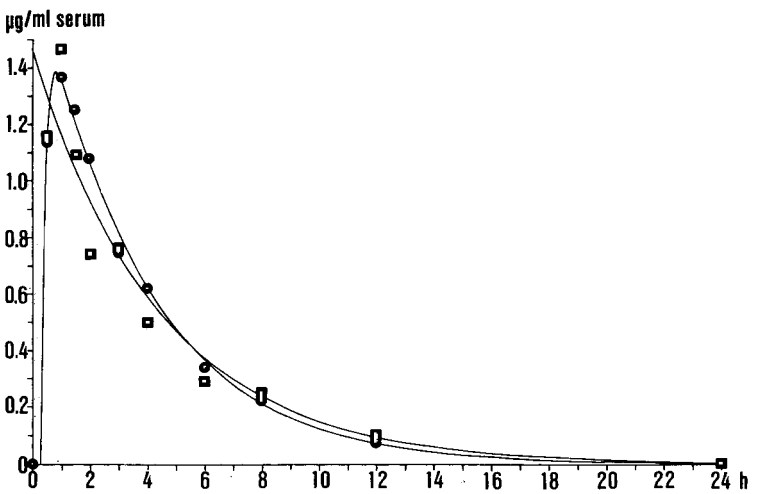


Fig. 7. Procainamide · HCl serum levels of subject 2 after a single dose of 250 mg intravenously (■) and orally (●).

*For levels greater than 200 μg/ml urine, smaller aliquots were used.

**Subject 3 was excluded since, following the oral dose, higher serum levels and a higher degree of renal excretion compared to the intravenous dose were observed.

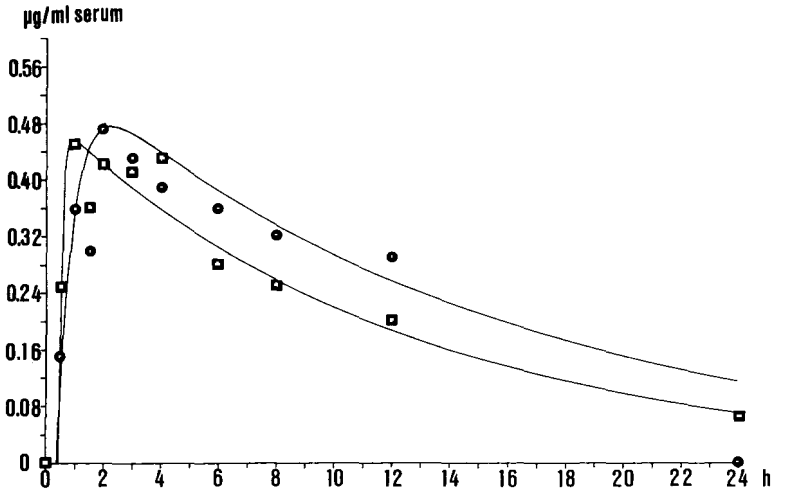


Fig. 8. N-Acetylprocainamide · HCl serum levels of subject 2 after a single dose of 250 mg procainamide · HCl intravenously (▪) and orally (•).

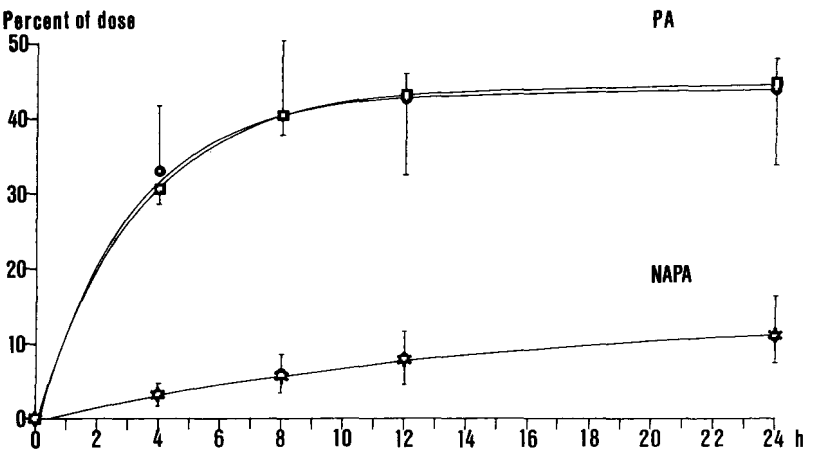


Fig. 9. Mean cumulative renal excretions after a single dose of 250 mg procainamide · HCl to five volunteers intravenously (PA, ▪; NAPA, *) and orally (PA, •; NAPA, ○).

Maximum serum levels of 0.5 ± 0.1 µg/ml were reached 1.6 ± 0.7 h post administration.

The elimination half-life of 8 ± 2 h and the area under curve of 5.8 ± 1.4 µg h/ml corresponded well with the data after intravenous dosage (see Tables III and IV).

From the cumulative renal excretions (Fig. 9), urinary half-lives were identical to serum half-lives (Table IV).

Cumulative renal excretions up to 24 h post procainamide administration are presented in Table V in comparison to the extrapolated amounts.

Renal clearances were calculated from data up to 24 h post administration. Values after oral dose were 385 ± 85 ml/min in the case of procainamide and

TABLE III

AREAS UNDER CURVES

Values are given in μg h/ml.

	Procainamide · HCl	N-Acetylprocainamide · HCl	Sum as procainamide · HCl
Intravenous	6.0 ± 0.9	4.8 ± 1.0	9.7 ± 1.4
Oral	4.3 ± 1.0	5.8 ± 1.4	9.9 ± 1.9

TABLE IV

HALF-LIVES

Values are given in hours.

	Procainamide · HCl		N-Acetylprocainamide · HCl	
	Serum	Urine	Serum	Urine
Intravenous	2.4 ± 0.4	2.4 ± 0.5	8 ± 2	8 ± 5
Oral	2.5 ± 0.3	2.3 ± 0.6	8 ± 2	10 ± 4

TABLE V

CUMULATIVE RENAL EXCRETION

Results* are expressed as percentage of dose administered.

	Procainamide · HCl	N-Acetylprocainamide · HCl	Sum
Intravenous dose			
$U^{24\ h}$	44 ± 4	11 ± 5	55 ± 8
U^{∞} (calc.)	44 ± 4	14 ± 9	58 ± 12
Oral dose			
$U^{24\ h}$	44 ± 10	11 ± 3	55 ± 11
U^{∞} (calc.)	44 ± 10	13 ± 4	57 ± 11

*U = urinary excreted drug.

TABLE VI

BIOAVAILABILITY OF PROCAINAMIDE FROM A 250-mg DRAGEE COMPARED WITH AN INTRAVENOUS DOSE

Ratio*	Drug (%)	Drug and metabolite (%)
$AUC^{\infty}_{p.o.}/AUC^{\infty}_{i.v.}$	81 ± 13	102 ± 11
$U^{\infty}_{p.o.}/U^{\infty}_{i.v.}$	100 ± 26	101 ± 26

*AUC = area under the curve, U = urinary excreted drug, i.v. = intravenous dose, p.o. = oral dose.

113 ± 36 ml/min in the case of N-acetylprocainamide corresponding to those after intravenous dose which were 316 ± 63 ml/min (drug renal clearance) and 161 ± 117 ml/min (metabolite renal clearance), respectively. Following oral and intravenous administration of 250 mg procainamide · HCl to five subjects, drug bioavailability was determined from individual ratios of serum areas under the curves and from cumulated renal excretions [27]. The average drug bioavailability was greater than 81%. Complete bioavailability can be calculated from both serum and urine when the data for the major equally potent metabolite are also considered (Table VI).

DISCUSSION

Analytical methods were developed for procainamide and N-acetylprocainamide in serum and also in urine. They proved to be practicable, selective, and sensitive enough to follow pharmacokinetics in serum and urine after a single intravenous or oral dose of 250 mg of procainamide · HCl to volunteers.

The HPLC method for serum of Su and Au [17] may be taken for comparison. They used 200-μl samples and, following back-extraction steps, could measure minimum serum levels of 300 ng/ml. For the TLC assay described here, even smaller samples of 100 μl suffice and, following one simple extraction step, improved detection limits of 60 ng/ml are obtained for both compounds. In general, from our experience, TLC may be used in most cases when low amounts of drug have to be determined. Since automatization of TLC [28] has become possible, even large series of samples can be processed with reasonable work and cost factors.

Pharmacokinetic data presented here corresponded well with values published previously. The drug volume of distribution (144 ± 20 l) and the distribution coefficient (2.1 ± 0.2 l/kg) agreed with the results of Galeazzi et al. (184 ± 24 l) [7] and of Koch-Weser and Klein (1.74—2.12 l/kg) [1], respectively. The drug half-life of 2.4 ± 0.4 h was in the range 2.5—4.7 h as reported by Koch-Weser and Klein [1]. Total clearance (711 ± 106 ml/min) was identical with data from Galeazzi et al. (851 ± 220 ml/min) [7] and Lima et al. (637 ± 215 ml/min) [8]. Drug renal clearance of 316 ± 63 ml/min was identical to the 341 ± 68 ml/min reported by Galeazzi et al. [7].

The metabolite distribution coefficient of 1.4 ± 0.1 l/kg was determined by Stec et al. [29] following single intravenous doses. The metabolite half-life of 8 ± 2 h from the present study agreed with that reported by Lima et al. (6 h) [8], Ludden et al. (7.5 h) [30] and Roden et al. (7.5 ± 2.2 h) [31]. The total clearance of 254 ± 15 ml/min from Stec et al. [29] was the same as that reported by Ludden et al. (283 ± 52 ml/min [30]. The renal clearance of 161 ± 117 ml/min from the present study agreed with that determined by Strong et al. (179 ± 40 ml/min) [6], Stec et al. (216 ± 25 ml/min) [29] and Ludden et al. (183 ± 50 ml/min) [30]. Deacetylation of N-acetylprocainamide was reported by Stec et al. [29] to be a minor route of elimination with a clearance of 6.5 ml/min ($\approx$ 2.8% of total clearance).

In the present study, within 24 h post administration, 44 ± 4% of the intravenous dose was accounted for as unchanged drug in urine compared with 11 ± 5% as metabolite. The same values, 43 ± 10% and 10 ± 5%, respectively, were observed by Galeazzi et al. [10] following a single intravenous dose of 500 mg of procainamide · HCl to four volunteers.

All subjects of the present study were slow acetylators [32].

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Journal of Chromatography, 277 (1983) 273—281
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1767

THIN-LAYER CHROMATOGRAPHIC DETERMINATION OF IMIPRAMINE AND DESIPRAMINE IN HUMAN PLASMA AND URINE AT SINGLE—DOSE LEVELS

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(First received August 4th, 1982; revised manuscript received May 6th, 1983)

SUMMARY

Thin-layer chromatographic methods were up-dated for pharmacokinetic studies of imipramine in plasma and urine. The free parent compound and its free desmethyl metabolite desipramine are determined in plasma. Conjugates of both compounds in urine are cleaved on treatment with glucuronidase/arylsulfatase. Following chromatography, intense yellow derivatives are obtained overnight on standing or by exposure to nitrous gases. Detection is performed in the visible range at 405 nm (plasma) or 460 nm (urine).

The methods are selective, accurate and sensitive, with detection limits for plasma of 2 ng/ml imipramine—HCl and 2 ng/ml desipramine—HCl, and 0.06 μg/ml total imipramine—HCl and 0.126 μg/ml total desipramine—HCl for urine. Pharmacokinetic data from plasma and urine results following single oral doses of 50 mg imipramine—HCl to eight volunteers were computed using one-compartment open models.

INTRODUCTION

Imipramine often serves as a standard reference substance in the research and development of new antidepressive drugs. Pharmacokinetic and metabolism data, predominantly in repeated-dose studies, have been reported in the literature [1—5]. By a first-pass effect, the potent desmethyl metabolite desipramine (Fig. 1) is formed to a larger extent following oral administration than after an intravenous dose [5—7]. The increased half-life leads to a higher degree of multiple dose cumulation as compared to imipramine.

In urine, approximately 70% of an oral dose is excreted [1—3], mostly as conjugated 2-hydroxy metabolites. Urinary levels of the free compounds imipramine and desipramine are in the range of the usual detection limits. On the other side, conjugates may be cleaved to allow for determination of total urinary imipramine and desipramine.

Specific single-dose pharmacokinetics could only be determined using gas

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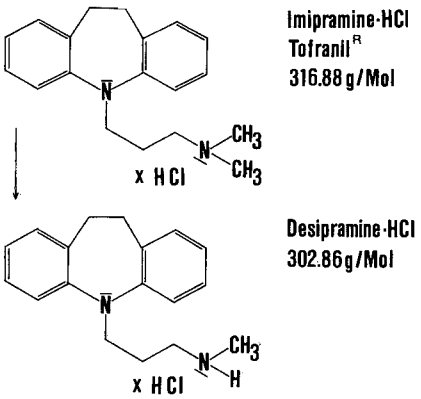

Fig. 1. Structures of imipramine—HCl and desipramine—HCl.

chromatography—mass spectrometry [8]. Using high-performance liquid chromatography (HPLC), Godbillon and Gauron [9] calculated detection limits of 5 ng/ml plasma for imipramine and 10 ng/ml plasma for desipramine, which were not sufficient to calculate single-dose pharmacokinetics. Also using HPLC, Reece et al. [10] reported detection limits of approximately 1 ng/ml for both compounds. Following a single oral dose of 50 mg of drug to one volunteer, plasma imipramine could be measured up to 28 h post administration, whereas only the peak level of 10 ng/ml desipramine (3.5 h post administration) could be quantified precisely (C.V. < 15%).

Using 5-ml plasma aliquots for thin-layer chromatography (TLC) [5], detection limits of 5 ng/ml for both compounds had been achieved. Up-dating the TLC methods improved the detection limits of imipramine and desipramine, allowing the determination of single-dose levels of both compounds in 1-ml samples of plasma and urine.

EXPERIMENTAL

Reagents

The reagents used were carbonate buffer, pH 10.9 (1 mol/l) AR, ethyl acetate AR, chloroform AR, methanol AR, concentrated ammonia solution (25%) AR, β-glucuronidase/arylsulfatase AR (Boehringer Mannheim, No. 15427) and acetate buffer, pH 5.5 (0.2 mol/l) AR. The solvent system was methanol—chloroform—concentrated ammonia (10:1:0.1).

Equipment

A Zeiss KM3 chromatogram spectrophotometer with microoptics and a Servogor® 210 (Metrawatt) recorder were used. Separation was performed on silica gel HPTLC plates without fluorescent indicator (No. 5641, E. Merck, Darmstadt, F.R.G.) in a Camag twin-trough HPTLC chamber 20 cm × 10 cm (No. 25254). For sample clean-up and spotting, a Vortex® mixer, a centrifuge, glass-stoppered tubes (ca. 8 ml), conical glass-stoppered tubes (ca. 8 ml) and a Desaga Autospotter® were used.

Sample preparation

Plasma. In a glass-stoppered tube, 1 ml of plasma was treated with 1 ml of carbonate buffer. The plasma was extracted with 5 ml of ethyl acetate for 30 sec on a Vortex mixer. The phases were separated by centrifugation (5 min), and 4 ml of the organic phase were transferred into a conical tube and evaporated to dryness at 40° C under a stream of nitrogen. The residue was dissolved in 100 µl of chloroform. Using the Desaga Autospotter*, 75 µl were transferred onto the HPTLC plate as a series of consecutive droplets of approximately 100 nl each. Since each of these drops evaporated before the next one was applied, narrow spots were obtained which were suitable for HPTLC.

Urine. In a glass-stoppered tube, 1 ml of urine was treated with 1 ml of acetate bufer and 20 µl of glucuronidase/arylsulfatase for 24 h at 37° C. The subsequent procedure was the same as described for plasma.

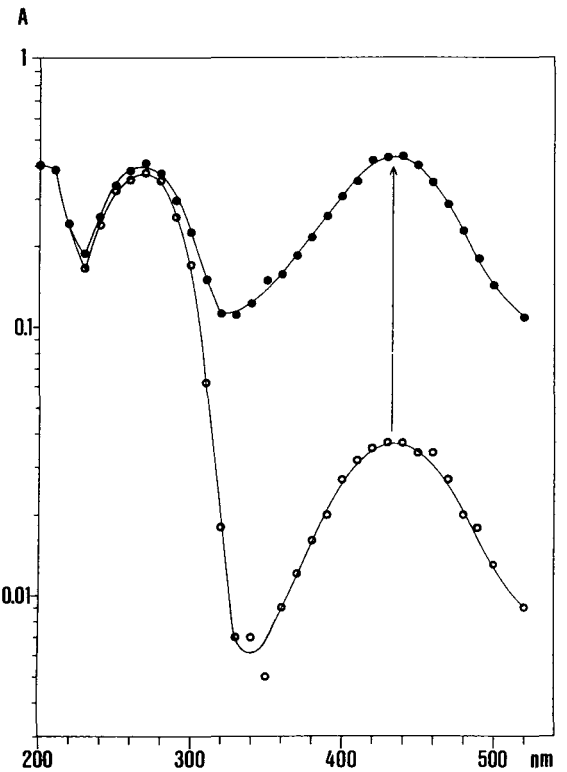

Fig. 2. In situ UV-VIS spectra of imipramine—HCl on a HPTLC plate, 0.7 µg/spot. (○), after chromatography; (●), overnight.

Chromatography

The twin-trough HPTLC developing chamber contained 11 ml of solvent in one compartment. After a 30-min preconditioning period, the plate was developed in the dark over a distance of 5 cm. Intense yellow derivatives (Fig. 2)

*Modified version: Tygon® tubes of larger diameter [Technicon, flow-rated, code 116-0549-09 (white)] and 60 cm long Hostaflon® tubes were used.

are formed on autoxidation overnight or, alternatively, by treatment with nitrous gases [5]. R_F values were imipramine 0.45 and desipramine 0.15.

Measurements were carried out in the direction of the solvent flow with an effective slit (microoptics) of 4.5 mm × 0.15 mm at a wavelength of 405 nm, in the case of plasma, scanning speed 50 mm/min and paper speed 240 mm/min. In the case of urine, measurements were performed at a wavelength of 460 nm in order to improve selectivity*. Peak heights (Figs. 3 and 4) were evaluated and quantified by means of a linear calibration graph based on parallel analysis of standards on the same plate.

RESULTS

Plasma

The compounds were admixed with blank plasma in five concentrations over the range 2—50 ng/ml plasma. Each admixture was split into six portions of 1 ml, so that six equal series were formed. Each series was then analyzed in turn, so that a total of six independent analytical results were available for each concentration.

TABLE I

IMIPRAMINE AND DESIPRAMINE DETERMINATION IN PLASMA BY TLC (RECOVERY, PRECISION AND ACCURACY)

n = 6 determinations, concentrations in ng/ml.

Imipramine—HCl			Desipramine—HCl		
Added	Found (mean ± S.D.)	Accuracy (ng/ml)	Added	Found (mean ± S.D.)	Accuracy (ng/ml)
50.0	50.0 ± 0.0	±0.0	50.0	49.8 ± 0.4	+0.2
20.0	19.7 ± 2.6	+0.3	20.0	20.5 ± 1.4	—0.5
10.0	9.8 ± 1.3	+0.2	10.0	9.8 ± 1.2	+0.2
5.0	5.2 ± 1.0	—0.2	5.0	4.7 ± 0.5	+0.3
2.0	2.0 ± 0.0	±0.0	2.0	1.7 ± 0.5	+0.3
Blank	0		Blank	0	

Quality criteria of an analytical method are selectivity, accuracy, precision and sensitivity [11, 12]. The corresponding parameters were derived from the analytical results given in Table I. As regards selectivity, the assay is free from interference for all substances (Fig. 3). Accuracy was considered to be the deviation (bias) of the mean value of the results from the amount added. For each substance, the average accuracy was approximately 0.1 ng/ml plasma. Regression coefficients were greater than 0.998.

Intra-assay precision was defined in terms of the standard deviation (S.D.), which was constant in the concentration range considered. The detection limit (D.L.) was taken as precision × 2: for imipramine—HCl, precision = 0.8 ±

*Coextracted matter from the enzyme mixture may cause minor interferences at λ = 405 nm, as we experienced.

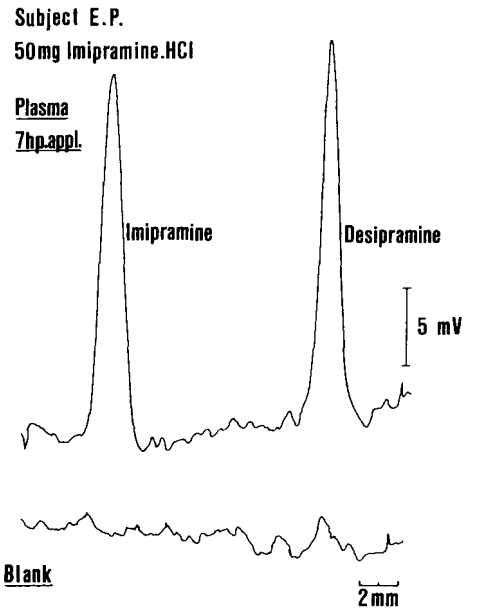

Fig. 3. Determination of imipramine—HCl (22 ng/ml) and desipramine—HCl (13 ng/ml) in plasma, compared to a blank.

1.0 ng/ml, D.L. = 2 ng/ml plasma; for desipramine—HCl, precision = 0.7 ± 0.5 ng/ml, D.L. = 2 ng/ml plasma.

The inter-assay precision was tested in two spiked control plasmas. From values obtained in May 1981, the following degrees of inter-assay precision were determined (ng/ml, mean ± S.D.): for control 1, imipramine—HCl = 21 ± 2, desipramine—HCl = 22 ± 3; for control 2, imipramine—HCl = 5 ± 1, desipramine—HCl = 5 ± 2.

Urine

The compounds were admixed to blank urine in five concentrations over the range 0.2—5 μg/ml urine. Each admixture was split into six portions of 1 ml, so that six equal series were formed. Each series was then analyzed in turn so that a total of six independent analytical results were available for each concentration. Quality criteria of the urine assay were defined by the corresponding parameters abstracted from the analytical results given in Table II. As regards selectivity, the assay is free from interference for both substances (Fig. 4).

For both imipramine and desipramine, the average accuracy was <0.03 μg/ml urine. Regression coefficients were greater than 0.998. The means of concentrations measured were linearly correlated to the S.D. values and in this way the intra-assay precision was defined.

The detection limit (D.L.) was taken as the intercept × 2: for imipramine—HCL, precision = 1.7% of the result + 0.03 μg/ml, D.L. = 0.06 μg/ml urine; for desipramine—HCl, precision = 1.2% of the result + 0.08 μg/ml, D.L. = 0.16 μg/ml urine.

The inter-assay precision was tested in two spiked control urines. From

TABLE II

IMIPRAMINE AND DESIPRAMINE DETERMINATION IN URINE BY TLC (RECOVERY, PRECISION AND ACCURACY)

n = 6 determinations, concentrations in μg/ml.

Imipramine—HCl			Desipramine—HCl		
Added	Found (mean ± S.D.)	Accuracy (μg/ml)	Added	Found (mean ± S.D.)	Accuracy (μg/ml)
5.00	5.00 ± 0.22	±0.00	5.00	5.10 ± 0.13	—0.10
2.00	2.00 ± 0.06	±0.00	2.00	2.00 ± 0.10	±0.00
1.00	0.98 ± 0.04	+0.02	1.00	1.03 ± 0.14	—0.03
0.50	0.53 ± 0.05	—0.03	0.50	0.50 ± 0.18	±0.00
0.20	0.20 ± 0.02	±0.00	0.20	0.22 ± 0.04	—0.02
Blank	0		Blank	0	

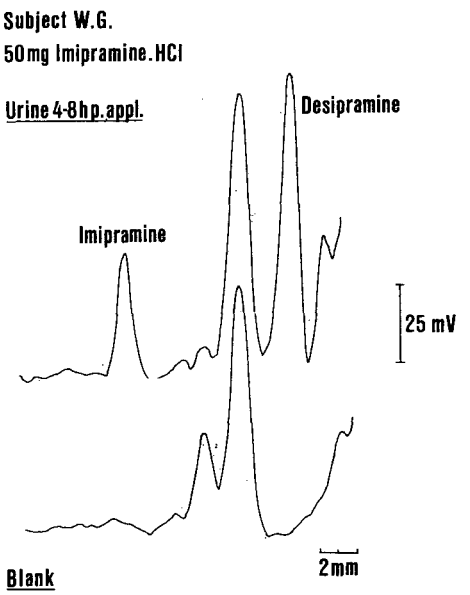

Fig. 4. Determination of total imipramine—HCl (0.7 μg/ml) and total desipramine—HCl (1.8 μg/ml) in urine, compared to a blank.

values obtained in May 1981, the following degrees of inter-assay precision were determined (μg/ml, mean ± S.D.): for control 1, imipramine—HCl = 2.0 ± 0.08, desipramine—HCl = 2.0 ± 0.12; for control 2, imipramine—HCl = 0.49 ± 0.08, desipramine—HCl = 0.51 ± 0.25.

Pharmacokinetics

Imipramine—HCl was administered in a single oral dose of 50 mg of Tofranil® to eight healthy male volunteers (age 21—42 years, height 172—190 cm, weight 63—83 kg). Blood was sampled up to 24 h and urine collected up to 48 h post administraton.

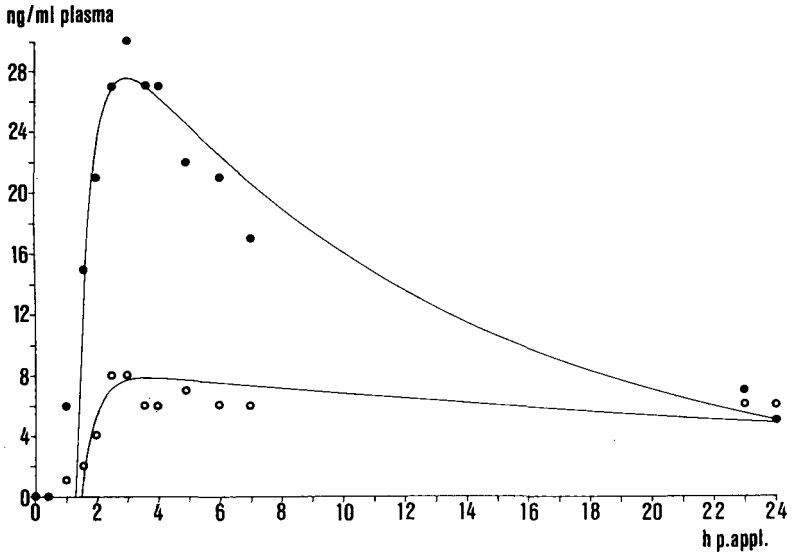

Fig. 5. Plasma pharmacokinetics from mean plasma levels of imipramine—HCl and desipramine—HCl after a single oral dose of 50 mg of imipramine—HCl to eight volunteers. (•), imipramine—HCl; (○), desipramine—HCl.

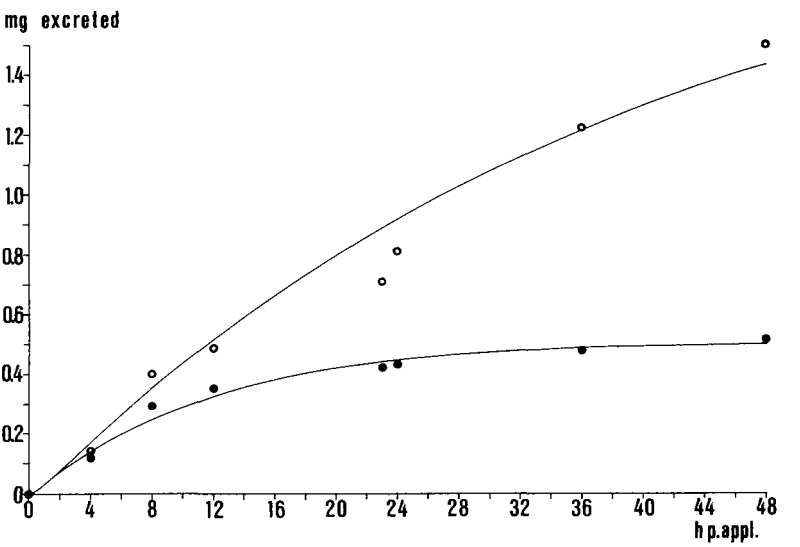

Fig. 6. Mean cumulative renal excretions of total imipramine—HCl and total desipramine—HCl after a single oral dose of 50 mg of imipramine—HCl to eight volunteers. (•) total imipramine—HCl; (○), total desipramine—HCl.

Using TLC, plasma levels were found in the ranges 0—80 ng/ml imipramine—HCl and 0—15 ng/ml desipramine—HCl, and urine levels were found in the ranges 0—1.0 μg/ml total imipramine—HCl and 0—3.0 μg/ml total desipramine—HCl.

Pharmacokinetic profiles were calculated based on one-compartment open

models. This is demonstrated by the plasma kinetics from the mean plasma levels in Fig. 5 and by the mean cumulative excretions shown in Fig. 6.

After a lag time of 1.3 ± 0.5 h, imipramine was absorbed with a half-life of 0.5 ± 0.3 h. Maximum plasma levels of 28 ± 17 ng/ml imipramine—HCl (range 10—83 ng/ml) were reached 3.4 ± 0.1 h post administration. The drug was eliminated with a half-life of 8.4 ± 1.1 h.

Desipramine plasma levels rose after a lag time of 1.5 ± 0.5 h with a half-life of 0.6 ± 0.3 h. Maximum levels of 8 ± 3 ng/ml desipramine—HCl (range 4—14 ng/ml) were reached 4.8 ± 1.5 h post administration. The metabolite was eliminated with a half-life of approximately 40 h.

Areas under the curves were: for imipramine—HCl 330 ± 184 ng h/ml (range 146—744 ng h/ml), for desipramine—HCl 123 ± 57 ng h/ml (range 58—208 ng h/ml). From these data, a steady-state level of approximately 60 ng/ml of both compounds may be expected for the oral doses of 3 × 50 mg imipramine—HCl per day. Renal clearance of both substances was calculated from data up to 24 h post administration as 24 ± 14 ml/min in the case of imipramine and 131 ± 63 ml/min in the case of desipramine.

From the mean cumulative excretions (Fig. 6), urinary half-lives were determined to be 8 h in the case of imipramine and 28 h in the case of desipramine. In Table III, the means of cumulative renal excretion 24 and 48 h post administration and the extrapolated values are given.

TABLE III

CUMULATIVE RENAL EXCRETION OF TOTAL IMIPRAMINE AND TOTAL DESIPRAMINE FOLLOWING A SINGLE ORAL DOSE OF 50 mg OF TOFRANIL® TO EIGHT VOLUNTEERS

	Total imipramine—HCl		Total desipramine—HCl		Sum of percentages of dose (mean ± S.D.)
	Amount excreted (mg, mean ± S.D.)	Percentage of dose (mean ± S.D.)	Amount excreted (mg, mean ± S.D.)	Percentage of dose (mean ± S.D.)	
24 h	0.4 ± 0.2	0.9 ± 0.4	0.8 ± 0.2	1.8 ± 0.4	2.6 ± 0.7
(range)	(0.2—0.9)		(0.6—1.2)		
48 h	0.5 ± 0.4	1.0 ± 0.8	1.5 ± 0.6	3.4 ± 1.4	4 ± 2
(range)	(0.2—1.4)		(0.8—2.7)		
Extrapolated values	0.5 ± 0.4	1.1 ± 0.8	2.0 ± 1.2	4 ± 3	5 ± 3
(range)	(0.2—1.5)		(0.8—4.2)		

DISCUSSION

The results were in accordance with values published previously. Following a single oral dose of 50 mg of imipramine—HCl to eight volunteers, peak levels of 28 ± 17 ng/ml plasma agreed with the results of Godbillon and Gauron [9] (18 ± 6 ng/ml). The drug plasma half-life of 8 h was in the range given by the same authors (approx. 6 h).

From single-dose data, the estimated steady-state plasma levels of approximately 60 ng/ml and approximately 60 ng/ml for drug and metabolite,

respectively, were in the ranges observed by Nagy and Treiber [5] (53 ± 30 ng/ml imipramine—HCl and 84 ± 49 ng/ml desipramine—HCl). Within 24 h post application, the cumulative renal excretion of 0.2—0.9 mg total imipramine—HCl and 0.6—1.2 mg total desipramine—HCl were in the ranges expected when considering those established by Crammer et al. [3] (0.6—0.7 mg total imipramine—HCl and 0.6—1.4 mg total desipramine—HCl).

Imipramine assay in plasma and urine was carried out to demonstrate quantitative TLC as a practicable, selective and sensitive analytical tool in drug/metabolite research. This technique may be used in most cases where low drug levels have to be determined. Since automatization of TLC [13] has become possible, even large series of samples can be processed with reasonable work and cost factors.

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Journal of Chromatography, 277 (1983) 282—286
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1772

Note

Comparison of high-performance liquid chromatographic, gas chromatographic—mass spectrometric, and fluorometric methods for the determination of homovanillic acid and 5-hydroxyindoleacetic acid in human cerebrospinal fluid

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(First received January 18th, 1983; revised manuscript received May 6th, 1983)

A number of analytical methods have been employed to determine 5-hydroxyindoleacetic acid (5HIAA) and homovanillic acid (HVA) in cerebrospinal fluid (CSF). These acid metabolites have been measured using fluorometry [1—9], gas chromatography—mass spectrometry (GC—MS) [10—13], and liquid chromatography with flow-through fluorometric [14—17], amperometric [18, 19] or combined fluorometric/amperometric [20] detectors. Relatively few of the procedures have been compared to alternative methods in a systematic fashion. The HPLC—fluorometric methods for 5HIAA have been compared to a GC—MS technique [17] and an amperometric method [20],

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and one of the fluorometric procedures for 5HIAA has been compared to a GC—MS procedure [21]. Two studies have compared the fluorometric determination of HVA to GC—MS assays [21, 22]. Here we present a four-way comparison of HPLC, fluorometric, and GC— MS methods for the determination of 5HIAA and HVA in human CSF.

METHODS

Lumbar CSF was obtained from subjects after administration of probenecid. Fluid from the first approx. 12 ml was mixed and 2—3-ml aliquots frozen without addition of preservative. Samples were stored at −80° C and analysed within two months.

HPLC

The CSF sample was briefly centrifuged (1 min, ca. 10,000 *g*) and 10—25 μl then injected directly into the HPLC system. The compounds of interest, along with tyrosine and tryptophan, were separated on a 10-μm average particle size 30 × 0.39 cm μBondapak C_{18} reversed-phase column. HVA was detected amperometrically while 5HIAA was measured with both amperometry and fluorometry [20].

GC—MS

One-ml samples were extracted with ethyl acetate after acidification and the addition of 100 ng/ml of dideuterated 5HIAA and HVA. The organic phase was removed and evaporated in a stream of nitrogen. The residue was then reacted with 100 μl of pentafluoropropanol—pentafluoropropionic anhydride (20:80). Following evaporation under nitrogen the residue was dissolved in 50 μl of hexane and 2—5 μl injected onto a ca. 2 m 3% OV-17 column. After separation and selected monitoring of ions derived from the proteo and dideutero (HVA-d_2 and 5HIAA-d_2) species, quantitation was performed by interpolation from a standard curve constructed from injection of known amounts of proteo and d_2 standards (0—500 ng d_0/100 ng d_2) [12]. The method was performed in two different laboratories (GC—MS-1 and GC—MS-2) using similar sample preparation procedures. Finnigan 3300 GC—MS instruments (electron impact mode) were used in both laboratories.

Fluorometric

The fluorometric (FLUOR) methods for 5HIAA [1, 6] and HVA [5, 10] both involve acidification, extraction into an organic solvent, and back-extraction. Fluorescence of 5HIAA is mesaured after addition of acid. HVA is determined fluorometrically after oxidation to a dimeric fluorophore using ferricyanide.

Samples were run in duplicate for all methods, except for the HPLC assay where the fluorometric and amperometric 5HIAA values were averaged. Samples were collected over a 14-month period and run with the normal workload of the laboratories.

RESULTS AND DISCUSSION

In Tables I and II the methods are compared, with the population means, correlation values and average percent differences being listed. Fig. 1a—f presents selected correlation diagrams obtained from the cross-comparisons.

TABLE I

STATISTICAL ANALYSIS OF CSF 5HIAA COMPARISON

Figure	Methods (y vs. x)	Average % difference	Mean y/x	r	Equation ($y = mx + b$)		n
					m	b	
1a	FLUOR vs. HPLC	16.5	106 /106	0.85	0.66	37	38
	GC—MS-1 vs. HPLC	18.2	97.6/ 92.4	0.74	0.75	29	14
	GC—MS-2 vs. HPLC	19.4	107 /100	0.65	0.67	40	13
	GC—MS-1 vs. FLUOR	17.7	97.6/100	0.71	0.70	27	14
	GC—MS-2 vs. FLUOR	16.3	107 /103	0.68	0.67	38	13
	GC—MS-1 vs. GC—MS-2	17.4	99.5/107	0.74	0.72	22	11
1b	HPLC FLUOR vs. EC	8.48	111 /115	0.95	0.94	10	33

TABLE II

STATISTICAL ANALYSIS OF CSF HVA COMPARISON

Figure	Methods (y vs. x)	Average % difference	Mean y/x	r	Equation ($y = mx + b$)		n
					m	b	
1c	FLUOR vs. HPLC	30.8	171/223	0.77	0.67	21	38
1d	GC—MS-2 vs. HPLC	11.1	232/223	0.94	1.04	0.50	37
	GC—MS-1 vs. HPLC	10.8	191/198	0.87	0.77	37.8	18
1e	GC—MS-1 vs. GC—MS-2	8.11	191/199.	0.96	0.94	3.6	18
	GC—MS-1 vs. FLUOR	22.8	191/166	0.72	0.66	81	18
1f	GC—MS-2 vs. FLUOR	34.8	232/171	0.69	0.88	82	38

For the determination of 5HIAA good agreement was observed between the population means (see Table I); however, the average percent differences of 16—19% and the low correlation coefficients (0.65—0.85) indicate only fair agreement across methods for individual samples. The small range of values for the samples (50—150 ng/ml) — and the small number of samples analysed by the GC—MS methods — account in part for the low r values. The high average percent difference between the two GC—MS methods (17.4%) and the high correlation (0.95) and low average absolute percent difference (8.48%) seen when the HPLC—amperometric and HPLC—fluorometric values were compared (Fig. 1b) suggest that the HPLC value is the most accurate estimation. This is further supported by the high correlation (0.99) of the HPLC—fluorometric and —amperometric methods for 5HIAA obtained in a previous comparison [20]. It should be pointed out this conclusion regarding the relative accuracies of the 5HIAA determinations is meant to apply to this study only.

For the analysis of HVA (Table II and Fig. 1c—f) the group means determined by fluorometry were significantly ($p < 0.01$) lower than those determined by HPLC (171 vs. 223), or the GC—MS methods (166 vs. 191 and 171 vs. 232). This underestimation of HVA has been previously reported [21, 22]

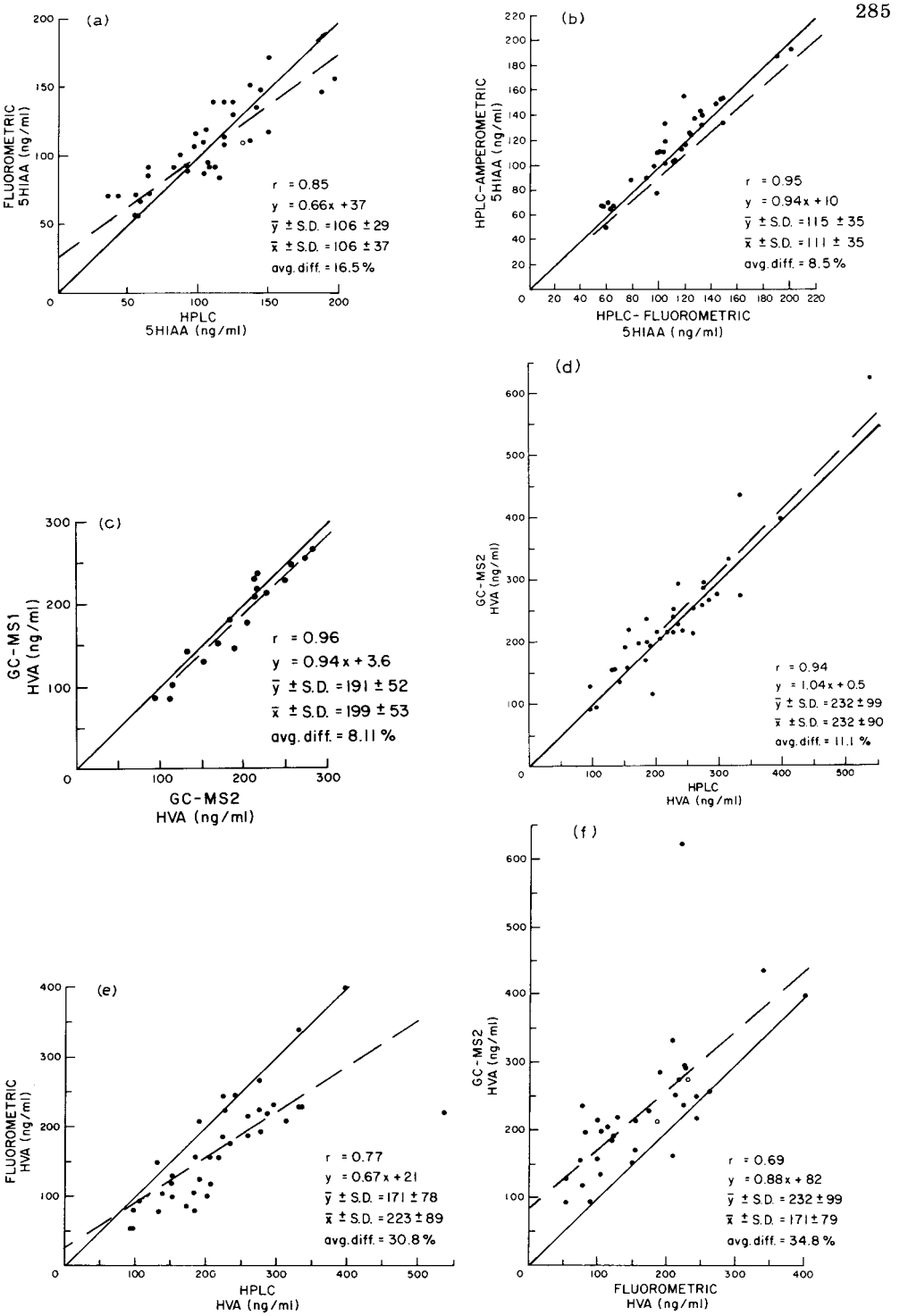

Fig. 1. (a–f) Scatter diagrams and regression statistics for method comparisons. See text for abbreviations and details of methods used. Points plotted as open circles comprise two samples. The regression line has been plotted as a broken line, while the solid line is the line of identity ($x = y$).

and has been suggested to be due to inefficient conversion of HVA to the dimeric fluorophore. The HPLC and GC—MS methods for HVA compared favorably to one another with population means differing by less than 5% and average absolute individual sample differences of 8—11%. While most of the 5HIAA and HVA levels were significantly elevated above the normal adult range (10—100 ng/ml) due to the administration of probenecid we believe the results of a comparison in an untreated population would be largely similar. However, the higher detection limits obtained for HVA and 5HIAA using the fluorometric (FLUOR) methods might compromise their use for less concentrated samples.

CONCLUSION

We have compared three different instrumental techniques for the determination of HVA and 5HIAA in human CSF. Good agreement between the HPLC and GC—MS methods was observed for HVA, as measured by correlation coefficients, population means and average individual sample differences. The fluorometric method for HVA gave significantly lower values and was less correlated. Population means for 5HIAA were similar, however agreement across methods was only fair for individual samples.

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Journal of Chromatography, 277 (1983) 287—291
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1782

Note

High-performance liquid chromatography of prostacyclin

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(First received February 8th, 1983; revised manuscript received May 6th, 1983)

The vascular endothelium synthesizes prostacyclin (PGI_2) from metabolism of arachidonic acid [1—4]. It is a highly active substance with strong inhibitory action on platelet aggregation through elevation of cyclic AMP, and at higher concentrations prevents platelet adhesion [5—7]. In addition, PGI_2 relaxes vascular smooth muscle and induces hypotension [8—10]. These effects have stimulated studies concerning the role of PGI_2 in maintaining homeostasis and vascular tone as well as investigation of its use as a therapeutic agent in cardiovascular disease and thrombotic disorders [11—13].

Prostacyclin is unstable in acidic or neutral aqueous solutions and rapidly hydrolyzes to an inactive compound, 6-keto-prostaglandin $F_{1\alpha}$ (6-K-$PGF_{1\alpha}$) [14, 15]. Under alkaline conditions, pH > 10, the rate of hydrolysis of the vinyl ether moiety of PGI_2 is substantially reduced. A reliable method for separation of PGI_2 and 6-K-$PGF_{1\alpha}$ under alkaline conditions would be valuable for assaying purity of standard solutions and determining PGI_2 levels for in vivo and in vitro pharmacology studies. High-performance liquid chromatography (HPLC) procedures have been reported for the separation of PGI_2 and 6-K-$PGF_{1\alpha}$ in standard solutions and biological extracts [16—19]. These methods utilize reversed-phase octadecyl silane columns with mildly alkaline, pH $\approx$ 9, solvent systems which compromise the stability of the PGI_2 and the life of the silica-based column packing. In this report a procedure is described for HPLC separation of PGI_2 and 6-K-$PGF_{1\alpha}$ on a reversed-phase styrene—

0378-4347/83/$03.00

divinylbenzene column with highly alkaline, pH > 11, solvent systems. Under these conditions there is no detectable hydrolysis of PGI_2, nor do the conditions cause deterioration of the column packing.

EXPERIMENTAL

Apparatus

A Varian Model 5020 liquid chromatograph (Varian, Palo Alto, CA, U.S.A.) was used for solvent delivery. Samples were injected with a manual 100-μl loop injector (Valco, Houston, TX, U.S.A.). Separations were performed on a 150 × 4.1 mm PRP-1 column (Hamilton, Reno, NV, U.S.A.) which contains a 10-μm particle size styrene—divinylbenzene copolymer. The effluent was continuously monitored with a Varichrome variable-wavelength detector (Varian).

Reagents

Acetonitrile and water were HPLC grade from Mallinckrodt (McGraw Park, IL, U.S.A.). Reagent grade sodium hydroxide and ammonium hydroxide (NH_3 29.8%) were also obtained from Mallinckrodt. Prostaglandins were a generous gift from Upjohn (Kalamazoo, MI, U.S.A.). The 6-keto-prostaglandin E_1, was a gift from Dr. P.K. Wong (New York Medical College, Valhalla, NY, U.S.A.). All solvents and reagents were used without further purification.

Procedure

Prostacyclin standard solutions were prepared in 0.01 *N* sodium hydroxide at a concentration of 1 mg/ml. Other prostaglandins were dissolved in acetonitrile or acetonitrile—water (90:10) at 1 mg/ml. Aliquots of standard solutions were evaporated to dryness and taken up in 0.01 *N* sodium hydroxide prior to analysis.

Chromatography of PGI_2 and 6-K-$PGF_{1\alpha}$ was studied with two alkaline mobile phases. Mobile phase I was a mixture of acetonitrile—0.01 *N* sodium hydroxide (21:79) with a measured pH of 12.3. Mobile phase II was a mixture of acetonitrile — 1% (v/v) ammonium hydroxide (17:83) with a pH of 11.0. A flow-rate of 1 ml/min was used for both mobile phases. Sample volumes of 20—100 μl were injected and the effluent continuously monitored at 206 nm. At the end of each day the column was washed with 40 ml of water followed by 20 ml of acetonitrile.

RESULTS AND DISCUSSION

Chromatography

Since the vinyl ether moiety of PGI_2 is subject to acid hydrolysis, chromatography should be performed with a highly alkaline solvent system to prevent hydrolysis. Bonded-phase silica HPLC columns degenerate rapidly when used with solvents with pH > 8 since alkaline solvents dissolve the silica and eventually strip the bonded phase from the packing. Styrene—divinylbenzene HPLC columns, however, can be used up to pH 13 without significant deterioration. The chromatography of PGI_2 and 6-K-$PGF_{1\alpha}$ was investigated on

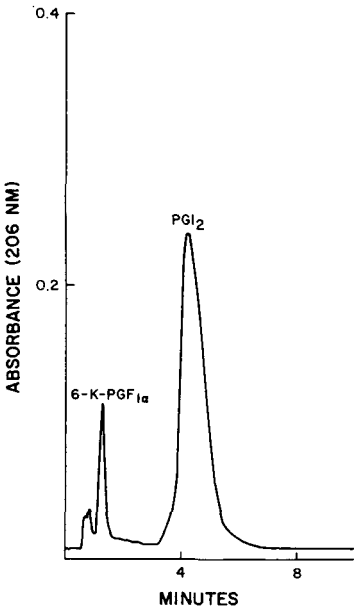

Fig. 1. HPLC of PGI_2 and 6-K-$PGF_{1\alpha}$ standards with mobile phase I.

TABLE I

CAPACITY FACTORS (k') FOR PROSTAGLANDINS

$k' = (V_r - V_0)/V_0$, where V_r is the elution volume and V_0 is the void volume.

Prostaglandin	Mobile phase I	Mobile phase II
PGI_2	6.4	8.5
6-K-$PGF_{1\alpha}$	1.6	1.3
6-K-PGE_1	1.6	1.3
PGA_2	8.1	9.5
PGB_2	10.9	11.3
PGD_2	3.7	4.2
PGE_2	3.0	3.5
$PGF_{2\alpha}$	2.4	3.5
TXB_2	2.0	2.7

a styrene–divinylbenzene reversed-phase HPLC column using two highly alkaline mobile phases. Fig. 1 is a chromatogram of 20 μg PGI_2 and 40 μg 6-K-$PGF_{1\alpha}$ injected in 20 μl of 0.01 N sodium hydroxide and eluted with mobile phase I. Chromatograms obtained with mobile phase II were essentially identical to those obtained with mobile phase I. The capacity factors, k', for PGI_2, 6-K-$PGF_{1\alpha}$ and other prostaglandins were determined by injecting 20 μg of each standard in 20 μl of 0.01 N sodium hydroxide and monitoring their elution at 206 nm. The values of k' for both mobile phases are listed in Table I.

Although mobile phase I had a significantly higher background absorbance than mobile phase II, the background was sufficiently low with both solvent systems to reliably monitor the effluent at 206 nm. Subsequent chromatography was run with mobile phase I primarily to avoid use of ammonium hydroxide because of its volatility.

Calibration

A standard calibration curve was prepared for PGI_2 by injection of 0.5—50 μg of standard in 100 μl of 0.01 *N* sodium hydroxide. The peak height response vs. amount injected was linear over the entire concentration range. The coefficient of variation for a 1-μg injection of PGI_2 was 7.3% (n = 7). Recovery from the chromatograph was determined by injecting 50 μg of PGI_2 and collecting the peak as it was eluted. The collected sample was diluted to 5.0 ml with 0.01 *N* sodium hydroxide and a 100-μl aliquot assayed by reinjection and quantitation using the calibration curve. The observed recovery was 99.1 ± 4.0% (n = 4). The quantitative recovery indicates that there is no detectable loss of PGI_2 due to either adsorption loss by the column or hydrolysis during the chromatography.

Incubation of [^{3}H]PGI_2 in plasma

Human and rabbit platelet poor plasma obtained from citrated blood were mixed with [^{3}H]PGI_2 (12 Ci/mmol, New England Nuclear, Boston, MA, U.S.A.), 4 ng/ml, and incubated at 37°C in a closed incubation system. Aliquots were withdrawn at 5-min intervals up to 25 min. The aliquots were immediately made alkaline with Na_2CO_3, 10 mg/ml, and the [^{3}H]PGI_2 extracted as previously described [16]. The extracted samples were run on the chromatograph to separate [^{3}H]PGI_2 from [^{3}H]6-K-$PGF_{1\alpha}$. Fig. 2 is a plot of the counts per min found for [^{3}H]PGI_2 vs. time of incubation with human and rabbit plasma. The data are normalized to the first sample withdrawn after mixing. The decrease in counts for [^{3}H]PGI_2 corresponded to an increase in counts for [^{3}H]6-K-$PGF_{1\alpha}$. The observed half-lives for [^{3}H]PGI_2 in human and rabbit plasma at 37°C were 11.2 min and 13.5 min, respectively.

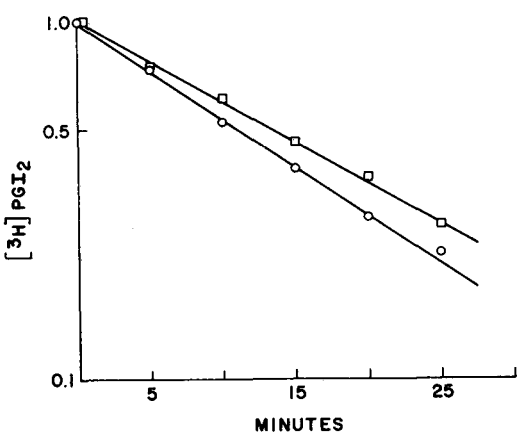

Fig. 2. Decrease in plasma concentration of [^{3}H]PGI_2 vs. time of incubation at 37°C with human (○) and rabbit (□) platelet poor plasma.

ACKNOWLEDGEMENT

This work is supported in part by Grant No. 79902 from The American Heart Association.

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Journal of Chromatography, 277 (1983) 292—299
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1794

Note

Quantitative analysis of pharmacological levels of vitamin K_1 and vitamin K_1 2,3-epoxide in rabbit plasma by high-performance liquid chromatography

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(First received February 15th, 1983, revised manuscript received May 6th, 1983)

Vitamin K_1 (2-methyl-3-phytyl-1,4-naphthoquinone) is a cofactor for the post-ribosomal γ-carboxylation of glutamyl residues in clotting factors II, VII, IX and X. Carboxylation of the glutamyl residues imparts calcium binding properties to these enzymes. During clotting factor precursor γ-carboxylation, vitamin K_1 is metabolised to vitamin K_1 2,3-epoxide [1—4]. The epoxide metabolite is reconverted back to vitamin K_1 by vitamin K_1 epoxide reductase. It has been proposed a cyclic interconversion of the vitamin and the epoxide, termed the vitamin K_1 K_1 epoxide cycle, is thought to have a role in preserving the body pool of the vitamin. Recent advances in vitamin K_1 biotransformations have been well reviewed [5, 6]. The interconversion of vitamin K_1 and its epoxide occurs in the liver and can be inhibited by coumarin anticoagulants such as warfarin [7, 8], acenocoumarin [9] and novel derivatives such as difenacoum [10].

Investigation of vitamin K_1, its metabolism and interaction with anticoagulants, has been carried out primarily in the rat [4, 11, 12] although recently the rabbit has been shown to be a good animal model with which to study the temporal changes and modifications of vitamin K_1 metabolism in vivo [13—15]. However, in these studies it was necessary to use radiolabelled vitamin K_1 and thin-layer chromatography (TLC) as a separative procedure to identify K_1 metabolites in biological fluids. This rather cumbersome method was used because of the lack of an alternative, reliable assay for vitamin K_1.

High-performance liquid chromatography (HPLC) has recently been developed to separate and quantify the various forms of vitamin K present in human biological fluids and green vegetables [16—19]. Extremely sensitive two-column HPLC systems have been developed by Lefevere and co-workers [19, 20] to measure physiological levels of vitamin K_1 in human serum with a

0378-4347/83/$03.00

sensitivity of 0.9 ng/ml. These methods involve fractionation of serum extracts using a silica adsorption column followed by reversed-phase separation of the appropriate reconstituted eluate on a C18 column. However, to continue our research we required a rapid, reproducible and reliable assay for vitamin K_1 and its metabolites in rabbit plasma after administration of a pharmacological dose of the vitamin. For this purpose it was only necessary to use a single-column HPLC separation because of the relatively high levels of vitamin K_1 in the plasma extracts and in order to reduce the assay time in view of the large number of samples measured. We report here both normal- and reversed-phase HPLC methods developed for this purpose.

EXPERIMENTAL

Apparatus

HPLC was carried out using the following components: an Altex 110A isocratic solvent delivery pump, an Altex 160 fixed-wavelength UV detector connected to a Gilson N1 potentiometric recorder. Glassware was first rinsed with 5% dimethyldichlorosilane in toluene and thereafter methanol-washed.

Reagents

Chemicals and reagents used were of analytical grade. All solvents used were HPLC grade (Rathburn Chemicals, Walkerburn, U.K.). Vitamin K_1 2,3-epoxide was synthesised by the method of Tishler et al. [21], the structure confirmed by UV absorbance between 200 and 400 nm and its purity verified by normal-phase HPLC. No residual vitamin K_1 was detected. Vitamin MK4 (2-methyl-3-farnesylfarnesyl-1,4-naphthoquinone) was a gift from Hoffman-La Roche (Basle, Switzerland). 2-Chloro-3-phytyl-1,4-naphthoquinone (Cl-K) was a gift from Sorex Laboratories (Widnes, U.K.).

Standard solutions

Solutions of 5, 50 and 500 μg/ml of vitamin K_1, its 2,3-epoxide, MK4 and Cl-K were prepared in hexane and stored protected from fluorescent light.

Blood sampling

Male New Zealand White rabbits (2.5—3.0 kg) were injected intravenously into the marginal ear vein with 1 mg/kg of phylloquinone (Konakion®) and blood was collected from the opposite ear at times 0.25, 0.5, 0.75, 1, 2, 3, 4, 5 and 6 h. Blood samples were centrifuged (2000 *g*) and plasma stored at −20°C until required for assay purposes.

Plasma extraction

An aliquot of the internal standard solution (either vitamin MK4 or Cl-K) was put into a 10-ml glass tube and blown dry under nitrogen. Plasma (1 ml) was then added, the tube vortexed for 30 sec and left at room temperature to equilibrate for 15 min. Following equilibration of the plasma an equal volume of methanol was added and the tube shaken mechanically for 2 min. Hexane (5 ml) was added and the tube shaken again for a further 5 min. To ensure complete separation of the methanol—water phase from hexane, the tube was centrifuged for 1 min at 500 *g*. The hexane layer was removed, blown dry

under nitrogen and redissolved in 100 μl of the appropriate eluent. A 20-μl volume of this solution was injected into the chromatograph.

Chromatographic conditions

Reversed phase. The mobile phase used was 30% dichloromethane in acetonitrile which was degassed by sonication prior to use; the flow-rate was 1 ml/min with a pressure of 35–100 bar, UV detection was effected at 254 nm at a sensitivity of up to 0.002 a.u.f.s.; quantification was by peak height comparison. Calibration graphs were calculated from the linear regression curves obtained from the ratio of the peak heights of 2-chlorovitamin K_1 to that of either vitamin K_1 or vitamin K_1 2,3-epoxide at concentrations over the pharmacological range observed in rabbit plasma. A reversed-phase column was used for the separation (Ultrasphere ODS C18 with 5 μm particle diameter, 25 cm × 4.5 mm I.D., Altex, Berkeley, CA, U.S.A.) protected by a guard column (2.5 cm × 4.5 mm I.D.) packed with Ultrasphere ODS C18, particle size 5 μm. Column efficiency was ⩾ 55,000 plates/m for the test compounds including the internal standard.

Normal phase. The mobile phase used in this system was 0.2% acetonitrile in hexane pumped at 2 ml/min (35—100 bar). UV detection was effected at 254 nm with a sensitivity of up to 0.002 a.u.f.s. Calibration curves were calculated as for the reversed-phase system but using vitamin MK4 as internal standard. The normal-phase system used a Partisil-10 column (25 cm × 4.5 mm I.D., 10 μm particle diameter, Whatman, Maidstone, U.K.) protected by a guard column (2.5 cm × 4.5 mm I.D.) packed with Partisil-10 silica gel. This guard column was repacked at 3-monthly intervals when column efficiency was seen to decline. Normal-phase column efficiency was typically greater than 20,000 plates/m for all test compounds including the internal standard. Both normal- and reversed-phase separations were run at ambient temperature.

RESULTS

Reversed phase

Fig. 1a shows a typical chromatographic separation of vitamin K_1, its 2,3-epoxide and Cl-K (internal standard).

Retention times were 8.2 min for vitamin K_1 2,3-epoxide, 10.5 min for Cl-K and 11.1 min for vitamin K_1 giving a run time of 15 min. Fig. 1b shows a rabbit extract taken 2 h after 1 mg/kg phylloquinone was administered. There was slight interference of the vitamin K_1 2,3-epoxide peak with background UV absorbing material. The calculated recoveries from plasma were 94.7 ± 4.3% for vitamin K_1, 91.7 ± 3.6% for vitamin K_1 2,3-epoxide and 94.1 ± 5.1% for Cl-K at a plasma concentration of 0.5 μg/ml. Plasma analysis was in the range of 0.1—10 μg/ml with a sensitivty of 100 ng/ml.

Linear regression lines obtained from the standard graphs were $y = 1.8739x + 0.0046$, $r = 0.993$ for vitamin K_1 and $y = 0.6543x + 0.0738$, $r = 0.997$ for vitamin K_1 2,3-epoxide. Intra-assay variation was calculated by the repeated chromatographing of a single-spiked plasma sample and gave a coefficient of variation of 3.9%. The coefficient of variation for the calculated slopes of the standard graphs was 7.7% ($n = 6$).

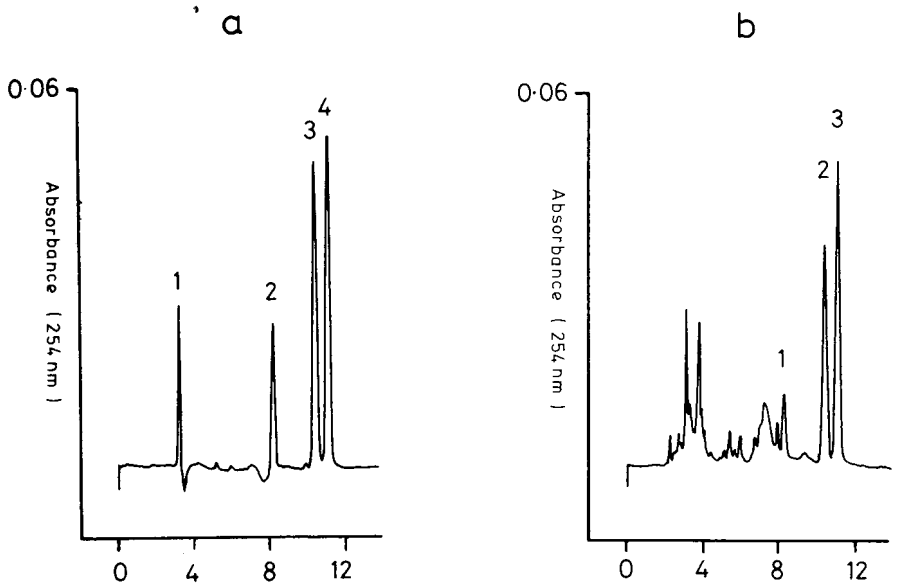

Fig. 1. Reversed-phase high-performance liquid chromatograms. (a) Separation of 300 ng vitamin K_1 (4), vitamin K_1 2,3-epoxide (2) and Cl-K (3); (1) is the solvent front. (b) Extract of 1 ml rabbit plasma taken 2 h following intravenous administration of phylloquinone (1 mg/kg). Peaks: 1 = vitamin K_1 2,3-epoxide; 2 = internal standard, Cl-K (1 μg/ml); 3 = vitamin K_1.

Normal phase

Fig. 2A shows a typical chromatographic separation of vitamin K_1, its 2,3-epoxide and MK4. Vitamin K_1 exists in the *cis* and *trans* isomeric forms at the 2′,3′ position of the phytyl side-chain. It can be seen that normal-phase HPLC clearly separates these geometric isomers of vitamin K_1 with retention times of 6.9 min for *cis*-vitamin K_1 and 7.8 min for *trans*-vitamin K_1. The retention time for vitamin K_1 2,3-epoxide was 8.7 min and for vitamin MK4, 11 min. Fig. 2B shows a chromatogram of rabbit plasma taken 4 h following intravenous administration of 1 mg/kg phylloquinone. There was no interference from UV absorbing material in rabbit plasma at sensitivities below 0.005 a.u.f.s. Plasma recoveries were greater than 90% for all standards at a concentration of 0.2 μg/ml and plasma analysis was in the range 0.02—10 μg/ml giving a sensitivity of 20 ng/ml. Linear regression lines were obtained from the standard graphs and were $y = 0.3597x + 0.0101$, $r = 0.994$ for *cis*-vitamin K_1, $y = 0.8939x + 0.0185$, $r = 0.993$ for *trans*-vitamin K_1 and $y = 0.4180x + 0.0284$, $r = 0.995$ for vitamin K_1 2,3-epoxide. Intra-assay variation, calculated from repeated sampling of a single-spiked plasma sample, gave a coefficient of variation of 3.6%. The coefficient of variation of the slopes of the standard graphs, calculated over a two-month period, was 7.0%.

Fig. 3 shows levels of vitamin K_1 in rabbits given 1 mg/kg phylloquinone intravenously. Plasma concentration—time curves were obtained for comparison using both reversed-phase and normal-phase HPLC. The plasma profiles are similar to those obtained using radiolabelled phylloquinone [15].

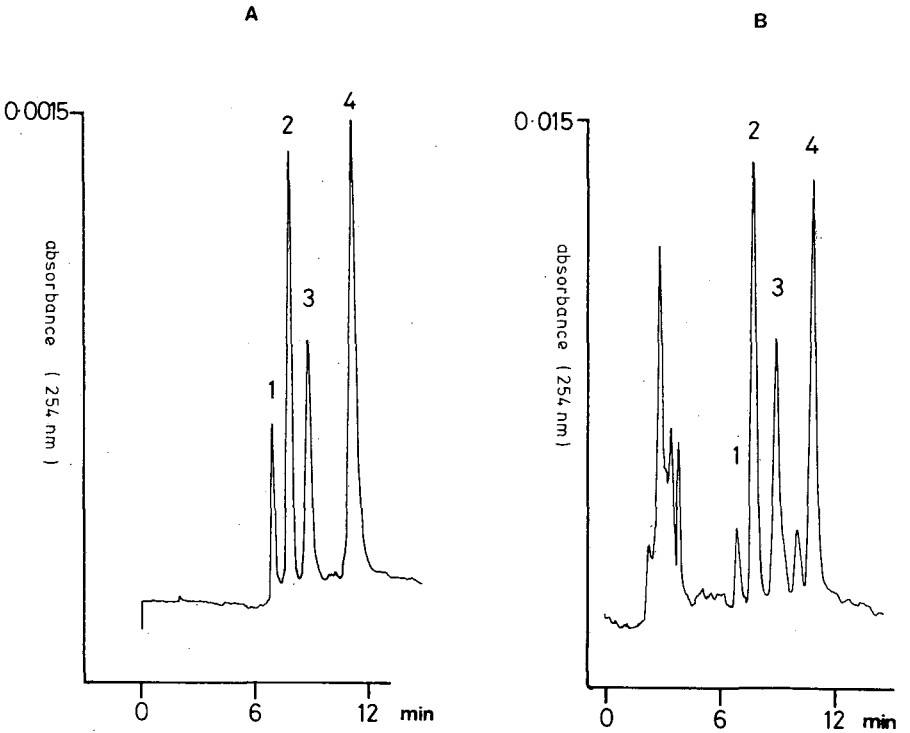

Fig. 2. Normal-phase high-performance liquid chromatograms. (A) 23 ng Vitamin K_1 (separation of the *cis* and *trans* isomers), vitamin K_1 2,3-epoxide and vitamin MK4. (B) Extract of 1 ml rabbit plasma taken 4 h after intravenous administration of phylloquinone (1 mg/kg). Peaks: 1 = *cis*-vitamin K_1; 2 = *trans*-vitamin K_1; 3 = vitamin K_1 2,3-epoxide; 4 = vitamin MK4 (internal standard added to a concentration of 0.5 μg/ml).

Commercial vitamin K_1 contains a mixture of the *cis* and *trans* geometric isomers that range from 12% *cis*-vitamin K_1 in Konakion (Hoffmann-La Roche, Nutley, NJ, U.S.A.) to 28% in phylloquinone (Sigma, St. Louis, MO, U.S.A.). Photoisomerisation of the *trans* to *cis* form of vitamin K_1 occurs especially in fluorescent light. A sample of chromatographically pure *trans*-vitamin K_1 underwent 20% isomerisation when exposed to fluorescent light for 40 h. As a precautionary measure plasma samples and solvent extracts were protected from light at all times by wrapping the tubes in aluminium foil.

DISCUSSION

The two HPLC assays reported here, the normal- and reversed-phase systems, both show good separation of vitamin K_1, its 2,3-epoxide metabolite and the internal standards used. The normal-phase HPLC system, however, is superior to the reversed-phase one because of its ability to separate the *cis* and *trans* isomers of vitamin K_1.

Cis-vitamin K_1 has far less biological activity than *trans*-vitamin K_1 in stimulating prothrombin synthesis and vitamin K_1 2,3-epoxide formation [22].

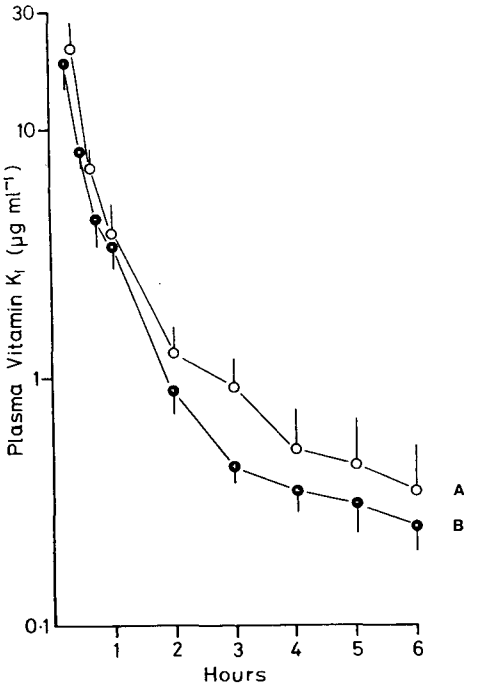

Fig. 3. Plasma concentration—time curves for vitamin K_1 in rabbit plasma following intravenous administration of phylloquinone (1 mg/kg). All vitamin K_1 levels were obtained using (A) reversed-phase and (B) normal-phase HPLC. Plasma levels in graph B refer to the sum of the *cis* and *trans* isomer of vitamin K_1. Values are given as mean ± S.D. (n = 4).

Also, following injection of a mixture of the *cis* and *trans* isomers, the disappearance of *cis*-vitamin K_1 from all subcellular fractions of rat liver is slower than that of the *trans* isomer [23]. Hence, for our research in the pharmacokinetics of vitamin K_1 and perturbations caused by anticoagulants, we require to measure and monitor the plasma decline of the active *trans* form of the vitamin following intravenous administration of the commercial preparations of vitamin K_1.

A previous normal-phase HPLC assay developed to separate and quantify endogenous physiological amounts of the geometric isomers of vitamin K_1 required a two-column system [19]. This assay, although very sensitive in its detection limit (500 pg/ml plasma), requires a complicated analytical procedure. The normal-phase system reported here, for the analysis of vitamin K_1 in rabbit plasma following pharmacological doses of the vitamin, is a simple single extraction, single-column chromatographic procedure which is rapid and affords high sensitivity. Interference with background UV absorbing material co-extracted from rabbit plasma is minimal and the normal-phase HPLC method quantitates 3 ng *trans*-vitamin K_1 injected on column. This system can be used for the rapid and routine analysis of plasma from the rabbit; an ideal animal model for the study of temporal changes to vitamin K_1 metabolism in vivo.

CONCLUSION

The determination of vitamin K_1 and vitamin K_1 2,3-epoxide, after single hexane extraction, was effected through isocratic HPLC. Both reversed-phase and normal-phase systems were used, the former using a C18 column as stationary phase with dichloromethane and acetonitrile as eluent and the latter a silica column with acetonitrile—hexane as the mobile phase. UV detection was at 254 nm; chlorovitamin K was used as internal standard for reversed-phase HPLC while the normal-phase system used vitamin K_2(MK4).

Normal-phase HPLC was found to be superior to the reversed-phase system with respect to the lack of interference from UV absorbing material co-extracted with vitamin K_1 and its 2,3-epoxide from rabbit plasma. The methods were used in the range 0.02—10 μg/ml, the sensitivity was 20 ng/ml for the normal phase system and the precision of the assay was in the range 3—7%. Plasma levels were measured using both HPLC systems as a comparison in rabbits after a pharmacological dose of vitamin K_1.

ACKNOWLEDGEMENTS

The work was supported by grants from the British Heart Foundation and the Mersey Regional Health Authority. We would like to thank Professor A.M. Breckenridge for his helpful interest in this work.

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Journal of Chromatography, 277 (1983) 300—304.
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1814

Note

Determination of 2,8-dihydroxyadenine in urine by high-performance anion-exchange liquid chromatography

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(First received March 8th, 1983; revised manuscript received June 3rd, 1983)

A renal calculus of 2,8-dihydroxyadenine (DOA) is a rare disease and studies are limited [1, 2]. Various analytical techniques such as UV spectroscopy and fluorometry were used to characterize DOA and distinguish it from other purine metabolites [3, 4]. Recently, Ericson et al. [5] reported the measurement of renal excretion of adenine and DOA in plasma, after administration of adenine, using a cation-exchange column. To determine the DOA in urine, the photometric method, primary purification and concentration procedure are comparatively complicated, not so accurate and time-consuming. We have developed a simple and efficient method for measuring DOA in urine using high-performance anion-exchange liquid chromatography (HPLC). This method was applied in a case of DOA stones with a partial deficiency of adenine phosphoribosyltransferase (APRTase) after renal transplantation. The HPLC technique is herewith described and the results in a rare case of DOA stones are compared with those of some normal subjects.

EXPERIMENTAL

Chemicals

DOA and adenine were obtained from Sigma (St. Louis, MO, U.S.A.). Ammonium acetate and acetic acid were purchased from Wako Pure Chemicals (Osaka, Japan).

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High-performance anion-exchange column chromatography

A high-pressure piston pump (Mitsumi Scientific Industry Co., Tokyo, Japan) served to pump the column eluent through a six-port sample injection valve with a 100-μl sample loop. A jacketed, stainless-steel column, 25 × 0.46 cm I.D., was used, and prepacked with Diaion CDR-10 anion-exchange resin with a mean particle size of 7 μm (Mitsubishi Kasei Co., Tokyo, Japan) [6]. The column temperature was kept at 60°C with a Haake constant-temperature circulator (Haake Co., Berlin, F.R.G.). All buffer solutions were delivered by an automated buffer exchanger (Hijiri Seiko Co., Tokyo, Japan). A JASCO UVIDEC-100 UV spectrophotometer (Japan Spectroscopic Co., Tokyo, Japan) was used at 305 nm. The UV signal was recorded using a JASCO RC-100 recorder (Japan Spectroscopic Co.) with a chart speed of 30 cm/h. Peak areas were determined with a digital integrator (Chromatopac C-E1B, Shimadzu Seisakusho Co., Kyoto, Japan).

Eluent and reagent solutions

All reagents were analytical grade, made up in double glass-distilled water. All aqueous reagents were filtered through a 0.45-μm microfilter (Fuji Photo Film Co., Tokyo, Japan), and degassed. Standard DOA solutions were made in 0.5 *M* hydrochloric acid at 5—20 μg/ml. A stepwise gradient for each 0.6 *M* rise in 6 *M* ammonium acetate buffer (pH 4.4) was used; the initial eluate was 0.006 *M* ammonium acetate buffer, and the final one was 6 *M* ammonium acetate buffer. Each buffer was pumped for 6 min through the column, which was operated at a flow-rate of 30 ml/h.

Sample preparation

Urine samples (24 h) from normal persons and patients were collected, acidified to pH 1—2 with hydrochloric acid and stored at —20°C until analysis.

RESULTS AND DISCUSSION

DOA is an adenine metabolite. Normally adenine is converted into adenylic acid (AMP) by APRTase and can again be used in the nucleotide metabolic cycle [7]. In the case of low activity or deficiency of APRTase, adenine accumulates, and excess adenine is changed to DOA by xanthine oxidase and excreted into the urine. But because of its poor solubility [4] a high level of DOA in the urine is known to cause calculi.

In this study, high-performance anion-exchange liquid chromatography proved to be useful for the determination of DOA in urine. The macroreticular anion-exchange resin column was used for the separation of urinary components [6]. The UV absorption spectra of DOA and adenine showed maxima at 305 nm and 260 nm, respectively (Fig. 1). The wavelength selected for the detector was 305 nm, corresponding to maximum UV absorption for DOA. This condition indicated that there was no detectable amount of adenine.

A chromatogram of normal urine spiked with DOA at a concentration of 1.5 μg per 100 μl, is presented in Fig. 2. The retention time of DOA was 16.4 ± 0.3 min (n=10). The calibration curve was plotted in the concentration range 5—20 μg/ml and found to be linear with a correlation coefficient of

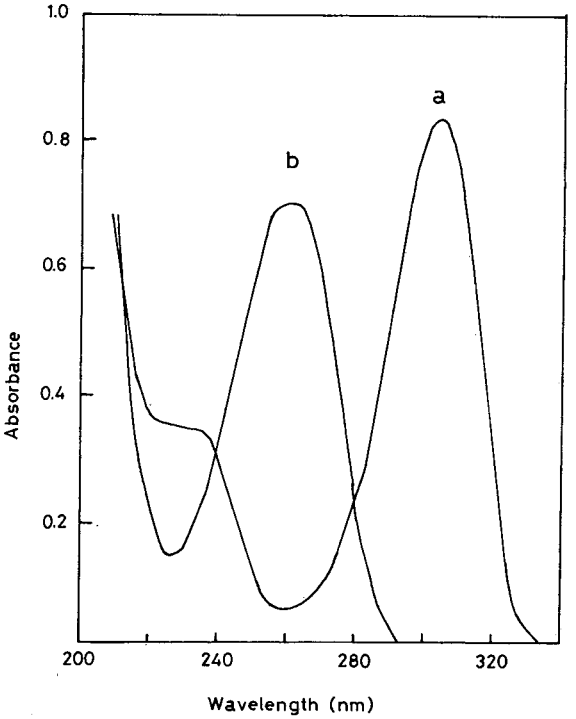

Fig. 1. The UV absorption spectra of DOA and adenine at a concentration of 10 μg/ml in 0.5 *M* HCl. Peaks: a = DOA, b = adenine.

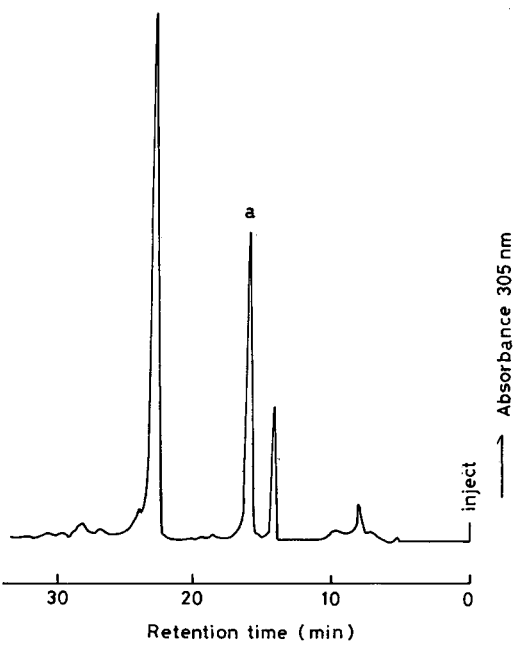

Fig. 2. Chromatogram of normal urine spiked with DOA, at a concentration of 1.5 μg per 100 μl. Chromatographic conditions: stationary phase, CDR-10 (average particle size, 7 μm), prepacked in a 25 × 0.46 cm I.D. stainless-steel column; mobile phase, 0.006—6 *M* ammonium acetate buffer (pH 4.4) with a stepwise gradient; flow-rate, 30 ml/h; detector, UV 305 nm; recorder chart speed, 12 cm/h. Peak: a = DOA.

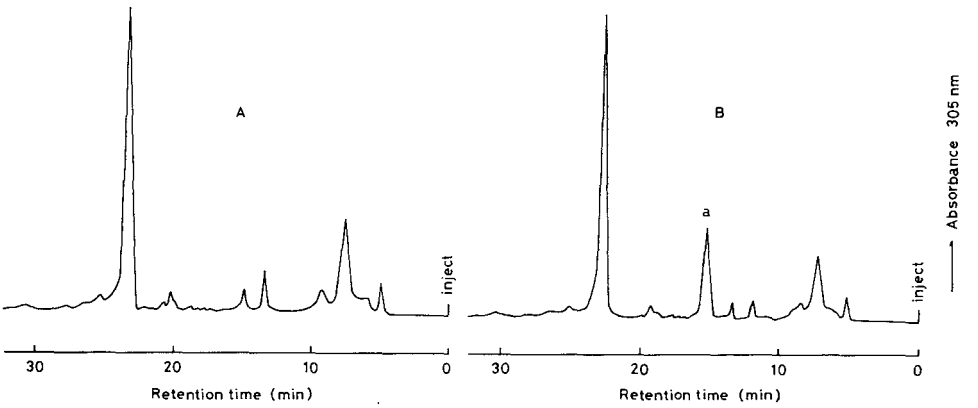

Fig. 3. Chromatograms of urine samples. The conditions are described under Experimental procedures. (A) A 100-μl aliquot of a normal urine was injected. (B) Urine of patient with a partial deficiency of APRTase after renal transplantation. Peak: a = DOA. The amount of DOA present = 14.3 μg/ml.

TABLE I

URINARY EXCRETION OF 2,8-DIHYDROXYADENINE

Sample*	Urinary excretion (mg/day)
1	40.6 ± 8.6** (mean ± S.D.)
2	N.D.***
3	N.D.

*1 = Patient with a partial deficiency of APRTase after renal transplantation; 2 = patient with normal APRTase after renal transplantation (n = 3); 3 = normal persons (n = 5).
**Urine of the same patient during 46 days (n=15).
***N.D. = not detected.

0.998. Chromatograms of urines of control and patient are shown in Fig. 3A and B, respectively.

Table I compares DOA levels in the urine of the patient with a partial deficiency of APRTase with that of a healthy person. DOA was not detected in the urine of either normal persons or patients with normal APRTase activity after renal transplantation. Excretion of DOA in the patient was observed to be 30—65 mg/day during 46 days immediately after renal transplantation. Since it was reported that the xanthine oxidase inhibitor allopurinol was effective for such therapeutic DOA [8], the present results suggest the possibility of recurrence of renal calculus and the necessity of prescribing xanthine oxidase inhibitor to prevent it.

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Journal of Chromatography, 277 (1983) 305—307
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1796

Note

Isocratic reversed-phase high-performance liquid chromatographic separation of deoxyribonucleosides and ribonucleosides

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(Received May 6th, 1983)

There is increasing evidence that the pattern of methylation in DNA bases influences gene expression in vertebrate cells. In addition, the methylation of DNA which occurs in animals treated with carcinogens is suspect in tumor formation. Since the methylated components could serve as an indicator of DNA function or damage, much research has focused on the determination of both major and minor fragments [1].

Many different analytical techniques have been used to determine the components of DNA. Reversed-phase high-performance liquid chromatography (RPLC) is found to be ideally suited for separation and quantitation of DNA fragments in its various forms [2—5]. However, the methods developed thus far have several disadvantages: (1) the time required for the separation of major and minor compounds is too long for routine application; (2) isocratic separation of these compounds from RNA fragments have been difficult to achieve; (3) it is difficult to assay DNA components in the presence of RNA components.

Therefore, we developed an isocratic RPLC separation of some important major and minor deoxyribonucleosides and ribonucleosides. The method is sensitive and selective for the determination of 5-methyldeoxycytidine (m_5dC),

0378-4347/83/$03.00

7-methyldeoxyguanosine (m_7dG), and 6-methyldeoxyadenosine (m_6dA) along with their ribonucleosides and other nucleic acid components.

EXPERIMENTAL

Apparatus

The chromatographic system was a Du Pont 8820 (Du Pont Instruments, Wilmington, DE, U.S.A.) which included a controlled-temperature column compartment, 254-nm fixed-wavelength detector, HP 3380A integrator (Hewlett-Packard, Avondale, PA, U.S.A.) and a strip-chart recorder (Houston Instruments, Austin, TX, U.S.A.). Separations were achieved with a Zorbax-TMS (Du Pont) column (250 × 4.6 mm I.D.) packed with 6-μm trimethylsilica.

Chromatographic conditions

Eluents were prepared with 2.0% methanol (glass-distilled, Burdick and Jackson Labs., Muskegon, MI, U.S.A.) in 4.0 m*M* $(NH_4)_2HPO_4$ and 4.0 m*M* $NH_4H_2PO_4$, pH 4.0 (v/v); the phosphate salts were the highest grade available from Fisher Scientific (Fair Lawn, NJ, U.S.A.). The flow-rate was 2.0 ml/min and the column temperature was 35°C.

Standards

The nucleic acid components were obtained from Vega Biochemicals (Tucson, AZ, U.S.A.) and Sigma (St. Louis, MO, U.S.A.); standard solutions were prepared in the mobile phase at concentrations of approximately 100 μM.

RESULTS AND DISCUSSION

Although the separation of deoxyribonucleosides and ribonucleosides has been reported using gradient elution with octadecylsilica reversed-phase columns [4, 5], we were able to use isocratic elution to achieve excellent resolution of the major deoxyribonucleosides and ribonucleosides together with some methylated analogues. The analysis of the majority of the nucleosides can be obtained in less than 14 min (Fig. 1). If, however, it is also important to determine the concentrations of the 6-methyl analogues of adenosine and the dimethyl analogues of guanosine, the analysis then requires approximately 40 min. In comparison to the previously published methods, the relatively short analysis time of our method is attributed to the use of the trimethylsilica reversed-phase packings. In addition, because we used isocratic elution, the total analysis time is even more rapid because the column need not be flushed and equilibrated between injections. In addition, detection limits are lower (ca. 5–15 pmol) with isocratic elution since there is no baseline drift.

Although the deoxy standards were clearly separated, interferences from contaminating RNA in biological samples can hamper the determination of concentrations of hydrolysates of DNA. Using the procedure described by Kuo et al. [4] to prepare DNA samples prior to chromatography, the resulting DNA/RNA enzymic hydrolysates were analyzed under our conditions. The uridine fractions from RNA were found to elute between the cytidine (C) and deoxycytidine (dC) peaks (Fig. 1). Some minor components present in

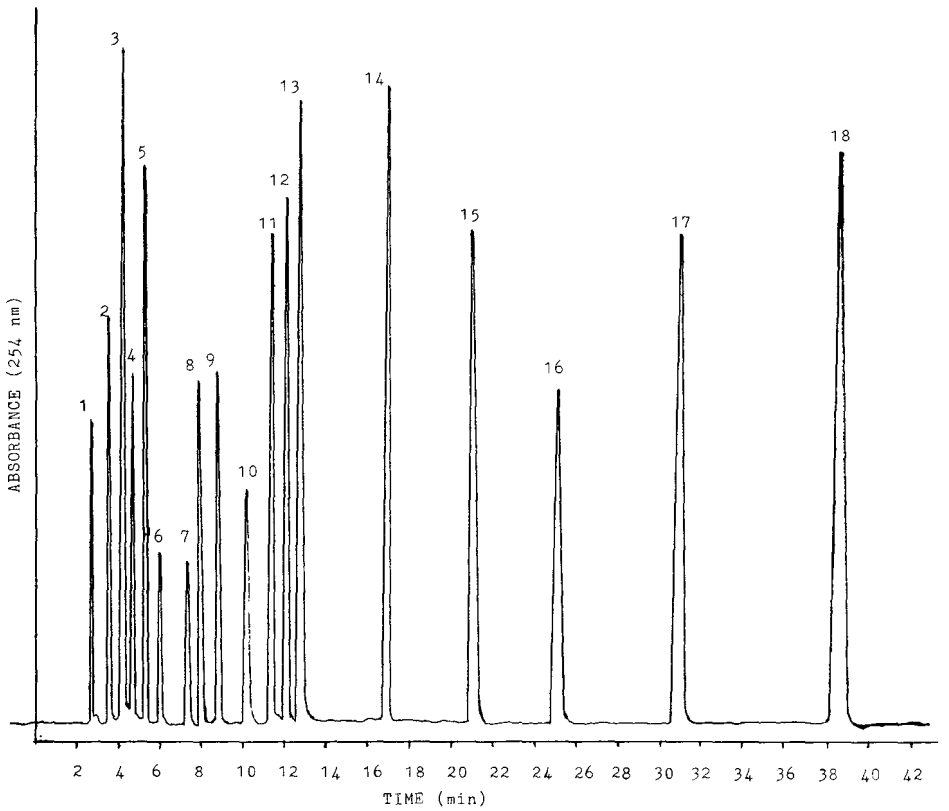

Fig. 1. Isocratic separation of major and minor deoxyribonucleosides and ribonucleosides. Conditions are given in the text. Peaks: 1 = C, cytidine; 2 = dC, deoxycytidine; 3 = I, inosine; 4 = G, guanosine; 5 = m_5dC, 5-methyldeoxycytidine; 6 = dI, deoxyinosine; 7 = dG, deoxyguanosine; 8 = m_7G, 7-methylguanosine; 9 = dT, thymidine; 10 = A, adenosine; 11 = m_1G, 1-methylguanosine; 12 = m_7dG, 7-methyldeoxyguanosine; 13 = dA, deoxyadenosine; 14 = m_1dG, 1-methyldeoxyguanosine; 15 = m_2^2G, 2,2-dimethylguanosine; 16 = m_6A, 6-methyladenosine; 17 = m_2^2dG, 2,2-dimethyldeoxyguanosine; 18 = m_6dA, 6-methyldeoxyadenosine.

RNA digests, such as 1-methylinosine, 2-methylguanosine and 1-methyladenosine, which interfere only with the resolution of ribonucleosides, do not interfere with the resolution of the deoxyribonucleosides. Therefore, in order to use this system for the assay of hydrolysates of RNA, conditions must be modified to obtain optimal resolution of the hydrolysates of interest. However, for routine assays of digests of DNA, the rapid and efficient isocratic separation as illustrated in Fig. 1 can be very useful in studies of DNA methylation and function.

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Journal of Chromatography, 277 (1983) 308—313
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1780

Note

Separation of bilirubin isomers and their conjugates by high-performance reversed-phase liquid chromatography

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(First received February 1st, 1983; revised manuscript received May 6th, 1983)

Recently, several methods have been reported for the direct separation of bilirubin (BR) species by means of high-performance liquid chromatography (HPLC) [1—4]. At present, we need a more sensitive and precise assay method for direct determination of each BR isomer and its conjugates to obtain accurate information on the conjugation mechanisms.

In the present report, we describe a sensitive method for the specific determination of three isomers of unconjugated BR (IIIα, IXα, and XIIIα BR), four isomers of BR mono-glucuronide (IIIα, endovinyl IXα, exovinyl IXα, and XIIIα BMG), three isomers of BR di-glucuronide (IIIα, IXα, and XIIα BDG), IXα BR mono- and di-glucosides, and IXα BR mono- and di-xylosides. This HPLC procedure is expected not only to serve as a valuable tool in the study of BR conjugation mechanisms but also to be clinically useful.

MATERIALS AND METHODS

Chemicals

Bilirubin (BR), UDP-glucuronic acid (UDPGa), UDP-glucose, UDP-xylose and glucaro-1,4-lactone were obtained from Sigma Chemical Company. IIIα, IXα and XIIIα isomers of BR were isolated from commercial BR and purified

0378-4347/83/$03.00

by the method of McDonagh and Assisi [5]. All other reagents were of analytical reagent grade. Glass plates precoated with silica gel (Merck, Kieselgel 60) were used for thin-layer chromatography.

High-performance liquid chromatography

The HPLC system was used was a Nippon Bunko (Tokyo, Japan) Tri-Rotar, equipped with a variable-wavelength spectrometric detector, Uvidex 100 III, and an electronic integrator, DP-L 200 data processor. The detector was set at 450 nm for analysis. Columns were of 4-mm bore polished stainless steel and 300 mm long. ODS gel, TSK-410 (particle size 5 μm, Toyo Soda, Japan) was used as packing material. Roughly 2 g of the packing material were suspended in 10 ml of chloroform, sonicated for 3 min and poured into the column. Then the packing material was packed with a constant pressure of 400 kg/cm^2 using methanol—water (1:1, v/v) as the pressuring liquid.

Two different solvent systems, A and B, were used. Solvent A, consisting of 0.05 *M* citrate buffer (pH 5)—methanol—acetonitrile (8:9:3, v/v), was used for the separation of BR conjugates. Solvent B, consisting of 0.05 *M* citrate buffer (pH 6.5)—methanol—acetonitrile (8:5:7, v/v), was used for the rapid analysis of unconjugated BR isomers. In most cases, after the elution of the conjugates with solvent A, the mobile phase was changed to solvent B for the separation of unconjugated BR isomers. The flow-rate was 0.6 ml/min and the paper speed 2 mm/min. All separations were carried out at room temperature (22—23°C).

Preparation of samples for HPLC

Amounts of 400 mg of crystalline ammonium sulfate, 20 mg of ascorbic acid and 0.2 ml of an extraction solvent consisting of isopropanol—methanol—dimethylsulfoxide (6:3:1, v/v) were added to 0.5 ml of each sample of bile and enzyme reaction mixture. The mixture was agitated vigorously on a Vortex mixer for 1 min, and centrifuged. An aliquot of the upper organic layer (5–20 μl) was injected into the column.

Biosynthesis of BR conjugates

One milligram of each of the purified BR isomers was dissolved in 0.05 *M* NaOH and the solution was diluted to the concentration of 200 μg/ml with 5.5% bovine serum albumin solution in 1.15% KCl. One volume of rat liver microsomal suspension (20 mg/ml in 1.15% KCl) was treated with 1 volume of digitonin solution (10 mg/ml in water), and the mixture was kept at 0°C for 30 min before preparation of the incubation mixtures.

The incubation mixture consisted of 0.05 ml of albumin-solubilized BR solution (final concentration 17.1 μM), 0.1 ml of digitonin-activated microsomal preparation (final protein concentration 2.0 mg/ml), 0.1 ml of aqueous solution containing UDPGa, $MgCl_2$ and glucaro-1,4-lactone (all final concentrations 10 m*M*), and 0.25 ml of 0.2 *M* phosphate buffer (pH 6.5). The mixture was incubated for 10 min at 37°C in a dark place. The reaction was stopped by adding 400 mg of ammonium sulfate and 20 mg of ascorbic acid, and the reaction tube was transferred into an ice bath. BR glucuronides biosynthesized were extracted with 0.2 ml of the extraction solvent. Glucose and xylose

conjugates of IXα BR were also synthesized enzymatically by the same procedure using UDP-glucose and UDP-xylose as the cofactors instead of UDPGa.

RESULTS AND DISCUSSION

The solvent systems A and B used here were adopted after a series of preliminary experiments. The solvent ratio in system A was chosen to obtain mainly the best resolution of the conjugates of BR isomers, and the ratio in solvent B was adopted to obtain a fast and good separation of unconjugated BR isomers.

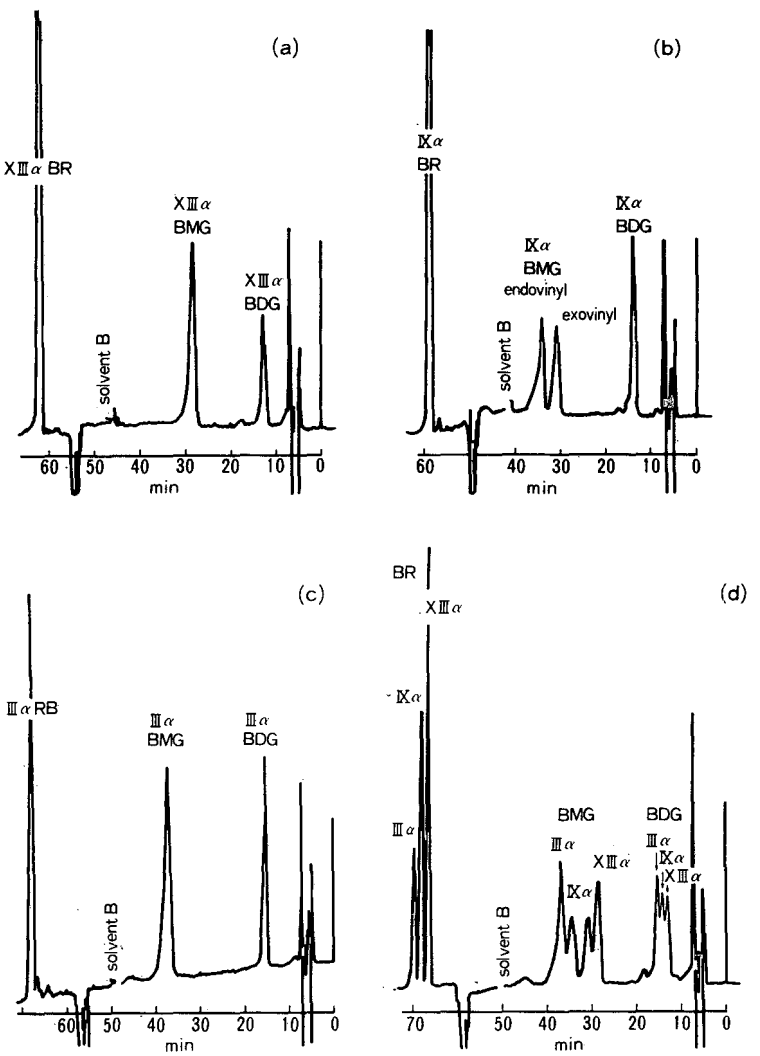

Fig. 1. HPLC separation of BR isomers and their glucuronides in enzymatic reaction mixture. (a) XIIIα species; (b) IXα species; (c) IIIα species; (d) mixture of XIIIα, IXα and IIIα species.

The elution order of BR isomers and their glucuronides was established by the standard samples synthesized enzymatically from specific substrates. Fig. 1 shows the HPLC analyses of three BR isomers and their mono- and di-glucuronides. It has been found that XIIIα isomers appeared first, followed by IXα and IIIα. IXα BMG was separated into two peaks corresponding to endovinyl and exovinyl isomers, respectively. The peak identification of the isomers was performed by subjecting the extract of BMG-enriched guinea-pig bile to HPLC analysis and azo pigment analysis [6]. As shown in Fig. 2, the guinea-pig bile extract mainly gave two peaks of IXα BMG isomers. The peak area of the first was about four times as large as that of the second. This was an identical value to the endovinyl/exovinyl isomer ratio obtained from the azo pigment analysis of the bile extract. From this, the former peak was identified as endovinyl isomer. Other investigators have also reported that the endovinyl isomer usually predominates in biological fluids [7].

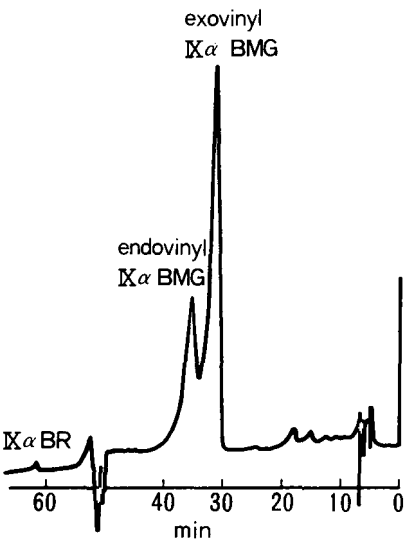

Fig. 2. Bilirubin metabolites in guinea-pig bile.

Fig. 3 shows HPLC analyses of IXα BR glucosides and xylosides. Mono-glucose and mono-xylose conjugates were clearly separated into two peaks corresponding to the endovinyl and exovinyl isomers.

The electronically integrated peak areas were compared with the amounts of each individual pigment. For each pigment, a linear relationship was observed between the amount of pigment injected (0—10 nmoles) and the corresponding peak area in the chromatogram. The repeatability of the peak areas for unconjugated BR isomers and their glucuronide species was also estimated. The relative standard deviation (S.D.) was less than 2% of each mean value (for five determinations) in all cases.

IXα BDG was the main component of human bile, and rat bile contained mainly IXα BMG and BDG (Fig. 4).

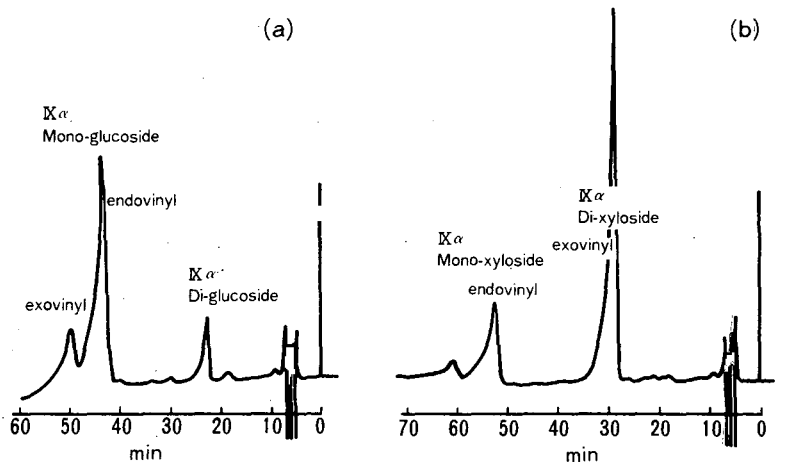

Fig. 3. HPLC separation of IXα BR glucosides and xylosides in enzymatic reaction mixture. (a) Glucosides; (b) xylosides.

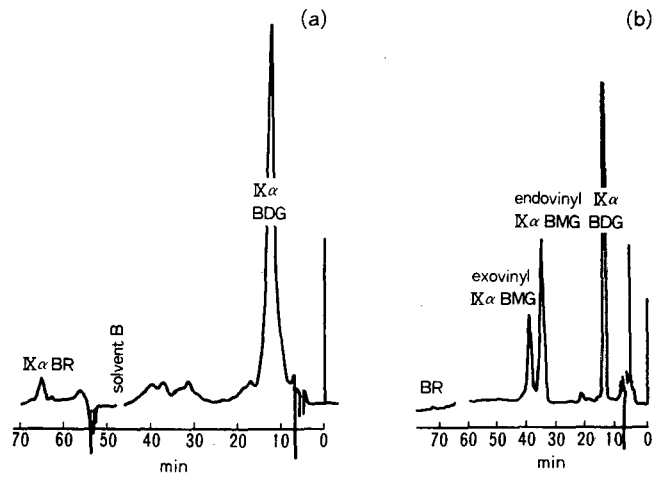

Fig. 4. Bilirubin metabolites in human and rat bile. (a) Human bile; (b) rat bile.

There is currently interest in the mechanisms of BR conjugation, especially BDG formation [8—13]. The HPLC method described here seems to be a useful tool to investigate these problems.

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Journal of Chromatography, 277 (1983) 314–320
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1799

Note

Thin-layer chromatographic determination of urinary testosterone, epitestosterone and androstenedione

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A thin-layer chromatographic (TLC) method for the separation of C_{19} steroids was combined with a spectrophotometric method for the determination of urinary testosterone, epitestosterone and androstenedione, instead of the previously used paper chromatography. A reproducible separation was obtained on silica gel F-254 precoated plates in the solvent system chloroform—diethyl ether, as well as easy identification due to the UV absorption of the Δ^4-3-keto group on a fluorescent background. The reliability of the modified method was evaluated from a study of sensitivity, precision, accuracy and comparison with radioimmunoassays (RIA).

MATERIALS AND METHODS

The method of acid hydrolysis, extraction, separation of ketonic from non-ketonic fraction and spectrophotometric measurement performed on a 24-h urine is adapted from that reported by Longhino et al. [1], except for the enzymatic hydrolysis and chromatographic separation of steroids. Steroids in the free form and after liberation by hydrolysis with beef liver β-glucuronidase (500 Fishman units per ml of urine; obtained from Pliva, Zagreb, Yugoslavia) and hydrochloric acid were extracted with diethyl ether. The combined ether

0378-4347/83/$03.00

extracts were evaporated and the residue was subjected to separation into ketonic and non-ketonic fractions using Girard T reagent.

TLC of testosterone (T), epitestosterone (ET) and androstenedione (A)

The ketonic fractions were dissolved in absolute ethanol (0.2 ml) and one half applied as small spots 2 cm apart on a starting line 2 cm from the lower edge of the plate. A mixture of standards T, ET and A, 2 μg of each dissolved in 0.2 ml of absolute ethanol, was applied to the middle and the sides of the plate. Before use the plates (0.25 mm thick, precoated silica gel F-254 plates, 20 × 20 cm, on glass support; obtained from Merck, Darmstadt, F.R.G.) were washed twice in ascending fashion with absolute ethanol, dried, activated at 105°C for 30 min and stored above silica gel in a desiccator. A 100-ml volume of the solvent system chloroform—diethyl ether (9:1, v/v) was mixed immediately before use and transferred to the chromatography chamber. The plate was developed to 15.0 cm from the starting line at room temperature [2].

T, ET and A standards were located and marked as UV-absorbing spots on a fluorescent background under a shortwave UV lamp in a dark room. The marked zones of silica gel (standards and corresponding zones of urinary steroids) were scraped into glass tubes and eluted by adding absolute ethanol (2 × 5 ml) and shaking vigorously. The silica gel was precipitated by centrifugation at 1500 *g* for a few minutes. The ethanol was transferred to clean tubes and evaporated under nitrogen.

The dry residues of the respective androgens produced color with sulphuric acid—ethanol reagent. The absorbance of the color read at 565, 600 and 635 nm (Gilford spectrophotometer, Model 250) against the reagent blank was corrected using Allen's formula. The concentrations of urinary T, ET and A were calculated by referring to the known amount of standard run on the same plate.

All solvents were reagent grade (Kemika, Zagreb, Yugoslavia).

RESULTS AND DISCUSSION

Separation of the steroids

The most common urinary C_{19} oxosteroids, such as androsterone (3α-hydroxy-5α-androstan-17-one, R_F = 0.42), dehydroepiandrosterone (3β-hydroxyandrost-5-en-17-one, R_F = 0.39), 11-oxoandrosterone (3α-hydroxy-5α-androstan-11,17-dione, R_F = 0.15), 11-oxoetiocholanolone (3α-hydroxy-5β-

TABLE I

R_F VALUES OF T, ET AND A OBTAINED BY TLC IN CHLOROFORM—DIETHYL ETHER (9:1)

The R_F values are the averages of ten chromatograms.

Steroid	R_F
Epitestosterone	0.21
Testosterone	0.30
Androstenedione	0.77

androstan-11,17-dione, R_F = 0.13), 11β-hydroxyandrosterone (3α,11β-dihydroxy-5α-androstan-17-one, R_F = 0.11), 11β-hydroxyethiocholanolone (3α,11β-dihydroxy-5β-androstan-17-one, R_F = 0.09) separate well from testosterone (T) (17β-hydroxy-4-androstene-3-one), epitestosterone (ET) (17α-hydroxy-4-androstene-3-one) and androstenedione (A) (4-androstene-3,17-dione) (Table I). Etiocholanolone (3α-hydroxy-5β-androstan-17-one, R_F = 0.29), which runs close to T, does not interfere, because it does not show any color reaction with the sulphuric acid—ethanol reagent.

Evaluation of the procedure

Sensitivity. The sensitivity of the modified method was evaluated by means of the recovery experiments as shown in Tables II—IV. The recovery of approximately 97.7% obtained for T (in the range 0.35—27.7 nmol/sample), 92.8% ET (in the range 0.7—8.7 nmol/sample) and 91.7% for A (in the range 0.9—24.4 nmol/sample) indicated that only negligible losses occurred during TLC. The silica gel blank carried through the procedure did not give any optical absorbance. The sensitivity is variable since it depends on the urine aliquot used. Under our working conditions, providing that we are dealing with one-third of 1 l of urine, it can be expected that reliable estimates will be obtained when the concentration of T is as low as 2.1 nmol/l (C.V. = 25.2%) and of A 5.2 nmol/l (C.V. = 28.8%), which is below the lowest values found for 24-h urine of healthy women according to Longhino et al. [1] (9.7—81.5 nmol/day for T, 11.2—86.6 nmol/day for A, and 4.2—34.5 nmol/day for ET). However, the equally high reliability of the ET estimates could be expected only at a concentration of 10.4 nmol/l, which is approximately twice as high as the lowest value indicated.

TABLE II

RECOVERY OF TESTOSTERONE AFTER TLC

Average recovery = 97.7%.

Testosterone (nmol/sample)			No. of determinations	C.V. (%)
Applied (nmol)	Recovered (mean ± S.D.)			
	nmol	%		
0.35	0.29 ± 0.073	82.9 ± 21.0	5	25.2
0.69	0.73 ± 0.17	105.8 ± 24.6	6	23.3
0.87	0.80 ± 0.17	92.0 ± 19.5	5	21.3
1.73	1.60 ± 0.31	92.5 ± 17.9	5	19.4
3.47	4.16 ± 0.76	119.9 ± 21.9	8	18.3
6.93	6.73 ± 1.21	97.1 ± 17.5	3	18.0
8.67	8.11 ± 1.42	93.5 ± 16.4	5	17.5
10.40	10.06 ± 1.25	96.7 ± 12.0	5	12.4
15.60	15.26 ± 1.84	97.8 ± 11.8	3	12.1
17.34	17.34 ± 1.95	100.0 ± 11.2	33	11.2
20.80	19.76 ± 2.32	95.0 ± 11.2	9	11.7
27.74	27.60 ± 1.84	99.5 ± 6.6	5	6.7

TABLE III

RECOVERY OF EPITESTOSTERONE AFTER TLC

Average recovery = 92.8%.

Epitestosterone (nmol/sample)			No. of determinations	C.V. (%)
Applied (nmol)	Recovered (mean ± S.D.)			
	nmol	%		
0.69	0.66 ± 0.35	95.6 ± 50.7	5	53.0
0.87	0.87 ± 0.34	87.4 ± 39.1	5	44.7
1.73	1.53 ± 0.45	88.4 ± 26.0	5	29.4
3.47	3.40 ± 0.87	98.0 ± 25.1	8	25.6
6.93	6.00 ± 1.84	86.6 ± 26.6	5	30.7
8.67	8.74 ± 2.43	100.8 ± 28.0	5	27.8

TABLE IV

RECOVERY OF ANDROSTENEDIONE AFTER TLC

Average recovery = 91.7%.

Androstenedione (nmol/sample)			No. of determinations	C.V. (%)
Applied (nmol)	Recovered (mean ± S.D.)			
	nmol	%		
0.87	0.59 ± 0.17	67.8 ± 19.5	4	28.8
1.75	1.96 ± 0.56	112.0 ± 32.0	4	28.6
3.49	2.97 ± 0.49	85.1 ± 14.0	4	16.5
7.00	6.28 ± 0.84	89.7 ± 12.0	12	13.4
12.22	11.31 ± 1.50	92.6 ± 12.3	5	13.3
17.46	17.32 ± 2.09	99.2 ± 12.0	22	12.1
20.95	18.99 ± 1.54	90.6 ± 7.4	7	8.1
24.44	23.67 ± 1.19	96.8 ± 4.9	4	5.0

Precision. The method of Snedecor [3] was used to give an estimate of the precision between the duplicate determinations obtained with a number of different urines. The coefficient of variation in the range 0.0—70.0 nmol/l is 11.0% for T, 20.9% for ET and 23.6% for A (Tables V—VII). This range covers the lowest levels of the respective androgens in 24-h urine of healthy women.

Accuracy. The accuracy of the modified method was measured by adding various amounts of T, ET and A to the ketonic fraction before TLC. A linear relationship was observed between the amounts added and the amounts recovered throughout the range examined, which is expressed by the regression equations: for T, $Y = 37.1 + 0.96X$, $r = 0.932$; for ET, $Y = 30.2 + 0.99X$, $r = 0.932$; for A, $Y = 51.0 + 0.92X$, $r = 0.918$, where Y is amount recovered, X is amount added and the intercept (a) is the endogenous amount of each respective androgen in the 24-h urine.

TABLE V

PRECISION OF DUPLICATE ESTIMATIONS OF URINARY TESTOSTERONE AT DIFFERENT RANGES

Range (nmol/l)	Mean ± S.D.	No. of determinations	C.V. (%)
0.0— 70.0	43.6 ± 4.8	32	11.0
70.0— 208.0	139.4 ± 14.6	28	10.5
138.0— 346.0	275.4 ± 18.8	24	6.8
346.0— 690.0	511.8 ± 23.6	28	4.6
1386.0—2080.0	1848.2 ± 38.8	12	2.1

TABLE VI

PRECISION OF DUPLICATE ESTIMATIONS OF URINARY EPITESTOSTERONE AT DIFFERENT RANGES

Range (nmol/l)	Mean ± S.D.	No. of determinations	C.V. (%)
0.0— 70.0	43.0 ± 9.0	26	20.9
70.0— 208.0	114.4 ± 16.0	20	14.0
208.0— 346.0	294.8 ± 18.8	26	6.4
346.0— 690.0	554.0 ± 24.2	26	4.4
1386.0—2428.0	2205.2 ± 46.4	10	2.1

TABLE VII

PRECISION OF DUPLICATE ESTIMATES OF URINARY ANDROSTENEDIONE AT DIFFERENT RANGES

Range (nmol/l)	Mean ± S.D.	No. of determinations	C.V. (%)
0.0— 70.0	47.4 ± 11.2	24	23.6
70.0— 208.0	123.6 ± 20.0	18	16.3
208.0— 416.0	322.0 ± 26.6	28	8.3
1040.0—1734.0	1410.6 ± 48.2	12	3.4

Comparison of the results obtained by TLC and RIA

In order to determine to what extent our results agree with those obtained by RIA, we carried out a comparative study on over 40 urines. One half of the ketonic fraction was subjected to the TLC separation and the other half of the same ketonic fraction was used for the determination of T (testosterone radioimmunoassay kit, from Biolab, Limal, Belgium) and A (androstenedione antiserum, from Biolab; [1,2,6,7-^{3}H]androst-4-ene-3,17-dione, from the Radiochemical Centre, Amersham) by RIA. The linear regression equations so obtained were for T (n = 43) $Y = 5.7 + 0.78X$, $r = 0.940$ ($P < 0.001$), and for A (n = 32) $Y = -5.7 + 0.81X$, $r = 0.976$ ($P < 0.001$), indicating a strong and significant correlation between the two methods (Figs. 1 and 2). RIA for ET was not available.

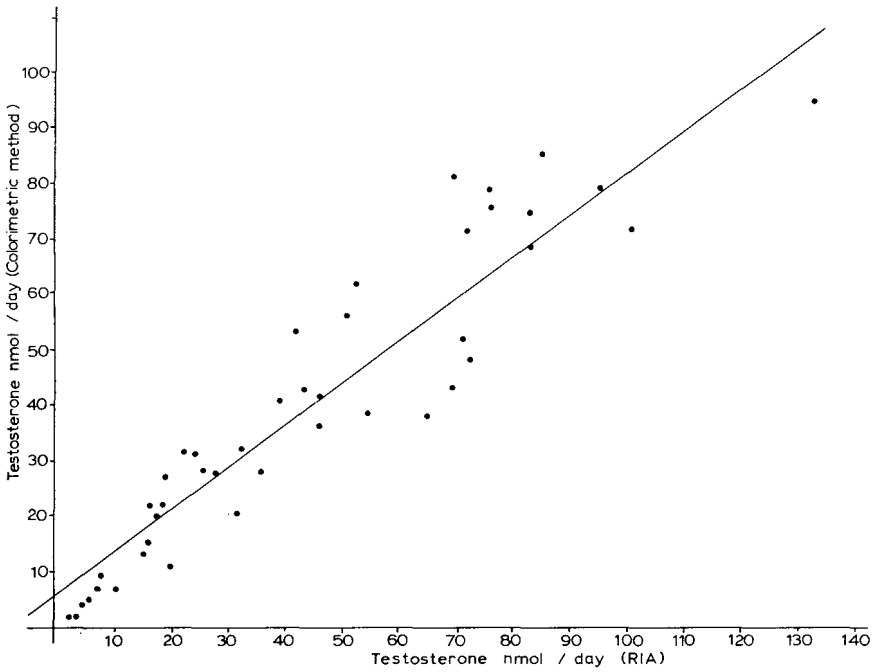

Fig. 1. Correlation between results for urinary testosterone obtained by TLC (Y) and by RIA (X). $Y = 5.7 + 0.78X$, $r = 0.940$, $P < 0.001$, $n = 43$.

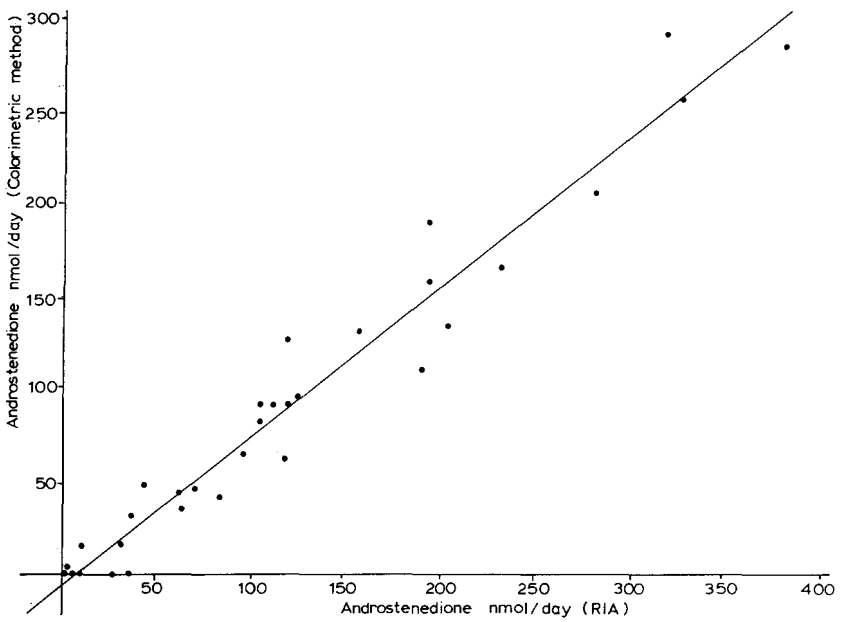

Fig. 2. Correlation between results for urinary androstenedione obtained by TLC (Y) and by RIA (X). $Y = -5.7 + 0.81X$, $r = 0.976$, $P < 0.001$, $n = 32$.

No significant average difference $P > 0.05$ was found between the results of the two methods either for T ($t = 0.641$; d.f. = 84) or for A ($t = 0.465$; d.f. = 62).

TLC shortens the already very long procedure by at least one working day. The other advantage of using TLC instead of paper chromatographhy to separate urinary T, ET and A is the relative simplicity of operation as well as reproducibility of separation, which makes it suitable for use in the routine clinical laboratory.

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Journal of Chromatography, 277 (1983) 321—327
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1775

Note

Rapid quantification on Chromarods of cholesterol, total bile salts and phospholipids from the same microliter sample of human gallbladder bile

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(First received January 24th, 1983; revised manuscript received April 13th, 1983)

A number of procedures exist for the analysis of bile, each involving a separate assay for the quantification of the three major bile components: cholesterol, bile salts and phospholipids. The major analytical methods include gas—liquid chromatography (GLC) and colorimetric assays for cholesterol [1—3], GLC and enzymatic assays for total bile acids [4, 5], and the determination of total phosphorus to quantify phospholipids [6, 7]. Though the process of bile lipid analysis has been automated [8, 9], each component must still be assayed separately.

A rapid technique, that couples thin-layer chromatography (TLC) with flame ionization detection (FID), is available for the quantification of lipid [10]. A number of investigators have quantified the response of cholesterol, phospholipids, and bile acids using this method with Chromarods (ref. 10 and references therein, and ref. 11). We wish to report a rapid technique for determining the concentration of these three components in human bile utilizing a single assay with a two-stage TLC system on Chromarods.

0378-4347/83/$03.00

MATERIALS AND METHODS

Chemicals

Cholesterol [5(6)-cholesten-3-ol], glycocholic acid (GC) (sodium salt), and bilirubin were purchased from Sigma (St. Louis, MO, U.S.A.) and found to be 99% pure by thin-layer chromatography (TLC). The A grade sodium salts of glycochenodeoxycholate (GCDC), taurodeoxycholate (TDC), and taurocholate (TC) were purchased from Calbiochem (La Jolla, CA, U.S.A.) and used without further purification. Egg phosphatidylcholine (PC) was prepared according to a method by Singleton et al. [12] and was found to be > 99% pure by TLC. Glass distilled solvents were of pesticide grade quality or better.

Preparation of standards and human bile for Iatroscan TH-10

A representative bile standard mixture (BSM) was prepared according to the method of Beke et al. [13] which consisted of a mixture of four bile salts in the following percentages: GCDC 44.9%, TC 17.9%, TDC 27.3%, and GC 9.9% (w/w). This mixture was combined with cholesterol and egg PC in chloroform—methanol (1:1) to produce a standard containing 1 μg/μl of each. This solution was serially diluted (0.25—8.0 μg) and applied to each Chromarod in 5-μl aliquots with a 5-μl microcapillary pipette (Arthur H. Thomas, Philadelphia, PA, U.S.A.) for maximum reproducibility [14].

Fresh human bile was obtained from patients undergoing elective cholecystectomy and was homogenized with a Brinkman polytron (Brinkman Instruments, Westbury, NY, U.S.A.) controlled by a Kinematic rheostat (Westbury, NY, U.S.A.) at a setting of 4 for 2 min. It was then divided into 1-ml aliquots and stored at −20°C. Aliquots (100 μl) of bile taken with a 100-μl capillary pipette (Fisher Scientific, Pittsburgh, PA, U.S.A.) were diluted 1:10, 1:15, 1:20, 1:25, or 1:30 with chloroform—methanol (1:1) then centrifuged at 2120 *g* in a clinical centrifuge for 2—5 min to sediment the bile proteins. An aliquot of the clear supernatant was removed from each and diluted 1:4 with chloroform—methanol (1:1) and 5 μl were spotted per Chromarod.

TLC of human bile, standard and bilirubin

Bile was diluted 1:10 with chloroform—methanol (1:1), centrifuged as above and 25 μl were spotted with a 25-μl capillary pipette (Fisher Scientific) on Silica gel 60 TLC plates (E. Merck, Darmstadt, F.R.G., distributed by Bodman Chemicals, Doraville, GA, U.S.A.) along with 50 μl (300 μg) BSM and 25 μl of a saturated bilirubin solution [2 mg bilirubin in 2 ml chloroform—benzene (1:1)].

Solvent systems for TLC and Iatroscan assays

TLC plates were run in a 20 × 26 × 9.5 mm glass chamber (Supelco, Bellefonte, PA, U.S.A.). Chromarods were run in small glass chambers [19 × 17.5 × 9.5 mm (Supelco)]. Paper wicks, made from Whatman 3 MM chromatography paper (VWR Scientific, Atlanta, GA, U.S.A.) and cut to fit the back chamber wall of each type of glass chamber, effected chamber saturation.

Two solvent systems were utilized for bulk component separation. Prior to running Chromarods with these systems, samples were focused to 1 cm above

the origin chloroform–methanol (1:1) and dried between systems according to the method of Harvey and Patton [14]. The first system consisted of chloroform–light petroleum–methanol–acetone (60:20:10:10) and the solvent front was run 8–10 cm (time, 30 min). Acetone–water (50:50) comprised the second system, the solvent front being run to 5 cm (time, 15 min). Each system was used for up to three consecutive analyses.

Operating conditions for Iatroscan TH-10

Samples for TLC–FID analysis were run in the Iatroscan TH-10 analyzer (Iatron Laboratories, Tokyo, Japan; marketing consultants Newman and Howell, Winchester, U.K.) on new type S Chromarods (Iatron Laboratories) and were activated by scanning through the hydrogen flame (30 sec per scan). After overnight hydration, as described by the manufacturer, rods were activated by scanning through the hydrogen flame (30 sec per scan). Integration was performed by a Hewlett-Packard 3390A integrator (Avondale, PA, U.S.A.) set at a threshold of 3 and 0.01 peak width. Hydrogen gas flow-rate to the flame ionization detector was 170 ml/min from a pressure of 0.8 kg/cm^2 and air flow-rate was 2 l/min. Scanning speed of Chromarods was 30 sec per scan and chart speed was 6 cm/min.

Statistical analysis

Statistical studies of correlations and differences between means were performed according to the method of Bailey [15]. The lipid concentrations of native bile were calculated by employing molecular weights of 498.9, 778.0, and 386.6 for bile salts, phospholipids, and cholesterol, respectively.

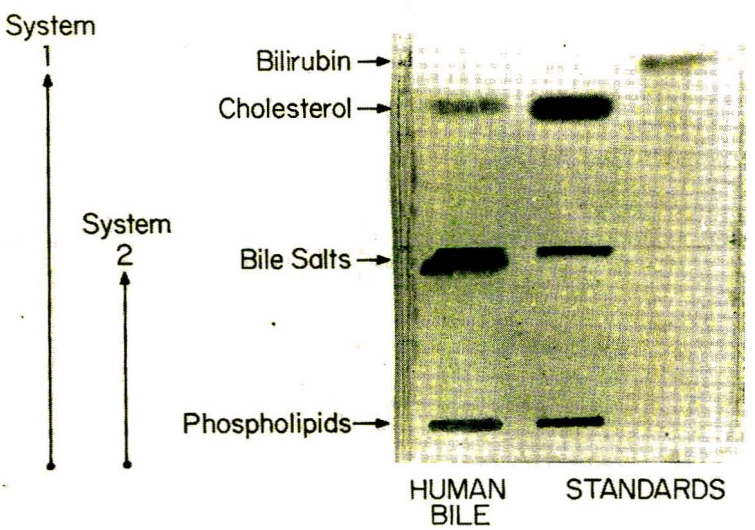

Fig. 1. Separation of bilirubin, cholesterol, bile salts and phospholipids by two solvent systems. System 1: chloroform–light petroleum–methanol–acetone (60:20:10:10) developed to 10 cm. System 2: acetone–water (50:50) developed to 5 cm. Spots visualized by charring with 40% sulfuric acid at 120°C for 5 min.

RESULTS

The separation of cholesterol, bile salts and egg PC by TLC is illustrated in Fig. 1. Neutral class components such as cholesterol and bilirubin migrated with the solvent front in the first system leaving bile salts and phospholipids 1 cm above the origin. In the second system only bile salts traveled with the solvent front, the polar phospholipids remained immobile. A similar procedure with acetone was utilized by Gentner et al. [16] for the separation of phospholipids and neutral lipids in milk.

Bile components (cholesterol, bile salts, phospholipids) were quantified from the area of three distinct peaks in the chromatogram. No quantifiable response occurred for bilirubin at physiological concentrations. All rods were repeatedly scanned (re-scanned) until no residual sample remained (2—3 times). Peak areas from consecutive scans were summed in order to calculate the total sample concentration. Above 3—4 μg per rod, cholesterol and phospholipids required two scans while 4.6 ± 1.29% and 1.2 ± 0.36% bile salts remained after the second and third consecutive scan.

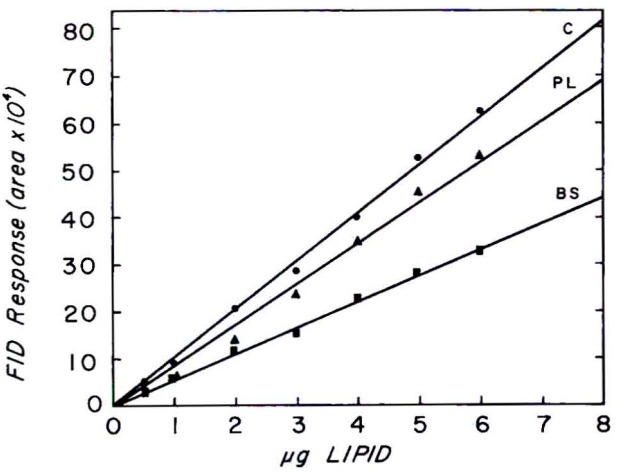

Fig. 2. Standard curve for increasing concentrations of cholesterol (•——•), egg PC (▲——▲), and a bile standard mixture (■——■). The sum of the initial and residual peak areas from consecutive scans was used to calculate the overall sample concentration. Each point represents the mean of six measurements.

A standard curve was generated over a range of concentrations (0.25—8.0 μg) as shown in Fig. 2. Linear regression analysis produced correlation coefficients of 0.998, 0.997 and 0.999 for cholesterol, egg PC and BSM indicating a high degree of reproducibility over the concentration range employed. Sample chromatograms of human bile are shown in Fig. 3. Variations in the quantities of cholesterol and phospholipid are evidenced by the changes in peak area. Results of bile analysis are shown in Table I. Each bile sample was assayed six times at three different dilutions in order to generate the data in Table I.

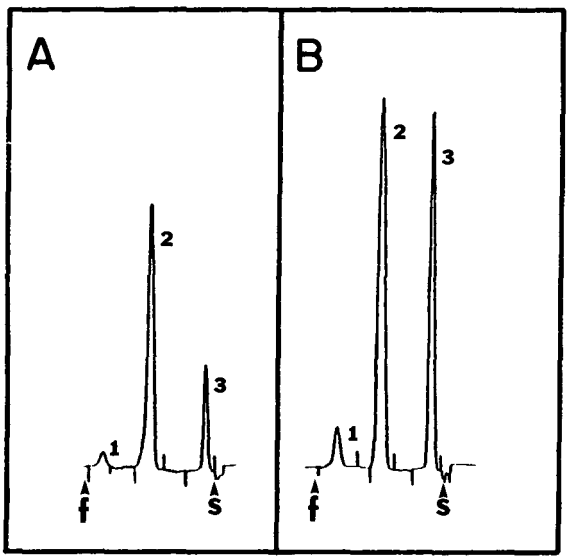

Fig. 3. Representative chromatograms of human bile. Trace A is from a sample with no visible gall stones. Trace B, with cholesterol type gallstones. Peaks: 1 = cholesterol, 2 = bile, 3 = phospholipids; f = solvent front, s = start.

TABLE I

BILIARY LIPID COMPOSITION

The data are means ± S.D. for six samples at three concentrations.

Sample	Composition (mmole/l)			Mole percent		
	Cholesterol (C)	Bile salts (BS)	Phospholipid (PL)	C	BS	PL
1	14.5 ± 1.62	206.6 ± 6.65	35.5 ± 1.51	5.7	80.5	13.8
2	7.0 ± 2.60	152.7 ± 3.03	19.1 ± 4.18	3.9	85.4	10.7
3*	18.0 ± 2.53	224.4 ± 9.88	66.2 ± 5.52	5.8	72.7	21.5
4	7.3 ± 2.24	66.2 ± 3.87	17.0 ± 1.56	8.1	73.1	18.8
5	24.7 ± 1.00	213.8 ± 14.00	72.0 ± 10.43	8.0	68.9	23.1
6	15.0 ± 0.45	140.5 ± 8.74	39.5 ± 2.58	7.7	72.0	20.3

*With cholesterol gallstones.

DISCUSSION

The procedure described here allows for the simultaneous analysis of the three major lipid components of bile. Ten samples could be examined in less than 1 h and only a small volume of bile is required. Since the actual amount of bile added per rod was between 0.045 μl (1:10 dilution) and 0.025 μl (1:30), smaller volumes could be sampled (< 50 μl bile) though care must be taken to minimize solvent evaporation.

The dilution range (1:10 to 1:30) was chosen due to the small amount of cholesterol and the large amount of bile salt in a sample. At the lowest concentration of cholesterol (7 mmole/l) only 0.125 μg were actually placed on a rod. Such a value approaches the detection limits of the system [13]. It was also desirable to keep the number of re-scans to a minimum. Re-scans were necessary for quantitative results since most of the samples, even at the highest dilution, contained overloading amounts of bile salt. Rods scanned over 170 times exhibited neither broadening nor tailing of peaks.

The composition of human bile varies depending on the physiological status of the gall bladder and bile duct, diet, sampling time, and the general health of the individual [17, 18]. The actual component percentages shown here are well within known ranges for individuals with and without gallstones [19, 20]. Cholesterol gallstone formation is thought to be caused by the supersaturation bile with cholesterol. According to Carey and Small [21] the total lipid concentration plus the bile salt:phospholipid ratio are the predominant determinants physiologically for cholesterol solubilization.

The present technique produced a low degree of variation per sample component over a wide range of clinical and standard concentrations. Current methodologies for the analysis of bile lipids utilize a series of time-consuming assays. Quantification of bile lipid classes by TLC—FID on Chromarods greatly simplifies the analysis of this important hepatic secretion.

ACKNOWLEDGEMENTS

We are grateful to H. Rodger Harvey for the purification of egg phosphatidylcholine and Pam Bruce for excellent technical assistance. This research was supported by United States Public Health Service Grants AM 01076 and AM 27304 awarded to J.S.P.

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Journal of Chromatography, 277 (1983) 328—332
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1766

Note

Isotachophoresis as a preseparation technique for liquid chromatography

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(First received December 20th, 1982; revised manuscript received May 6th, 1983)

High-performance liquid chromatographic (HPLC) profiles of uremic serum ultrafiltrate are rather complex [1]. For purposes of identification and characterization of the HPLC peaks, information may be obtained from chromatographic retention data, on-line and off-line HPLC—mass spectrometric analysis, off-line (Fourier) infrared analysis and to a certain extent from UV-ratio monitoring at multiple wavelengths [2]. However, it is desirable to decrease the complexity of the profiles, especially in view of the spectrometric identification techniques, where peak impurities might obscure the spectra. For this reason uremic serum ultrafiltrate was preseparated by isotachophoresis [3], the advantages of which are as follows. (1) The concentration effect of dilute samples. (2) The self-sharpening effect of zone boundaries. (3) The possibility of selecting a discrete amount of anions or cations by a proper choice of electrolyte conditions. (4) The length between leading zone and terminating zone (sample) is constant at the moment the terminator has passed the injection point. The steady-state therefore need not to be reached for sample collection. (5) Using valves for sample introduction even allows the collection of non-ionic compounds, as they remain in the valve during the isotachophoretic separation.

EXPERIMENTAL

Isotachophoresis

Separations were performed on an LKB Tachophor isotachophoretic analyzer (LKB, Bromma, Sweden) at 60 μA (stabilized current, end voltage 9 kV), in a 0.4 mm I.D. PTFE capillary instead of the original separating capillary plate. Test runs were also done in home-made equipment [3] using both UV and conductivity detection.

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Amaranth red and fluorescein were used as coloured markers in the initial experiments. Hard-cutting of the zone train migrating between amaranth red and fluorescein or terminator was done by means of a razor blade [4].

In the preparation runs, 1 μl of uremic serum ultrafiltrate was injected into the isotachophoretic analyzer. The volume collected by hard-cutting (5 μl, 4 cm) was transferred to a conical microvial (Chrompack, Middelburg, The Netherlands) and injected into the liquid chromatograph using a 10-μl syringe (Glenco, Chrompack, Middelburg, The Netherlands).

So far no hard-cutting has been performed using a PTFE valve as described by Kenndler and Kaniansky [5].

Liquid chromatography

The equipment used consisted of two Model 100 A pumps, a Model 321 controller, and a Model 160 fixed-wavelength UV detector, all from Beckman (Berkeley, CA, U.S.A.). A 1-μl aliquot of serum ultrafiltrate was diluted to 5 μl and injected into the liquid chromatograph. Further experimental conditions for isotachophoresis and liquid chromatography are given in Table I.

In this way the same absolute amounts of (anionic) solutes in the serum with and without isotachophoretic preseparation are loaded on the HPLC column. Additional experimental conditions for isotachophoresis and liquid chromatography are given in Table I.

TABLE I

OPERATIONAL SYSTEM FOR ISOTACHOPHORETIC AND LIQUID CHROMATOGRAPHIC ANALYSES

Isotachophoresis		
	Electrolyte	
	Leading	Terminating
Anion	Chloride	HEPES*
Concentration	0.025 *M*	0.025 *M*
Counter-ion	Histidine	Sodium
pH	6	9.5
Solvent	H_2O	H_2O
Liquid chromatography		
Mobile phase	100% solvent I to 100% solvent II	
Gradient	Within 30 min Solvent I: 0.05 *M* ammonium formate pH 4—methanol (95:5, v/v) Solvent II: methanol	
Column	25 cm × 4.6 mm, stainless steel, packed with Polygosil-60, C18, 5-μm particles**	
Detection	UV at 254 nm	
Flow-rate	1 ml/min	

*HEPES = N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid, sodium salt (Sigma, St. Louis, MO, U.S.A.).

**Polygosil (Machery-Nagel & Co., Düren, F.R.G.).

Uremic serum ultrafiltrate

Uremic serum ultrafiltrate was obtained from Prof. S. Ringoir, Department of Nephrology, University Hospital of Ghent, Belgium. The filtrate became available during a sequential ultrafiltration—hemodialysis artificial kidney treatment of a uremic patient. Large molecules such as proteins are rejected by the artificial kidney membrane (molecular weight cut-off 10,000) and consequently are not present in the serum ultrafiltrate.

RESULTS

Fig. 1 shows an isotachophoretic test run of a uremic serum ultrafiltrate sample. Test runs were performed on home-made equipment using both UV and conductivity detection. From these runs zone train lengths between amaranth red as frontal coloured marker and fluorescein, useful as a terminal marker, or terminator were simply determined. Therefore, in the isotachopherogram of Fig. 1 only amaranth red is present.

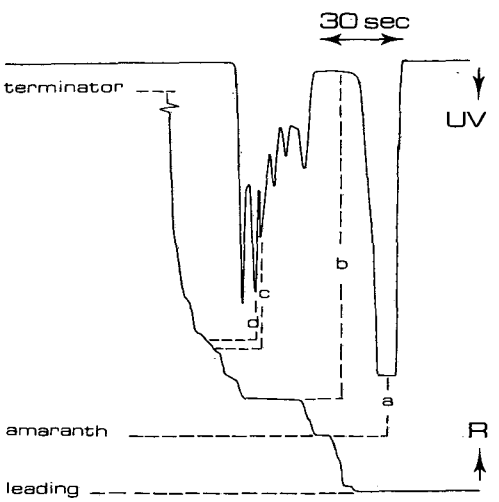

Fig. 1. Anionic separation of uremic serum ultrafiltrate (0.5 μl injected) by isotachophoresis (test run). UV absorption and conductivity detector traces are shown (R = resistance). a = Amaranth red; b = phosphate; c = hippurate; d = urate. Conditions are given in Table I.

Hard-cutting was done on the 0.4 mm I.D. capillary in the LKB apparatus, between amaranth red and the terminator, of which the position of the zone boundary was calculated from the test runs performed. Fluorescein was not used here to prevent interference in the HPLC analysis.

In Fig. 2 the HPLC profiles of uremic ultrafiltrate with and without anionic preseparation are compared. After anionic preseparation a number of peaks in the HPLC profiles have disappeared. These are either cationic or neutral constituents.

From the chromatographic retention data tentative peak assignments have been made for some major peaks, as given in the figure legend.

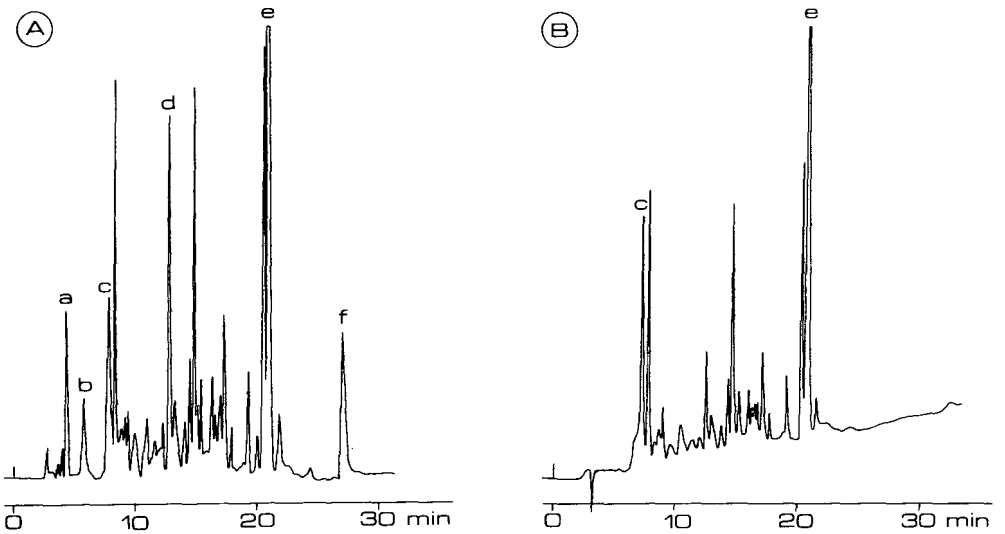

Fig. 2. HPLC profiles of uremic serum ultrafiltrate without (A) and with (B) anionic preseparation by isotachophoresis. Conditions are given in Table I. Tentative assignments: a = creatinine; b = uracil; c = uric acid; d = xanthine; e = hippuric acid; f = caffeine. These peaks have been identified by spiking with appropriate standards.

DISCUSSION

From the experiments it can be concluded that the combination of isotachophoresis and liquid chromatography can give valuable information about the identity or character of solutes in a complex diverse matrix such as biological fluids.

In this study proteins were not present in the samples, but they can be readily separated from the anionic or cationic low molecular weight solutes in the sample in the same isotachophoretic (pre)separation run [6], by choosing suitable operational conditions.

Combination of the selectivities of isotachophoresis and HPLC makes a powerful combination. In isotachophoresis a choice is made between anionic and cationic preseparation. Variation of the pH of the leading electrolyte influences the mobility of the different species, as they have different pK values. In HPLC selectivity can be influenced by the nature of both the mobile phase and the stationary phase in a most flexible way.

Direct transfer of the aqueous samples from isotachophoresis to HPLC imposes the use of reversed-phase liquid chromatography. However, isotachophoresis in non-aqueous media, which is at present being developed [7], will be compatible with normal-phase liquid chromatography as well. With some technical developments that are available or will be available in the near future [5, 8] it might be possible to select more discrete regions of the migrating zone train. These regions in capillary isotachophoresis necessarily represent small sample volumes ($< 1\ \mu l$). Combination with microbore liquid-chromatography columns (e.g. 1 mm I.D.) therefore seems promising. As the chromatographic

dilution in the microbore columns (1 mm I.D.) is much less than in wide-bore columns (4.6 mm I.D.), the former will have a higher mass sensitivity by a factor of 20. This will be an advantage in those cases where only small sample volumes are available, because in wide-bore columns larger sample volumes can be injected.

The on-line coupling of the techniques of isotachophoresis and microbore liquid chromatography and microbore liquid chromatography and mass spectrometry are at present under investigation.

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Journal of Chromatography, 277 (1983) 333—339
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1798

Note

Resolution of the seven isomeric ring-hydroxylated propranolols as *tert.*-butyldimethylsilyl derivatives by capillary gas chromatography—mass spectrometry

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(Received February 24th, 1983)

Three previously unknown ring-hydroxylated urinary metabolites of the β-adrenergic antagonist drug propranolol were recently identified by Walle et al. [1]. In rats and in man, 2-hydroxypropranolol (2-HO-P), 5-HO-P, and 7-HO-P were found, as well as the previously known 4-HO-P [2—6], which is the major monohydroxylated urinary metabolite of propranolol in both species, and the only hydroxylation product identified in the dog. Briefly, these compounds were identified in the following fashion. Urine samples were treated enzymatically to hydrolyze glucuronide conjugates; then the monohydroxypropranolols were extracted and analyzed as trimethylsilyl (TMS) and trifluoroacetyl (TFA) derivatives by gas chromatography (GC) and gas chromatography—mass spectrometry (GC—MS) using packed column techniques (3% OV-1). Identification of the isomers was based upon comparison of retention times and mass spectra with those of derivatized synthetic monohydroxypropranolols [1, 7]. Two derivatization techniques were employed because, under the conditions of the analyses, 2-HO-P and 8-HO-P were unseparated as TMS derivatives, and 4-HO-P and 5-HO-P were unseparated as TFA derivatives. Another complication encountered was that as TFA derivatives the propranolol peak overlapped with the 2-OH-P peak, necessitating the use of selected ion monitoring to distinguish these two compounds. Furthermore, since TFA derivatives partially decompose on columns previously used with TMS derivatives, separate columns had to be used for the two types of derivatives.

The purpose of the work presented here was to develop a simpler technique for separating and identifying the seven isomeric ring-hydroxylated propranolols

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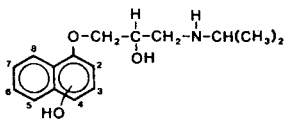
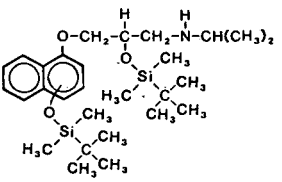
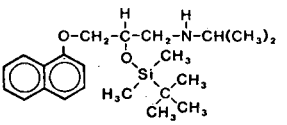
in order to facilitate future studies with these compounds. Such a technique has been developed, using a relatively new silylating reagent, N-methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA), which produces volatile *tert.*-butyldimethylsilyl (TBDMS) derivatives of aliphatic and aromatic hydroxyl groups [8], yielding mono-derivatized propranolol (TBDMS-P) and di-derivatized hydroxypropranolols (TBDMS-HO-P).



Ring-Hydroxylated Propranolol



TBDMS-Hydroxypropranolol



TBDMS-Propranolol

When pyridine is used as a solvent for the derivatization, secondary amines are derivatized very slowly by MTBSTFA [8]; and, with the TBDMS group attached to the aliphatic oxygen which occurs far more rapidly in pyridine [8], derivatization of the secondary amine group is further sterically hindered. No tri-derivatized hydroxypropranolols were observed during the course of this study. (It may be possible to produce the tri-TBDMS derivatives with acetonitrile as the solvent [8—10] but these derivatives would be expected to be less volatile than the di-TBDMS derivatives.) Separations were carried out using glass capillary GC—MS. The applicability of the method was demonstrated by analysis of a urine sample from a rat dosed with (±)-propranolol and identification of the ring-hydroxylated metabolites.

MATERIALS AND METHODS

Chemicals

(±)-Propranolol hydrochloride was obtained from Sigma (St. Louis, MO, U.S.A.). 2-HO-P and 4- through 8-HO-P were synthesized as their hydrochloride salts in these laboratories as previously described by Oatis et al. [7]; 3-HO-P (the least stable isomer) was freshly synthesized as the free base according to the previously described procedure [7]. N-Methyl-N-(*tert.*-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA) containing 1% *tert.*-butyldimethylsilylchlorosilane was obtained from Regis Chemical (Morton Grove, IL, U.S.A.), and pyridine from Fisher Scientific (Fair Lawn, NJ, U.S.A.).

Equipment

Capillary GC—MS analyses were performed using a Varian 3700 gas chromatograph equipped with a split injector. The column, a Grade A, 60 m × 0.25 mm I.D. SP-2100 wall coated open tubular (WCOT) glass capillary obtained from J & W Scientific (Orangeville, CA, U.S.A.), was interfaced through an open-split interface to a Finnigan MAT-212 mass spectrometer equipped with a Spectrosystem SS200 data system. A Fisher Recordall® Series 5000 strip-chart recorder was used during selected ion monitoring in analogue mode.

Preparation of TBDMS derivatives of propranolol and monohydroxypropranolols

A 1—1.5 mg sample of each compound was dissolved in 200 μl of pyridine; 20 μl of MTBSTFA were added, and the reaction mixture was tightly capped and heated at 100°C for 2 h. Derivatization of 8-HO-P is apparently sterically hindered, and requires 1—2 h heating to drive it to the di-derivatized product. The other compounds were more readily derivatized, but all of the individual samples were treated identically. A mixture of propranolol and the seven monohydroxypropranolols (1—1.5 mg of each) was similarly derivatized using 100 μl of pyridine and 120 μl of MTBSTFA; this mixture required heating for 16 h at 100°C to complete the derivatization of 8-HO-P. The derivatives were stable in solution for at least 2 weeks.

Biological sample

The hydroxypropranolol metabolites from a 2-ml aliquot of a 24-h urine collection (total urine volume was 26 ml) from a male Sprague—Dawley rat injected intraperitoneally with 10 mg/kg (±)-propranolol were enzymatically hydrolyzed and extracted according to the method described by Walle et al. [1]. The extracted metabolites were derivatized using 100 μl of pyridine and 20 μl of MTBSTFA with 16 h heating at 100°C.

Capillary GC—MS resolution of propranolol and the monohydroxypropranolols

The TBDMS derivatives of propranolol and the seven isomeric monohydroxypropranolols were resolved under the following conditions: injector temperature 240°C; split ratio 1:350, column temperature programmed from 240 to 255°C at a rate of 0.2°C/min; helium carrier gas average linear velocity 23—25 cm/sec; open-split interface temperature 260°C; line-of-sight temperature 250°C; ion source temperature 240°C; ionization energy 70 eV. During the analyses of synthetic samples the mass spectrometer was scanned repetitively from 30 to 550 a.m.u. at a rate of 3 sec per decade with an interscan time of 1 sec. Selected ion monitoring was used as the detection system during the analysis of the biological sample because of the enhanced sensitivity of this technique. The mass spectrometer was set to monitor *m/z* 217 using a direct-probe sample of TBDMS-derivatized 2-HO-P for calibration. All of the compounds eluted before the final column temperature was reached.

RESULTS

The TBDMS derivatives of propranolol and the hydroxypropranolols (with the exception of 8-HO-P) were readily prepared using MTBSTFA with pyridine as the solvent. 8-HO-P formed the di-derivatized product less rapidly than the other hydroxypropranolols, but was driven to completion with heating at 100°C for up to 16 h. With insufficient heating, two peaks were observed corresponding to mono- and di-derivatized 8-HO-P, with the mono-derivatized product eluting first. With sufficient heating, only the later eluting peak was observed.

Table I presents the elution order and the retention times of the separately derivatized synthetic compounds; the retention times presented have been

TABLE I

ELUTION ORDER OF THE TBDMS DERIVATIVES OF PROPRANOLOL AND THE MONOHYDROXYPROPRANOLOLS ON AN SP-2100 WCOT CAPILLARY COLUMN

The column temperature was programmed from 240 to 255°C at a rate of 0.2°C/min.

Derivatized compound	Adjusted retention time* (min, ± 0.1)	Relative retention time** (± 0.01)
Propranolol	14.7	1.00
8-HO-P	34.4	2.34
2-HO-P	36.1	2.46
3-HO-P	46.1	3.14
7-HO-P	49.0	3.33
4-HO-P	54.1	3.68
6-HO-P	59.4	4.04
5-HO-P	60.0	4.08

*The elution time of a non-retained compound (butane) was subtracted from the absolute retention times to obtain the adjusted retention times.
**Relative to TBDMS-propranolol.

adjusted for the void volume of the system. A representative chromatogram of the results obtained when all eight compounds are derivatized as a mixture is presented in Fig. 1, as a mass chromatogram of the sum of *m/z* 72, 217, and 274. Proposed structures of these ionic species are depicted in Fig. 2. A profile essentially identical with that in Fig. 1 was obtained for *m/z* 72 alone, and the same profile minus the propranolol peak was obtained for *m/z* 217 or

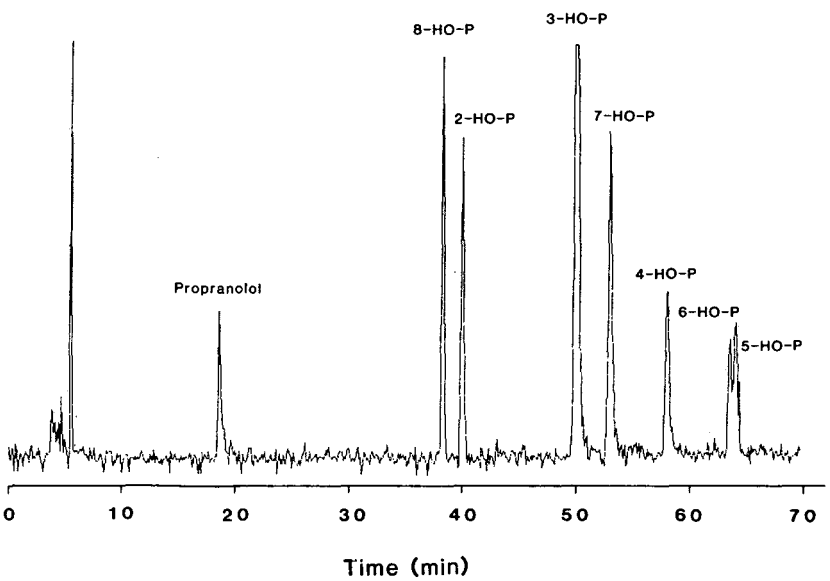
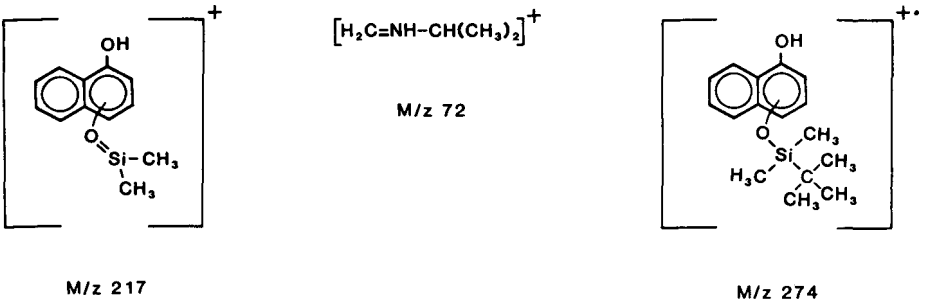


Fig. 1. Capillary GC—MS chromatogram of the TBDMS derivatives of propranolol and ring-hydroxylated propranolols, as a retrospective combined plot of three ionic species, *m/z* 72, 217, and 274 (see Fig. 2). The sample size was 3 μl (0.1 mg). See text for GC—MS parameters.



M/z 217

M/z 274

Fig. 2. Proposed structures of the ionic species traced in Fig. 1.

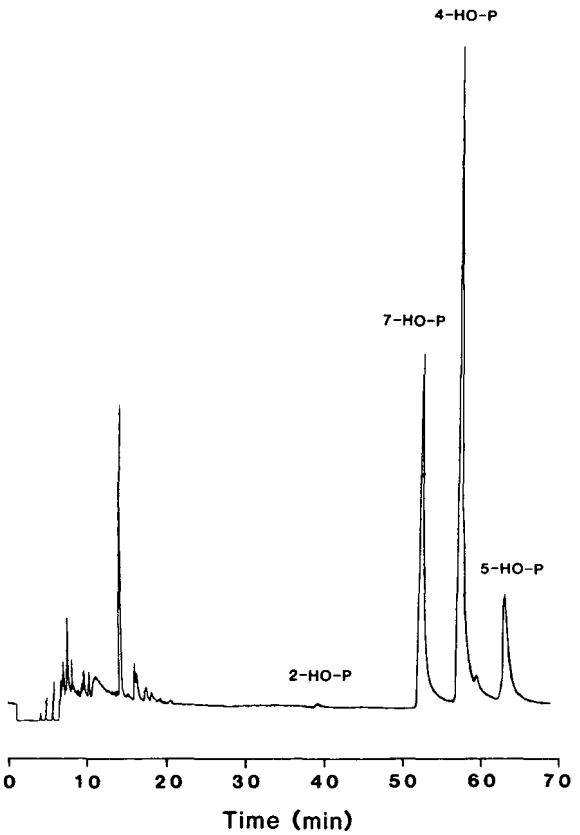


Fig. 3. Selected-ion monitoring (*m/z* 217) capillary GC—MS chromatogram of the ring-hydroxylated metabolites of (±)-propranolol extracted from rat urine. The sample size was 5 μl (10 μg). Peaks are labeled in accordance with the retention times of derivatized synthetic compounds chromatographed on the same day. The apparent peak tailing is due primarily to filtering in the analogue recording electronics. For GC—MS conditions, see text.

274 alone. All three of these ions are abundant in the mass spectra of the hydroxypropranolols, and each is potentially suited for selected ion monitoring. The *m/z* 217 and 274 ions are more specific for the hydroxypropranolols, as the *m/z* 72 ion is subject to interference from smaller molecular weight species likely to be present in a biological sample. Alternatively, the molecular

ion (*m/z* 503) could be monitored to enhance specificity for the hydroxypropranolols, but with a sacrifice in sensitivity.

The TBDMS derivatives of propranolol and the hydroxypropranolols were well resolved on an SP-2100 WCOT capillary column, with the exception of 5- and 6-HO-P, which were separated by only 0.5—0.6 min with an overall retention time of about 1 h. This degree of resolution was obtained only when care was taken to ensure that the initial average linear velocity was only slightly above the optimal average linear velocity for the carrier gas (helium), so that the system would approach maximum efficiency at the higher elution temperatures. Under a given set of flow conditions, capillary GC—MS retention times were reproducible to within ± 0.1 min.

A selected ion monitoring (*m/z* 217) chromatogram of the TBDMS derivatives of the ring-hydroxylated propranolol metabolites extracted from the urine of a rat dosed with (±)-propranolol is presented in Fig. 3. The retention times of the peaks observed from this sample were consistent with the observed retention times of derivatized synthetic 2-, 7-, 4-, and 5-hydroxypropranolol run on the same day. The species identified by these techniques, and their relative proportions, are consistent with the results previously obtained in rats by Walle et al. [1].

DISCUSSION

Propranolol and the seven isomeric ring-hydroxylated propranolols can be separated and reliably identified as TBDMS derivatives using capillary GC—MS techniques. The identification methodology presented here affords significant advantages over the techniques used in the past [1]. The TBDMS derivatives are well-suited for several detection systems, including flame ionization (data not shown), repetitive scanning mass spectrometry, and selected ion monitoring MS. Only one derivatization technique is employed, eliminating the necessity for using two separate columns. TBDMS-propranolol is well separated from the other seven compounds, so that special techniques for distinguishing the parent compound from one of its metabolites are not necessary.

The only major problem encountered using the techniques presented here was the moderate peak overlap between the TBDMS derivatives of 5- and 6-OH-P. In order to ensure reliable identification of these two compounds, care must be taken to ensure that the capillary GC system is operating at near-maximal efficiency. Problems concerning the identity of either or both of these two compounds in a mixture may be overcome by adding a small quantity of derivatized synthetic 5- or 6-HO-P and comparing relative peak heights with and without the added compound. The method presented here should facilitate further studies of ring oxidation metabolic pathways for propranolol, and should also be applicable to isomeric metabolites of related compounds.

ACKNOWLEDGEMENTS

This work was supported in part by U.S. Public Health Service grants GM 20387 and RR 01239. Kevin D. Ballard is a recipient of a South Carolina Graduate Student Assistantship.

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Journal of Chromatography, 277 (1983) 340—346
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

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Note

Quantitation of (*d,l*)-3-ethyl-2,6-dimethyl-4,4*a*,5,6,7,8,8*a*,9-octahydro-4*a*,8*a*-*trans*-1H-pyrrolo[2,3-*g*]isoquinolin-4-one in human plasma by gas chromatography—chemical ionization mass spectrometry

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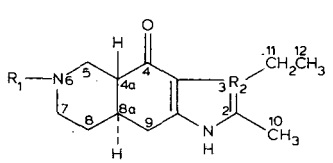
(First received February 22nd, 1983; revised manuscript received May 6th, 1983)

The compound (*d,l*)-3-ethyl-2,6-dimethyl-4,4*a*,5,6,7,8,8*a*,9-octahydro-4*a*,8*a*-*trans*-1H-pyrrolo[2,3-*g*]isoquinolin-4-one [I] is one of a number of pyrrolo-[2,3-*g*]isoquinolines which exhibit neuroleptic activity and are being investigated as possible antipsychotic agents [1]. A high-performance liquid chromatographic (HPLC) method [2] for determination of [I] lacked the sensitivity (25 ng ml^{-1}) necessary for monitoring [I] in plasma following a single therapeutic dose of the drug. This paper describes a gas chromatography—chemical ionization mass spectrometry (GC—CIMS) assay which can quantitate between 2 and 50 ng ml^{-1} of [I] in plasma. The sensitivity of the assay is sufficient to measure the concentration of the drug in human plasma for 4—8 h after the oral administration of a single 5-mg dose of the drug.



Compond	R	R
[I]	$-CH_3$	^{12}C
[II]	$-CD_3$	^{12}C
[III]	$-CH_3$	^{12}C

EXPERIMENTAL

Materials

Compounds [I] and [II] were synthesized by Dr. G.L. Olson, Chemical Research Department and compound [III] was synthesized by the isotope synthesis group, Hoffmann-La Roche Inc. (Nutley, NJ, U.S.A.). The specific

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activity of [III] was 0.046 μCi mg^{-1}. Methanol, chloroform and ethyl acetate were purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Phosphate buffer (1.0 *M*, pH 11) was prepared by mixing 530 ml of 1 *M* $K_2HPO_4 \cdot 3H_2O$ and 470 ml of a saturated solution of sodium phosphate, and adjusting the solution to pH 11 with 1 *M* $KH_2PO_4 \cdot 3H_2O$.

Analytical procedure

Stock solutions (1 mg ml^{-1}) of compounds [I] and [II] were prepared in methanol. Standard solutions were prepared by dissolving the stock solution in methanol to give final concentrations of 4—100 ng of [I] per 100 μl. For preparation of standard curves, 100 μl of the standard solutions were added to 2 ml of the control plasma containing 40 ng of compound [II] and either 4, 10, 20, 40 or 100 ng of compound [I]. The spiked plasma samples were then taken through the complete analytical procedure described below. The experimental plasma samples were thawed, and 2-ml aliquots of each were pipetted into 16-ml screw-capped tubes (Pyrex 9825) with Teflon®-lined screwcaps. A 40-ng amount of the internal standard [II] was added to each plasma sample. A 2-ml volume of 1 *M* phosphate buffer pH 11 and 6 ml of chloroform were added and the tubes were shaken on a variable-speed reciprocating shaker (Eberbach, Ann Arbor, MI, U.S.A.) for 15 min. The sample tubes were centrifuged, the aqueous layer was discarded, the organic phase was transferred to a 5-ml centrifuge tube (Pyrex No. 8061), and the organic solvent was evaporated at 50°C under a stream of nitrogen. The residue was reconstituted in 50 μl of ethyl acetate and a (5-μl) aliquot of this solution was injected into the GC—MS system.

Stability study

Sufficient compound [I] was added to several control plasma samples to give a drug concentration of 3 ng ml^{-1}. The fortified plasma samples were stored at —20°C in 16-ml tubes with Teflon-lined screw-caps.

Efficiency of extraction

The recovery of compound [I] was determined by adding known amounts of ^{14}C-labelled compound [III] to three individual 2-ml plasma control samples and extracting the samples using the procedure described. The radioactivity in the organic layer was counted on a Mark III liquid scintillation spectrometer (Searle Analytic, Des Plaines, IL, U.S.A.) using 10 ml of Aquasol (New England Nuclear, Boston, MA, U.S.A.) as counting solution.

Effect of the blood collection container on extractability of [I]

Control human blood (100 ml) was spiked with approximately 700 ng of compound [III] (7 ng ml^{-1}). The stoppers of six Vacutainers (B & D., Vacutainer No. 6527) were removed. Aliquots (5 ml) of spiked blood were transferred into the Vacutainers which were then restoppered. Additional 5-ml aliquots of blood were transferred to six of the same 16-ml culture tubes (Pyrex No. 9825) which were used for plasma extractions. The culture tubes were capped and all twelve tubes were placed on a horizontal shaker and gently shaken for 30 min. Three of the Vacutainer blood samples and three

of the culture tube blood samples were centrifuged and plasma collected. A 1-ml aliquot each of the six plasma samples and 1 ml each of the remaining whole blood samples were radioassayed according to the procedure described previously.

Instrumentation

GC—MS analysis was performed using a Finnigan Model 1015 mass spectrometer operated in the CI mode. Data were acquired using a Finnigan 6000 data system with revision I software. GC separations were performed on a glass column (1 m × 1 mm I.D.) packed with 3% OV-17 on 120—140 mesh Gas-Chrom Q (Applied Science Labs., State College, PA, U.S.A.). Prior to use, the column was conditioned with no flow for 2 h at 300°C and with a 60 ml min^{-1} helium flow overnight at 280°C. The column was mounted in a Finnigan 9500 gas chromatograph and was connected to the mass spectrometer via a direct transfer line. Methane was used as carrier gas at a pressure of 1.2 kg m^{-2}. The ion source pressure of methane was approximately 53 Pa. The reagent gas (ammonia) was added using the direct insertion probe gas inlet to give a total ion source pressure of 80 Pa. The temperatures of the injection port, GC oven and transfer line were 275°C, 280°C and 250°C, respectively. Under these conditions, compound [I] had a retention time of 1.3 min. The filament was operated at an ionization energy of 70 eV and an emission current of 0.92 MA. The voltage to the conversion dynode was supplied by a Hewlett-Packard Model 6516A, 0—3000 V, power supply. Typically, the conversion dynode electron multiplier was operated at 1.9 kV. The electron multiplier was operated at 1.8 kV and the preamplifier was set to 10^{-8} A/V.

The slope (m) and intercept (b) values from a least-squares analysis of the m/z 247 to m/z 250 ion ratio versus amount added data from the calibration curve samples were used to calculate the amount (x) of [I] in an experimental sample from the measured ion ratio (R) using the equation $x = (R - b)/m$.

RESULTS AND DISCUSSION

Ammonia—methane CIMS spectra of [I] and [II] are shown in Fig. 1. As expected, the mass spectra consist principally of MH^+ ions. Fig. 2 shows typical selected ion current profiles from the analysis of plasma taken from a subject both before and after a dose of [I]. The analysis of the pre-dose plasma gave no response in the selected ion current profile corresponding to [I].

Linear calibration curves (correlation coefficients >0.99) were observed for the determination of between 2 and 50 ng/ml of [I] using 20 ng ml^{-1} of [II] as the internal standard. The precision and accuracy of the method were determined by analyzing samples of human control plasma which were fortified to give concentrations of [I] of 2, 5, 10, 20 and 50 ng ml^{-1}. The samples were prepared and analyzed in duplicate on three different days. The inter-assay precision of calibration curves is shown in Table I. The mean relative standard deviation for all the concentrations used to prepare the calibration curve was 4.7%. Mean intra-assay precision was 4.7% (Table II). The lower limit of quantitation of the assay is 2 ng ml^{-1} using 2.0 ml of human plasma. The extractability of [I] from human plasma was 88—89%.

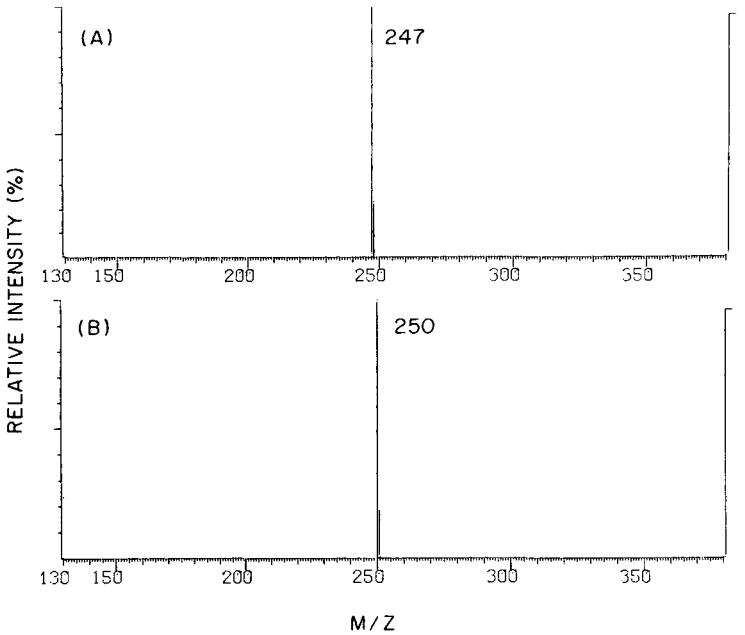

Fig. 1. Ammonia—methane CI mass spectra of [I] (A) and its trideuterated analogue [II] (B).

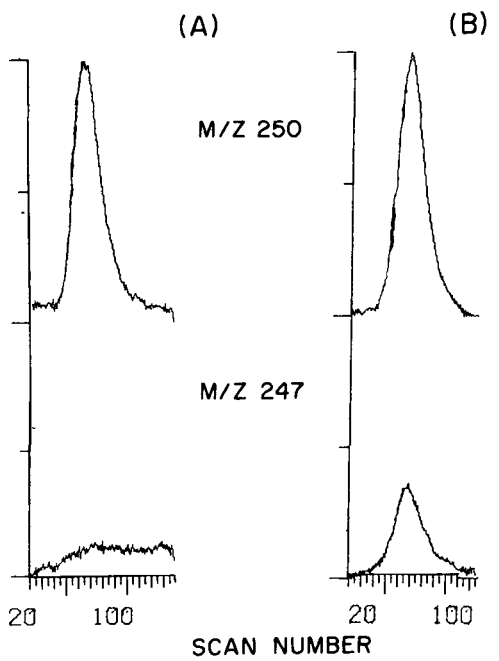

Fig. 2. Ion chromatograms from the analysis of 1 ml of plasma from a subject who had received a single 5-mg oral dose of [I]. (A) Plasma taken just prior to dosing; (B) plasma taken 30 min post dosing. The concentration of [I] in this last sample was 8.2 ng ml^{-1}. The ions at *m/z* 247 and 250 represent [I] and [II], respectively. For these ion chromatograms, 1 μl out of 50 μl was inejcted.

TABLE I

INTER-ASSAY VALIDATION DATA FOR [I] IN PLASMA

Calibration curve	Concentration (ng ml^{-1})				
	2	5	10	20	50
1	2.0	5.1	9.4	19.0	51.6
	2.0	5.8	9.6	19.3	50.5
2	1.9	4.9	9.3	20.4	50.2
	2.3	5.0	9.9	19.9	50.2
3	2.1	5.0	9.8	20.4	49.6
	1.8	5.0	10.3	20.4	49.6
Mean	2.0	5.1	9.7	19.9	50.3
± S.D.	0.17	0.33	0.37	0.62	0.74
R.S.D.* (%)	8.5	6.5	3.8	3.1	1.5
			Mean R.S.D. =		4.7

*R.S.D. = relative standard deviation.

TABLE II

INTRA-ASSAY VALIDATION DATA FOR [I] IN PLASMA (n=3)

Concentration added (ng/ml^{-1})	Calculated concentration (ng/ml^{-1} ± S.D.)	R.S.D. (%)
2	2.3 ± 0.12	5.2
5	4.7 ± 0.51	10.9
10	9.7 ± 0.31	3.2
20	19.0 ± 0.43	2.3
50	51.3 ± 1.06	2.1
	Mean R.S.D. =	4.7

The effect of the collection container (B & D, Vacutainer No. 6527) on assay accuracy was evaluated. For blood samples fortified with 7 ng ml^{-1} of [I], the mean concentration of [I] (± S.D.) determined was 6.6 ± 0.1 ng ml^{-1} for samples exposed to the culture tubes and 6.6 ± 0.2 ng ml^{-1} for samples exposed to the Vacutainers. In plasma, the mean [I] concentration (± S.D.) determined was 5.2 ± 0.2 ng ml^{-1} for the blood samples exposed to the culture tubes and 5.2 ± 0.2 ng ml^{-1} for the blood samples exposed to the Vacutainers. Thus, the exposure of the blood samples to the Vacutainers caused no artifactual increase or decrease in the concentration of [I]. Assuming a hematocrit (Ht) of 0.45, the red blood cell/plasma partition ratio for [I] was calculated to be approximately 1.6.

The stability of [I] in plasma on storage at −20°C over a 2-week storage period was demonstrated by reanalysis of the plasma samples fortified with [I]. Table II contains the results of ten repeat determinations of the plasma concentration of [I] assayed on three separate days. The mean (± S.D.) concentration of [I] determined was 3.3 ± 0.2 (R.S.D. = 6.1%). Compound [I] was stable in plasma for at least 2 weeks.

TABLE III

PLASMA CONCENTRATION (ng ml^{-1}) OF [I] FROM THE REPEAT ANALYSES OF A FORTIFIED HUMAN PLASMA SAMPLE

Control human plasma was pooled (100 ml) and spiked with 300 ng of [I] (i.e. 3 ng ml^{-1}).

Date	No.	[I] found (ng ml^{-1})	$\frac{\text{Found} - \text{mean}}{\text{mean}} \times 100$
11/9/82	1	3.5	+6.1
	2	3.5	+6.1
	3	3.4	+3.0
	4	3.6	+9.1
11/10/82	1	3.3	0
	2	3.1	–6.1
	3	3.1	–6.1
	4	3.3	0
11/23/82	1	3.3	0
	2	3.2	–3.0
	Mean ± S.D. = 3.3 ± 0.2		(R.S.D. = ±6.1)

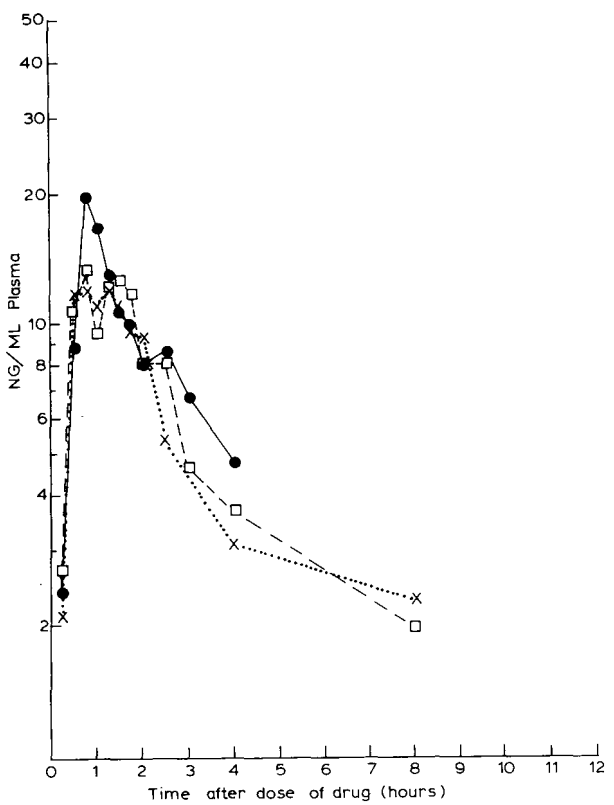

Fig. 3. Plasma concentration–time curves of [I] after oral administration of a 5-mg oral dose of [I] to three healthy volunteers.

The concentrations of [I] in the plasma of three human subjects given single 5-mg oral doses of drug are shown in Fig. 3. The mean (± S.D.) peak concentration of [I] was 17.5 ± 2.3 ng ml^{-1}. The peak plasma concentrations were observed at 1—2 h after drug administration and fell to below the limit of quantitation of the assay (<2 ng ml^{-1}) from 4—8 h post-dose. The half-life in these three subjects was approximately 2 h.

To summarize, a GC—MS method for [I] was developed which is sensitive to 2 ng ml^{-1} of plasma. The assay was used to determine [I] in plasma for 4—8 h following a single oral dose of the drug.

ACKNOWLEDGEMENTS

The author wishes to thank Dr. W.A. Garland for his kind encouragement and Ms. K. Schreck for her assistance in the preparation of this manuscript.

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Journal of Chromatography, 277 (1983) 347—351
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1813

Note

Gas chromatographic routine analysis of five tricyclic antidepressants in plasma

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(First received March 7th, 1983; revised manuscript received June 3rd, 1983)

Since the beginning of the clinical trials with tricyclic antidepressants, clinicians have been looking for a simple and fast procedure for monitoring the plasma levels of these drugs. Several analytical methods have been proposed for the determination of drugs and their metabolites, as reported in a recent review [1]; routinely, they are most frequently assayed by gas—liquid chromatography (GLC) [2—5] or, more recently, by high-performance liquid chromatography (HPLC) [6—10].

Gas chromatographic procedures, however, often yield variable results because of some problems in concentration of organic extracts or in derivatisation of demethylated metabolites. We therefore report a simple and reliable gas chromatographic method with nitrogen detection which allows the determination of plasma therapeutic concentrations of amitriptyline, nortriptyline, imipramine, desipramine or clomipramine and requires no secondary amine derivatisation or organic solvent evaporation.

EXPERIMENTAL

Chemicals and reagents

Amitriptyline and nortriptyline were supplied by Merck, Sharpe and Dohme Research Lab. (Rahway, NJ, U.S.A.), imipramine, desipramine, maprotiline and clomipramine by Ciba-Geigy (Basle, Switzerland).

The reagents used in the extraction procedure were of analytical grade. *n*-Heptane was purified as reported in a previous paper [2].

Stock standard solutions were prepared by dissolving in 10 ml of water, 10 mg, calculated as free base, of amitriptyline, nortriptyline, imipramine, desipramine, maprotiline or clomipramine. These solutions are stable at 4°C for at least six months.

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Maprotiline or desipramine were used as internal standards: working solution was prepared by diluting the stock solution in water (100 ng in 10 μl).

Gas chromatography

The analysis was carried out on a Fractovap Series 2200 chromatograph (Carlo Erba) equipped with a nitrogen—phosphorus detector and Minigrator digital integrator.

The column was a 120 cm × 3 mm I.D. silanized glass column rinsed with methanolic potassium hydroxide 0.1% (v/w) and packed with 10% OV-17 on Gas-Chrom Q (100—120 mesh) washed with methanolic potassium hydroxide 0.1% (v/w).

The instrument was set at the following conditions: column temperature, 235°C, detector temperature 500°C, injection port temperature 290°C, hydrogen flow 3 ml/min, carrier gas (helium) flow 40 ml/min, air flow 200 ml/min.

Extraction procedure

All glassware used was rinsed with methanol containing 0.1% of potassium hydroxide. To 1 ml of serum or plasma 10 μl of internal standard, 1 ml of 0.5 *M* sodium hydroxide, 6 ml of *n*-heptane and 0.3 ml of isoamyl alcohol were

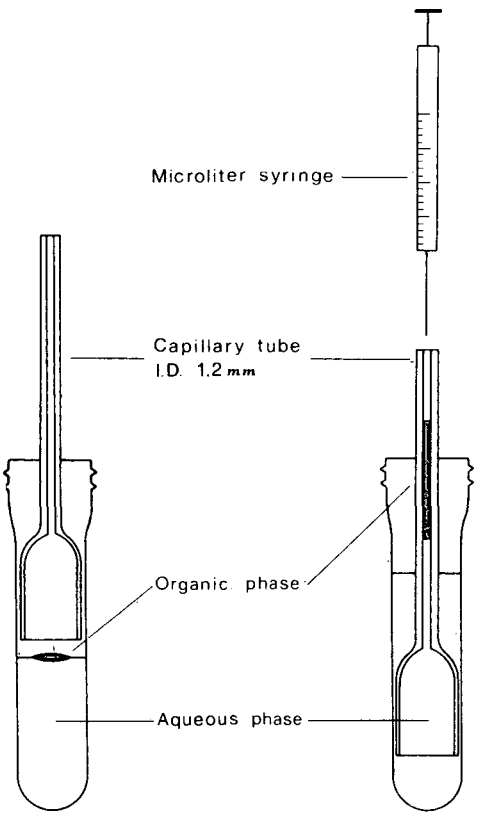

Fig. 1. Apparatus for the recovery of a small volume of organic phase.

added. After extraction for 2 min in a vortex mixer and centrifugation at 2500 *g* for 10 min, the test tube was placed in glycol antifreeze solution at −25°C. The organic phase, separated from the frozen aqueous phase, was reextracted with 2.5 ml of 0.2 *M* hydrochloric acid for 1 min and centrifuged. The acid extract was transferred to a 15-ml specially designed test tube and after addition of 2.5 ml of 0.5 *M* sodium hydroxide and 25 μl of *n*-heptane, was extracted for 1 min. After centrifugation, a bubble cap with a capillary collector (Fig. 1) was inserted and 10 μl of *n*-heptane were injected into the chromatograph.

The concentrations of drugs in unknown samples were determined by calculating the ratios of each drug peak area with that of the internal standard. These ratios were then compared with equivalent ratios obtained from a chromatogram of known quantities of a standard mixture, extracted and separated using the described procedure.

RESULTS AND DISCUSSION

A typical chromatogram of a plasma sample obtained from an amitriptyline-treated depressed in-patient, is shown in Fig. 2.

The detection limit for all the antidepressants studied was between 10 and 20 ng: the retention times of the compounds studied are reported in Table I.

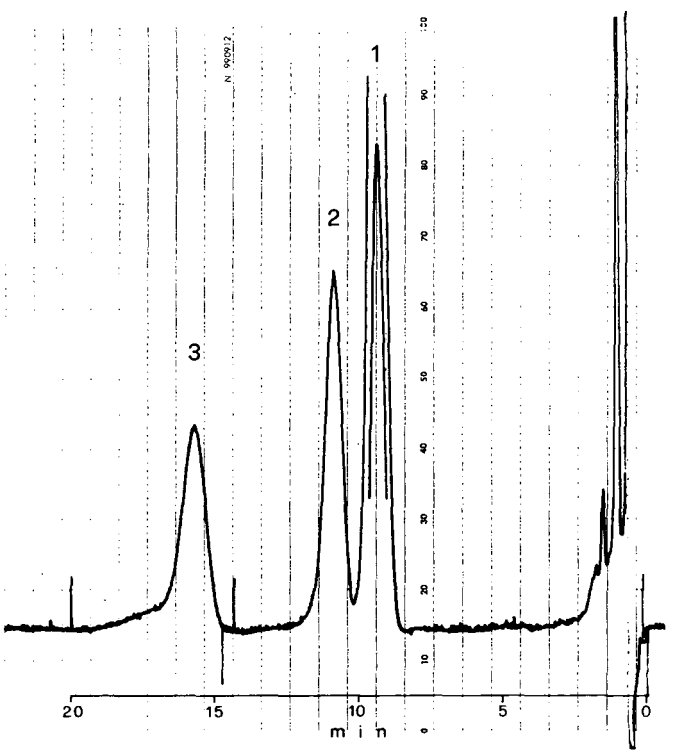

Fig. 2. Chromatogram of a plasma sample from a depressed in-patient treated with amitriptyline (50 mg, intramuscularly, twice a day): 1 = amitriptyline, 308 ng; 2 = nortriptyline, 168 ng; and 3 = internal standard (maprotiline, 100 ng). Chromatographic conditions: see text.

Drug-free plasma samples of healthy subjects and of untreated in-patients were extracted and analyzed for possible interference by endogenous constituents: no background interference was observed. Furthermore, we also noted that the commonly used benzodiazepines (nitrazepam, flurazepam) did not interfere with the analysis.

Table II gives the results obtained when the described procedure was applied to drug-free blood samples spiked with increasing amounts of amitriptyline, nortriptyline or imipramine, desipramine with maprotiline as internal standard or with clomipramine using desipramine as internal standard.

The assay precision calculated from six samples had a coefficient of variation ranging from 3.1% to 10.5%.

TABLE I

RETENTION TIMES OF STANDARDS

Compound	Time (sec)
Amitriptyline	573
Imipramine	621
Nortriptyline	666
Desipramine	731
Maprotiline	972
Clomipramine	1090

TABLE II

PRECISION OF THE ASSAY IN THE DETERMINATION OF AMITRIPTYLINE, NORTRIPTYLINE, IMIPRAMINE, DESIPRAMINE AND CLOMIPRAMINE ADDED TO HUMAN PLASMA SAMPLES

n=6.

Compound	Amount added (ng/ml)	Amount found (ng/ml, mean ± S.D.)	C.V. (%)
Amitriptyline*	50	50.3 ± 4.97	9.87
	100	91.9 ± 5.86	6.37
	200	201.0 ± 14.12	7.24
Nortriptyline*	50	49.8 ± 4.78	9.59
	100	98.2 ± 3.41	3.46
	200	198.8 ± 11.09	5.57
Imipramine*	50	50.8 ± 3.95	7.77
	100	100.0 ± 5.50	5.50
	200	198.2 ± 15.27	7.70
Desipramine*	50	52.0 ± 1.60	3.07
	100	99.2 ± 4.80	4.83
	200	197.6 ± 10.00	5.06
Clomipramine**	50	49.1 ± 2.21	4.49
	100	101.9 ± 10.70	10.49
	200	202.0 ± 13.11	6.48

*Internal standard: maprotiline.
**Internal standard: desipramine.

TABLE III

PLASMA CONCENTRATION OF AMITRIPTYLINE AND NORTRIPTYLINE IN CHRONICALLY TREATED* DEPRESSED WOMEN

Day of treatment	Patient No:	Amitriptyline (ng/ml)						Nortriptyline (ng/ml)					
		1	2	3	4	5	6	1	2	3	4	5	6
7		70	90	95	120	145	280	20	30	20	85	30	40
14		80	140	140	130	190	410	20	60	25	150	50	110
21		70	130	140	120	185	435	20	50	30	155	50	160
28		55	120	120	100	190	445	20	55	30	125	45	135

*For treatment conditions see text.

The described method was applied to the determination of plasma concentrations of amitriptyline and nortriptyline of several depressed in-patients chronically treated with amitriptyline 50 mg intramuscularly, twice a day. Blood samples were drawn in the morning, 10 h after the last administration. The data are reported in Table III.

In conclusion, although other analytical methods for tricyclics appear to be adequate, the modification presented in this report resulted in an improved system. First, the organic solvent does not need to be evaporated since the concentration of drug in the small final volume is sufficient for detection. Second, the evaluation of the demethylated metabolites nortriptyline and desipramine is performed without derivatisation. Finally, drugs are evaluated in a small blood sample and in a total assay time of approximately 2 h.

ACKNOWLEDGEMENT

This work was supported in part by CNR Grant No. 82.01068.83: research project "Monitoraggio degli aspetti biologici degli psicofarmaci".

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Journal of Chromatography, 277 (1983) 352—355
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1801

Note

Determination of ethoheptazine in human post mortem material

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(First received March 1st, 1983; revised manuscript received May 6th, 1983)

Ethoheptazine, or 1-methyl-4-carboxy-4-phenylhexamethylene imine, is a synthetic analgesic which has been used clinically for over a decade in the treatment of mild to moderate pain of varied etiology. Reports [1—5] indicate that ethoheptazine is an effective analgesic which is well tolerated and non-addictive. The few clinical studies suggest that ethoheptazine provides effective analgesia following administration of doses ranging from 50 to 150 mg three of four times a day. After oral therapy the incidence of side effects is relatively low. The metabolic fate of ethoheptazine in man is unknown.

Evidence for metabolic pathways is mainly obtained from animal studies. Ethoheptazine is metabolized by at least three routes, including hydrolysis to the corresponding acid, oxidation to the hydroxy derivative which may further undergo hydrolysis, and a possible N-desmethylation to the corresponding nor-derivative which subsequently may be hydrolysed. Biotransformation was established by identifying respective metabolites by either tracer techniques, paper chromatography, infrared spectroscopy or electrophoresis.

In this paper a fatal case of drug overdose is described. An unknown amount of ethoheptazine was involved. No data have been reported on the concentrations of ethoheptazine in human biological material (plasma, urine, tissue) after therapeutic or toxic dosage. A gas chromatographic method was used to determine ethoheptazine in post mortem blood, liver, kidney, spleen and brain.

0378-4347/83/$03.00

MATERIALS AND METHODS

Apparatus

An Intersmat gas chromatograph Model 120 F was used, equipped with an alkali flame detector. The column was a 2 m × 3.2 mm I.D. glass column with 3% OV-17 on Chromosorb W HP. Chromatograms were recorded on a Varian instrument Model A 25.

Conditions

Column temperature 225°C; injector temperature 240°C; detector temperature 290°C; gas-flow 30 ml N_2/min.

ANALYTICAL PROCEDURE

1. Prepare the following solutions:
   (A) Dissolve 230 mg of pethidine HCl as internal standard in 100 ml of distilled water. Dissolve 453.5 mg of ethoheptazine citrate in 100 ml of distilled water. Dilute 1 ml of this solution with 100 ml of distilled water.
   (B) Extraction solvent. Mix diethyl ether—*n*-hexane—isopropanol (4:1:0.1).
   (C) Ammonium hydroxide. Dilute 25 ml of 25% (w/v) NH_4OH to 100 ml with distilled water.
2. Homogenize about 2 g of minced tissue with 5 ml of the internal standard at 0°C with a Potter Elvejehm homogeniser. Measure the volume of the homogenate (I).
3. Centrifuge at 3000 *g* for 10 min. Transfer to a clean test tube the clear supernatant and measure its volume (II).
4. Add 0.25 ml of the diluted ammonia solution for each milliliter of the supernatant (II), and 6.0 ml of the extraction solvent mixture.
5. Vortex for 2 min. Centrifuge at 3000 *g* for 10 min.
6. Take an aliquot of the organic layer and evaporate to dryness at room temperature. Dissolve the residue in 200 μl of ethanol and inject 1 μl into the gas chromatograph.

Calibration curve

Control specimens (2 g or 2 ml) of liver, kidney, spleen, brain and plasma containing 2—20 μg of ethoheptazine were prepared and analyzed according to the described method. Peak height ratios of ethoheptazine to the internal standard were calculated and plotted against the concentration of ethoheptazine. The equation of the calibration curve was $Y = 0.102X - 0.057$ with a correlation of 0.993.

RESULTS AND DISCUSSION

Using the described procedure the peaks of ethoheptazine and internal standard were completely separated from those of the control plasma and solvent. Fig. 1 shows a typical chromatogram of ethoheptazine (a) and

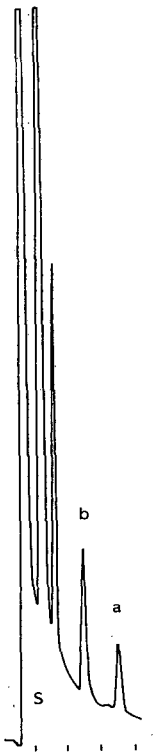

Fig. 1. Chromatogram of extract from plasma. S = solvent, b = pethidine, a = ethoheptazine.

TABLE I

CONCENTRATIONS OF ETHOHEPTAZINE FOUND IN VARIOUS POST MORTEM SPECIMENS

Tissue	Ethoheptazine (μg/g)
Liver	10.0
Brain	4.5
Spleen	3.1
Kidney	2.4
Blood	15.0

pethidine (b) in plasma. Peaks not assigned are from biological matrix or solvent.

The minimum detectable concentration of ethoheptazine in plasma was 1.0 μg/ml. The coefficients of variation over the concentration range 1.0—10 μg/ml plasma were measured to be 8.2—3.5%.

Recoveries of ethoheptazine from spiked plasma samples over the calibration range were 85 ± 7%. Also recovery studies were performed in the following tissues: liver, spleen, kidney and brain. After homogenizing and centrifuging the respective tissues a solid and a liquid layer are obtained. After extraction of

both the aqueous phase and the solid layer for each tissue, total recovery of ethoheptazine was 80 ± 18%.

The described method proved to be adequate to study the post mortem distribution of ethoheptazine in biological material. In our case, the respective concentrations of the drug in the different post mortem specimens are shown in Table I. Neither chromatographic nor mass spectrometric research revealed any possible metabolites or degradation products of ethoheptazine.

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Journal of Chromatography, 277 (1983) 356—360
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1810

Note

New method for the gas chromatographic determination of valproic acid in serum

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(First received January 4th, 1983; revised manuscript received June 3rd, 1983)

Valproic acid is an anticonvulsant drug which is widely used in epilepsy. Determination of valproic acid in serum is required in epilepsy therapy for efficient control of seizures. Many gas chromatographic methods have been described for its determination. In some methods, valproic acid has been analysed after derivatization such as methylation [1—4], butylation [5], phenacylation [6, 7] and trimethylsilylation [8], and in others assayed in free form [9—16]. We have developed a novel method for measuring valproic acid in serum, in which valproic acid is derivatized into the hexafluoroisopropyl ester. This method gives a sharp peak of valproic acid on a gas chromatogram at low temperature, 80°C. It is possible to determine less than 0.5 μg of valproic acid using 0.1 ml of serum sample.

EXPERIMENTAL

Reagents and standards

Derivatizing reagents were hexafluoroisopropanol and trifluoroacetic anhydride which were obtained from Tokyo Kasei (Tokyo, Japan). The internal standard solution was octanoic acid (*n*-caprylic acid), 5 μg/ml in hexane. Standard solution of sodium valproate was prepared containing 5, 10, 20, 30, 50 and 100 μg/ml as valproic acid in water. For the extraction step hexane and 6 *N* hydrochloric acid were used.

0378-4347/83/$03.00

Apparatus and conditions

A Shimadzu GC-4BPF gas chromatograph equipped with a hydrogen flame ionization detector and a glass column 2.0 m × 3 mm I.D. packed with 3% OV-1 on 80—100 mesh were used, with a column temperature of 80°C, a detector temperature of 120°C and a carrier gas (nitrogen) flow-rate of 40 ml/min.

Standard procedure

To 0.1 ml of serum sample in a 1.5-ml centrifuge tube were added 0.3 ml of water, 1 drop of 6 *N* hydrochloric acid and 0.8 ml of hexane solution containing octanoic acid as an internal standard. The tube was shaken vigorously for 1 min and centrifuged at 7000 *g* for 4 min. The lower layer was removed. The hexane layer was dried over a small amount of anhydrous sodium sulphate, transferred to a tapered tube and evaporated just to dryness. To the residue were added 0.1 ml of hexafluoroisopropanol and 0.02 ml of trifluoroacetic anhydride, and the mixture was allowed to stand at room temperature for 30 min. One to 3 μl of the reaction mixture were injected into the gas chromatograph.

Drug-free serum spiked with standard solution of valproic acid (0.5—10 μg) was treated similarly to the standard procedure to establish calibration curves. The unknown concentration was determined by comparison of the valproic acid/octanoic acid peak height ratios with those of the calibration curve.

Procedure without derivatization (outline)

To 0.5 ml of serum were added 0.5 ml of water, 0.1 ml of 6 *N* hydrochloric acid and 0.8 ml of a mixture of chloroform—isobutanol (9:1, v/v) containing an internal standard (biphenyl). The mixture was vortexed for 1 min and then centrifuged at 1500 *g* for 10 min. The aqueous (upper) layer was removed and an aliquot (1—2 μl) of the organic phase was injected directly into the gas chromatograph. Gas chromatographic conditions: 10% SP-1000 (1-m long column), 160°C.

RESULTS AND DISCUSSION

A mixture of hexafluoroisopropanol and trifluoroacetic anhydride was used successfully for the derivatization of carboxylic acid where it was converted into hexafluoroisopropyl ester [17]. This technique was applied to the gas chromatographic determination of homovanillic acid [18, 19], vanillylmandelic acid [18, 19], bile acids [20, 21] and captopril [22]. In this reaction, trifluoroacetic anhydride acts as a catalyst, therefore the presence of a small amount of trifluoroacetic anhydride must be enough for derivatization. In most cases except one report [22], however, the proportion of trifluoroacetic anhydride to hexafluoroisopropanol in the reagent mixture is the same or twice as much. It should be noted that use of too much trifluoroacetic anhydride may result in a by-product. In the case of valproic acid, it had a tendency to give a side-peak on the chromatogram with increasing concentration of trifluoroacetic anhydride in the reagent mixture. This side-peak showed the same retention time as that in a case treated only with trifluoroacetic anhydride.

An appropriate concentration ratio of hexafluoroisopropanol/trifluoroacetic anhydride was 5:1.

Hexafluoroisopropyl ester has been considered to be superior with regard to simplicity of preparation, volatility and absence of artifacts. However, in most reports except for bile acids [20, 21], high reaction temperatures (50—75°C) have been used. It should be noted that a higher temperature does not give reproducible results, probably because of loss of reagents during derivatization. Esterification of valproic acid and octanoic acid with the reagent mixture proceeded readily at room temperature and was completed in 10 min. The derivatives were stable for at least one day at room temperature.

A mixture of 2,2,3,3,3-pentafluoro-1-propanol and pentafluoropropionic anhydride (1:4, v/v) was used in the gas chromatographic determination of indomethacin [23]. Recently, we found that a mixture of 2,2,2-trifluoroethanol and trifluoroacetic anhydride (5:1, v/v) also reacted readily with valproic acid to produce the corresponding ester, and no by-products were formed during derivatization. Compared with hexafluoroisopropanol, 2,2,2-trifluoroethanol is inexpensive, thus much more economical.

We have evaluated a number of solvents — hexane, ethyl acetate, benzene, chloroform and their mixtures — for the extraction of the drug. It was found that hexane was the most satisfactory solvent because the best yield (about 70%) was obtained by a single extraction without salting-out.

TABLE I

COMPARISON OF THE PROPOSED PROCEDURE (METHOD A) WITH THE PROCEDURE IN THE UNDERIVATIZED FORM (METHOD B) FOR THE DETERMINATION OF VALPROIC ACID

Serum samples	No.	Valproic acid added (μg/ml)	Valproic acid found (μg/ml)	
			Method A	Method B
Drug-free sera	1	12	13.5	12.5
	2	24	28.8	26.4
	3	48	48.9	47.2
	4	54	54.5	52.8
	5	105	110.7	108.4
	6	135	135.2	136.4
	7	144	144.0	147.5
	8	150	151.5	163.6
	9	162	159.0	168.5
	10	180	171.6	181.5
Patients' sera	1		32.9	30.7
	2		33.3	33.3
	3		33.9	33.8
	4		39.0	33.9
	5		46.6	41.8
	6		46.9	45.5
	7		58.3	51.1
	8		62.6	60.0
	9		68.9	64.7
	10		69.2	68.2

The next step, evaporation of the extract to dryness, was effective with regard to concentrating the drug. In order to determine small amounts of valproate, the extract was evaporated to dryness under reduced pressure at room temperature by means of a rotatory evaporator, and the residue was derivatized. Valproic acid is volatile and we must be careful about loss by evaporation. However, use of octanoic acid, which has similar properties to valproic acid, as an internal standard can avoid the problem derived from loss by evaporation.

Linear calibration curves passing through the origin were obtained for plots of valproic acid/internal standard peak height ratio versus concentration in the range 0.5—10 μg per 0.1 ml of serum. The correlation coefficient was 0.9996 using the method of least squares. The minimal concentration detectable for valproic acid was 2 μg/ml of serum. The reproducibility of the method at the level 5 μg/ml of serum was evaluated by replicate analyses of an identical sample. The coefficient of variation was less than 3%.

Human drug-free serum samples spiked with valproic acid (range of concentrations: 12—180 μg/ml) were measured by the proposed procedure (Method A), and the values measured are compared with those obtained with a procedure without derivatization (Method B) in Table I. The comparison shows no significant difference in accuracy between results obtained by the two methods. The coefficient of correlation is 0.9986.

This method was applied to measure routine serum levels of valproic acid in some epileptic patients who were receiving sodium valproate. The results

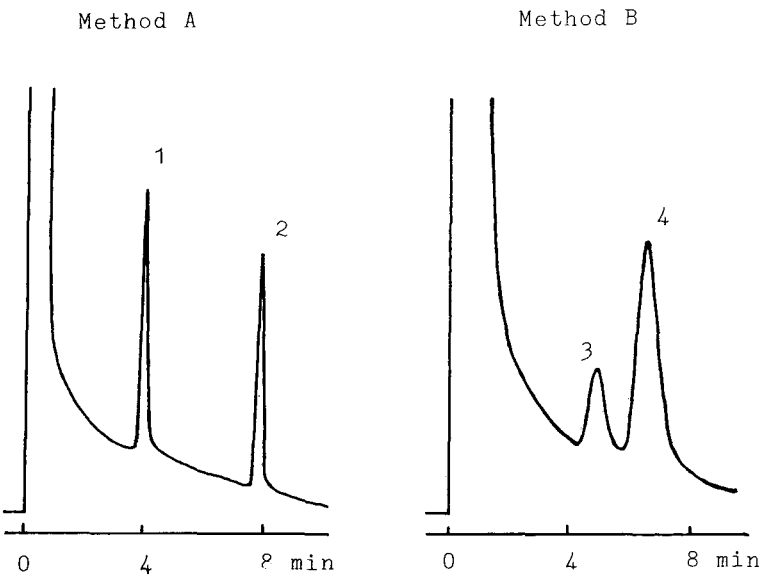

Fig. 1. Chromatograms from analyses for sodium valproate in a patient's serum by the proposed procedure (Method A) and the procedure in the underivatized form (Method B). Peaks: 1 = hexafluoroisopropyl valproate, 2 = hexafluoroisopropyl octanoate (internal standard), 3 = free valproic acid, 4 = biphenyl (internal standard). Peaks 1 and 3 correspond to about 33 μg of valproic acid in 1.0 ml of serum. Conditions: Method A, 3% OV-1, column 2 m long, 80°C, 0.1 ml of serum; Method B, 10% SP-1000, column 1 m long, 160°C, 0.5 ml of serum.

are also compared with those obtained with Method B in Table I and they almost agreed.

Fig. 1 shows chromatograms of a patient's serum by Method A and Method B. Compared with the procedure in the underivatized form, the proposed method was a little more time-consuming for routine use, but produced sharp, well-shaped peaks, improved the chromatographic properties of valproic acid and resulted in a reduction in the volume of serum samples required for analysis. The determination of valproic acid in serum at concentrations down to 0.2 μg per 0.1 ml was achieved. The method is therefore also applicable if the available sample volume is severely limited and can thus be used for determination of the drug in children, to whom it is frequently given.

A great improvement in sensitivity with electron-capture detection was expected for the derivatization. But, the sensitivity of the derivative toward electron-capture detection was found not to be as much as expected compared with that using the hydrogen flame ionization detector.

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Journal of Chromatography, 277 (1983) 361—367
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1787

Note

Simultaneous derivatisation of carboxyl and hydroxyl groups of a new antiphlogistic drug for its determination by electron-capture gas chromatography

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(First received February 9th, 1983; revised manuscript received May 20th, 1983)

CGP 6258 (5-benzoyl-2,3-dihydro-6-hydroxy-1H-indene-1-carboxylic acid) is a new compound with analgesic and antiphlogistic properties. A simple, but sensitive gas chromatographic (GC) method for its quantitative determination in plasma and urine has been developed. The method described here is based on the single-step derivatisation of hydroxy acids described by Brooks et al. [1] and also for amino acids by Wilk and Orlowski [2]. The reaction is based on the simultaneous esterification of the carboxyl group and the acylation of the hydroxy group. The alcohol used for the esterification was 2,2,3,3,3-pentafluoro-1-propanol (PFP). Heptafluorobutyric anhydride (HFBA) is the acylating agent, but it also catalyzes the esterification. The resulting derivative is stable, volatile and highly sensitive when determined using an electron-capture detector. Due to the selectivity of this derivatisation procedure, no purification steps are necessary. The method consists of only three steps: extraction, derivatisation and chromatography.

EXPERIMENTAL

Reagents and chemicals

The following were used: CGP 6258 ($C_{17}H_{14}O_4$; mol. wt. 282.30) and CGP 7726 (internal standard) ($C_{18}H_{16}O_4$; mol. wt. 296.33), both as solutions in 0.01 mol/l NaOH (for structures see Fig. 1); 1 mol/l HCl (Laboratory grade Ciba-Geigy); toluene and *n*-heptane, both distilled over a 1-m Vigreux column; 2,2,3,3,3-pentafluoro-1-propanol (PCR Research Chemicals, Gainsville, FL, U.S.A.); heptafluorobutyric anhydride (Fluka; distilled over P_2O_5); pH 5 phthalate buffer solution (0.100 mol potassium hydrogen phthalate, 0.050 mol sodium hydroxide per liter); β-glucuronidase—arylsulfatase (Boehringer, Mannheim, F.R.G.).

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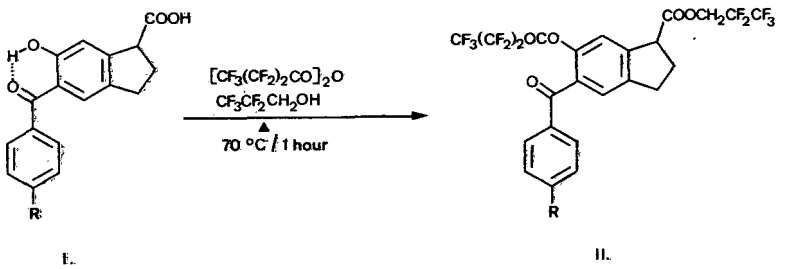

I. II.

Fig. 1. Structures of CGP 6258 (I; R = H), CGP 7726 (I; R = CH_3) and the respective derivatives (II).

Procedure

Plasma. A 1-ml plasma sample, 0.5 ml of internal standard solution (0.675 nmol CGP 7726 in 0.01 mol/l NaOH), 1 ml of 1 mol/l HCl and 5 ml of toluene are shaken for 15 min at 200 rpm on a mechanical rotary shaker. After brief centrifugation the organic phase is transferred into a clean vial and evaporated to dryness under a stream of nitrogen. To the dry residue, 0.1 ml of hepta-

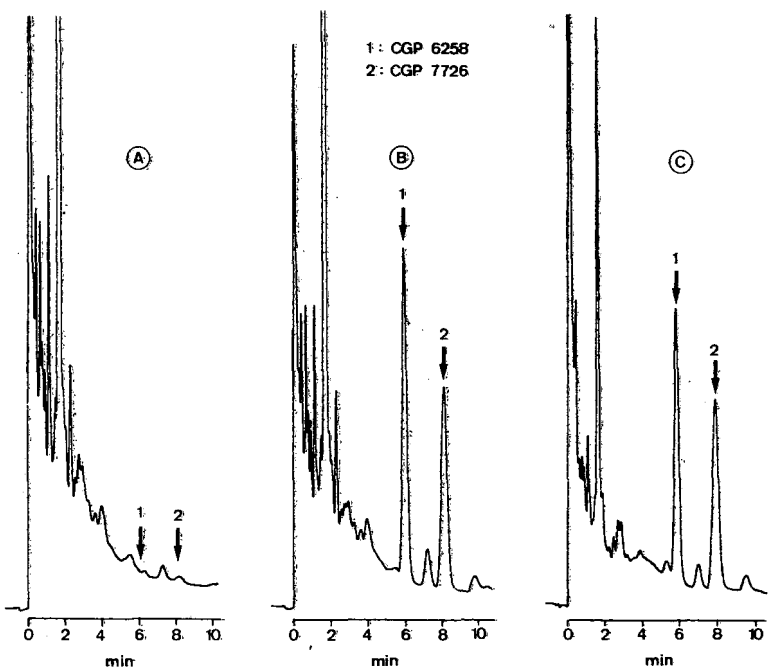

Fig. 2. Chromatograms of plasma extracts: (A) blank plasma (0.5 ml); (B) plasma (0.5 ml) spiked with 0.708 nmol of CGP 6258 and 0.675 nmol of internal standard; (C) plasma from a volunteer collected 10 h after a single oral dose of 300 mg of CGP 6258; 0.05 ml of plasma and 0.675 nmol internal standard were used for the determination. The sample contains 0.616 nmol of CGP 6258 which corresponds to 12.3 μmol/l.

fluorobutyric anhydride and 0.05 ml of pentafluoropropanol are added. The vial is stoppered and heated to 70°C for 1 h. Excess reagents are removed by evaporation to dryness under a stream of nitrogen at 40°C. The residue is redissolved in 1 ml of *n*-heptane and aliquots of 3—5 μl are injected into the gas chromatograph.

Urine (free and conjugated drug). Up to 0.5 ml of urine, 1 ml of phthalate buffer (pH 5) and 30 μl of β-glucuronidase—arylsulfatase are incubated by agitation in a waterbath at 37°C for 15 h. After hydrolysis, the internal standard is added and the sample processed as described for plasma.

Gas chromatography

The GC analysis was carried out on a Pye GCV instrument equipped with a ^{63}Ni electron-capture detector.

The column used was a 1.5 m × 4 mm I.D. Pyrex glass column packed with 3% OV-225 on Supelcoport 80—100 mesh. Temperatures were: column oven 220°C; injector 200°C; detector 350°C. The nitrogen carrier gas flow-rate was 40 ml/min.

Chromatograms of blank plasma and urine extracts (Figs. 2 and 3) illustrate that no biological constituents interfere with the quantitation of CGP 6258. Chromatograms of plasma and urine extracts, spiked and from pharmacokinetic studies are shown in Figs. 2 and 3.

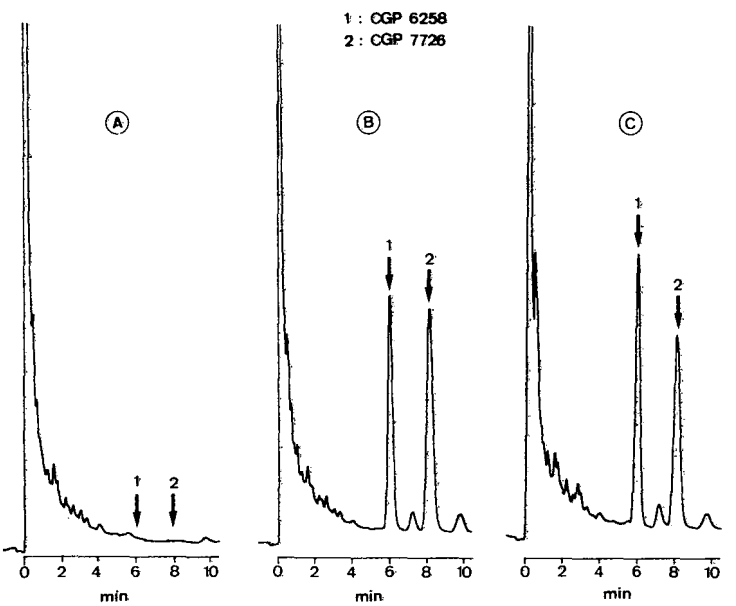

Fig. 3. Chromatograms of urine extracts: (A) blank urine (0.5 ml), after hydrolysis; (B) urine (0.5 ml) spiked with 0.708 nmol each of CGP 6258 and 0.675 nmol of internal standard, after hydrolysis; (C) urine fraction (8—10 h) from a volunteer after a single oral dose of 300 mg of CGP 6258; 0.2 ml of urine (diluted × 100) and 0.675 nmol of internal standard were used for the determination. The sample contains 0.762 nmol of CGP 6258 which corresponds to 381 μmol/l.

Calibration curves

Calibration curves for plasma and urine determinations were prepared as follows. Blank plasma or urine samples were spiked with solutions of CGP 6258 in 0.01 mol/l NaOH (0—0.708 nmol/sample). The samples were then processed as described. The peak height of the CGP 6258 derivative was divided by the peak height of the internal standard derivative and the ratio (H_x) plotted against initial CGP 6258 concentrations.

Calculation of the linear regressions [3] resulted in coefficient of correlation values (r) of 0.9940 for plasma and 0.9942 for urine. Standard errors of estimation (S_e) were 0.0574 H_x for plasma and 0.0496 H_x for urine.

Hydrolysis

Hydrolysis of conjugates was optimized by incubation of urine samples obtained from volunteers who had been treated with oral doses of CGP 6258, for various time periods and with various amounts of enzyme. The enzyme preparation β-glucuronidase—arylsulfatase (from *Helix pomatia*) gave optimal results. No increase of the free CGP 6258 was observed if additional enzyme was added after 7 h or if incubation time was prolonged.

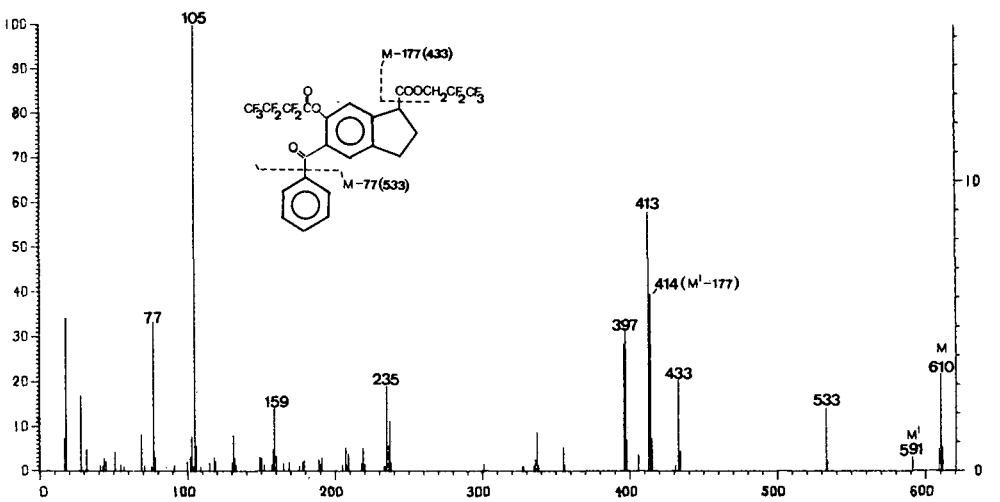

Fig. 4. Mass spectrum of the derivative formed by treatment of CGP 6258 with pentafluoropropanol and heptafluorobutyric anhydride. A molecular ion was observed at m/z 610. The pattern of the fragmentation is illustrated.

Stability

Plasma and urine samples remain unchanged for at least six months at —20°C in the dark. Stock solutions of CGP 6258 and internal standard in 0.01 mol/l NaOH are stable at 4°C for at least one week. The derivatives, in heptane, are stable for at least one week at 4°C.

Derivatisation

The reaction is based on the procedure described by Wilk and Orlowski [2] and was optimized for CGP 6258 (Fig. 1). Maximum response was achieved after reaction for 45 min at 70°C. An additional hour of reaction time has

no influence on the recovery. Thus, 1 h was chosen as the reaction time. The structure of the derivative was verified by mass-spectrometry (Fig. 4). Overall yield (extraction and derivatisation) was about 80%.

Recoveries, precision and limit of quantitation

Recovery of CGP 6258 and precision were evaluated by analysing spiked plasma and urine (with hydrolysis) samples. Forty samples each were prepared with CGP 6258 concentrations between 0.071 and 1.010 nmol/sample. The differences between the found and the initial concentrations ranged from −12% to +15% for plasma and from −7% to +16% for urine.

Calculation of the linear regression [3] between given and found concentrations resulted in coefficients of correlation (r) of 0.9949 for plasma and 0.9967 for urine. Standard errors of estimation (S_e) were 0.0290 nmol/sample for plasma and 0.0134 nmol/sample for urine.

The limit of quantitation is about 35 nmol/l.

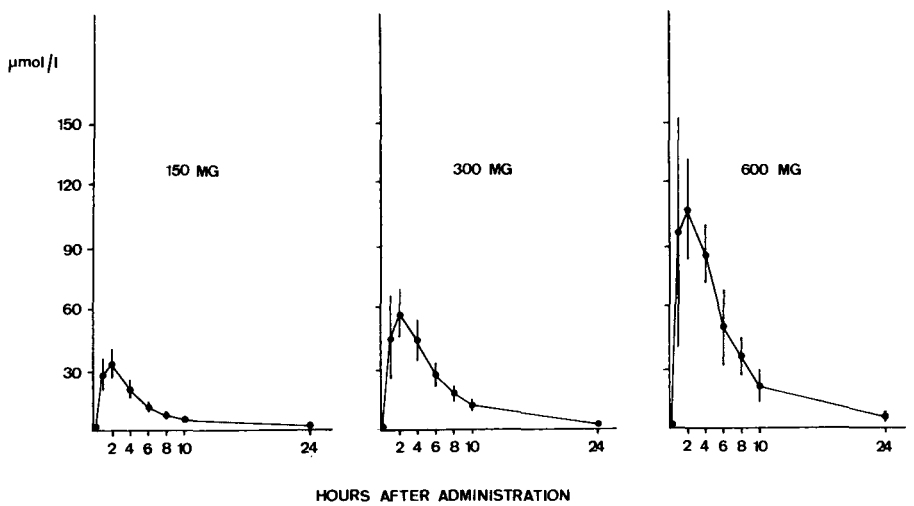

Fig. 5. Mean plasma concentration curves after 150, 300 or 600 mg oral doses in six healthy volunteers.

TABLE I

MEAN (± S.D.) AREAS UNDER THE CONCENTRATION CURVES (AUC), CALCULATED BY THE TRAPEZOIDAL RULE AND MAXIMAL CONCENTRATIONS (C_{max})

Dose (mg)	AUC (0—24 h) (µmol l^{-1} h)	C_{max} (µmol l^{-1})
150	198.7 ± 31.9	34.0 ± 5.3
300	408.4 ± 86.4	59.9 ± 11.0
600	797.0 ± 148.8	121.1 ± 41.1

Application

Six healthy volunteers received single oral doses of 150, 300 and 600 mg of CGP 6258 in a randomized cross-over study. Mean plasma levels (Fig. 5) show a linear dose—response relationship. Mean areas under the plasma concentrations curves (AUC) gave the following ratios: 1.00:2.01:4.00 (Table I). Biological half-lives in plasma (3—3.3 h) were independent of the dosage. Urinary excretion of total (free and conjugated) CGP 6258 was also found to give a linear dose—response (Fig. 6).

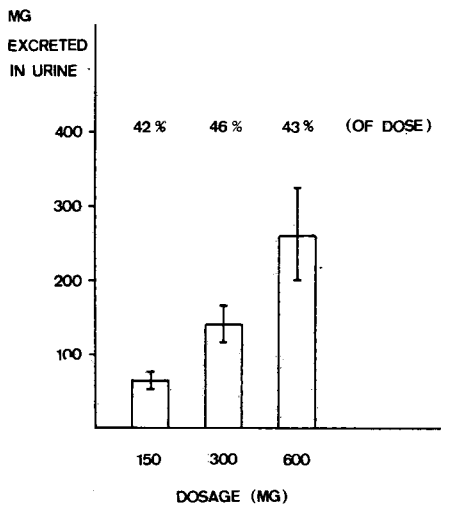

Fig. 6. Mean total (free and conjugated) CGP 6258 amounts excreted in 24-h urine of six healthy volunteers following single oral doses of 150, 300 or 600 mg of CGP 6258.

DISCUSSION

CGP 6258, being a highly polar compound, could not easily be chromatographed without prior derivatisation. The simultaneous acylation—esterification with reagents containing fluorine not only reduces polarity but drastically enhances volatility of a derivative with a molecular weight of 610. The introduction of fourteen fluorine atoms improves the electrophore properties of the derivative. Although benzophenones are known to have high electron-capture response [4, 5] this derivatisation increases sensitivity, thermal stability and selectivity.

A single extraction step is sufficient to extract enough of the CGP 6258 to ensure accurate quantitative determination. The use of a very favourable internal standard, differing only by one CH_2 group, allows correction of the losses incurred during extraction and derivatisation to 100% recovery.

The peaks following both the CGP 6258 and the internal standard derivative peaks (retention times approx. 7 and 10 min) are of unknown origin. Other reaction conditions did not affect these two peaks. None of the biological constituents interfere with the determination despite the simple, one-step extraction procedure.

The detector temperature was not optimized for maximum sensitivity due to the fact that extracts from biological material would contaminate the ^{63}Ni-cell at temperatures below 350°C after a few injections. The sensitivity is excellent, less than 1% of the peak plasma level after the lowest dose and thus more than sufficient for pharmacokinetic purposes.

ACKNOWLEDGEMENTS

Thanks are due to Dr. G. Haas, Ciba-Geigy Limited, Basle, for the supply of internal standard, and to Dr. P. Imhof, Ciba-Geigy Limited, Basle, for providing the biological samples.

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Journal of Chromatography, 277 (1983) 368—373
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1773

Note

Gas chromatographic determination of indomethacin and its O-desmethylated metabolite in human plasma and urine

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(First received June 9th, 1982; revised manuscript received May 16th, 1983)

Indomethacin (I) is one of the most widely used non-steroidal anti-inflammatory agents [1—3]. However, its precise pharmacokinetic parameters are still poorly known and recently they have been shown to be largely influenced by the administration time [4—6].

In order to determine if in humans these circadian changes in the pharmacokinetics of I would be related to an alteration in its biotransformation rate, it was necessary to measure simultaneously plasma and urine concentrations of I and of its major metabolite, O-desmethylindomethacin (DMI) [7, 8].

Several methods have already been described for the measurement of I in biological fluids including spectrofluorimetry [9, 10], gas chromatography (GC) [11—16], GC—mass spectrometry [17], high-performance liquid chromatography (HPLC) [18—24], thin-layer chromatography [25, 26] and radioisotopic dilution [7]. Most of them were devoted to the determination of I only. Recently, three HPLC methods [19—21] were developed which allow the quantification of I and its three metabolites, DMI, deschlorobenzoylindomethacin (DBI) and O-desmethyldeschlorobenzoylindomethacin (DMBI). However, these methods exhibited some drawbacks due to interferences of endogenous compounds in plasma and urine [19], or to the complexity of the whole procedure [21], or to the need of the use of an expensive gradient analysis system. Since the metabolic studies of I in man [7, 8] showed that demethylation followed by deacylation is the major pathway while direct deacylation of I is a minor one, it seems that DMI concentration could be an accurate index of the biotransformation rate of I in man.

Therefore we thought it useful to develop an easy to use GC method with electron-capture detection which could allow the measurement of I and DMI

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with a high sensitivity and specificity in plasma as well as in urine. The application of this technique to the pharmacokinetic study of I, given as a single oral dose, is shown.

EXPERIMENTAL

Standards and reagents

I and DMI were supplied by Merck Sharp and Dohme—Chibret Laboratories (Paris, France). Penfluridol [(chloro-4-α,α,α-trifluoromethyl-*m*-tolyl)-4-bis(*p*-fluorophenyl)-4,4-butyl-1-piperidinol-4] used as internal standard, was a generous gift of Janssen Pharmaceutica (Beerse, Belgium).

Acetone, diethyl ether and hexane (Merck, Darmstadt, F.R.G.) were distilled before use. Diazomethane as a methylating agent was extemporaneously generated from N-methyl-N-nitroso-*p*-toluenesulfonamide (Merck).

Diethyl ether and a mixture of diethyl ether—hexane (50:50) were used as extraction solvents for plasma and urine, respectively.

The stock solutions of I, DMI and internal standard were prepared in acetone (100 μg/ml) and were found to be stable for at least six months at +4°C. Working solutions were prepared weekly by diluting the stock solutions in acetone.

Gas chromatographic conditions

Analyses were performed under isothermal conditions on a Hewlett-Packard Model 5710 A gas chromatograph equipped with a ^{63}Ni linear electron-capture detector. The glass column (1 m × 2 mm I.D.) was packed with 3% OV-17 (Pierce, Rockford, IL, U.S.A.) on Chromosorb W HP (80—100 mesh) (Pierce), and conditioned for 2 h at 270°C (argon—methane, 90:10, carrier gas flow-rate, 10 ml/min), 4 h at 320°C (no gas flow) and 24 h at 270°C (carrier gas flow-rate, 30 ml/min). The column temperature was 260°C, injection port and detector temperature 300°C and the carrier gas (argon—methane, 90:10) flow-rate was at 60 ml/min.

Sample preparation

Extraction procedure. In a glass-stoppered centrifuge tube 0.2 ml of internal standard solution (10 μg/ml) was introduced. The solvent was evaporated to dryness under a gentle stream of nitrogen in a 37°C water-bath, then 1 ml of plasma or urine, 1 ml of 0.1 *N* hydrochloric acid and 8 ml of extraction solvent were added. The mixture was shaken for 15 min on a rotating mixer (60 rpm) and then centrifuged at 4000 rpm (1800 *g*) for 15 min at 4°C. A 7-ml volume of the organic layer was transferred into a conical test-tube and the solvent was evaporated to dryness under the conditions already described.

Derivatization. To the dry plasma or urine extract, 2 ml of acetone were added and the formation of methyl esters of I and DMI was performed directly by passing a stream of diazomethane into the solution until the occurrence of a yellow coloration which indicated the presence of an excess of reagent [27]. The solvent was evaporated to dryness and the methylated extract was dissolved in 100—500 μl of acetone; 4 μl of this solution were injected into the gas chromatograph.

Standard curves

Using the analytical procedure described above, standard curves were obtained by running human biological samples spiked with I (25—5000 ng/ml and 100—5000 ng/ml for plasma and urine, respectively) and DMI (10—500 ng/ml and 100—5000 ng/ml for plasma and urine, respectively) and with internal standard at a fixed concentration of 2000 ng/ml. The ratios of the peak heights of I and DMI to internal standard were plotted against the concentrations of I and DMI, respectively.

RESULTS AND DISCUSSION

Typical chromatograms obtained from blank plasma and blank urine before and after spiking with known amounts of I and DMI, and a plasma from a patient having received 75 mg of I, 12 h before sampling, are shown in Fig. 1. The retention times were at 5.6, 8.2 and 12.6 min for I, DMI and internal standard, respectively.

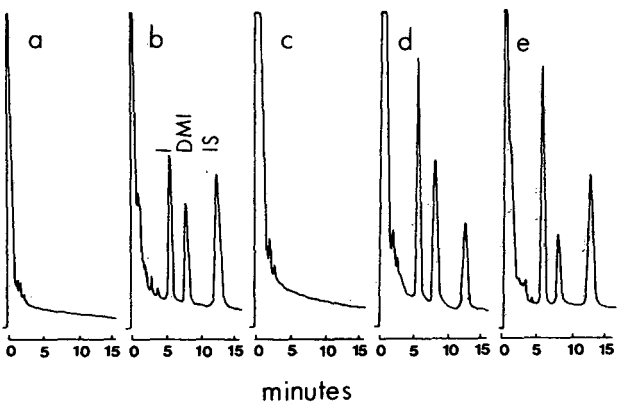

Fig. 1. Gas chromatograms of plasma and urine extracts: (a) control plasma; (b) plasma spiked with I (500 ng/ml), DMI (125 ng/ml) and internal standard (IS, 2000 ng/ml); (c) control urine; (d) urine spiked with I (500 ng/ml), DMI (500 ng/ml) and IS (2000 ng/ml); (e) plasma from a patient having received a single oral dose of 75 mg of I, 12 h before sampling.

No interferences from endogenous substances were observed. In addition, the possible interference of several drugs which are currently administered together with I, such as other non-steroidal anti-inflammatory agents (ketoprofen, naproxen, ibuprofen, phenylbutazone, diclofenac), analgesics (aspirin, paracetamol), tranquillizers (diazepam, lorazepam, oxazepam, nitrazepam) have been tested. These compounds did not exhibit any peak in the retention time range of the analysis, since most of them were not detectable with the electron-capture detector and the others appeared in the solvent peak. Compared to plasma, in the case of urine, instead of pure diethyl ether, a mixture of diethyl ether—hexane (50:50) was used as extraction solvent. This modification was necessary to reduce the front solvent chromatographic peak in order to quantify amounts of I and DMI smaller than 100

ng/ml. For this, 50% of hexane appeared sufficient to remove a large proportion of the interfering endogenous substances within the limits of the extraction capacity of the solvent.

The calibration graphs obtained after extraction of I and DMI from plasma and urine were found to be linear in the range of the concentrations studied. For plasma $Y = 0.00205X - 0.0184$ and $Y = 0.00131X - 0.0004$, and for urine $Y = 0.00122X - 0.0286$ and $Y = 0.00080X - 0.0037$ for I and DMI, respectively, with a regression coefficient $r^2 = 0.999$ in all cases.

The absolute sensitivity of the electron-capture detector was near 40 pg for I and 75 pg for DMI, which allowed the detection of plasma concentrations of 2 ng/ml and 4 ng/ml for I and DMI, respectively. In spite of the use of hexane in the extraction solvent, for urine the sensitivity limits were slightly higher than for plasma, being 10 ng/ml for both compounds.

The interassay reproducibility of the method was checked by analysing plasma and urine samples spiked with several concentrations of I and DMI. The results listed in Tables I and II show that the coefficients of variation do not exceed 7.0 and 4.9% for I and 9.2 and 6.1% for DMI in plasma and urine, respectively.

TABLE I

REPRODUCIBILITY OF I AND DMI MEASUREMENT IN HUMAN PLASMA

Amount added (ng/ml)		Mean of ten assays (ng/ml, ± S.D.)		Coefficient of variation (%)	
I	DMI	I	DMI	I	DMI
25	10	22.4 ± 1.5	9.2 ± 0.9	6.5	9.2
100	25	100.8 ± 7.0	25.3 ± 1.5	7.0	6.1
500	50	494.9 ± 22.5	49.7 ± 2.5	4.5	5.1
1000	100	1004.2 ± 36.8	98.9 ± 5.9	3.7	6.0
2000	250	2004.7 ± 81.0	250.1 ± 11.7	4.0	4.7
5000	500	4999.8 ± 122.6	501.2 ± 17.8	2.5	3.5

TABLE II

REPRODUCIBILITY OF I AND DMI MEASUREMENT IN HUMAN URINE

Amount added (ng/ml)		Mean of six assays (ng/ml, ± S.D.)		Coefficient of variation (%)	
I	DMI	I	DMI	I	DMI
100	100	100.2 ± 4.1	105.0 ± 5.1	4.1	4.8
250	250	245.8 ± 10.1	259.3 ± 9.8	4.1	3.8
500	500	508.5 ± 10.3	498.2 ± 30.2	2.0	6.1
1000	1000	1004.2 ± 49.2	973.8 ± 14.6	4.9	1.5
2000	2000	2012.5 ± 26.9	2005.2 ± 46.1	1.3	2.3
5000	5000	5012.5 ± 52.2	5001.0 ± 86.6	1.0	1.7

Compared to HPLC methods, the derivatization step during GC methods could be considered a drawback in terms of reproducibility and time consumption. However, through the classical methylation procedure used, it appeared that the esterification of the studied carboxylic acids was complete, rapid (only a few minutes are required for the treatment of each sample) and reproducible as confirmed by the low coefficients of variation given above.

In addition, the sensitivity obtained with the electron-capture detector is ten times higher than that of the HPLC methods. Another advantage of the described GC method is the minimal plasma or urine background, even when very low concentrations of I and DMI have to be detected

Fig. 2 illustrates the time course of plasma levels of the two compounds observed in a patient after a single oral dose of 75 mg of a slow-release form of I. These curves clearly indicate that the analytical method described above is suitable for a pharmacokinetic study of I and DMI.

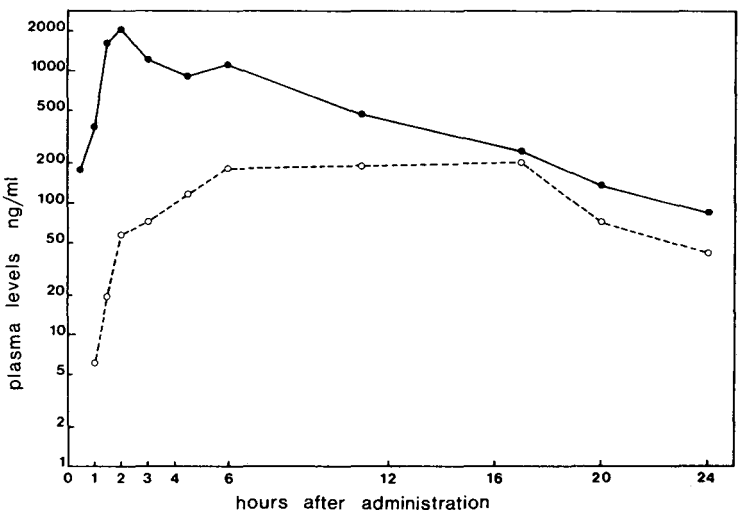

Fig. 2. I (•) and DMI (○) plasma concentration—time curves in a patient having received at 8 p.m. a single oral dose (75 mg) of a slow-release form of I.

CONCLUSIONS

The method described is simple, specific, reliable and highly sensitive. The time of analysis needed for a batch of twelve to fifteen samples is about 6 h. The stability of the chromatographic system is remarkably high since no change could be observed after routine analysis of 1000 samples within a period of one year.

The sensitivity and the accuracy of the method appear suitable to follow plasma and urine concentrations of I and DMI after a single therapeutic dose of I.

ACKNOWLEDGEMENT

The authors thank Dr. C. Le Louarn (Merck Sharp and Dohme–Chibret Laboratories) for the generous gift of indomethacin and its demethylated metabolite.

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Journal of Chromatography, 277 (1983) 374—380
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1812

Note

Gas chromatographic determination of busulfan in plasma with electron-capture detection

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(First received March 7th, 1983; revised manuscript received June 3rd, 1983)

Busulfan, an alkytating agent of the bis-(methanesulfonate) ester type, is frequently used in the treatment of chronic myelogenous leukemia. Recently a gas chromatographic (GC) procedure for the determination of busulfan in plasma was presented for the first time [1], which comprised an initial extraction of plasma with dichloromethane followed by evaporation and derivatization of busulfan using sodium iodide in acetone. The formed derivative was purified by solvent extraction. The GC step utilized a packed column in conjunction with selected ion monitoring (SIM).

In the present paper a new derivatization technique is given, comprising derivatization and extraction of busulfan from plasma in a single step. High separation efficiency and sensitivity are obtained by the use of a capillary column in combination with electron-capture detection (ECD).

EXPERIMENTAL

Gas chromatography

The GC analysis was performed using a Varian 3700 instrument, equipped with a constant-current ^{63}Ni electron-capture detector. An OV-1 fused-silica capillary column (25 m × 0.3 mm I.D.) with a film thickness of 0.52 μm (Hewlett-Packard) was used. The original injector was substituted by an injection splitter (Model A, Cat. No. 8045) obtained from Chrompack, Middelburg, The Netherlands.

The original flow regulator was replaced by a Brooks 8601 pressure regulator (0—2.5 bar). The instrument was operated isothermally with the oven, detector and injector port temperatures at 145°C, 250°C and 200°C, respectively. The carrier gas (helium) flow-rate was 2 ml/min, with an inlet pressure of 0.4 bar.

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Make up gas (nitrogen), 30 ml/min, was added through the hydrogen inlet in the detector base. A split ratio of 1:10 was used unless otherwise stated.

A Varian 1400 instrument equipped with a flame ionization detector and a 1.5 m × 2 mm I.D. column packed with 10% SP 2401 on Supelcoport 100—120 mesh was used. The instrument was operated isothermally with oven, detector and injector port temperatures of 130°C, 260°C and 200°C, respectively. The carrier gas (nitrogen) flow-rate was 30 ml/min.

The GC—mass spectrometric analysis was carried out as described in ref. 1 using an LKB 2091 instrument. The liquid chromatographic (LC) conditions are described in ref. 1.

Reagents and chemicals

Busulfan was obtained from EGA-Chemie (Steinheim, F.R.G.) and 1,5-bis(methanesulfonoxy)pentane was prepared according to the method reported previously [1]. Sodium iodide and *n*-pentadecane were obtained from Merck (Darmstadt, F.R.G.). All solvents used were of analytical grade.

Reaction conditions

Busulfan was dissolved in acetone (0.1 ml) and mixed with phosphate buffer pH 7.0, ionic strength (I) = 0.1 to give a final busulfon concentration of 1.0 mg/ml. The aqueous phase was mixed with sodium iodide and *n*-heptane containing the internal standard (*n*-pentadecane 0.3 mg/ml). The reaction was carried out at 70°C under stirring with a micro-magnet in the tube. The sodium iodide concentration was varied within the range 1—4 M, having a constant phase ratio V_{org}/V_{aq} = 1. At appropriate times samples (0.1 ml) were withdrawn from the organic phase for analysis.

The influence of the ratio V_{org}/V_{aq} on the reaction rate was studied as above using 4 M sodium iodide and phase ratios V_{org}/V_{aq} within the range 0.2—5. The analysis was performed using GC—flame ionization detection (FID).

Determination of partition coefficients

The partition coefficients for busulfan and 1,4-diiodobutane were determined as described in ref. 1. The distribution of 1-methanesulfonoxy-4-iodobutane was studied using equal volumes of phosphate buffer pH 7.0 (I = 0.1) and *n*-heptane (equilibrium time 30 min at 25.0 ± 0.1°C). The initial concentration of 1-methanesulfonoxy-4-iodobutane in the aqueous phase and the concentration in the aqueous phase at the equilibrium stage were determined by LC.

Determination of busulfan in plasma

Plasma (1.00 ml) was mixed with 1,5-bis(methanesulfonoxy)pentane (0.10 ml, 1.0 μg/ml) in acetone and sodium iodide in water (1.0 ml, 8 M). After addition of *n*-heptane (0.40 ml), the reaction was carried out at 70°C for 40 min under stirring. Part of the organic phase (1—2 μl) was injected into the GC—ECD system using a split ratio of 1:10.

RESULTS AND DISCUSSION

Conversion of busulfan to 1,4-diiodobutane in aqueous solution

The reaction of busulfan with sodium iodide in acetone has been described recently [1]. In the present paper the reaction with sodium iodide was studied in aqueous solution with the ultimate goal of performing the derivatization directly in plasma to obtain a simplified work-up procedure preceding the GC step. The reaction of busulfan in aqueous solution using 4 *M* sodium iodide at 70°C gave a yield of 50% of 1,4-diiodobutane in about 15 min.

However, it was not possible to obtain a quantitative conversion to 1,4-diiodobutane, probably due to subsequent hydrolysis of the derivative formed. By performing the reaction in the presence of an organic solvent, the stability of 1,4-diiodobutane could be increased considerably. For example, 1,4-diiodobutane was stable for at least 50 h at 70°C in a two-phase system comprising equal volumes of phosphate buffer pH 7.0 and *n*-heptane, which must be attributed to the high partition of 1,4-diiodobutane to the organic phase ($K_d > 100$).

A quantitative yield of 1,4-diiodobutane was obtained in a two-phase system after about 30 min using 4 *M* sodium iodide (Fig. 1). The reaction was slower using 1 *M* and 2 *M* sodium iodide and the final yields were lower (75.5% and

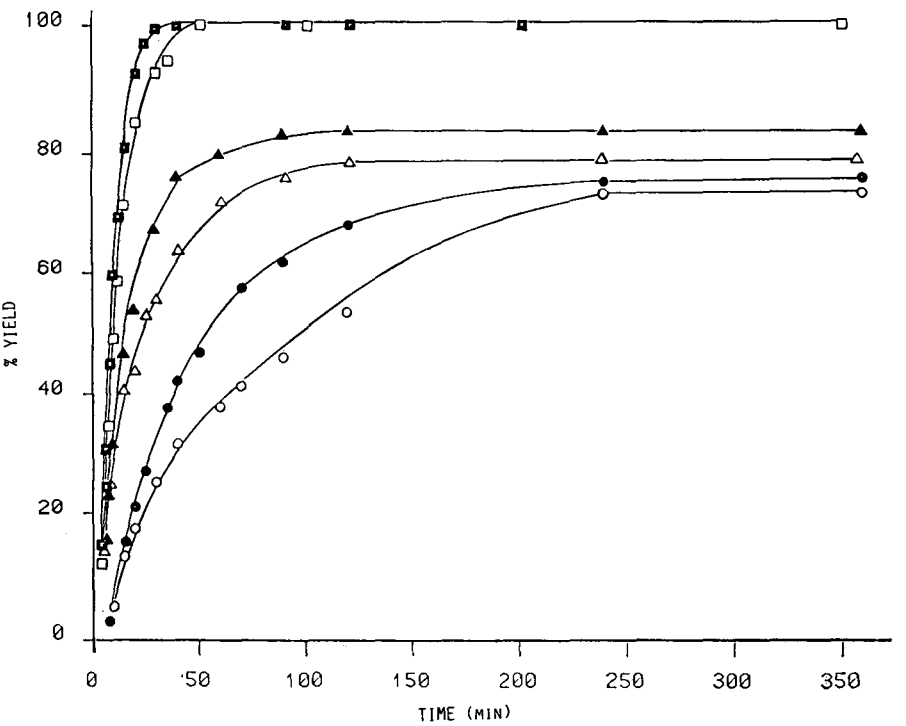

Fig. 1. Influence of sodium iodide concentration on the formation of 1,4-diiodobutane and 1,5-diiodopentane. Temperature: 70°C. The yields were calculated using 1,4-diiodobutane and 1,5-diiodopentane as reference. Key: sodium iodide 1 *M* (●), 2 *M* (▲), 4 *M* (■) with 1,4-diiodobutane as reference; sodium iodide 1 *M* (○), 2 *M* (△), 4 *M* (□) with 1,5-diiodopentane as reference.

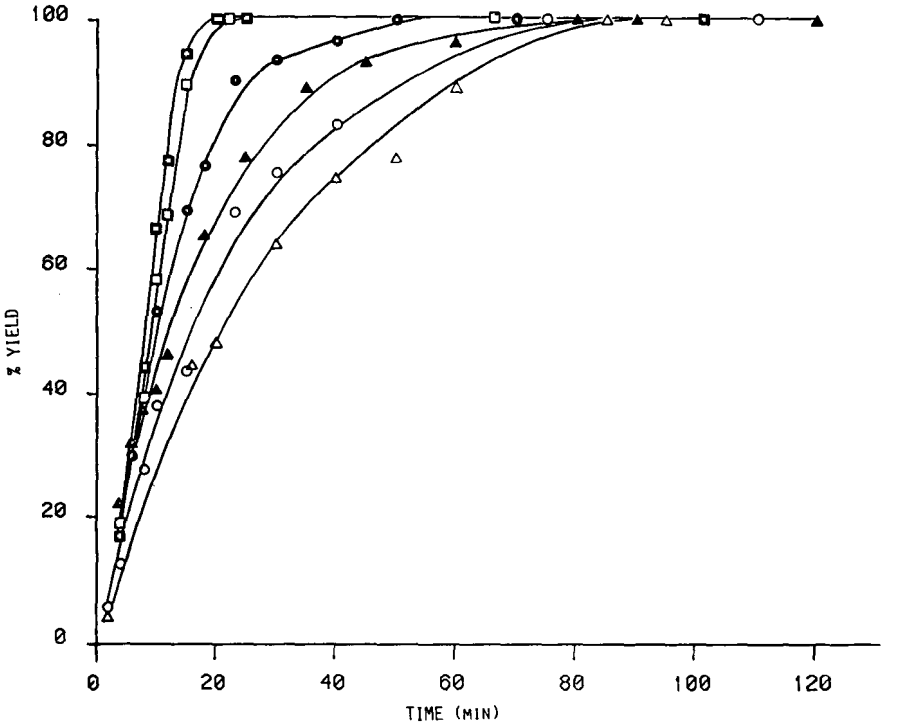

Fig. 2. Influence of ratio V_{org}/V_{aq} on the formation of 1,4-diiodobutane and 1,5-diiodopentane. Temperature: 70°C. The yields were calculated using 1,4-diiodobutane and 1,5-diiodopentane as reference. Key: V_{org}/V_{aq} ratio of 0.2 (▪), 1 (•), 5 (▲) with 1,4-diiodobutane as reference; V_{org}/V_{aq} ratio of 0.2 (□), 1 (○), 5 (△) with 1,5-diiodopentane as reference.

84.2%, respectively). A high concentration of iodide is obviously required to obtain a rapid and complete conversion of busulfan to 1,4-diiodobutane.

The reaction of busulfan with iodide takes place in the aqueous phase. Consequently, the overall reaction rate in the two-phase system should be affected by the fraction of busulfan and the intermediate (1-methanesulfonoxy-4-iodobutane) present in the aqueous phase.

The influence of the ratio V_{org}/V_{aq} on the rate of formation of 1,4-diiodobutane is illustrated in Fig. 2. An increased reaction rate is observed when the ratio is decreased. The major portion of busulfan is partitioned to the aqueous phase ($K_d < 0.01$) and hence a change of the ratio V_{org}/V_{aq} in the range studied (0.2—5) should only slightly affect the rate of formation of 1-methanesulfonoxy-4-iodobutane. However, 1-methanesulfonoxy-4-iodobutane is considerably more lipophilic than busulfan ($K_d = 2.0$) and a decreased ratio V_{org}/V_{aq} should increase its rate of conversion to 1,4-diiodobutane. For example, a change of the ratio from 5 to 0.2 will change the fraction of 1-methanesulfonoxy-4-iodobutane present in the aqueous phase from 30% to 90%.

The internal standard 1,5-bis(methanesulfonoxy)pentane has a lower reaction rate than busulfan. This is probably due to its more lipophilic character which will give a higher partition to the organic phase (Figs. 1 and 2).

Reaction of busulfan in plasma

The reaction of busulfan and the internal standard was carried out in a two-phase system using plasma containing 4 *M* sodium iodide as an aqueous phase and *n*-heptane as an organic phase at 70°C for 40 min. The yields of 1,4-diiodobutane and 1,5-diiodopentane were 91.0 ± 3.2% (C.V., $n = 5$) and 92.0 ± 3.5% (C.V., $n = 5$), respectively. A ratio V_{org}/V_{aq} of 0.2 is used in the general procedure for the determination of busulfan in plasma to give a rapid formation of 1,4-diiodobutane. Furthermore, a ratio V_{org}/V_{aq} of 0.2 gives a higher concentration of 1,4-diiodobutane in the organic phase as compared to the initial plasma concentration of busulfan, making further concentration steps unnecessary prior to the GC analysis.

Injection technique

Splitless injection [2, 3] is one of the most used techniques in quantitative trace analysis by capillary GC [4]. Generally the technique is performed isothermally using a high-boiling alkane as a solvent, when combined with ECD [5—7]. The quantitation of busulfan using the splitless technique with *n*-decane as the solvent resulted in a number of interfering peaks in the chromatograms even when the solvent was redistilled. However, no interfering peaks were found when *n*-heptane was used as a solvent in combination with a split injection technique.

A poor reproducibility was obtained when the peak heights from a number of identical injections were compared using split of ratios 1:2—1:40 (Table I). However, very good precision was obtained in the peak height ratios 1,4-diiodobutane/1,5-diiodopentane (Table I). The column efficiency was constant ($H = 0.77 \pm 0.04$ mm, S.D.) using split ratios in the range 1:5—1:40 but it decreased by about 50% using a split ratio of 1:2.

TABLE I

EFFECT OF SPLIT RATIO ON PRECISION

Split	1,4-Diiodobutane peak height (mm) ± S.D.	1,5-Diiodopentane peak height (mm) ± S.D.	Peak height ratio ± S.D.
1:2	396 ± 63	463 ± 73	0.85 ± 0.01
1:5	227 ± 26	248 ± 28	0.91 ± 0.01
1:10	126 ± 11	137 ± 12	0.92 ± 0.01
1:15	90 ± 11	98 ± 12	0.92 ± 0.01
1:25	70 ± 5	76 ± 5	0.91 ± 0.01
1:40	42 ± 7	46 ± 8	0.92 ± 0.01

Detection, selectivity and precision

The minimum detectable concentration [8] obtained by ECD is given in Table II. The value for 1,4-diiodobutane corresponds to a minimum detectable quantity of 30 fg (retention time 8 min). The sensitivity of the EC detector in combination with the high separation efficiency of the capillary column, makes the determination of busulfan in patient plasma possible in the low ng/ml level without any interfering peaks (Fig. 3). The standard curve using plasma was linear within the range studied, 5—300 ng/ml. A least-squares

TABLE II

MINIMUM DETECTABLE CONCENTRATION (MDC) OF 1,4-DIIODOBUTANE AND 1,5-DIIODOPENTANE

Compound	MDC* $\times 10^{16}$ mol/sec
1,4-Diiodobutane	0.44
1,5-Diiodopentane	0.54

*Signal-to-noise ratio = 3.

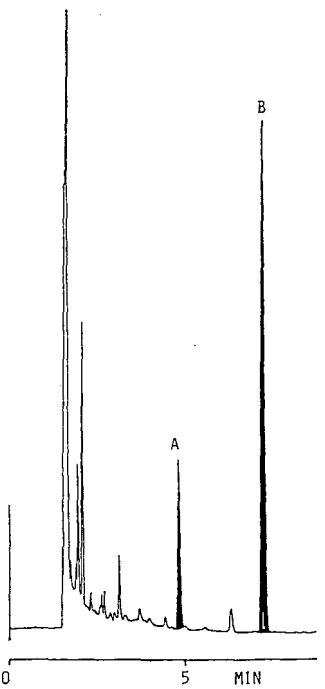

Fig. 3. Chromatogram obtained from patient plasma containing 14 ng/ml busulfan. Split: 1:10. Key: A = busulfan as 1,4-diiodobutane; B = 1,5-bis(methanesulfonoxy)pentane as 1,5-diiodopentane.

analysis gave a slope of $1.64 \times 10^{-2} \pm 0.04 \times 10^{-2}$ (S.E.M.), an intercept of $9.70 \times 10^{-2} \pm 5.50 \times 10^{-2}$ (S.E.M.) and a correlation coefficient of 0.9988.

The precision of the analytical method was ± 3.9% (C.V.) at the 10 ng/ml level (n = 5) and ± 2.3% (C.V.) at the 100 ng/ml level (n = 5).

Evaluation of the method

The prechromatographic procedure is more rapid and labour saving than in the GC—SIM method [1], and the technique requires less-expensive instrumentation.

The accuracy of the method was corroborated by analysis of patient plasma using GC—SIM [1]. The results obtained by GC—ECD were in good agreement

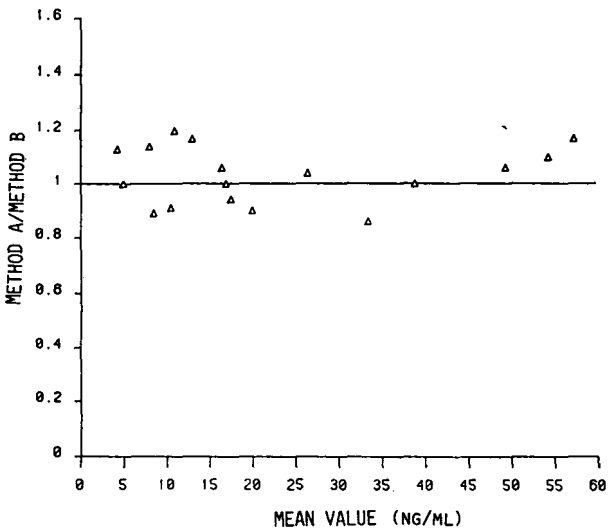

Fig. 4. Method-comparison analysis. Method A: data from analysis by GC—ECD. Method B: data from analysis by GC—SIM.

with those obtained by GC—SIM, when plotted according to ref. 9 (Fig. 4). The quotient of ECD values/SIM values was 1.043 ± 0.111 (S.D.). It should be pointed out, however, that the metabolic pattern of busulfan is poorly characterized, and since metabolites of busulfan might be converted to 1,4-diiodobutane by performing the derivatization directly in the biological material, the accuracy of the GC—ECD method if applied to analysis of, for example, other human tissues or biological material from other species should be evaluated by the GC—SIM method.

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Journal of Chromatography, 277 (1983) 381—384
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1769

Note

Sensitive high-performance liquid chromatographic method for the determination of metronidazole and metabolites

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(First received December 23rd, 1982; revised manuscript received May 6th, 1983)

Metronidazole (Flagyl, Clont; 1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole) is effective in the treatment of anaerobic infections [1]. Metronidazole is also gaining wider use in the treatment of Crohn's disease, a debilitating enteritis [2].

Previous methods for the determination of metronidazole and its metabolites in biological fluids have utilized bioassays [3], gas—liquid chromatography (GLC) [4], polarography [5], thin-layer chromatography (TLC) [6, 7] and high-performance liquid chromatography (HPLC) [7—10]. Bioassays and polarography are nonspecific and time-consuming. GLC involves the organic extraction of metronidazole, with subsequent derivatization, and does not include metabolites. The HPLC assays described do not incorporate an internal standard and can be contaminated by protein precipitates leading to poor resolution and shorter column life.

The HPLC method described here is rapid, involving no organic extraction, utilizes an internal standard to minimize day-to-day variability, does not have problems with protein precipitation, and simultaneously measures metronidazole and its major metabolites (of which hydroxymetronidazole is biologically active) in biological fluids.

MATERIALS AND METHODS

Chemicals

Metronidazole, 1-(2-hydroxyethyl)-2-hydroxymethyl-5-nitroimidazole (hydroxymetronidazole), and 1-acetic acid-2-methyl-5-nitroimidazole (metronidazole acetic acid) were kindly provided by Bayer AG, Leverkusen, F.R.G. Tinida-

0378-4347/83/$03.00

zole, used as an internal standard, was provided by Pfizer GmbH, Karlsruhe, F.R.G. All other chemicals were of HPLC grade or better.

High-performance liquid chromatograph

A Waters (Milford, MA, U.S.A.) high-pressure liquid chromatography system was used for all determinations. This system included a Model M6000-A high-pressure pump, a Model 450 variable-wavelength UV detector, and a Wisp 710 A automatic injector. A Hewlett Packard (Avondale, PA, U.S.A.) Model 3380A integrator was used for data processing. The carrier was a solution of 0.005 *M* KH_2PO_4 (pH 4.5)—methanol—tetrahydrofuran (82.6:16.5:0.9) pumped at a flow-rate of 1.4 ml/min through a reversed-phase μC_{18} Bondapak column (30 cm × 3.9 mm; average particle size 10 μm; Waters Associates), with the effluent monitored at 324 nm.

Sample preparation

To a 1.5-ml conical microfuge tube (Eppendorf GmbH, Hamburg, F.R.G.) were added 200 μl of plasma or urine (urine being previously diluted 1:10 with distilled water), 50 μl of ethanol containing 10 mg per 100 ml tinidazole, and 50 μl of 0.1 *M* $ZnSO_4$. The tubes were capped, vortexed for 15 sec, and placed in a refrigerator for 15 min. The tubes were then centrifuged for 2 min in an Eppendorf microfuge. An aliquot (10 μl) of the superantant was injected into the liquid chromatograph.

For the measurement of the glucuronide conjugates of metronidazole and hydroxymetronidazole in urine, 1 ml of urine was mixed with 4 ml of acetate buffer (0.2 *M*, pH 4.5). To this mixture were added 50 μl of glucuronidase—sulfatase (10,000 Fishman units/ml; Sigma GmbH, Munich, F.R.G.). This solution was incubated at 37°C for 16 h. Following incubation, 1 ml of the incubate was mixed with 1 ml of distilled water. A 0.2-ml aliquot of this mixture was prepared as described above for the plasma samples.

RESULTS AND DISCUSSION

With the system described, the retention times for metronidazole acetic acid, hydroxymetronidazole, metronidazole, and tinidazole (internal standard) were 2.4, 3.0, 4.7, and 6.7 min, respectively (Fig. 1). Interfering peaks from pooled plasma, urine, and urine samples prepared for glucuronide analysis were not observed. As seen in Fig. 1, no peak tailing was found, a problem reported earlier [9], enabling the use of peak height or peak area in the calculation of standard curves. The calibration graph was linear over a concentration range of 1—40 μg/ml when plasma and urine were spiked with the substances measured. The limit of detection was 0.05 μg/ml for metronidazole and metabolites. The accuracy of the method was tested by adding the compounds of interest to plasma and urine and extracting ten samples. The percentage coefficient of variation (C.V.) of extracted plasma samples at a concentration of 2 μg/ml metronidazole, hydroxymetronidazole, and metronidazole acetic acid was 4.1, 4.3 and 4.3%, respectively. From urine, the C.V. values were 5.1, 5.9, and 4.4%, respectively. Day-to-day variability was tested by extracting frozen plasma and urine samples previously spiked with metronidazole and metab-

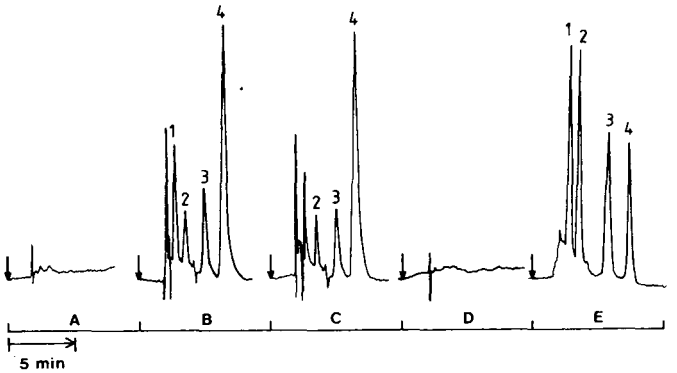

Fig. 1. HPLC chromatograms from: (A) extract of blank plasma; (B) extract of blank plasma to which metronidazole (2 μg/ml) and metabolites (2 μg/ml each) and internal standard (5 μg) had been added; (C) extract of plasma obtained from a patient receiving metronidazole; (D) extract of blank urine; (E) extract of urine obtained from a patient receiving metronidazole. The peaks indicated are (↓) injection point; (1) metronidazole acetic acid; (2) hydroxymetronidazole; (3) metronidazole; (4) tinidazole (internal standard).

olites over a three-week period. The C.V. of plasma samples was 5.6% for metronidazole, 4.5% for hydroxymetronidazole, and 3.5% for the acid metabolite. Similar results were found for the extraction of urine samples.

This method has been used to study the pharmacokinetics of metronidazole following intravenous or oral single dosing (Table I) and multiple dosing [11]. The method is accurate, as indicated by the low C.V. of extracted samples, and enables the simultaneous measurement of metronidazole and major metabolites in plasma and urine. Bioassays have proved to be inaccurate due to the antimicrobial action of hydroxymetronidazole [12] which is also found in plasma. Polarography suffers from the same problem, due to the reduction of all nitroimidazoles present, metronidazole and its metabolites, which measures the total amount of nitroimidazole present, but does not differentiate between metronidazole and its metabolites [5]. The described method utilizes no organic extraction, as do the GLC and TLC methods [4, 6, 7] and one of the HPLC methods [10].

TABLE I

URINARY EXCRETION OF METRONIDAZOLE AND ITS METABOLITES IN TWO PATIENTS FOLLOWING A SINGLE 400-mg ORAL DOSE OF METRONIDAZOLE

Excretion is expressed as percentage of dose eliminated in 48 h.

Compound	Patient I	Patient II
Metronidazole (free)	9.5	2.5
Metronidazole (conjugated)	6.1	3.6
Metronidazole 1-acetic acid	26.9	10.2
Hydroxymetronidazole (free)	38.1	8.5
Hydroxymetronidazole (conjugated)	9.9	12.2

The previously described HPLC methods used no internal standard, the use of which increases accuracy [8, 9, 13]. In addition, protein precipitation was reported to be a problem if samples were left standing for long periods of time, as would be the case when automatic injectors are used [9]. The use of $ZnSO_4$ and cooling described here has eliminated this problem, with no additional precipitation being seen over 6 h. Also, by not using perchloric acid [8] or trichloroacetic acid for protein precipitation, column life is greatly extended.

In conclusion, the described method is rapid and can be used for plasma and urine without carrier modification. Metronidazole is assayed simultaneously with its major metabolites in plasma and urine [11], with an increased accuracy due to the use of an internal standard.

ACKNOWLEDGEMENT

This work was supported by the Sandoz Stiftung für Therapeutische Forschung.

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Journal of Chromatography, 277 (1983) 385—390
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1774

Note

High-performance liquid chromatographic analysis of isoniazid and acetylisoniazid in biological fluids

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Plasma concentrations of isoniazid and acetylisoniazid may be valuable in monitoring compliance with an efficacy of therapy in tuberculosis. They have also been used to measure the acetylator status of patients in order to assess relative risk of development of adverse effects to several drugs.

Several methods are available for the measurement of isoniazid in biological fluids. The most commonly used methods are listed in Table I but each have disadvantages in terms of sensitivity, specificity or time of analysis. Two selective, although relatively insensitive high-performance liquid chromatographic (HPLC) methods have been described [1, 2]. We therefore describe an HPLC method which is ten-fold more sensitive and which has provided superior resolution of isoniazid and acetylisoniazid in our hands.

0378-4347/83/$03.00

TABLE I

SUMMARY OF METHODS AVAILABLE

Type of assay	Biological specimen*	Specificity	Estimated sensitivity (μg/ml)
Colorimetric [5]	S U	Isoniazid	>5
Microbiological [7]	S U	Isoniazid and metabolites	<0.1
Fluorimetric [6]	S U	Isoniazid	0.01
Gas chromatographic [8]	U	Isoniazid and metabolites	<0.5
Gas chromatographic—mass spectrometric [3]	P	Isoniazid and metabolites	0.01
Radioimmunological [9]	S	Isoniazid	<0.05
Liquid chromatographic [1]	P U	Isoniazid and acetylisoniazid	<0.2
Liquid chromatographic [2]	S	Isoniazid and acetylisoniazid	0.3
HPLC (present method)	B S P U	Isoniazid and acetylisoniazid	0.02

*B = blood, S = serum, P = plasma, U = urine.

EXPERIMENTAL

Reagents

Isoniazid and iproniazid phosphate were supplied by Aldrich (Gillingham, U.K.). Acetyl isoniazid was prepared using a modification [3] of the method of Fix and Gibas [4]. The acetonitrile was of HPLC quality and was supplied by Fisons (Loughborough, U.K.). All other solvents and reagents were of analytical quality.

Sample preparation

A schematic representation of the procedure for the analysis of isoniazid and acetylisoniazid is shown in Fig. 1. Heparinised plasma (1 ml), heparinised blood (1 ml) or a 1-ml dilution of urine (1:10, v/v) was placed in a PTFE-lined screw-capped culture tube, and 50 μl of the internal standard solution (containing 2 μg iproniazid), 500 μl phosphate buffer (0.5 *M*, pH 7.4) saturated with sodium chloride, and 10 ml chloroform—butane-1-ol (70:30) were added. The samples were extracted by gentle mixing for 10 min followed by filtration through Whatman IpS phase separating paper. The organic phase was back extracted with 500 μl of 0.05 *M* phosphoric acid by gentle mixing for 10 min. After brief centrifugation, 20 μl of the aqueous phase were applied to the chromatograph.

Chromatography

The HPLC system used was supplied by Laboratory Data Control (Stone, U.K.). The system comprised a Constametric III high-pressure solvent delivery system equipped with a Model 7125 Rheodyne valve and fitted with a Spherisorb nitrile column (250 × 4.5 mm I.D.; particle size 5 μm). The absorbance was measured at 266 nm, at 0.01 a.u.f.s. deflection using a Spectromonitor III variable-wavelength detector. The mobile phase used was 0.01 *M* phosphoric acid in acetonitrile—water (20:80) at a flow-rate of 2 ml/min.

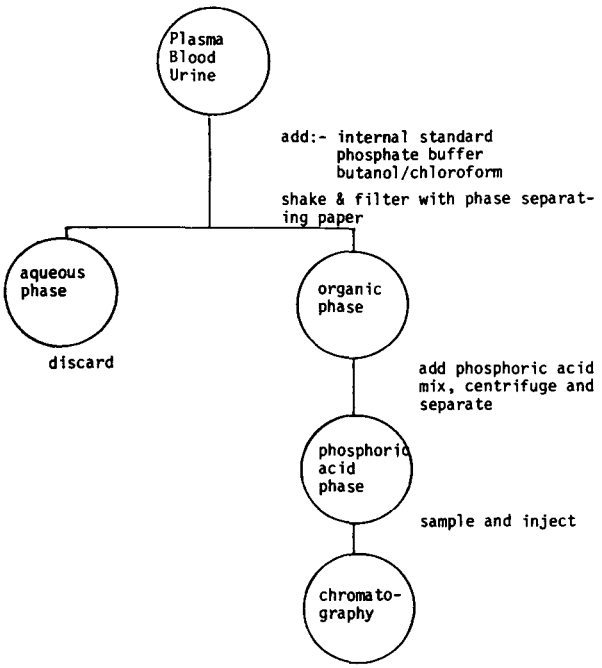

Fig. 1. Flow diagram of the sample preparation for the HPLC analysis of isoniazid and acetylisoniazid.

Chromatograms were recorded and the peak heights integrated using a Chromatography Control Module equipped with a printer-plotter.

Calibration and accuracy

Calibration curves were constructed by adding known masses of internal standard and isoniazid to plasma, blood or urine, and plotting the peak height ratios of isoniazid to internal standard against the amount of isoniazid added. Peak height ratios were used to calculate the amount of isoniazid in unknown samples, and the standard deviation of the normalized peak height ratios was used to determine the accuracy of the method over the range of isoniazid standards employed. The precision of the method was also studied by submitting four replicate plasma samples containing 1 and 10 μg/ml isoniazid to the entire procedure. The same approach was used to calibrate and determine the precision of acetylisoniazid estimations.

To estimate the recoveries for the method, the peak heights of analysed 1-ml samples containing known amounts of isoniazid, acetylisoniazid, and the internal standard were compared to the respective peak heights obtained by injecting equal amounts directly into the chromatograph.

RESULTS AND DISCUSSION

Isoniazid and acetylisoniazid were efficiently extracted from plasma, urine and blood. Salting out of the drug using sodium chloride improved extraction efficiency, whereas ammonium chloride which was previously used by

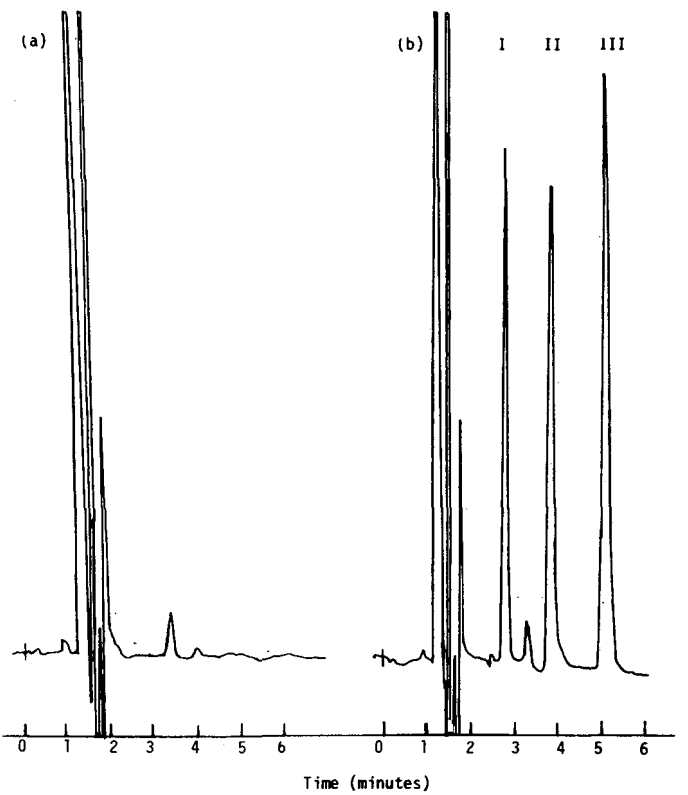

Fig. 2. Chromatograms of (a) control plasma and (b) plasma containing known amounts of (I) acetylisoniazid, (II) isoniazid and (III) the internal standard (iproniazid).

TABLE II

ACCURACY, RECOVERY AND PRECISION OF THE METHOD FOR DETERMINING ISONIAZID AND ACETYLISONIAZID IN BIOLOGICAL FLUIDS

Drug	Biological fluid	Concentration range (μg/ml)	Coefficient of variation of normalized peak height ratios (%)	Recovery (%)
Accuracy and recovery				
Isoniazid	Plasma	0.1—15	4.6	71
Isoniazid	Blood	0.2—15	5.2	68
Isoniazid	Urine	2 —100	3.7	77
Acetylisoniazid	Plasma	0.2—15	3.7	75
Acetylisoniazid	Blood	0.2—15	8.3	72
Acetylisoniazid	Urine	2 —100	4.1	79
Precision (n = 4)				
Isoniazid	Plasma	10	0.9	
Isoniazid	Plasma	1	1.8	
Acetylisoniazid	Plasma	10	4.0	
Acetylisoniazid	Plasma	1	4.1	

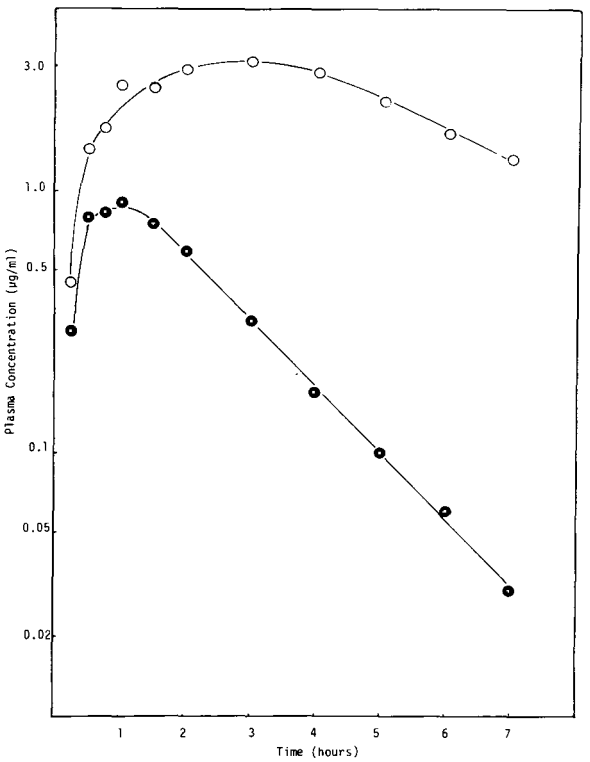

Fig. 3. Semi-logarithmic plot of the plasma concentrations of isoniazid (•) and acetylisoniazid (○) in a healthy male subject for 7 h after a single oral dose of 200 mg of isoniazid.

other authors [1, 5] was found to be much less effective and interfered with subsequent chromatography.

Isoniazid, acetylisoniazid and the internal standard (iproniazid) were well separated by the method described, as were two peaks associated with extraction from plasma (Fig. 2). Retention time for the longest retained compound (iproniazid) was only 6.5 min. The method was accurate and reproducible (see Table II) and could detect as little as 0.02 μg of isoniazid or acetylisoniazid per ml plasma.

The method gave satisfactory results when used to determine kinetic parameters after oral administration of only 200 mg isoniazid to a normal individual (Fig. 3). None of the drugs (ethambutol, streptomycin, rifampicin, pyrazinamide or pyridoxine) often given to patients receiving isoniazid therapy interfered with the estimation of isoniazid or acetylisoniazid. Because of adequate sensitivity of the method the oral isoniazid dose could be reduced by 70% for determining the pharmacokinetic parameters, thus reducing the nausea associated with larger doses. Also only 1 ml of plasma was required for the estimation rather than 3 or 4 ml [1, 5].

Many of the methods described in Table I measure only isoniazid and do not measure its acetylated metabolite [1, 5, 6, 7]. The microbiological method although sensitive, also measures metabolites with anti-tuberculous activity

and determinations take approximately 10 days [7]. The liquid chromatographic method of Saxena et al. [1] can accurately measure acetylisoniazid as well as the parent compound but it is relatively insensitive and uses the method of difference after spiking the samples with additional amounts of drug and metabolite. A recent hydrophilic ion-pair reversed-phase HPLC method is even less sensitive, although faster than the method of Saxena et al. (see Table I). A major disadvantage is a large lump due to serum components which occurs 20 min after injection and could thus interfere with subsequent chromatograms [2].

The gas chromatographic method of Nota et al. [8] does measure acetylisoniazid as well as parent compound but has poor sensitivity. The gas chromatographic—mass spectrometric method [3], although sensitive, requires very expensive equipment.

We have found that the method we describe can be performed on conventional HPLC equipment and up to 40 samples can be processed in a working day.

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Journal of Chromatography, 277 (1983) 391—395
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1783

Note

High-performance liquid chromatographic determination of cimoxatone and its O-demethyl metabolite in plasma

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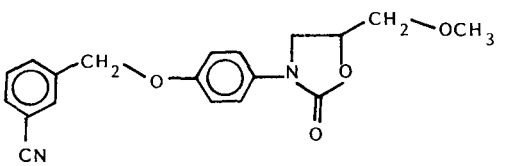
(First received February 11th, 1983; revised manuscript received April 7th, 1983)

Cimoxatone (I) (MD 780515, 3-[4-(3-cyanophenylmethoxy)phenyl]-5-(methoxymethyl)-2-oxazolidinone, Fig. 1), is a new, selective and reversible inhibitor of type A monoamine oxidase (MAO) both in rat [1, 2] and man [3]. Cimoxatone is extensively metabolized in man [4] and its major plasma metabolite, MD 770222 (II) {3-[4-(3-cyanophenylmethoxy)phenyl]-5(hydroxymethyl)-2-oxazolidinone}, is also a selective and reversible type A MAO inhibitor, but 7—8 times less inhibitory than the parent compound [5].

(I)



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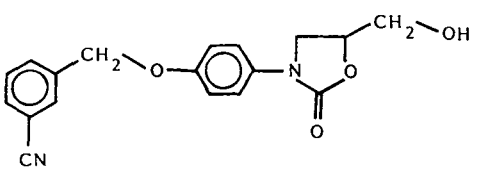

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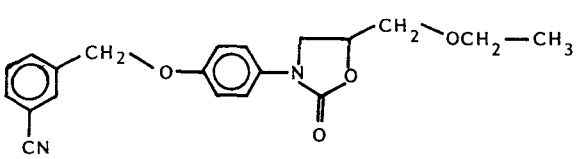

Fig. 1. Structures of cimoxatone (I), the O-demethyl metabolite, MD 770222 (II) and the internal standard (III).

0378-4347/83/$03.00

A high-performance liquid chromatogrpahic (HPLC) method was previously developed in our laboratory using a column packed with silica gel, whose limit of sensitivity was 0.03 mg/l for both compounds and which required a 5—6 ml plasma sample. The measurement of I and II in plasma requires a more sensitive analytical procedure, thus a technique using reversed-phase HPLC with automatic injection was developed and is described in this paper.

MATERIALS AND METHODS

Chemicals and solvents

Pure standards of I, II and MD 780918 (III) {3-[4-(3-cyanophenylmethoxy)-phenyl]-5(ethoxymethyl)-2-oxazolidinone} were synthesized in the Department of Organic Chemistry at Delalande Research Centre; III was used as an internal reference standard.

A solution of water—acetonitrile (90:10, v/v) containing 5 mg/l of III (0.5 mg in 100 ml) was prepared.

All the solvents and chemicals were of analytical grade: toluene, *n*-heptane, acetonitrile (SDS), methanol (Carlo Erba), potassium dihydrogen phosphate and phosphoric acid (Merck).

Extraction procedure

A 100-μl volume of the internal standard solution and 1 ml of plasma were added to a conical tapered tube. After mixing on a Vortex, 5 ml of toluene were added. Samples were extracted on a mechanical shaker (Luckham) for 30 min and the two phases were then separated by centrifugation at 4°C (1000 *g* for 10 min). The organic phase was transferred into a second tube and evaporated to dryness under nitrogen at 40°C. The dry extract was dissolved in 500 μl of *n*-heptane; the solution was shaken on a Vortex mixer, then 500 μl of water—acetonitrile (90:10, v/v) were added. The biphasic solution was vigorously shaken on a Vortex mixer for 45 sec and after centrifugation (5 min at 1000 *g*) the aqueous phase (470 μl) was transferred into a vial, the volume was adjusted to 850 μl with water and injected onto the HPLC column. Automatic injection of samples was carried out every 20 min.

High-performance liquid chromatography

Analyses were carried out with a Micromeritics 750 liquid chromatograph equipped with a 786 UV—visible spectrophotometer and a 725 automatic injector (500-μl injection loop). The ultraviolet (UV) detector was set at 240 nm which corresponds to a relative maximum of absorption for cimoxatone. The UV signal (0.005 a.u.f.s.) was recorded on a 3390A Hewlett-Packard integrator. A stainless-steel column was packed at 400 bar (isopropanol) with 5 μm ODS Spherisorb (batch 18/109). Methanol—phosphate buffer (55:45, v/v) was used as a mobile phase (1 ml/min at 20°C). The buffer was prepared from 0.05 m*M* KH_2PO_4 and the pH adjusted to 4 with phosphoric acid.

Calibration curves

A 1 mg amount of both I and II was dissolved in 100 ml of a solution of water—acetonitrile (90:10, v/v). The solution containing 10 mg/l of the two

compounds (solution A) was then diluted to 1 mg/l (solution B) and to 0.1 mg/l (solution C). The calibration curve was prepared with solution C (0.01, 0.02 and 0.05 mg/l), solution B (0.1 and 0.5 mg/l) and solution A (1 mg/l). The aqueous solutions of the standards were stored at 4°C and renewed every month; no degradation of I, II and the internal standard occurred during this period.

The samples were then processed according to the extraction procedure and to the HPLC sections.

RESULTS AND DISCUSSION

Extraction of I and II from plasma was assayed with toluene, diethyl ether, chloroform and ethyl acetate. A single extraction with toluene provided the cleanest extract. A further purification of the plasma extracts was obtained by dissolution in *n*-heptane followed by extraction with water—acetonitrile (90:10, v/v) which was then injected into the HPLC system.

As expected from their chemical structures, pH changes did not affect the recovery of I and II which, with toluene, was near 90% for both compounds. The extraction of I and II at alkaline pH (>12) led to the degradation of both compounds. In fact, 2-oxazolidinones are not stable at alkaline pH and are hydrolysed to the corresponding β-amino-alcohol derivatives [6]. The extraction from plasma was carried out at pH 7.4.

Plasma extracts from healthy adult volunteers after a single oral dose of the drug showed that I, II and the internal standard were well separated using an ODS Spherisorb reversed-phase column with acetonitrile—0.05 m*M* phosphate buffer pH 5.5 (45:55, v/v) as a mobile phase. No interference occurred from any endogenous compound. However, when the method was applied to monitor plasma concentrations of I and II in depressed patients, it was found that oxazepam interfered with the analysis of the metabolite. Anxiolytics and hypnotics are currently co-administered during chronic treatment with antidepressant drugs. The chromatographic conditions were therefore modified and the mobile phase was replaced by methanol—0.05 m*M* phosphate buffer pH 4.0 (55:45, v/v). Under these conditions, oxazepam, diazepam, demethyl diazepam, lorazepam, chlordiazepoxide and flunitrazepam did not interfere with the analysis of I, II and III. Chromatograms of plasma extracts from a depressed patient treated with I and lorazepam are shown in Fig. 2.

The calibration curves $Y = aX + b$ were drawn by least-squares linear regression analysis of spiked plasma concentration of I or II (X) versus the peak height ratio (Y) of I(II)/internal standard. One sample was prepared for each concentration. A linear response was observed in a range of concentrations between 0.01 and 1.0 mg/l for both compounds. The limit of detection was 0.005 mg/l for both I and II. The results from 16 calibration curves performed during a three-months period showed correlation coefficients (r^2) always > 0.999; the slope of the calibration curves ranged from 3.321 to 4.044 for I and from 5.304 to 7.186 for II. If compared with the results obtained from the grouped data (Table I) the variability of the slope for each single calibration curve exceeded the 95% confidence interval for both compounds. These day-to-day variations were attributed to the extraction procedure and/or to

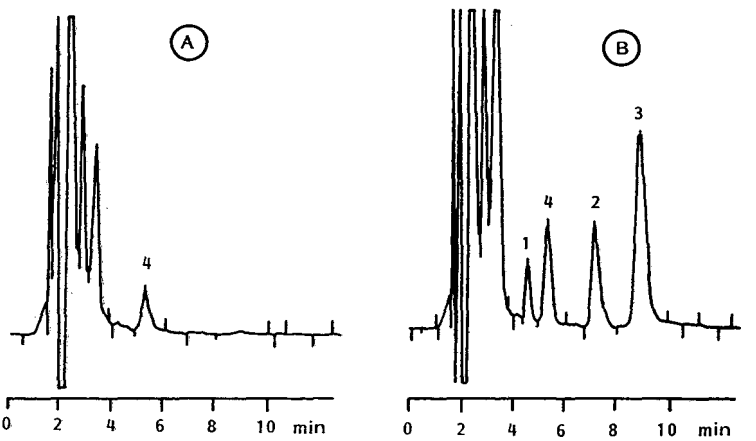

Fig. 2. HPLC chromatograms of plasma extracts obtained from a depressed patient before drug treatment (A) and 24 h after the first 40-mg dose (B). Peaks: 1 = II (0.064 mg/l); 2 = I (0.176 mg/l); 3 = internal standard (0.5 mg/l); 4 = lorazepam.

TABLE I

ACCURACY AND PRECISION OF GROUPED DATA

Cimoxatone (I)				MD 770222 (II)			
Spiked conc. (mg/l)	Found conc. (mg/l)	S.D./X (%)	No. of measurements	Spiked conc. (mg/l)	Found conc. (mg/l)	S.D./X (%)	No. of measurements
0.010	0.009	11	14	0.010	0.011	9	14
0.020	0.020	10	13	0.020	0.021	10	13
0.050	0.050	4	16	0.050	0.051	4	16
0.100	0.098	4	16	0.100	0.097	2	16
0.500	0.508	2	15	0.500	0.501	2	15
1.000	0.996	1	16	1.000	1.000	1	14

Linear least-squares regression analysis (grouped data)

	Equation ($Y = aX + b$)	Slope (a) 95% confidence intervals	Intercept (b) 95% confidence intervals
I	$Y = 3.558X + 0.0045$	3.504/3.612	−0.0216/0.0305
II	$Y = 5.978X$ 0.0051	5.817/6.138	−0.0768/0.0667

the chromatographic analysis, and showed that it is preferable to repeat a calibration curve for each set of samples rather than use the mean calibration curve which gives only the accuracy and the precision of the grouped data. The reproducibility of the technique is also confirmed by the linear relationship between the slope values of the single calibration curves for I and II ($r^2 = 0.935, p < 0.001$).

Plasma concentrations of I and/or II may occasionally exceed 1 mg/l at the doses used in clinical studies, but it was demonstrated that the calibration curve is linear up to 1.5 mg/l for each compound. An example of the plasma concentration—time curve determined in one healthy adult after a single oral dose of a 40-mg tablet of I is given in Fig. 3.

In conclusion, the full automatic procedure makes the technique suitable for routine analyses. This simple and sensitive analytical method has been ex-

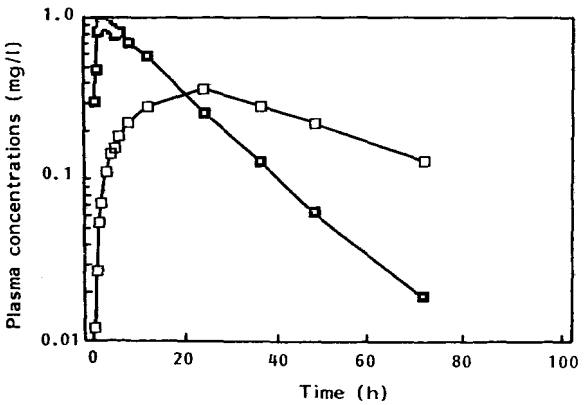

Fig. 3. Plasma concentrations of I (■) and II (□) from one subject after oral administration of the drug (40-mg tablet).

tensively used for the investigation of the pharmacokinetics of I and its major plasma metabolite II, as well as for plasma monitoring in depressed patients following repeated dose treatment.

ACKNOWLEDGEMENTS

The authors wish to thank M. Strolin Benedetti for discussion of this work, J.P. LeMoing for his skilful technical assistance, and M. Farny for preparation of the manuscript.

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Journal of Chromatography, 277 (1983) 396—400
Biomedical Applications
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CHROMBIO. 1784

Note

Determination of ketanserin and its major metabolite (reduced ketanserin) in human plasma by high-performance liquid chromatography

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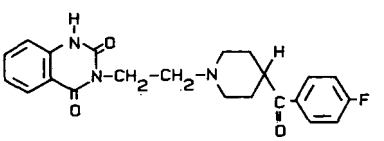
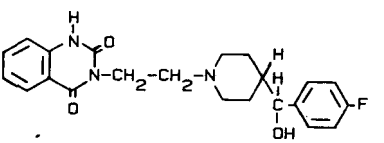
(First received February 11th, 1983; revised manuscript received April 7th, 1983)

Ketanserin (R-41,468), a quinazoline derivative (Fig. 1), is a potent inhibitor of the contractile responses to serotonin (5-HT) of isolated rat caudal, canine basilar, carotid, coronary and splenic arteries. Besides its strong affinity to 5-HT_2 receptors and its inaffinity to 5-HT_1 receptors, it has a five-fold lower affinity for histamine H_1 receptors and adrenergic α_1 receptors [1].

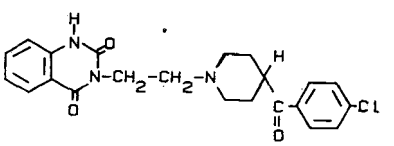
Clinically, ketanserin in a dose of 10 mg given intravenously causes a distinct fall in supine systemic arterial, right-atrial and pulmonary capillary wedge



Ketanserin



Reduced Ketanserin



R-46,594

Fig. 1. Chemical structures of ketanserin, reduced ketanserin and the internal standard, R-46,594.

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pressures, whereas cardiac output, renal blood flow, and glomerular filtration rate show no persistent changes [2]. Ketanserin further improved cardiac output in patients with severe cardiac failure, who had already been treated with digitalis and diuretics, it reduced both pre- and after-load and caused a significant fall in blood pressure while cardiac output and stroke work indices both increased [3], and in patients with thrombophlebitis it reduced pain and swelling [4]. Ketanserin may also be useful in the treatment of Raynaud's phenomenon [5] and in the treatment of postoperative hypertension following coronary artery bypass surgery [6].

Previously a rapid method for the determination of ketanserin by high-performance liquid chromatography (HPLC) was described [7]. One of the metabolites of ketanserin is reduced ketanserin (R-46,742) (Fig. 1). It is not yet understood whether this metabolite is pharmacologically active or not [8]. In this present article a method is described for the determination of both ketanserin and reduced ketanserin using HPLC and ultraviolet (UV) absorbance detection at a fixed wavelength of 214 nm.

EXPERIMENTAL

Chemicals and reagents

Ketanserin, {3-[2-[4-(4-fluorobenzoyl)-1-piperidinyl] ethyl]-2,4(1H,3H)-quinazoline dione}, reduced ketanserin {3-[2[4-[(4-fluorophenyl)hydroxy-methyl]-1-piperidinyl] ethyl]-2,4(1H,3H)-quinazoline dione} and the internal standard {3-[2-[4-(4-chlorobenzoyl)-1-piperidinyl] ethyl]-2,4(1H,3H)-quinazoline dione} (R-46,594) (Fig. 1) were provided by Janssen Pharmaceutica (Beerse, Belgium). R-46,594 was chosen as internal standard because of its structural similarity to ketanserin. HPLC-grade acetonitrile and HPLC-grade water were obtained from J.T. Baker Chemicals (Deventer, The Netherlands). *n*-Heptane spectroscopic grade was obtained from E. Merck (Darmstadt, F.R.G.). All other chemicals were reagent grade.

Instrumentation

The HPLC system was manufactured by Waters Assoc. (Milford, MA, U.S.A.) and consisted of an M-45 solvent delivery system, a Waters intelligent sample processor Model 710 B, and a Model 441 UV absorbance detector with the wavelength fixed at 214 nm. The recorder was a Philips PM 8220 pen recorder with a 10-mV output. For the extractions a Vortex mixer (Wilton & Co, Etten-Leur, The Netherlands) was used.

Chromatographic conditions

The column was a Chrompack CP^{tm} Spher C8 standard column, 25 cm × 4.6 mm I.D., with particle size of 8 μm. The flow-rate was set at 1 ml/min which resulted in a precolumn pressure of 68.9 bar. Detection was at 214 nm with sensitivity at 0.02. The chart speed was 0.5 cm/min.

Mobile phase preparation

To prepare the mobile phase, 550 ml of acetonitrile were filtered through a type FH 0.5-μm Millipore filter and 450 ml of water were filtered through

a type HA 0.45-μm Millipore filter. These liquids were combined and then were added 2 ml of a buffer solution, pH 5.8, consisting of 30% acetic acid and 100% diethylamine (10:4.25). The mobile phase was placed in an ultrasonic bath for 15 min to remove dissolved air.

Extraction procedure

Plasma (2 ml) was pipetted into 15-ml glass centrifuge tubes and spiked with 0.2 μg of the internal standard. Then 0.5 *M* sodium hydroxide (100 μl) was added and the plasma extracted twice with *n*-heptane—isoamyl alcohol (95:5, 2 ml) by vortexing for 1 min, and centrifuged at 4500 *g* for 5 min. The organic layers were combined and extracted with 0.05 *M* sulfuric acid (4 ml) by vortexing for 1 min, and centrifuged at 4500 *g* for 5 min. The aqueous layer was removed and, after adjusting the pH to 9 by adding 25% ammonium hydroxide (200 μl), extracted with *n*-heptane—isoamyl alcohol (95:5, 4 ml) by vortexing for 1 min, and centrifuged at 4500 *g* for 5 min. The organic layer was transferred into a conical tube and evaporated to dryness under a nitrogen stream in a waterbath at a temperature of 50°C. The residue was dissolved in the mobile phase (100 μl) and a 25-μl aliquot was injected into the HPLC apparatus.

Preparation of calibration standards

Ketanserin, reduced ketanserin and the internal standard were dissolved in methanol to produce stock solutions containing 1 mg/ml of these compounds. These stock solutions were further diluted with methanol to produce standard solutions containing 1 μg/ml. The stock solutions were stored in the dark at 4°C for one month; the standard solutions were prepared freshly when needed.

RESULTS AND DISCUSSION

The retention times under the conditions described were 4.9 min for reduced ketanserin, 6.4 min for ketanserin and 8.1 min for the internal standard. In Fig. 2 chromatograms are shown of control plasma (Fig. 2a), control plasma spiked with ketanserin, reduced ketanserin and the internal standard (Fig. 2b), and of a plasma sample obtained from a patient receiving three tablets of ketanserin 40 mg daily (Fig. 2c). The sample was drawn about 4 h after ingestion of the last tablet. (The patient was a 44-year-old white female who suffered from Raynaud's phenomenon and was on this medication for eight days; concurrently she was receiving nitrazepam.) The calculated concentration of ketanserin was found to be 0.169 mg/l and the concentration of reduced ketanserin was 0.103 mg/l.

The standard curves for ketanserin and reduced ketanserin were obtained in a series of experiments in which varying amounts of ketanserin and reduced ketanserin ranging from 0.0125 to 0.200 μg/ml and a fixed concentration of 0.1 μg/ml of the internal standard were added to plasma obtained from healthy human volunteers. After the extraction procedure described above, the samples were injected into the HPLC apparatus and the ratio of the peak height of ketanserin and reduced ketanserin to internal standard was plotted against the known concentrations of each substance. Both plots were linear with $r = 0.999$ for ketanserin and $r = 0.991$ for reduced ketanserin. A concentration of 0.01 μg/ml can easily be measured.

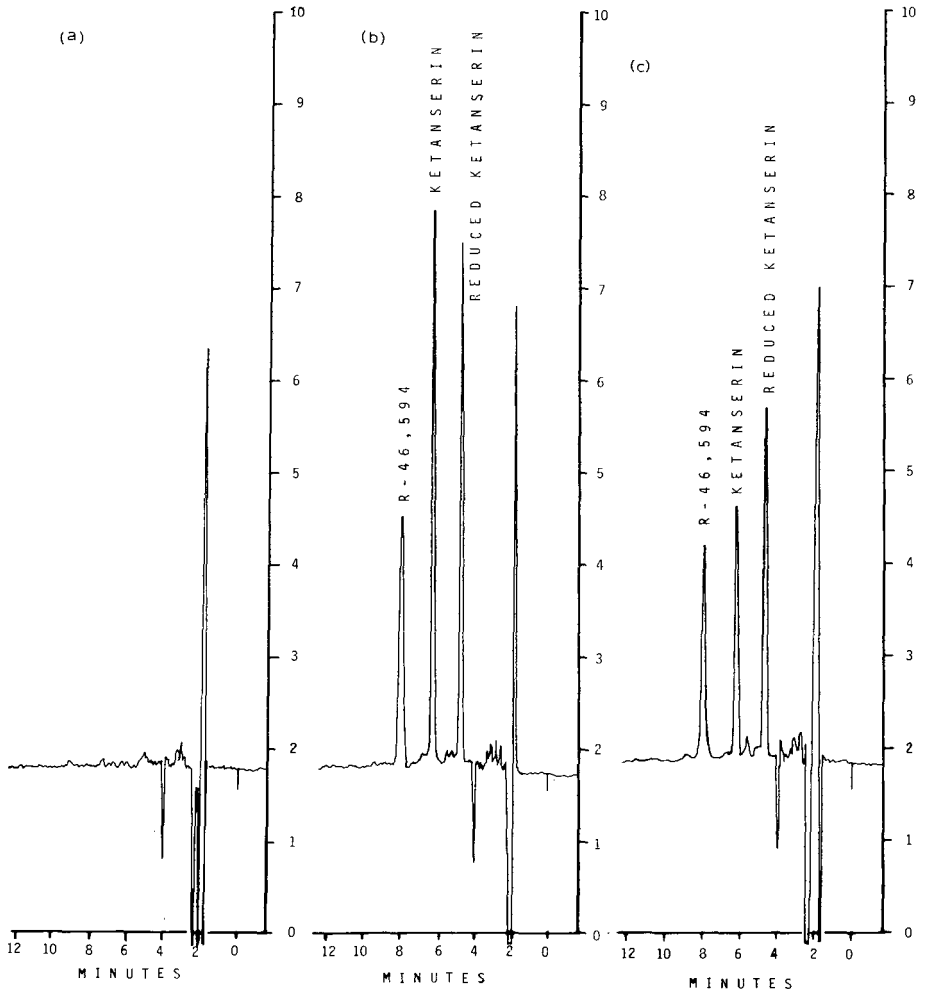

Fig. 2. High-performance liquid chromatograms of (a) control plasma, (b) control plasma spiked with ketanserin, reduced ketanserin (both 0.200 μg/ml) and internal standard (0.100 μg/ml), and (c) a patient's plasma sample.

The extraction efficiency was determined by comparing the peak height of ketanserin and reduced ketanserin extracted from plasma with the peak height of a solution of ketanserin and reduced ketanserin in the mobile phase (the internal standard was added after extraction). The recovery of ketanserin was 82.7 ± 7% and of reduced ketanserin 81.0 ± 8% (n = 10).

The reproducibility of the method was tested by determining the intraassay coefficient of variation; at a concentration of 0.100 μg/ml this was 3.2% for ketanserin and 3.6% for reduced ketanserin (n = 10). Also the interassay coefficient of variation was determined over a period of two months; at a concentration of 0.100 μg this was 4.2% for ketanserin and 4.9% for reduced ketanserin (n = 10).

The method reported here is useful for determination of the clinical phar-

macokinetics of ketanserin, as both the concentrations of ketanserin and reduced ketanserin can be measured in one run.

ACKNOWLEDGEMENTS

The author wishes to thank Janssen Pharmaceutica, Beerse, Belgium, for kindly supplying ketanserin, reduced ketanserin and the internal standard R-46,594, Mrs. Ina Bult for typing the manuscript and Mr. Jan Doorenspleet for drawing the figures.

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Journal of Chromatography, 277 (1983) 401—407
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1786

Note

New, accurate semi-automatic high-performance liquid chromatographic method for routine monitoring of amiodarone plasma levels

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(First received February 14th, 1983; revised manuscript received May 6th, 1983)

Amiodarone is a benzofuran derivative used in the treatment of supraventricular and ventricular arrhythmias [1—3]. The relationship between pharmacokinetic parameters and therapeutic use in man has been scarcely investigated, because, for many years, only non-selective and unpractical radioactive methods were available for detecting this drug in human plasma [4].

More recently some selective high-performance liquid chromatographic (HPLC) methods have been proposed for the measurement of amiodarone in plasma and other tissues [5—7]. This work reports a simple and sensitive HPLC procedure, using an original and more suitable internal standard. Optimal conditions for extraction and chromatography were selected in order to have both a satisfactory recovery and a high sensitivity. Furthermore, the use of a computerized HPLC apparatus equipped with an automatic sampler makes this method reliable for routine monitoring of plasma levels.

EXPERIMENTAL

Reagents and chemicals

Diethyl ether for extraction was analytical grade, filtered on ferrous sulphate; methanol for the chromatography was HPLC grade (LiChrosolv); all other reagents were analytical grade products. All reagents were obtained from Merck (Darmstadt, F.R.G.).

Amiodarone hydrochloride was kindly supplied by Sigma-Tau (Pomezia, Italy). The internal standard, 2-ethyl-3-benzofuranyl-[4-(2-(di-methylamino)-propoxy)-3,5-diiodophenyl]methanone*, was synthesized in our laboratory

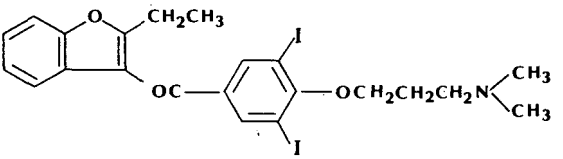
*The internal standard is available free upon request.

0378-4347/83/$03.00

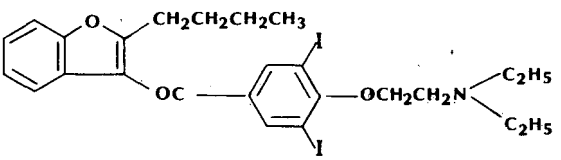
from benziodarone, $NaOCH_3$ and 3-dimethylamino-1-propyl chloride in toluene under reflux for 2 h. Its hydrochloride was crystallized from acetone and showed a melting point of 169–170°C (Fig. 1). The composition of the product $C_{22}H_{24}ClI_2NO_3$ is the following:

	C (%)	H (%)	N (%)	I (%)
Calculated	41.07	3.71	2.00	39.92
Found	41.30	3.62	2.18	39.67

Infrared (IR Spectrophotometer Model Microlab 620 MX, Beckman, Fullerton, CA, U.S.A.) and nuclear magnetic resonance (NMR Model EM 360 L, Varian Instruments Division, Palo Alto, CA, U.S.A.) spectra confirmed the structure.



Internal Standard



Amiodarone

Fig. 1. Structural formulae of amiodarone and internal standard.

Standard solutions

A stock solution of amiodarone hydrochloride was prepared (1.0 mg/ml) in methanol–water (1:1) and stored at 4°C in the dark. Appropriate volumes of methanolic working solutions prepared daily were added to drug-free human plasma to obtain five plasma standard samples at concentrations 0.05, 0.1, 0.5, 1.0 and 2.0 μg/ml.

A stock solution of internal standard (1 mg/ml) was prepared by dissolving 25 mg of the compound in 25 ml of methanol–water (1:1); a working solution was prepared daily by diluting (1:20) the stock solution with methanol.

Sample preparation

A 2-ml volume of 0.2 *M* acetate buffer (pH 3.8) and 20 μl of internal standard working solution were added to 0.5 ml of the plasma sample. The extraction was performed with 4 ml of diethyl ether and carried out in screw-capped

conical glass tubes (16 × 200 mm) using a rotary mixer for 15 min. After centrifugation at 750 *g* for 5 min, the upper organic phase was aspirated and extraction repeated with 4 ml of fresh diethyl ether. After a second centrifugation, the two organic phases were pooled and evaporated under vacuum, at room temperature, using a Vortex evaporator (Buchler Instruments, Fort Lee, NJ, U.S.A.).

The residue was redissolved in 500 μl of the mobile phase for chromatography and transferred in a crimp seal vial (Supelco Inc., Bellefonte, PA, U.S.A.); 50 μl of this solution were automatically sampled and injected for chromatography.

HPLC conditions

A Hewlett-Packard Model 1084-B liquid chromatograph, equipped with an automatic variable-volume injector and a variable-wavelength UV detector, was used for all analyses. The apparatus was controlled by a computer terminal (Hewlett-Packard Model 79850 B LC).

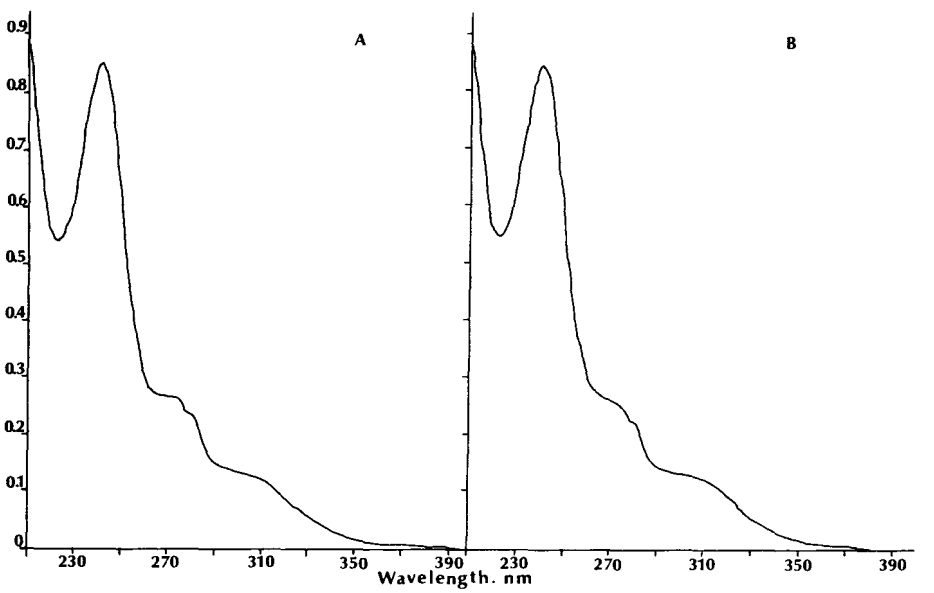


Fig. 2. UV spectra of amiodarone (A) and internal standard (B) dissolved in mobile phase.

The LiChrosorb SI-100 column (HIBAR RT), 5-μm particle size (stainless steel, 250 mm × 4 mm I.D.) was supplied by Merck.

Samples were eluted isocratically at a constant flow-rate of 2.0 ml/min. The mobile phase was methanol—diethyl ether (70:30, v/v) containing 0.02% perchloric acid. The solution was filtered through a 0.5-μm Millipore filter and degassed. The eluent was monitored continuously at 242 nm with a 2^3 attenuation. Printing of chromatograms, integration of peaks and all calculations were performed by the computer terminal of the HPLC apparatus. The peak area ratios of amiodarone to the internal standard were plotted against the

drug concentrations to obtain the standard curve. All glassware was ultrasonicated and carefully cleaned with a sulfuric–dichromate solution and washed with distilled water, methanol and diethyl ether.

UV spectra

To determine suitable detection wavelengths, the absorbance spectra of amiodarone and internal standard were performed preliminarily using a Cary 219 (Varian Instruments Division, Palo Alto, CA, U.S.A.).

The compounds were dissolved in mobile phase at a concentration of about 15 mg/l. Both the amiodarone and the internal standard show the maximum absorbance peak at 242 nm, with an $E^{1\,cm}_{1\%}$, respectively, of 0.562 and 0.581 (Fig. 2).

Furthermore, UV spectra of peaks were checked during the chromatographic procedure by the stop-flow method using the variable-wavelength detector of the HPLC apparatus.

RESULTS AND DISCUSSION

The efficiency and the reproducibility of the extraction were very satisfactory under our conditions (Table I). The diethyl ether was found to be the most suitable solvent for a rapid and complete extraction of amiodarone from the plasma; the steps of decantation and evaporation of the solvent appear to take place more easily. The most critical point of the extraction is the fixing of the pH, which must range between 3.6 and 4.2 for the complete recovery of amiodarone and of the internal standard. We have always used diethyl ether stabilized with 7 ppm of 2,5-di-*tert.*-butyl-4-methylphenol (BHT).

TABLE I

EXTRACTION EFFICIENCY EXPRESSED AS A PERCENTAGE OF PEAK AREA OF ALCOHOLIC STANDARD SOLUTIONS

Amiodarone concentration (μg/ml)	*n*	Mean ± S.D.
0.25	10	96.7 ± 4.6
1.00	10	98.3 ± 3.3

Five plasma samples spiked with amiodarone (0.05, 0.1, 0.5, 1.0 and 2.0 μg/ml) were chromatographed to test the linearity of the method. Amiodarone/internal standard peak area ratios were plotted against drug concentrations and linear regression analysis was performed by the least-squares method, using a Hewlett-Packard 9885 desk-computer. The straight line equation fitting the experimental points was: $Y = 0.523\,X - 0.006$ with a correlation coefficient $r = 0.999$ ($P < 0.01$).

The use of a variable-wavelength UV detector allows the eluate to be monitored at 242 nm with a sensitivity nearly double that of the detector with

a fixed wavelength of 254 nm [6]. Fig. 3 shows the chromatograms of drug-free plasma extracts, and extracts of plasma spiked with amiodarone (1 μg/ml) and internal standard (1 μg/ml), and the extract of a plasma sample of a patient treated with amiodarone.

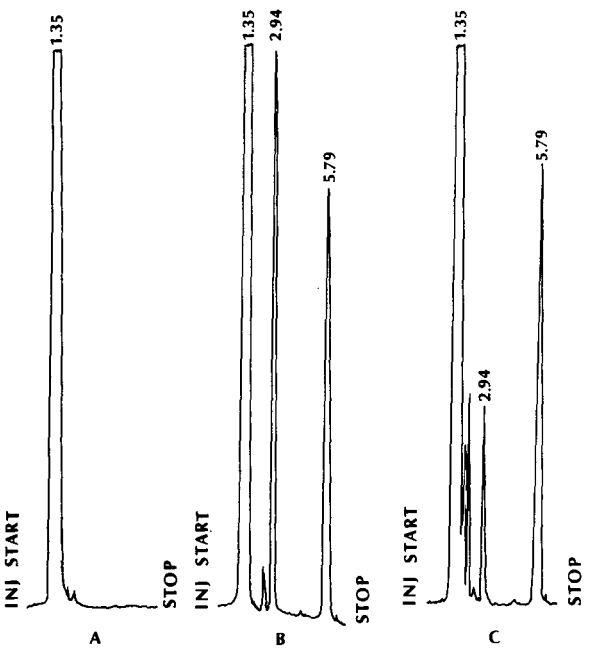


Fig. 3. Chromatograms of a blank plasma (A), a plasma sample spiked with standard amiodarone 1 μg/ml (B), and a patient plasma sample with 0.22 μg/ml amiodarone (C). See text for chromatographic conditions.

Under the chromatographic conditions described above it was possible to achieve a good separation of amiodarone from the internal standard. The retention time was 2.94 min for amiodarone and 5.74 min for the internal standard. The eluent mixture we used allowed symmetric and sharp peaks to be obtained, clearly separated from the solvent front and from a probable amiodarone metabolite, with a retention time of 2.09 min. The percentage of perchloric acid in the eluent mixture is critical for elimination of peak tailing. The retention times are shortened either by a major quantity of perchloric acid or by reduction of the amount of diethyl ether in the mixture. The retention times are negatively influenced by the solvent effect.

Injections of extracts redissolved only in methanol show a drastic reduction in retention times, while the extracts redissolved in diethyl ether or diisopropyl ether show rather longer retention times. Under the analytical conditions indicated, the life of a column is about 800 analyses with only a slightly decreased efficiency.

The UV spectra of the chromatographic peaks were scanned by the stop-flow method, in order to check possible interferences during the analysis of plasma

samples of patients treated with other drugs. The administration of one or more of following drugs is frequently associated with amiodarone treatment: digoxin, quinidine, procainamide, lidocaine, benzodiazepine, dipyridamole, isosorbide dinitrate, theophylline, caffeine, furosemide. In no case have we noted any variation in the UV spectrum or in any ratio other than 3.79 between the absorbance at 242 nm and that at 280 nm.

The sensibility and the linearity of the method (from 0.020 to 5.00 μg/ml) allows suitable monitoring of patients chronically treated with amiodarone, the haematic levels of which ranged between 0.15 and 0.50 μg/ml.

The reproducibility of the method was determined at two different concentrations of amiodarone. The intraday and interday coefficients of variation were calculated for spiked plasma samples containing 1.00 and 0.25 μg/ml amiodarone (Table II).

TABLE II

REPRODUCIBILITY DATA FOR THE DETERMINATION OF AMIODARONE IN HUMAN PLASMA

Amiodarone concentration (μg/ml)	*n*	Intraday		Interday	
		Mean ± S.D. (μg/ml)	C.V. (%)	Mean ± S.D. (μg/ml)	C.V. (%)
1.00	10	0.976 ± 0.0360	3.68	1.011 ± 0.0305	3.02
0.25	10	0.247 ± 0.0106	4.29	0.248 ± 0.0114	4.62

With the method reported above all the critical parameters for accurate measurement of the amiodarone plasma levels were optimized. The method, besides being automated as much as possible with respect to extraction, injection, integration and calculation phases, offers some advantages compared with the methods previously published. The most important advantages are higher sensitivity, higher extraction recovery, more rapid chromatographic separation and optimization of UV detection, compared with the methods of Flanagan et al. [5], Lesko et al. [6] and Covelli et al. [7].

Our chromatograph, suitably programmed for all the parameters, is fit for running automatically sets of 60 samples at a time. We have been using this method for more than a year for monitoring the amiodarone plasma levels. A technician can easily analyse more than 50 samples a day.

The sensitivity of the method is high enough to allow us to perform pharmacokinetics studies which we are now carrying out in our laboratories and will publish as soon as possible. However, the sensitivity of our method could still be improved by the use of unstabilized ethyl ether both for the eluent mixture and for the extraction, and by increasing the initial volume of the plasma to be tested.

ACKNOWLEDGEMENT

This work was supported by the CNR (Consiglio Nazionale delle Ricerche) grant RN-18350/A.

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Journal of Chromatography, 277 (1983) 408—413
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1795

Note

Analysis of sulfinpyrazone and its metabolites in human plasma and urine by high-performance liquid chromatography

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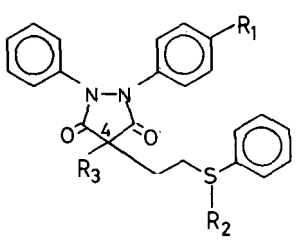
(Received March 9th, 1983)

Sulfinpyrazone {1,2-diphenyl-4-[2-(phenylsulfinyl)ethyl]-3,5-pyrazolidinedione} (Anturane®) (Fig. 1, 1) was used initially as an uricosuric drug [1]. Recently it has been studied as a platelet-aggregation inhibitor and in the prevention of sudden death after myocardial infarction [2, 3]. Sulfinpyrazone is transformed into a series of metabolites (2—6) of which the sulfide (2) is thought to be the pharmacologically active compound [4]. Clinical pharmacological studies on drug interactions [5] and on the induction of drug-metabolizing enzymes by sulfinpyrazone after single and chronic administration [6] have prompted us to develop a high-performance liquid chromatographic (HPLC) method for the determination of the parent drug (1) and metabolites



	R_1	R_2	R_3
1	H	O	H
2	H	—	H
3	H	O_2	H
4	OH	O	H
5	OH	—	H
6	OH	O_2	H
7	H	O	OH

Fig. 1. Structure of sulfinpyrazone (1) and metabolites: (2) sulfide, (3) sulfone, (4) *p*-hydroxysulfinpyrazone, (5) *p*-hydroxysulfide, (6) *p*-hydroxysulfone, (7) 4-hydroxysulfinpyrazone.

0378-4347/83/$03.00

2 to 6 in human plasma and urine; solvent extraction of the samples, followed by HPLC gradient elution on a C_{18} reversed-phase column and UV detection was used. Colorimetric [7], gas chromatographic [8, 9] as well as liquid chromatographic methods [10—17] have been described but none of these has measured simultaneously sulfinpyrazone and its metabolites in plasma and urine with non-radioactive techniques.

MATERIALS AND METHODS

Apparatus

A system consisting of two Model 6000A pumps, a WISP Model 710B automatic injector, a Model 660 solvent programmer, a Model 440 UV detector (all from Waters, Königstein, F.R.G.) and a Model C-R1B data processor plotter (Shimadzu, Düsseldorf, F.R.G.) were used for chromatography and calculations.

Reagents

All test substances were of analytical grade; sulfinpyrazone (1) and substances 2 to 7 were gifts from Ciba Geigy (Basel, Switzerland) and Ciba Geigy Pharmaceutical Division (Horsham, U.K.). Naproxen was from Chemie Grünenthal (Stolberg/Rhdl., F.R.G.). Acetonitrile (for spectroscopy, Merck, Darmstadt, F.R.G.) was used without purification; deionized water was purified with Norganic cartridges (Millipore, Neu-Isenburg, F.R.G.). Standard solutions of sulfinpyrazone (1), metabolites 2 to 6 and naproxen (1 g/l) in methanol were kept at 4°C in the dark.

Procedure

Plasma and urine from healthy male volunteers were collected at specified time intervals, after 400-mg doses of sulfinpyrazone [18]. Blood was collected in heparinized tubes, centrifuged and the plasma was frozen at −23°C. Sodium bisulfite (1 mg/ml) was added to urine samples and kept at the same temperature; they were thawed and centrifuged before analysis.

Plasma extraction. A 1-ml aliquot of plasma was spiked with 10 μg of naproxen, and then 1 ml of 1 *N* hydrochloric acid, 0.5 ml of sodium sulfite (20 mg/ml) and 4 ml of solvent [1-chlorobutane—dichloromethane (1:3, v/v)] were added. After 15 min shaking and 10 min centrifugation at 2000 *g*, 3 ml of the organic phase were transferred to a 15-ml conical test tube and evaporated under a stream of nitrogen at 37°C. The residue was dissolved in 100 μl of 2% sodium sulfite with vortexing, and 10 μl were injected in the HPLC system.

Urine extraction. A 0.5-ml volume of urine was mixed with 0.5 ml of water; 25 μg of naproxen were added and extracted as for plasma.

Calibration curves. For each analysis series a five-point calibration curve for each of substances 1 to 6 was run in parallel with the samples. To 15-ml extraction tubes known amounts of the substances (for concentration ranges see Table I) dissolved in methanol were added and evaporated under nitrogen. Blank plasma (1 ml) or urine (0.5 ml) was added and the extraction procedure was performed as described above. The slope and *y* intercept values were

evaluated for each substance and further used for calculations of the concentrations of the samples.

Chromatographic conditions

The mobile phase consisted of: eluent A, 0.1 *M* ammonium acetate—acetonitrile (780:220, v/v); eluent B, acetonitrile. The solvents were filtered and degassed before use. For the gradient elution, convex curve 7 program from 0% to 100% of eluent B in 15 min was used. The solvent rate was 2.0 ml/min and the pressure varied from 70 to 140 bar (7—14 mPa). A reversed-phase C_{18} column (μBondapak C_{18}, Waters; 300 × 3.9 mm I.D.; 10-μm irregular particles) and a guard column (Bondapak C_{18} Corasil, Waters; 25 × 4 mm I.D.; 35—50-μm spherical particles) were used for the separation. After a series of analyses the columns were washed with water and methanol. UV detection was at 254 nm and a sensitivity of 0.1 a.u.f.s. was used. Quantitation was effected by electronic area integration and the calibration curves were calculated from the ratio of peak area of the substance (1 to 6)/area of internal standard vs. added concentration (mg/l). Sample analysis time was 15 min followed by 5 min reequilibration before the next sample injection. Chromatograms were run at room temperature.

RESULTS

Fig. 2a shows a chromatogram of plasma spiked with sulfinpyrazone (1) and metabolites 2 to 6. Fig. 2b displays a HPLC separation of a plasma extract and Fig. 2c is of a urine extract obtained from volunteers after drug administration. Peaks were sharp and well separated, and blank plasma or urine did not show interfering peaks in the chromatogram. Several drugs were also tested: anti-

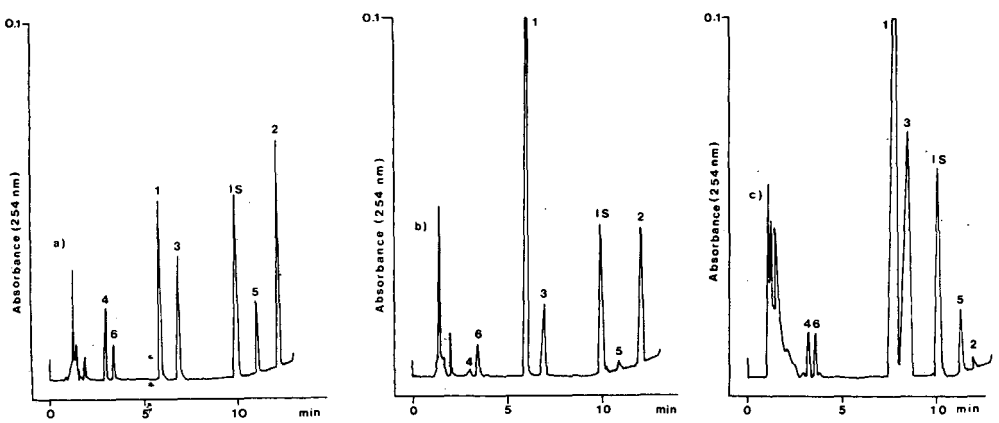


Fig. 2. (a) Chromatogram of an extract from blank plasma spiked with sulfinpyrazone (1) (5 mg/l, retention time, RT, 5.86 min, k' 3.34), (4) (0.5 mg/l, RT 2.91 min, k' 1.16), (6) (0.5 mg/l, RT 3.37 min, k' 1.50), (3) (4 mg/l, RT 6.86 min, k' 4.08), IS (internal standard) (10 mg/l, RT 9.85 min, k' 6.30), (5) (1 mg/l, RT 10.81 min, k' 7.01), and (2) (5 mg/l, RT 12.06, k' 7.93). The number above each peak corresponds to the formula in Fig. 1. (b) Chromatogram of plasma extract from a volunteer 2 h after ingestion of a 400-mg sulfinpyrazone tablet. (c) Extract of 2—4 h urine fraction after 400-mg oral sulfinpyrazone administration.

pyrine and metabolites (nor-antipyrine, 4-hydroxyantipyrine, 3-hydroxymethylantipyrine [19]), cimetidine, digoxin, acetylsalicylic acid, ampicillin, azlocillin, cotinine, heparin, hippuric acid, 1-(β-hydroxypropyl)theobromine, lidocaine, metoprolol, methaqualone, mezlocillin, nicotine, neostigmine bromide, pindolol, procainamide, propranolol, quinidine, salicylic acid, salicylamide, secobarbital, theobromine, theophylline, uric acid, vitamin B complex, vitamin C, and warfarin did not interfere. Phenprocoumon interfered with the internal standard and antipyrine with metabolite 6. 4-Hydroxysulfinpyrazone (7) which is considered an artefact [16] was not determined and eluted after the sulfide (2) (k' = 8.63). Treatment of urine with β-glucuronidase—arylsulfatase yielded concentration values which did not differ significantly from those of untreated samples.

Accuracy and precision of the analysis for plasma and urine are shown in Table I. The calibration curves were found to be linear in the ranges indicated in Table I. Sensitivity was 0.1 mg/l for plasma and 0.5 mg/l for urine. Calculated recoveries were 90—100% for all substances except for 4 which was 60%.

TABLE I

REPRODUCIBILITY OF PLASMA AND URINE ANALYSIS FOR SULFINPYRAZONE AND METABOLITES

Substance	Conc. added (mg/ml)	Conc. found (mg/ml)	C.V.* (%)	Conc. added (mg/ml)	Conc. found (mg/ml)	C.V.* (%)
1	0.50	0.58	1.5	5.0	5.2	6.3
	5.0	4.9	2.2	50.0	48.7	1.2
	30.0	30.0	3.4	100.0	100.1	1.6
2	0.50	0.53	2.1	2.0	2.1	6.3
	2.00	1.93	2.3	10.0	9.8	2.9
	4.0	4.0	3.9	50.0	50.1	0.8
3	1.00	0.94	3.5	0.50	0.46	6.1
	4.0	4.0	3.9	2.0	2.0	1.7
4	0.50	0.52	9.8	5.0	4.7	7.5
	1.50	1.49	6.4	20.0	20.0	2.7
5	1.00	0.89	8.3	0.50	0.53	9.6
	4.0	4.0	6.0	2.0	2.0	1.0
6	0.20	0.20	10.4	5.0	4.7	8.6
	2.0	1.9	3.9	20.0	20.0	2.0

*C.V. = coefficient of variation (n = 5).

DISCUSSION

Different extraction procedures, stationary phase packings and elution procedures have been published [11—17]. The method described in this paper

simplifies the extraction step, allows the simultaneous determination of sulfinpyrazone (1) and metabolites 2 to 6 in both plasma and urine, and possesses higher peak resolution and sharpness. The chromatographic system is very stable (more than one thousand injections can be done with the same column) and endogenous compounds and numerous drugs do not interfere. Up to 50 extractions and analyses can be done in 24 h. The precision and accuracy (Table I) are adequate.

After drug administration, sulfinpyrazone (1) is the main substance in plasma, the sulfone (3) and the sulfide (2) are the main metabolites and 4, 5 and 6 minor ones. In some volunteers 5 could not be detected (Fig. 3). The decline of the plasma concentrations of the metabolites parallels that of the parent compound with the exception of the sulfide whose maximum concentration appears much later [14, 15, 20] (Fig. 3). The delay in the peak plasma concentration of the sulfide is probably due to reduction of sulfinpyrazone by the gut microflora following enterohepatic circulation [21]. The 4-C glucuronides of 1, 2 and 3 which have been described [16, 17] in urine were not quantified as these derivatives were unavailable, and also because of their highly polar character which precludes their extraction in the solvents used. Dieterle and co-workers [16, 17] detected them after administration of radioactively labeled sulfinpyrazone and using radioactivity detection techniques.

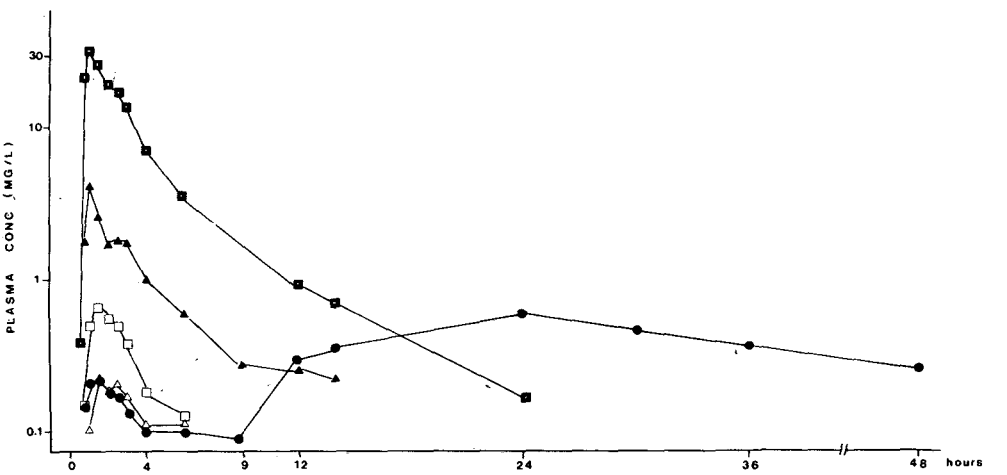


Fig. 3. Plasma concentration—time curve for sulfinpyrazone (1, ▪) and metabolites 2 (●), 3 (▲), 4 (□) and 6 (△) after a single oral dose of 400 mg of sulfinpyrazone.

The described method is simple, specific and sensitive for the analysis of sulfinpyrazone and metabolites in human blood and urine, and may be used in human pharmacokinetic studies, in the drug level monitoring of patients and in animal exprimentation. It has been used routinely during the last six months and the results of these investigations will be published elsewhere.

ACKNOWLEDGEMENTS

This work was carried out with support by the Deutsche Forschungsgemeinschaft (Bonn-Bad Godesberg, F.R.G.). N.S. Wang was a fellow from the Deutsche Akademische Austauschdienst (Bonn-Bad Godesberg). We thank Miss B. Koroknay for technical help.

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Journal of Chromatography, 277 (1983) 414—418
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1805

Note

High-performance liquid chromatographic determination of bromazepam in human plasma

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(First received March 2nd, 1983; revised manuscript received May 25th, 1983)

Since the early 1960s, benzodiazepines have been widely used as minor tranquillizers, sleep inducers and muscle relaxants. Bromazepam is a member of the 1,4-benzodiazepine class of compounds and was synthesized by Fryer et al. [1]. A usual daily therapeutic dose of bromazepam is 3—8 mg. Such a low therapeutic dose is associated with the strong action of this drug and results in low concentrations in plasma.

Analytical methods such as gas—liquid chromatography (GLC) [2—4] and thin-layer chromatography (TLC) [5] have been reported for determining bromazepam and its metabolites in body fluids. There has been no literature available concerning the determination of bromazepam in the plasma or serum by high-performance liquid chromatography (HPLC). It was the aim of this work to establish the assay method for the quantitation of bromazepam in human plasma by HPLC. The method will be applied for pharmcokinetic and bioavailability study of the drug.

0378-4347/83/$03.00

EXPERIMENTAL

Reagents

Reagents used were of analytical grade. Bromazepam [7-bromo-1,3-dihydro-5-(2-pyridyl)-2H-1,4-benzodiazepin-2-one] was obtained from Kodama (Tokyo, Japan). Tetra-*n*-butylammonium hydroxide (TBAH) 10% in water was obtained from Tokyo Kasei Industries (Tokyo, Japan).

Borate buffer (1 *M*) was adjusted to pH 8.0 with sodium hydroxide (0.1 *M*). Standard solutions of bromazepam were prepared in methanol at concentrations of 10.3 and 103.0 ng/ml. A methanol solution of carbamazepine (5H-dibenzo[*b*,*f*]azepine-5-carboxamide) was prepared at a concentration of 51.0 ng/ml and used as the internal standard.

Mobile phase

Twenty milliliters of 10% TBAH in water were added to 1 l of water and the pH was adjusted to 7.5 with phosphoric acid. The mobile phase was prepared by adding 700 ml of the above TBAH solution and 20 ml of methanol to 300 ml of acetonitrile.

High-performance liquid chromatography

The chromatograph consisted of a Hitachi 638-30 fitted to a variable-wavelength UV monitor, Hitachi 635-0900, operated at a maximum sensitivity of 0.005 a.u.f.s. (230 nm). Injection was made by a 50-μl micro-syringe via a Hitachi 638-0801 injector. The column was 30 cm × 4 mm I.D., prepacked with μBondapak C_{18}, particle size 10 μm (Waters Assoc., Milford, MA, U.S.A.). Chromatography was performed in reversed-phase mode at a flow-rate of 1 ml/min at room temperature. The chart speed was 5 mm/min.

Extraction procedure

One milliliter of plasma, 1 ml of internal standard solution, 2 ml of borate buffer (pH 8.0), and 20 ml of toluene were combined in a glass-stoppered extraction tube. The tube was then shaken for 10 min. After centrifugation, the toluene phase was transferred to a flask and evaporated to dryness under reduced pressure.

The residue was allowed to stand at −20°C for 2 h and redissolved with 3 ml of ethanol—water (90:10, v/v). The solution was transferred to a 10-ml tube. The tube was again allowed to stand at −20°C for 2 h. The ethanol solution was then centrifuged at 1500 *g* for 10 min and decanted into a 10-ml flask and evaporated to dryness under reduced pressure. The residue was resuspended in 100 μl of acetonitrile—TBAH (60:40, v/v), pH 7.5, and a 40-μl portion was injected into the chromatograph.

RESULTS AND DISCUSSION

Direct GLC measurements of underivatized bromazepam [2, 3] or the TLC method [5] do not provide accurate and sensitive analysis of this compound in biological fluids. The methylation of bromazepam to its N^1-methyl derivative, however, results in a significant improvement of the GLC measurements

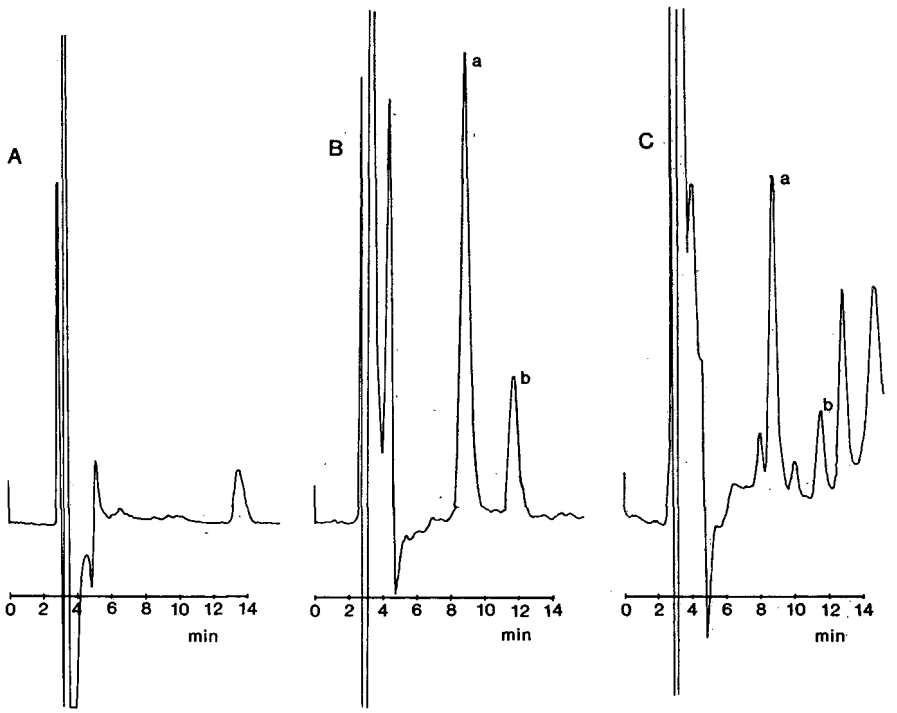

Fig. 1. (A) Chromatogram of an extracted blank plasma. (B) Chromatogram of an extracted standard plasma (1.0 ml) spiked with 103 ng of bromazepam (peak a) and 51 ng of internal standard (peak b). (C) Chromatogram of an extracted plasma (1.0 ml) from a patient dosed with a 5-mg bromazepam tablet.

and the method was successfully applied for pharmacokinetic studies in man [4]. The present HPLC method does not require a derivatization procedure and the sample can be directly injected onto the HPLC column after the initial extraction procedure.

A typical chromatogram of bromazepam, obtained after processing 1 ml of plasma spiked with 103.0 ng of the drug is shown in Fig. 1 together with chromatograms of an extracted blank plasma and a patient's sample. Peaks a and b correspond to bromazepam and the internal standard, respectively. The respective retention times are 8.5 and 11.5 min. There were no interfering peaks originating from endogenous compounds in plasma. Other drugs, such as neostigmine methyl sulfate, pentazocine, atropine sulfate and thiopental sodium, commonly used concomitantly with bromazepam, were found not to interfere with the bromazepam analysis.

Calibration curve

Calibration curves of plots of peak height ratios against concentrations were linear ($Y = 0.135 + 0.0271X$; $r = 0.999$) over the concentration range 10—200 ng/ml.

Analytical recovery

Known amounts (20.6, 51.5 and 103.0 ng) of bromazepam and a constant amount (51.0 ng) of internal standard were added to 1-ml portions of pooled plasma and the samples were processed as described. The analytical recovery of bromazepam at these concentrations was between 93.7 and 108.7% (Table I).

TABLE I

RECOVERY OF BROMAZEPAM FROM PLASMA

Drug added to 1 ml plasma (ng)	No. of determinations	Drug recovered (ng)	Recovery (%)	Mean recovery (%)	C.V.* (%)
20.6	1	19.3	93.7	98.4	4.72
	2	20.6	100.0		
	3	19.5	94.7		
	4	21.7	105.3		
51.5	1	50.8	98.6	101.8	5.31
	2	48.8	94.8		
	3	56.0	108.7		
	4	54.1	105.0		
103.0	1	111.6	108.3	103.1	4.70
	2	102.1	99.1		
	3	100.3	97.4		
	4	110.6	107.4		

*C.V. = coefficient of variation.

Reproducibility

Day-to-day reproducibility of the method was evaluated by analyzing a pooled plasma containing bromazepam in a concentration of 51.5 ng/ml on five different days. A calibration curve was prepared on each assay day. Each calibration curve was constructed by determining two low concentrations (10.3 and 20.6 ng/ml) and two high concentrations (103.0 and 206.0 ng/ml). The mean value for the drug concentration determined was 50.2 ng/ml and the coefficient of variation was 5.5%.

Precision at low concentrations

Plasma samples with three different low concentrations of bromazepam, 5.2, 10.3, and 20.6 ng/ml, were analyzed in one assay run. The results are summarized in Table II. The mean peak height ratio and the coefficient of variation at a concentration of 5.2 ng/ml were 0.248 and 5.6%, respectively. A strict linearity was observed at these concentrations and the method provided satisfactory precision at concentrations as low as 5 ng/ml.

TABLE II

PRECISION OF ASSAY METHOD AT LOW CONCENTRATIONS

Drug added to 1 ml plasma (ng)	Peak height ratio						C.V.* (%)
	Individual values				Mean	S.D.	
5.2	0.250	0.262	0.225	0.254	0.248	0.0138	5.6
10.3	0.369	0.379	0.436	0.452	0.409	0.0356	8.7
20.6	0.658	0.692	0.663	0.722	0.684	0.0256	3.7

*C.V. = coefficient of variation.

ACKNOWLEDGEMENT

The excellent technical assistance of Mrs. N. Saito is gratefully acknowledged.

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Journal of Chromatography, 277 (1983) 419—422
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1806

Note

High-performance liquid chromatographic procedure for the determination of probucol in human plasma

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Probucol, 2,2-bis(3,5-di-*tert.*-butyl-4-hydroxyphenylmercapto)propane, has been reported to have a potent cholesterol-lowering activity. Its effectiveness has been demonstrated in normal animals, experimental hypercholesterolaemic animals [1—3], healthy volunteers [4, 5] and lipaemic patients [6]. The compound has been reported to be a unique drug whose nature is different from that of clofibrate and colestipol, which are reference drugs [7], in that it produced a significant decrease in serum low density lipoprotein cholesterol level and exerted little influence on the serum triglyceride level [4].

However, little work has been done with respect to the determination of its plasma levels which are supposed to be indexes for the evaluation of its effectiveness and safety in man. The only quantitative analysis of probucol in plasma has been performed by mass fragmentography [8], and few reports are available on its pharmacokinetic profile [9].

Therefore, an attempt was made to develop a method for the quantitative determination of plasma probucol concentrations that would be simple, suitable for routine analysis and highly sensitive. In this paper, we describe a simple method for the determination of probucol in human plasma using high-performance liquid chromatography (HPLC). The results of the HPLC assay for plasma probucol concentrations after a single oral dose of 250 mg to healthy male subjects are also described.

EXPERIMENTAL

Probucol (Fig. 1a) and the internal standard, 2,2-bis(3,5-di-*tert.*-butyl-4-

0378-4347/83/$03.00

Fig. 1. Chemical structure of probucol (a) and internal standard (b).

hyroxyphenylmercapto)pentane (Fig. 1b), were supplied by the Dow Chemical Company (Indianapolis, IN, U.S.A.). Acetonitrile, methanol and ethyl ether were purchased from Wako Pure Chemical Industries (Tokyo, Japan).

The HPLC separations were carried out using a Waters Assoc. ALP/GPC 244 Compact System equipped with 254 nm filter kit. A μBondapak C_{18} reversed-phase column (30 cm × 3.9 mm I.D., particle size 10 μm, Waters Assoc.) was used with the mobile phase acetonitrile—water (85:15, v/v) at a flow-rate of 2.0 ml/min.

Sample preparation

To 1.0 ml of human plasma the internal standard and ethanol were added. The contents were stirred on a Vortex mixer and centrifuged for 10 min at 1700 *g*. A portion of the supernatant was transferred to a centrifuge tube and the ethanol was evaporated under a stream of air. To the residue 0.2 *N* NaOH and diethyl ether were added and the mixture was shaken and centrifuged for 10 min at 1700 *g*. The ether layer was evaporated to dryness under a stream of air. The residue was redissolved in methanol (100 μl) and an aliquot (40 μl) was injected into the HPLC system and analyzed.

The calibration curve was constructed at probucol concentrations of 0.25—25 μg/ml plasma.

Healthy volunteers received a single 250-mg oral dose of probucol. Blood samples were collected at scheduled intervals and were centrifuged at 1700 *g* for 10 min to obtain plasma samples.

RESULTS AND DISCUSSION

Chromatograms obtained by the above procedures using the plasma samples with and without probucol and the internal standards are given in Fig. 2. No significant interference was observed in the regions for probucol and the internal standard on the chromatogram. A good chromatographic separation was obtained with apparent retention times for probucol and the internal standard of 7.0 and 10.4 min, respectively, a separation factor (α) of 1.055 and a resolution (R_s) of 1.36.

The linearity of the calibration curve constructed for the determination of probucol at concentrations of 0.25—25 μg/ml is demonstrated in Table I. At a concentration of 0.25 μg/ml the peak height ratio was 0.084 ± 0.003 with a coefficient of variation (C.V.) of 3.9%. At concentrations higher than 0.25 μg/ml the C.V. was smaller than 3.9% showing very little deviation of the peak height ratio. A higher extraction ratio for probucol was obtained with a value greater than 89%. The equation for the resulting line was $Y = 0.31795X -$

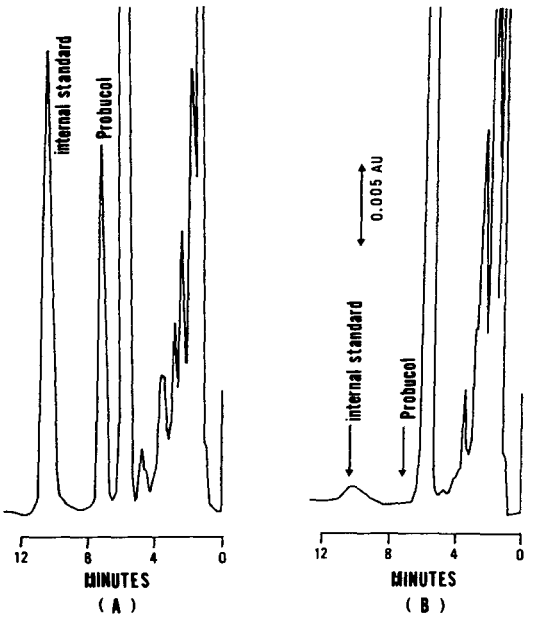

Fig. 2. (A) Representative chromatogram of probucol and internal standard in human plasma. Concentrations in plasma: probucol 2.5 μg/ml, internal standard 4.5 μg/ml. (B) Chromatogram of a blank human plasma.

TABLE I

LINEARITY OF HPLC PROCEDURE FOR PROBUCOL IN HUMAN PLASMA BY INTERNAL STANDARD METHOD

Theoretical plasma probucol conc. (μg/ml)	Mean peak height ratio ± S.D. (± C.V.%)*	Recalculated conc. (μg/ml)	Percentage of theory	Extractability (%)
0.25	0.084 ± 0.003 (± 3.9)	0.270	108	90
0.50	0.163 ± 0.005 (± 3.3)	0.518	104	93
1.00	0.319 ± 0.003 (± 0.8)	1.008	101	94
2.50	0.789 ± 0.012 (± 1.5)	2.486	99	89
5.00	1.593 ± 0.013 (± 0.8)	5.015	100	94
10.00	3.170 ± 0.019 (± 0.6)	9.976	100	94
250.00	7.949 ± 0.103 (± 1.3)	25.007	100	99

*Results from four (0.25 μg/ml) and five (0.50, 1.00, 2.50, 5.00, 10.00, 25.00 μg/ml) replicate samples were used.

0.00186 with a correlation coefficient of 0.999. Plasma concentrations of probucol calculated from the calibration curve were comparable to the corresponding theoretical values, being 99—108% of the theoretical values.

Plasma concentrations of probucol were determined as described above. Fig. 3 shows the time-course of plasma probucol concentrations after a single dose of 250 mg. The plasma levels at 4 h were 1.17 μg/ml and reached a peak level of 5.04 μg/ml at 18 h, after which they declined to a level of 1.13 μg/ml

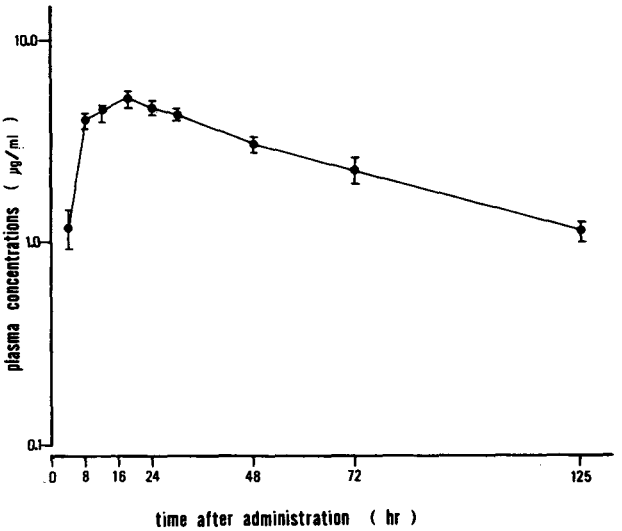

Fig. 3. Plasma concentrations of probucol after oral administration of probucol (250 mg/body) in healthy volunteers.

at 125 h with an apparent biological half-life of 49.7 h. The area under the curve (AUC) from time 0 to 125 h was 331.3 $\mu g\ ml^{-1}$ h.

The detection limit (0.25 μg/ml) of this procedure was considered sufficient to determine plasma probucol concentrations, since the dose of 250 mg used in the present study was about half its intended clinical dose [7] whose plasma concentrations were within the range (0.25—25 μg/ml) of the calibration curve constructed here.

In view of these results, it was concluded that HPLC is a simple, highly sensitive and reproducible procedure for the determination of plasma concentrations of probucol and, therefore, a valuable tool in the investigation of the clinical pharmacokinetics and bioavailability of the compound.

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Journal of Chromatography, 277 (1983) 423—426
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1808

Note

Determination of salbutamol in plasma by high-performance liquid chromatography with fluorescence detection

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(Received April 13th, 1983)

Salbutamol, 1-(4-hydroxy-3-hydroxymethylphenyl)-2-*tert.*-butylaminoethanol, also known as albuterol, is a β_2-adrenoceptor agonist widely used for the treatment of bronchial asthma and in obstetrics for the prevention of premature labour [1]. Previously reported assays for salbutamol have used liquid scintillation spectrometry [2], gas chromatography—mass spectrometry [3, 4] and more recently, high-performance liquid chromatography (HPLC) with amperometric detection [5]. However, these methods suffer from either a lack of adequate sensitivity [2], the use of time-consuming derivatization procedures [3, 4] or the need for elaborate equipment not routinely available in most laboratories [3—5]. We report a sensitive yet simple assay for salbutamol that uses ion-pair extraction and HPLC with detection by means of the drug's endogenous fluorescence. The method has been applied to a study of the plasma concentrations of salbutamol in pregnant women receiving the drug for the prevention of premature labour.

0378-4347/83/$03.00

EXPERIMENTAL

Reagents

Salbutamol sulphate was kindly supplied by Glaxo Australia (Melbourne, Australia). Di(2-ethylhexyl) phosphate (DEHP) was obtained from Sigma (St. Louis, MO, U.S.A.). All other chemicals were of analytical reagent grade and obtained from Ajax Chemicals (Sydney, Australia). They were used as received. A phosphate buffer of pH 7.2 was used with a strength of 0.42 *M*.

Chromatographic system

A Laboratory Data Control Constametric IIG pump (Riviera Beach, FL, U.S.A.), a Rheodyne Loop injector No. 7120 (200 μl loop attached) (Berkeley, CA, U.S.A.) and a Zorbax® 5 μm ODS reversed-phase column (25 cm × 4.6 mm, DuPont, Wilmington, DE, U.S.A.) with pre-column (packed with Co:Pell ODS, Whatman, Clifton, NJ, U.S.A.) were used with a Perkin-Elmer 3000 fluorescence spectrometer (Norwalk, CT, U.S.A.) and Omniscribe (Houston Instrument, Austin, TX, U.S.A.) chart recorder. Maximum salbutamol fluorescence would be expected on excitation at 220 nm in accordance with UV spectrometry experience, however due to the limitations of the particular fluorimeter on hand, excitation was performed at 230 nm (15 nm slit) with emission being detected at 309 nm (20 nm slit).

The mobile phase was composed of 8% v/v acetonitrile in distilled water containing 0.15% v/v phosphoric acid BP. The flow-rate was 1.7 ml/min.

Plasma samples

Drug-free venous blood was obtained from healthy human subjects receiving no medication. Blood was also obtained from inpatients receiving salbutamol for the prevention of premature labour. Blood was collected into plastic tubes containing lithium heparin and centrifuged for 10 min at 1000 *g* in a refrigerated centrifuge. Plasma was separated and stored between −2 and −8°C in plastic tubes until assayed. A stability study revealed no degradation on storage after a 31-day period.

Ion-pair extraction of salbutamol

Plasma (1 ml) in a stoppered 10-ml glass tube was buffered at pH 7.2 using 0.2 ml of the buffer and extracted with a solution of 0.1 *M* DEHP in chloroform (6 ml) by vortexing for 2 min. The phases were separated by centrifugation (1000 *g* for 10 min) and the chloroform phase transferred to a clean tube containing 500 μl of 0.5 *M* hydrochloric acid into which salbutamol was extracted by vortexing for 2 min followed by centrifuging (1000 *g* for 5 min). A 200-μl aliquot of the acid phase was injected directly into the chromatograph.

RESULTS AND DISCUSSION

Salbutamol isolated from plasma chromatographed with a retention time of 6.0 min and was not interfered with by any peaks present in control plasma (Fig. 1). Although there is a peak at 23 min in the chromatogram from blank

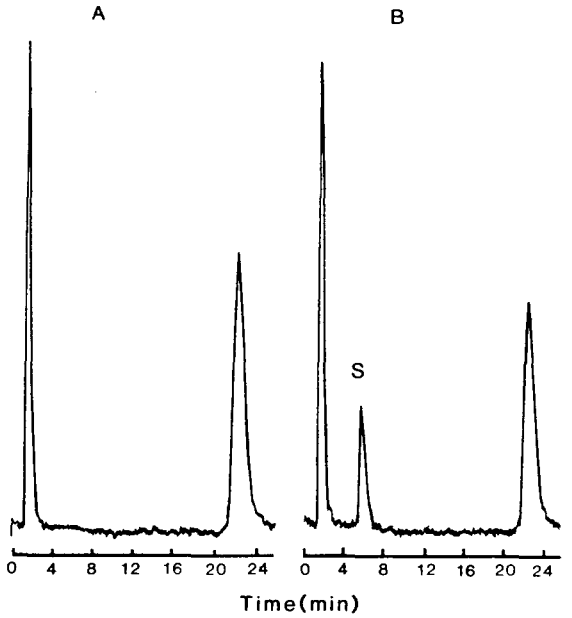

Fig. 1. Chromatograms obtained from plasma samples of patients. A: Blank; B: sample containing salbutamol (S), 12 ng/ml.

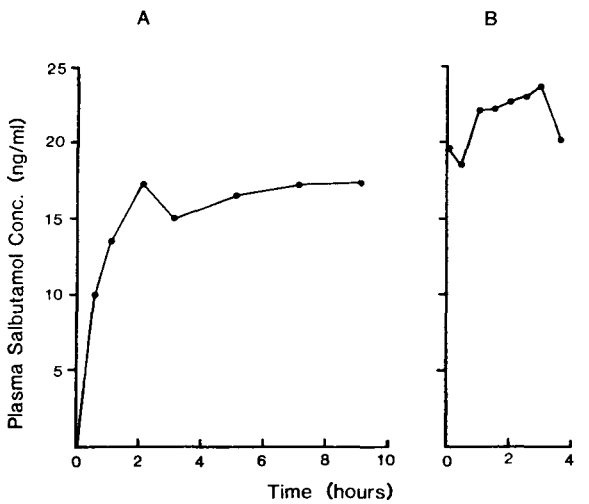

Fig. 2. Plasma salbutamol concentrations in a patient receiving the drug to arrest premature labour. (A) Intravenous infusion rate of salbutamol was 15.8 μg/min for the first 2 h, thereafter 7.8 μg/min; (B) subsequent oral maintenance therapy of 4 mg salbutamol 4-hourly.

plasma the analysis time can be reduced to about 15 min by injecting every second sample immediately after salbutamol elutes. Recovery of salbutamol, assessed by the injection of known amounts of salbutamol onto the chromatographic column, averaged 84 ± 3% (S.D.). The standard curve of peak height versus known concentration of salbutamol was linear over the range 0 to 100 ng/ml and the detection limit was 1.0 ng/ml (signal-to-noise ratio = 2:1).

Precision of the assay was assessed by replicate assays of known standards added to control plasma. At a plasma salbutamol concentration of 100 ng/ml the coefficient of variation (C.V.) was 2.5% (n=8) while at 5 ng/ml, the C.V. was 3.2% (n=7). The day-to-day C.V., assessed at 40 ng/ml, was 11% (n=5).

The assay was used to measure plasma salbutamol concentrations in pregnant women receiving the drug to arrest premature labour. Typical data, from a patient who received the drug intravenously and later orally, are shown in Fig. 2. No interference with our assay method was observed from the following drugs when they were given to patients whose salbutamol concentrations were monitored: betamethasone, chloral hydrate, paracetamol, pethidine, prochlorperazine mesylate, morphine, papaveretum, hyoscine HBr, aspirin, codeine and dexamethasone. Furthermore, theophylline, added to control plasma at a concentration of 20 μg/ml, also produced no interference. The data in Fig. 2 demonstrate that the method can accurately quantitate plasma salbutamol concentrations arising from both chronic intravenous and chronic oral dosage, with no interference from metabolites. The method possesses similar sensitivity but superior precision at low concentration than the other recently reported methods [3—5] and should therefore be equally useful in measuring plasma salbutamol concentrations after single intravenous or oral doses of the drug as those methods.

In summary, the assay described has the advantage over existing assays of being simple and not requiring elaborate and relatively expensive equipment. The use of the more widely available fluorimetric detection method should facilitate the acquisition of pharmacokinetic data on salbutamol.

ACKNOWLEDGEMENT

The authors wish to thank Sister E. Evered-Wilson for assistance with the collection of clinical samples.

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Journal of Chromatography, 277 (1983) 427—432
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1815

Note

High-performance liquid chromatography of lonidamine in human plasma and urine

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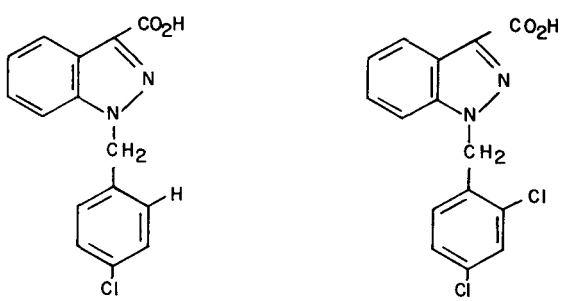
(First received October 8th, 1982; revised manuscript received June 6th, 1983)

Lonidamine is a substituted indazole carboxylic with anticarcinogenic, antispermatogenic and embryotoxic properties [1—4]. In vitro studies using testicular germinal cells [3] and Ehrlich's ascitic tumor cells indicate that lonidamine affects energy metabolism causing a decrease in cellular oxygen consumption mediated by a specific effect on mitochondrial function [5, 6].



AF 1312/TS (internal standard)

LONIDAMINE

Fig. 1. Chemical structures of lonidamine and AF 1312/TS used as internal standard.

0378-4347/83/$03.00

In view of the antitumor effects of lonidamine in the Lewis lung tumor and sarcoma 180 [7], of its unique mechanism of action and of the suggestive lack of overlapping toxicity with other chemotherapeutic drugs we have undertaken a clinical phase I pharmacokinetic and toxicologic study of lonidamine which will be reported separately. Lonidamine (L) and AF 1312/TS (IS) have been previously assayed by a fluorometric method in serum and testes after extraction with *n*-heptane [7]; recoveries reported were 54% and 63% respectively, and sensitivity limits were 1 μg/ml of serum and 2 μg/g of tissue. This report describes a sensitive and selective high-performance liquid chromatography (HPLC) assay for the quantitative determination of lonidamine from urine and plasma samples using its analogue AF 1312/TS as the internal standard (Fig. 1). The technique uses an ultraviolet detector set at 300 nm which is the maximum absorbance wavelength of lonidamine and permits the detection of concentrations of 200 ng/ml of biological sample.

MATERIALS AND METHODS

Reagents

All solid chemicals were of reagent-grade; anhydrous diethyl ether used for extraction was of analytical reagent grade; acetonitrile for HPLC was of chromatoquality (99%) grade (BDH Chemicals, Montreal, Canada).

Columns

The column used was 250 mm × 4.6 mm I.D. stainless-steel containing a 5-μm Spherisorb ODS packing purchased from Chromatographic Sciences (Montreal, Canada).

Instruments

A high-performance liquid chromatograph equipped with two Constametric III pumps (LDC) and a Rheodyne injector permitting injection of volumes between 1 and 20 μl, was used. The detector was a Perkin-Elmer Model LC-55 set at 300 nm and the recorder a Perkin-Elmer Model 56 set at either 1, 2 or 5 mV depending on the concentration of the sample assayed. Peak areas were calculated with a Varian integrator Model 485 equipped with filtering and baseline tracking devices.

Mobile phase

Throughout the procedures, the isocratic mobile phase contained a mixture acetonitrile—0.1 *M* acetate buffer, pH 3.50 (50:50). The flow-rate was 2.0 ml/min keeping the pressure between 80 and 120 bars, well below the critical pressure of 205 bars.

Analytical standards

Lonidamine and AF 1312/TS were generously supplied by the Angelini Research Institute of Rome, Italy.

Preparation of standards

Stock solutions of lonidamine and AF 1312/TS were prepared by diluting

10 mg of pure substance in 100 ml of methanol; these solutions were stable for more than six months when kept refrigerated at 3—4°C.

Buffer

An acetate buffer of 0.1 *M* was made by adding 6 ml of glacial acetic acid to 500 ml of bidistilled water, the pH was adjusted to 3.50 with 1.0 *M* sodium hydroxide, and the volume completed to 1 l with bidistilled water.

Extraction procedure

To 15-ml PTFE screw-cap tubes containing 1.0 ml of plasma or urine, 5.0 μg of AF 1312/TS were added as internal standard followed by 0.4 ml of 1.0 *M* hydrochloric acid. The samples were then vortexed for 15 sec in order to ensure homogeneity and the extraction was performed with 5.0 ml of anhydrous diethyl ether by agitation for 10 min on an Eberbach reciprocal shaker. The samples were then centrifuged for 10 min at 800 *g* and the organic layer was removed. The extraction procedure was repeated on the residual plasma or urine fraction with 3.0 ml of anhydrous diethyl ether. The two organic fractions were combined and evaporated to dryness under dry nitrogen at 45°C. The residue was dissolved in 200 μl of acetonitrile and 20 μl were used for the assay.

Calibration curves

Plasma. To aliquots of 1 ml of plasma were added amounts ranging from 0 to 25 μg of lonidamine followed by 5.0 μg of AF 1312/TS as internal standard. Each of these concentrations was assayed in triplicate.

Urine. To aliquots of 1 ml of urine were added amounts ranging from 0 to 40 μg of lonidamine followed by 5.0 μg of AF 1312/TS as internal standard. Each concentration was assayed in triplicate.

RESULTS

The mean values obtained for the calibration curves appear in Table I for plasma and urine. In both cases, the standard deviations estimated for each concentration studied were less than 10% of the mean. The regression coefficients were 0.99 for both plasma and urine calibration curves. Routine analysis, performed on different days, on plasma and urine samples spiked with known amounts of lonidamine demonstrated that the reproducibility of this analysis procedure is within 5%. To allow for a more quantitative determination of lonidamine concentration and to circumvent biases induced by possible variations of peak shape over time, surface areas instead of peak heights were used for the quantitative determination of lonidamine. Comparison of peak areas obtained from the extraction of plasma and urine samples spiked with known amounts of lonidamine and AF 1312/TS with those obtained by the injection of standards under the same conditions indicated a recovery of 90 ± 4% for both substances in plasma and urine. Furthermore, the recovery is constant for lonidamine over the range of concentrations studied. This range covers the maximum concentrations obtained during the acute pharmacokinetic studies in man using doses of 300 and 600 mg of lonidamine.

TABLE I

SURFACE RATIOS OF PLASMA AND URINE LONIDAMINE OVER INTERNAL STANDARD OBTAINED FOR CALIBRATION CURVES

Each concentration has been studied in triplicate.

Amount of lonidamine added to plasma (μg/ml)	Mean surface ratios (± S.D.)	Amount of lonidamine added to urine (μg/ml)	Mean surface ratios (± S.D.)
0	0	0	0
0.5	0.06 ± 0.006	0.5	0.09 ± 0.006
1.0	0.12 ± 0.01	1.0	0.18 ± 0.002
1.5	0.18 ± 0.01	2.0	0.38 ± 0.03
2.0	0.29 ± 0.03	4.0	0.77 ± 0.08
4.0	0.68 ± 0.04	6.0	0.96 ± 0.10
6.0	0.98 ± 0.06	8.0	1.53 ± 0.03
8.0	1.56 ± 0.06	10.0	2.03 ± 0.05
10.0	1.78 ± 0.07	12.5	2.70 ± 0.16
12.5	2.14 ± 0.07	15.0	3.04 ± 0.12
15.0	2.56 ± 0.11	20.0	3.45 ± 0.06
17.5	3.07 ± 0.06	25.0	4.60 ± 0.01
20.0	3.29 ± 0.10	30.0	5.37 ± 0.20
22.5	3.84 ± 0.06	35.0	6.43 ± 0.41
25.0	4.41 ± 0.23	40.0	7.28 ± 0.62

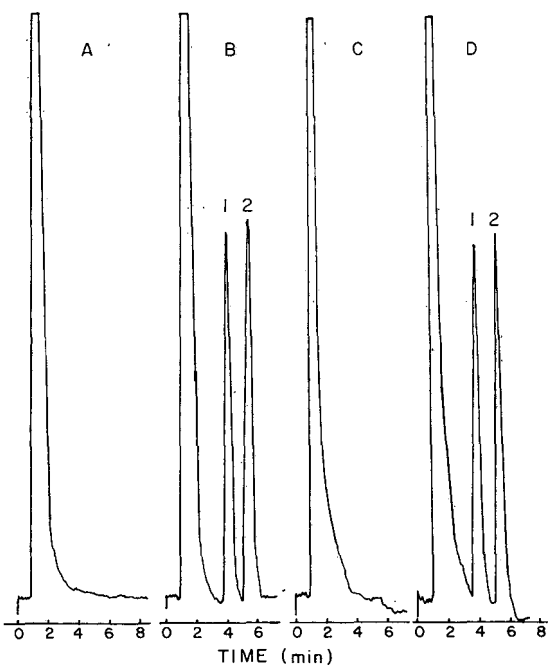


Fig. 2. Typical chromatograms of control human plasma (A), control plasma (B) spiked with AF 1312/TS (1) and lonidamine (2), control human urine (C) and control urine (D) spiked with AF 1312/TS (1) and lonidamine (2).

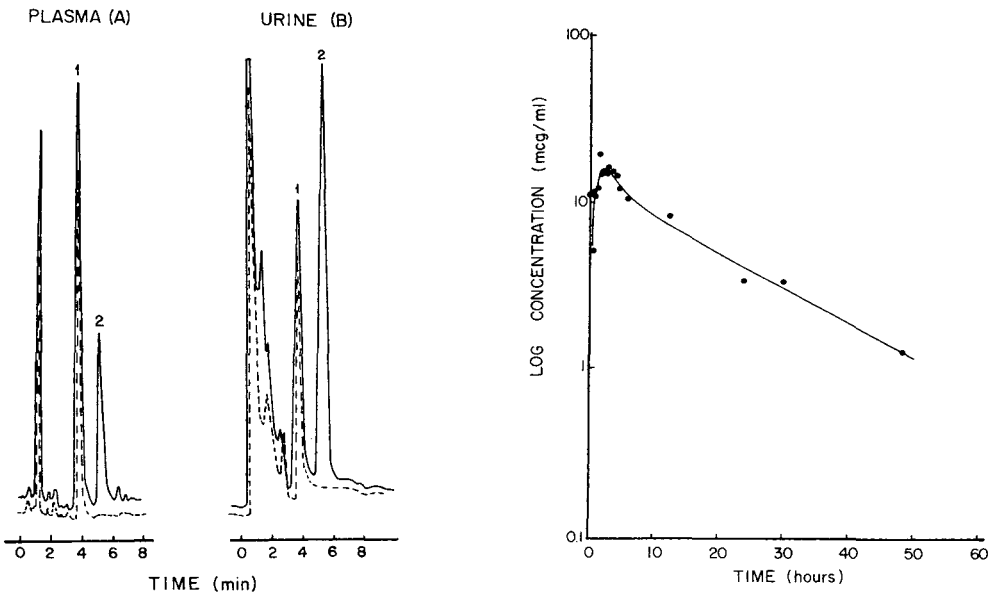


Fig. 3. (A) Chromatogram of plasma samples obtained before (· · · ·) and 1.5 h after (——) oral administration of lonidamine (600 mg) to a cancer patient. (B) Chromatogram of urine samples obtained before (· · · ·) and from 3 to 6 h after (——) drug administration to the same patient.

Fig. 4. Lonidamine plasma concentrations after a single oral dose of 600 mg; the black cicles (•) represent the experimental values and the solid line (—) represents the least square regression curve. Dose, 600 mg; lag time, 0.24 h; K_a, 1.03 h^{-1}; β, 0.048 h^{-1}; K_{12}, 0.26 h^{-1}; K_{21}, 0.21 h^{-1}; $T_{1/2}$, 14.4 h; *VD*, 43.4 l; *TBC*, 2.09 l/h.

Fig. 2 illustrates typical chromatograms obtained by the extraction of blank and spiked plasma and urine samples. The peaks are well defined with no extraneous substances interfering with the assay. The respective retention times for IS and L are 4 and 5.5 min in the conditions already described. Fig. 3 shows chromatograms obtained from plasma and urine samples collected 1.5 h after administration to a cancer patient of a single oral dose of 300 mg. Lonidamine concentrations were 2.70 μg/ml and 7.5 μg/ml for plasma and urine, respectively. Fig. 4 illustrates plasma concentrations measured in one patient who received two capsules of 300 mg of lonidamine. Those preliminary results suggest that lonidamine's single-dose pharmacokinetics should be explained by a bicompartmental open model with first order absorption and a lag time.

DISCUSSION

AF 1312/TS was chosen as the internal standard in view of its very similar chemical structure and of its retention time which permits a clear separation from lonidamine and interfering substances. Our analytical conditions allow the elution of both substances in a region of the chromatogram free from interferences (Fig. 2). The method outlined is rapid and accurate with a sen-

sitivity limit of approximately 0.2 μg/ml of plasma or urine. The analytical procedure reported in this paper differs greatly from the method published by Catanese et al. [8]. Firstly, the extraction procedure, using diethyl ether in acidic medium, permits a better recovery (90%) compared to 54 ± 3% with *n*-heptane. Secondly, this HPLC assay permits the separation of lonidamine from its metabolites (Fig. 3). It also allows the use of an internal standard for the assay which in turn insures the control of possible biases induced by variations in the recovery during extraction and in the volume injected for the assay. The fluorometric assay used by Cantanese et al. [8] does not have these features. Lobl et al. [9] have developed an assay using uniformally tritiated compound. However, this assay is more suitable for the study of the cellular distribution of the indazole carboxylic acid derivatives.

According to our results, the use of reversed-phase HPLC seems to be a method of choice for the evaluation of indazole carboxylic acid derivatives in biological samples since clear separations can be achieved with very little differences in chemical structures. Our analytical method provides the means to study the kinetic parameters of lonidamine. The procedure, rapid and practical, is particularly suitable for multiple sample analysis and is currently used in our laboratory to determine single and chronic pharmacokinetics of lonidamine in cancer patients.

ACKNOWLEDGEMENTS

This work has been supported by Inter-Canada Pharmaceuticals and the F. Angelini Research Institute of Rome, Italy.

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Journal of Chromatography, 277 (1983) 433—435
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1778

Note

Determination of aclacinomycin A by reversed-phase high-performance liquid chromatography

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(First received January 31st, 1983; revised manuscript received May 12th, 1983)

Aclacinomycin A (ACM) is an anthracycline antibiotic isolated from *Streptomyces galilaeus* by Oki et al. [1]. Its chemical structure is shown in Fig. 1. Antitumor activity has been shown in several experimental tumor models as well as in phase I and phase II clinical trials in man [2, 3]. It was the aim of the following study to develop a sensitive detection method for ACM in biological material.

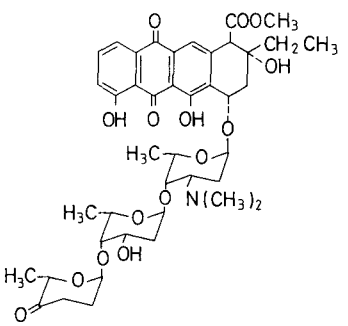

Fig. 1. Chemical structure of aclacinomycin A.

METHOD

Extraction

ACM (Behring, Marburg, F.R.G.) was added to pooled human plasma at concentrations of 10 μg/ml (12.3 μM) and 500 ng/ml (0.62 μM) and allowed to equilibrate for 1 h at room temperature. Plasma was deproteinized by adding an

0378-4347/83/$03.00

equal volume of 300 m*M* trichloroacetic acid (Merck, Darmstadt, F.R.G.). After centrifugation at 9980 *g* for 2 min the serum was applied to a C_{18} Sep-Pak® cartridge (Waters, Königstein, F.R.G.) which was then flushed with 5 ml of water and 5 ml of methanol—water (1:4, v/v). ACM was extracted with acetonitrile—methanol (1:1, v/v) (Merck) in a volume of 0.7 ml; 200-μl aliquots of the eluate were injected onto the column.

Patient samples (after bolus injection of 25 mg of ACM per m^2 body surface) were prepared by the same procedure, 10 ml of serum being applied to the C_{18} cartridge.

Liquid chromatography

The chromatographic system consisted of a μBondapak C_{18} column (300 × 4 mm, particle size 10 μm, Waters) as the support and acetonitrile—methanol—water (40:90:70, v/v) acidified with orthophosphoric acid (85%, 1.5 ml per 100 ml) as the mobile phase. The flow-rate was 2 ml/min, the temperature was ambient (about 25°C). Detection was performed at 254 nm. Quantitation is based on peak area as well as on peak height measurement.

Associated equipment comprised an M-45 solvent delivery system, an absorbance detector M 440 (Waters), and a BBC Metrawatt 10 mV writer (BBC, Nürnberg, F.R.G.).

RESULTS AND DISCUSSION

Under the conditions described ACM had a retention time of 5.9 min. As the ACM in 10 ml of serum is concentrated on the C_{18} cartridge the detection limit of ACM has been found to be 10.8 ng = 13.3 pmol per ml plasma.

A linear correlation between peak area as well as peak height and ACM injected from 25 ng to 5 μg (30.6 pmol, 6.2 nmol) has been demonstrated.

As the chromatographic system used is a modification of another one which was developed for the identification of other anthracyclines such as doxorubicin and daunorubicin [4], a good separation between these drugs and ACM could be established. This allowed the use of daunorubicin as internal standard (Fig. 2a and b).

The extraction method described had a recovery of 85 ± 7% in a series of six experiments in which ACM was added to pooled human plasma (concentration 10 μg/ml = 12.3 μM). The recovery for 500 ng/ml (= 0.62 μM) was 81 ± 9%. The inter-assay coefficient of variation for the ACM determination in general was 12.7% for 500 ng/ml (0.62 μM) samples (n = 14) and 6.9% for 10 μg/ml (12.3 μM) samples (n = 9). The intra-assay values were 8.5% (n = 6) and 4.7% (n = 6), respectively.

The advantage of using the preformed C_{18} cartridges is the rapid sample preparation. Moreover, concentration of ACM from serum samples containing low amounts of the drug is possible. This results in an improved sensitivity for pharmacokinetic studies.

In conclusion, the HPLC detection method as well as the extraction procedure described in this paper allow the collection of pharmacokinetic data in patients undergoing chemotherapy with ACM (Fig. 2b).

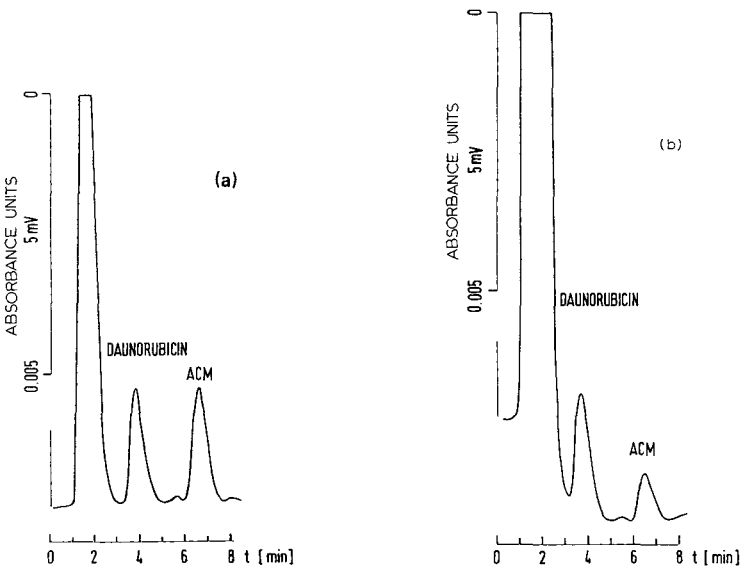

Fig. 2. (a) 1 ml of pooled human plasma was spiked with 750 ng (1.3 nmol) of daunorubicin and 400 ng (0.5 nmol) of ACM. After deproteinization and preparation on the C_{18} cartridge as described in Methods, a 200-μl aliquot was injected. (b) 10 ml of a patient serum, drawn 24 h after bolus injection of 25 mg per m^2 body surface, was spiked with 750 ng (1.3 nmol) of daunorubicin. The total 10-ml sample was deproteinized and prepared on the C_{18} cartridge; 200 μl of the eluate were injected. The ACM peak represents a plasma concentration of 17.2 ng/ml (21 pmol/ml).

ACKNOWLEDGEMENTS

Aclacinomycin A was a gift of Behring, Marburg, F.R.G. Some of the equipment was made available by the Deutsche Leukämieforschungshilfe.

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Journal of Chromatography, 277 (1983) 436—438
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1763

Letter to the Editor

Creatinine

XIII. Micro high-performance liquid chromatographic assay of creatinine in biological fluids using fixed- or variable-wavelength UV detector

Sir,

Endogenous creatinine has been widely employed to estimate the glomerular filtration rate for the study of renal function or for modifying drug dosages in renal patients [1—4]. Earlier, a simple, micro high-performance liquid chromatographic (HPLC) method using a variable-wavelength UV detector (set at 215 nm) was developed in our laboratory [5] for the assay of creatinine in plasma, serum and urine. Since variable-wavelength detectors have been generally much more expensive to purchase and maintain than commonly available fixed-wavelength (such as 254-nm) UV detectors, it would then seem highly desirable to develop a method which can also utilize the fixed-wavelength detector for quantitation. The purpose of this communication is to report results of perfection of such a method. The sensitivity of the present assay is also higher than that reported earlier [5].

The previous method [5] involved quick deproteinization of plasma (0.01—0.1 ml) with 2.5 (2.0 is also sufficient; higher ratios may be used for lower volumes of sample in the present assay) volumes of acetonitrile, and injection of "protein-free" supernatant (50—100 μl) to the 25-cm cation-exchange column (Whatman Partisil PXS 10/25 SCX). The mobile phase was 0.1 *M* monobasic ammonium phosphate acidified to pH 2.6. In order to increase the absorption of creatinine at 254 nm [5] in the present assay the pH of mobile phase was adjusted to 4.8 with 0.5 *N* NaOH, and the resultant shorter retention time (also causing interferences from endogenous compounds), due to the basicity of creatinine [5], was then offset by using lower strengths (0.012—0.035 *M* dependent on columns used) of monobasic ammonium phosphate. With a flow-rate of 2—3 ml/min the creatinine retention time is usually 4—6 min. Using a sensitivity setting of 0.002 or 0.005 a.u.f.s. at 254 nm (Model 440 from Waters Assoc. or Model 160 from Beckman) the detection limit based on a signal-to-noise ratio of 3 could be estimated to be 0.4 μg/ml or 0.04 mg% which is much lower than the normal clinical range (0.3—1.5 mg%). For urine analysis (lower detector sensitivity settings required) a 0.1-ml sample was first diluted with 4

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ml of water—acetonitrile (20:80, v/v), and then centrifuged before injection to the column [5]. Coefficients of variation of response factors from spiked plasma (0.5—10 mg%) and urine (50—400 mg%) samples were 1.42 and 0.46%, respectively. Coefficients of variation for inter- or intra-assays of actual human plasma and urine samples were between 1.2 and 3.0%.

The specificity of the assay has been reported earlier [5]. This is further substantiated by the identical results in the analysis of 15 human urine samples obtained based on the present and previous [5] assays using two different wavelengths. No interferences with the creatinine analysis were found in our extensive studies of samples from humans [6, 7] and animals (dogs and rabbits used). Measurements of creatinine in serum samples containing either low [7] or high [8] levels of creatinine based on the conventional automated picrate method were found generally to result in an overestimate (as much as 30—60% in some samples) of creatinine when compared to our HPLC methods.

The creatinine eluted from the present mobile phase can also be quantitated by a variable-wavelength detector set at 235 nm (SpectroMonitor III from Laboratory Data Control, Riviera Beach, FL, U.S.A.), and the detection limit was found to be about 0.02 mg%. Our attempt to find a suitable internal standard has thus far been unsuccessful although a total of about 30 potential compounds have been tested. This is, however, not critical in view of the low variability of our method.

The present assay appears to offer some advantages in many respects over several other HPLC methods published. For example, in a recent publication [9] the method required 1 ml of serum sample, a more complicated gradient system and a variable-wavelength UV detector. The higher pH of mobile phase (up to 7.1) used may also shorten the life span of the column. Furthermore, the more "troublesome" [5] trichloroacetic acid was used in their deproteinization [9]. In this respect, our laboratory appears to be the first to extensively utilize acetonitrile for deproteinization in the HPLC analysis (refs. 5, 10—12, and many others). Its advantages over other commonly employed methods have been fully discussed [5]. The method of Brown et al. [13] involved direct injection of biological samples (may not be good for the column) and post-column reaction of creatinine with the picrate reagent. Furthermore, their detection limit was only 0.3 mg%. The method of Soldin and Hill [14] required a variable-wavelength UV detector (set at 200 nm) and a stabilization period of 4 h prior to analysis (less than 30 min is usually required in our assay). The method of Spierto et al. [15] required 0.5 ml of serum, a variable-wavelength UV detector (set at 236 nm), and a more time-consuming, expensive ultrafiltration procedure. No applications to urine analysis were shown in three [9, 13, 15] of the above four HPLC assays. It should be noted that the mobile phase employed in our assay also appears to be simpler and cheaper when compared to others [9, 13, 14], and no internal standard was used in most other assays [9, 13, 14].

It was reported recently from our laboratory that creatinine might be extensively secreted and reabsorbed simultaneously by renal tubules in both humans [16, 17] and animals [17, 18], and there might also be a significant nonrenal elimination in normal humans [18, 19] and normal rabbits [18]. Potential implications of these findings remain to be fully explored.

ACKNOWLEDGEMENT

The above research was in part supported by a National Institute of Cancer grant, CA 29754-02.

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(Received April 29th, 1983)

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INFORMATION FOR AUTHORS

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Types of Contributions. The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications:* Regular research papers (Full-length papers), Short communications and Notes. Short communications are preliminary announcements of important new developments and will, whenever possible, be published with maximum speed. Notes are usually descriptions of short investigations and reflect the same quality of research as Full-length papers, but should preferably not exceed four printed pages. For review articles, see page 2 of cover under Submission of Papers.

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ANNOUNCEMENT

ANALYTICAL METHODS AND PROBLEMS IN BIOTECHNOLOGY

An International Symposium, Noordwijkerhout, The Netherlands, 17-19 April 1984

(Organized under the auspices of the Section on Analytical Chemistry of the Royal Netherlands Chemical Society and the Netherlands Biotechnological Society)

Scope of the Symposium

The development of analytical methods for biotechnological applications is an area of growing importance. Analytical methods currently available are now being adapted for practical use in biotechnological research, development and industrial production. But a large gap remains to be bridged between experts in analytical methodology and experts in biotechnology.
The purpose of the Symposium is to outline the problems faced in this field and to describe the rapid developments taking place. It is aimed at an interdisciplinary audience of those involved in industrial and academic biotechnology, as well as at analytical chemists themselves.

Scientific Programme

The Symposium is expected to cover a wide and representative range of current research activity on all aspects of analytical chemistry related to biotechnology. Analytical tools will be presented for process control in industrial biotechnology, for environmental biotechnology, and for fundamental research. The Programme will consist of invited plenary lectures, invited and submitted research papers and discussion sessions. The official language will be English and the following sessions are planned: Analytical Strategies, Analytical Techniques, Process Control.

Further Information

Those wishing to receive the first circular, which will include the topics of the Symposium, should contact: W.A. Scheffers, Symposium Analytical Methods and Problems in Biotechnology, Delft University of Technology, Laboratory of Microbiology, Julianalaan 67A, NL-2628 BC Delft, The Netherlands. Tel: (015)-78 24 11.