ISSN 0378–4347

VOL. 162 NO. 1 JANUARY 1, 1979
(Biomedical Applications, Vol. 4, No. 1)

JOURNAL OF CHROMATOGRAPHY
BIOMEDICAL APPLICATIONS

ELSEVIER SCIENTIFIC PUBLISHING COMPANY
AMSTERDAM

PUBLICATION SCHEDULE FOR 1979

Journal of Chromatography (incorporating Chromatographic Reviews) and Journal of Chromatography, Biomedical Applications

MONTH	D 1978	J	F	M	A	M	J	J	A	S	O	N	D
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Printed in The Netherlands

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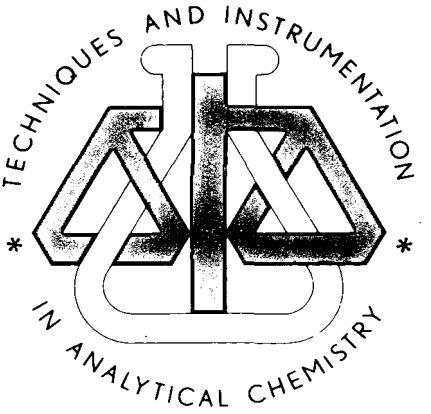

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JOURNAL OF CHROMATOGRAPHY

VOL. 162 (1979)

(Biomedical Applications, Vol. 4)

JOURNAL *of* CHROMATOGRAPHY

INTERNATIONAL JOURNAL ON CHROMATOGRAPHY, ELECTROPHORESIS AND RELATED METHODS

BIOMEDICAL APPLICATIONS

VOL. 4 1979

ELSEVIER SCIENTIFIC PUBLISHING COMPANY
AMSTERDAM

Printed in The Netherlands

Journal of Chromatography, 162 (1979) 1—6
Biomedical Applications

CHROMBIO. 235

ASSESSMENT OF SERUM CHOLESTEROL BY TWO METHODS: GAS—LIQUID CHROMATOGRAPHY ON A CAPILLARY COLUMN AND CHEMICAL IONIZATION-MASS FRAGMENTOGRAPHY WITH ISOTOPIC DILUTION OF [3,4-^{13}C]CHOLESTEROL AS INTERNAL STANDARD

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(Received May 26th, 1978)

SUMMARY

A gas—liquid chromatographic (GLC) method and an isotopic dilution—mass fragmentographic (ID—MF) procedure using the same capillary chromatographic separation are described for serum cholesterol assay. GLC included silylation and separation on a highly efficient glass capillary column which allowed the separation of cholesterol from cholestanol and the use of epicoprostanol as internal standard. The concentrations were calculated from the areas of the signals and digitalized by a reporting integrator. The reproducibility was 0.5% and the correlation with the ID—MF technique was 0.997. The ID—MF technique was characterized by the use of [3,4-^{13}C]cholesterol as the labelled standard and a chemical ionization mode. The reproducibility was 0.8%.

INTRODUCTION

Isotope dilution—mass fragmentography (ID—MF), developed from the work of Sweely et al. [1] and Hammar et al. [2], is ideally suited as a definitive method in clinical chemistry. As Cali [3] pointed out, "the isotope dilution aspects of the procedure are done on a weight basis and involve straightforward stoichiometric operations, and the mass spectrometric determinations involve only measurement ratios".

Three ID—MF techniques for plasma cholesterol assay have been proposed [4—6]. They all involve gas—liquid chromatography (GLC) on a liquid phase packed column, use either ^{14}C- or deuterium-labelled cholesterol as the internal standard, and mass spectrometry in the electron impact ionization mode.

In order to enhance the performance of the method we have developed a new technique characterized by the following salient features: a capillary column which allows the separation of cholesterol from very similar compounds; use of [3,4-^{13}C]cholesterol, so that ^{13}C, a non-radioactive isotope, does not induce radiolysis, exchange processes or isotopic effects; the use of a chemical ionization mode which leads to a simple fragmentation with a great relative abundance of the high-mass ions.

In the course of improving the chromatographic separation, a new capillary GLC technique was set up and compared with the ID—MF technique.

MATERIALS

Serum. Specimens were collected from adult patients after an overnight fast and stored at - 20° or analyzed within a few hours.

Chemicals. All chemicals were from Merck (Darmstadt, G.F.R.) with the exception of cholesterol (SRM 911a, National Bureau of Standards, Washington D.C., U.S.A.), [3,4-^{13}C]cholesterol (isotopic labelling; C_3, 83%; C_4, 87.5%, from Commissariat à l'Energie Atomique, Saclay, France) 5α-cholestan-3β-ol (cholestanol), epicoprostanol and 5α-cholestane (Sigma, St. Louis, Mo., U.S.A.), N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) (Supelco, Bellefonte, Pa., U.S.A.).

Chromatograph. A Packard-Becker 427 gas chromatograph (Delft, The Netherlands) equipped with a flame ionization detector and an SE-30 (LKB, Bromma, Sweden) capillary column (Type 2101-203, 25 m × 0.22 mm I.D., 120,000 theoretical plates) was used. Chromatograms were recorded on an (HP 3380A) reporting integrator (Hewlett-Packard, Washington D.C., U.S.A.).

Mass spectrometer. A Finnigan 3300 mass spectrometer equipped with the 6100 Interactive Data System (Sunnyvale, Calif., U.S.A.) was used.

METHODS

Gas—liquid chromatography

Saponification and extraction. These were performed according to the reference technique of Abell et al. [7], with slight modification. In a PTFE-lined, screw-capped, 15-ml glass tube were mixed 1 ml of a 0.25 m*M* solution of epicoprostanol in absolute ethanol, 0.1 ml of aqueous potassium hydroxide (6.0 m*M*) and 50 μl of serum. The tube was left for 1 h at 60° and, after cooling, 10 ml of a 0.025 m*M* solution of 5α-cholestane in hexane were added. After mixing, 2 ml of bidistilled water were added and the tube was shaken for 1 min on a Vortex mixer. A 2-ml aliquot of the hexane phase was transferred in a 2-ml glass-tube and evaporated under nitrogen at 60°.

Silylation. A 0.2-ml volume of a freshly prepared mixture of BSTFA and TMCS (4:1, v/v) was added to the dry residue. After 1 h at 60°, 0.8 ml of hexane was added and 1 μl of the cooled mixture was iniected into the chromatograph with an all-glass solid injector. The temperature of the injector and

detector was 290°, that of the column was programmed from 250° to 285° at 1°/min. The carrier gas was nitrogen and the flow-rate 3 ml/min. The peak area ratios epicoprostanol:cholestane and cholesterol:epicoprostanol were calculated. The reproducibility of the extraction yield was checked by the epicoprostanol:cholestane ratio, the serum cholesterol concentration was calculated by comparison of the cholesterol:epicoprostanol ratios obtained with the serum and a 5 m*M* standard cholesterol solution.

Isotope dilution—mass fragmentography

The chromatographic step was similar but cholestane was omitted and epicoprostanol replaced by 1 ml of a 0.25 m*M* solution of [3,4-^{13}C¼cholesterol dissolved in absolute ethanol.

The SE-30 capillary column was coupled directly to the ion source of the mass spectrometer by an all-glass connection without any separator. The chemical ionization mode was used with methane as the reagent gas at a pressure of 0.9 Torr. Relative intensities of the *m/e* 443.5 and *m/e* 445.5 ions were recorded. These fragments resulted from the loss of a methyl group from the molecular ion of the trimethylsilyl ether of [^{12}C]cholesterol and [3,4-^{13}C]-cholesterol, respectively. The 443.5:445.5 ratio was calculated for: (i) serum and the 5 m*M* cholesterol solution, each with the added amount of [3,4-^{13}C]cholesterol; (ii) serum treated without the addition of the labelled cholesterol; and (iii) the [3,4-^{13}C]cholesterol solution alone. The concentration of the serum cholesterol was calculated using the following equation in which, in order to avoid interference of background variation, all terms were expressed as ratios of the areas of the signals 443.5:445.5.

$$x = \frac{a - d}{1 - a/c} \cdot \frac{1 - b/c}{b - c} \cdot k$$

where x = concentration of serum cholesterol, k = concentration of the cholesterol standard solution, a = 443.5:445.5 for serum plus [3,4-^{13}C]cholesterol, b = 443.5:445.5 for the standard solution plus [3,4-^{13}C]cholesterol, c = 443.5:445.5 for serum without labelled cholesterol, and d = 443.5:445.5 for the [3,4-^{13}C]cholesterol solution alone.

RESULTS AND DISCUSSION

Gas—liquid chromatography

A typical chromatogram is shown in Fig. 1. The use of a highly efficient capillary column resulted in the separation of the trimethylsilyl ether of cholesterol from silylated derivatives of very similar compounds. Epicoprostanol is a sterol very closely related to cholesterol, having a hydroxyl group at the same position, a similar solubility in organic solvents, and similar reactivity towards silylating agents. Thus, since it is well separated, it could be used as an ideal secondary standard, treated from the very beginning of the assay in the same way as cholesterol. Conversely, 5α-cholestane, used as a secondary standard in all the previous techniques, except for that of Hindriks et al. [8], was added to the extraction solvent. Not being derivatized, in our method it only confirmed the reproducibility of the extraction yield of silylated epico-

prostanol. The high resolution of the chromatographic procedure gave a complete separation of cholestanol from cholesterol (Fig. 2). This sterol is present in serum, usually at a low concentration of about 25 μM [9], but it is increased in one type of hyperlipidemia, cerebrotendinous xanthomatosis [9].

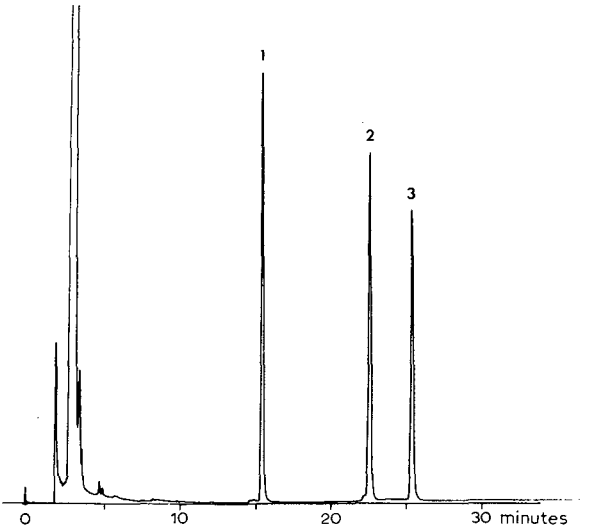

Fig. 1. GLC of serum. Cholesterol (3) concentration was 4.87 mM. No cholestanol was detected in this sample. Cholestane (1) and epicoprostanol (2) were added as described in the text.

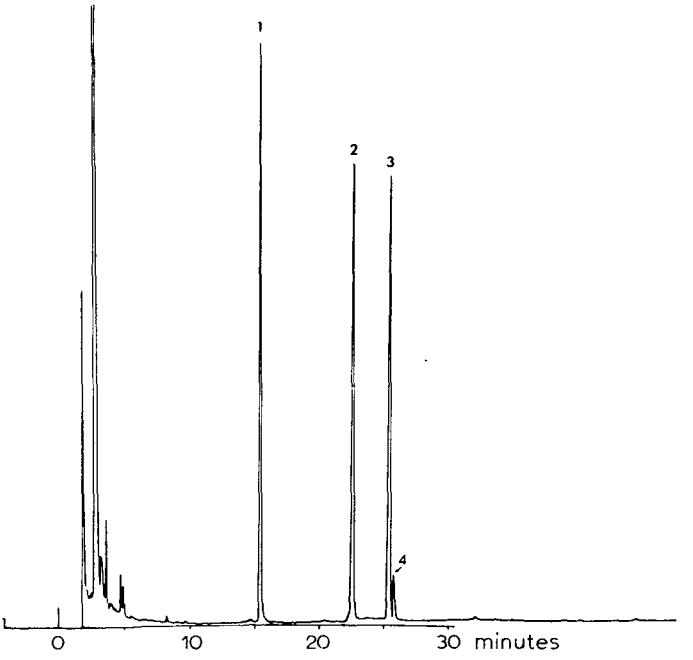

Fig. 2. GLC of a solution of 5 mM cholesterol (3) and 0.4 mM cholestanol (4) solution, (1) and (2) as in Fig. 1.

Thus it is a source of error in packed-column chromatographic assays which cannot separate it from cholesterol [10].

Contrary to usual practice, the cholesterol concentration was calculated from the areas of the signals, measured by a reporting-integrator, and not from their heights. This mode of calculation and the use of a glass capillary column appreciably improved the reproducibility, as can be observed by the following examples, The coefficient of variation (n=28) was 0.8% for the capillary column, and 1.6% for the packed column with measurement of peak height, but only 0.5% with the combined capillary column-reporting integrator.

The procedure was linear up to 18 mM and the limit of detection was 2.5 μM, a concentration which corresponds to an injection of 0.125 pmole of cholesterol. The accuracy of the GLC method was evaluated by comparison with the ID—MF method. The correlation coefficient (n=35) between the two was 0.997. This high accuracy resulted from the specificity of the chromatographic separation, the use of a standard reference material as primary standard and the use of a secondary standard, epicoprostanol, very similar to cholesterol.

Isotope dilution—mass fragmentography

The linearity of the technique was tested up to 18 mM and the coefficient of variation (n=25) was 0.8%. Compared with the relevant publications, this technique relies on several improvements. The chromatographic separation was carried out in a glass capillary column, the efficiency of which has already been indicated. The total resolution of cholestanol from cholesterol was an absolute requirement since cholestanol and [3,4-^{13}C]cholesterol (or cholesterol labelled by one ^{14}C atom) have the same molecular weight and a very similar mass-fragmentation pattern.

Mass fragmentography was conducted in the chemical ionization mode. Whereas the electron impact ionization mode, which leads to multiple fragments, is particularly interesting for structure elucidation studies, chemical ionization is more suitable for fragmentographic methods since it produces preponderantly few high-mass ions. Three main fragments (F) appear on the mass spectrum of cholesterol with methane as the ionizing reagent gas: M—H.

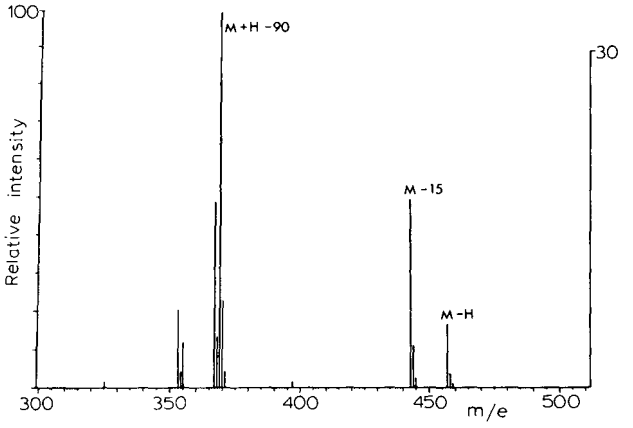

Fig. 3. Mass spectrum of trimethylsilyl ether of unlabelled cholesterol (m/e 458). Chemical ionization mode (for experimental details, see Materials and methods).

M-15 and M+H-90 (Fig. 3). The M-15 fragment, *m/e* 443.5 for cholesterol and *m/e* 445.5 for [3,4-^{13}C]cholesterol, was chosen because of the absence of a doublet arising from the two fragmentation paths F+H and F-H. In the case of M+H-90 the isotopic ion (M+2) which gives (M+2)-H-90, will bring a mass contribution to the fragment M+H-90. The stable isotope ^{13}C was preferred to ^{14}C since, as it is a non-radioactive isotope and does not induce radiolysis of the standard nor contaminate the mass spectrometer. Added to the fact that [3,4-^{13}C]cholesterol is at present 25 times less expensive by weight than [^{14}C]cholesterol, this allowed the use of a concentration of internal labelled standard within the concentration range of unlabelled serum cholesterol. Thus, the errors due to the use of different amplifications on each channel were avoided and the sensitivity was increased. In the case of deuterium-labelled compounds, the lability of the C—H bond will be reflected by an exchange of the label. Such risks are fully avoided by the use of ^{13}C-tagged compounds.

CONCLUSIONS

Although theoretically ideal as a definitive method, the ID—MF method has suffered from technical limitations [11]. The innovations presented, i.e., separation on a capillary column, use of ^{13}C as the labelling isotope and chemical ionization, were factors for improvement of the accuracy and reproducibility of ID—MF serum cholesterol assay. The internal standard [^{13}C]cholesterol could be used in an amount similar to that of serum cholesterol, a highly desirable situation for accurate ID—MF analysis.

The capillary GLC technique described, which has comparable reproducibility and accuracy, has the great advantage of being much more easily set up. It should be useful either as a reference method in some clinical laboratories or for specific investigations such as cholesterol assay in lipid metabolic disorders.

ACKNOWLEDGEMENTS

This work was supported by the Centre National de la Recherche Scientifique (ERA 267), Institut National de la Santé et de la Recherche Médicale (FRA 9) and Société Française de Biologie Clinique.

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CHROMBIO. 240

ANALYSIS OF CYSTEINYLDOPAS, DOPA, DOPAMINE, NORADRENALINE AND ADRENALINE IN SERUM AND URINE USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND ELECTROCHEMICAL DETECTION

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(Received March 20th, 1978)

SUMMARY

The catecholic amino acids, dopa, 2-S- and 5-S-cysteinyldopa, and 2,5-S,S-dicysteinyldopa were determined qualitatively in serum from patients with malignant melanoma by reversed-phase high-performance liquid chromatography, using electrochemical detection. In urine the catecholamines dopamine, noradrenaline and adrenaline were also determined qualitatively, as well as the above-mentioned compounds, in a single chromatographic run. The conditions were optimized by changing the pH of the mobile phase and by the addition of methanesulphonic acid. A comparison was made between the performance of four commercial reversed-phase packing materials containing chemically bonded octadecyl groups, using a standard mixture of catecholic amino acids. The influence of ionic strength, pH and amount of methanesulphonic acid on retention was investigated.

INTRODUCTION

5-S-Cysteinyldopa is a newly discovered amino acid occurring in both normal subjects and, in increased amounts, in patients with malignant melanoma. Its concentration in the urine has been determined to obtain information on the degree of dissemination of the tumour. The fluorometric method [1] used for this determination is sensitive enough to detect this amino acid in serum

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only in advanced cases. The separation and quantitative analysis of this compound and dopa in serum by liquid chromatography was recently described by the present authors [2]. An amperometric detector according to Kissinger [3] was used which permitted detection of very small amounts, i.e. 25 pg, of each compound. The separation was performed on a Nucleosil C_{18} phase and the mobile phase consisted of 0.5% (v/v) methanol in water, containing 2.9 g of phosphoric acid per litre. This packing material contains chemically bonded octadecyl groups on microparticulate silica.

A useful means of varying the retention in such reversed-phase systems was described by Knox and Jurand [4], who added anionic detergents to the mobile phase. This method was named "soap chromatography", and applied to the separation of catecholamines and their metabolites. The retention mechanism in this kind of chromatography has recently been discussed [5, 6]. Another example of the utility of chemically bonded reversed-phase packing materials for the analysis of catecholamines and related substances is given by Molnár and Horváth [7], who separated acidic and basic catecholamine metabolites by varying the pH and ionic strength of the mobile phase. High-performance ion-exchange chromatography has also been shown to be useful for the analysis of dopa and catecholamines, i.e. dopamine, noradrenaline and adrenaline [8, 9], in therapeutic serum and urine (see Fig. 1).

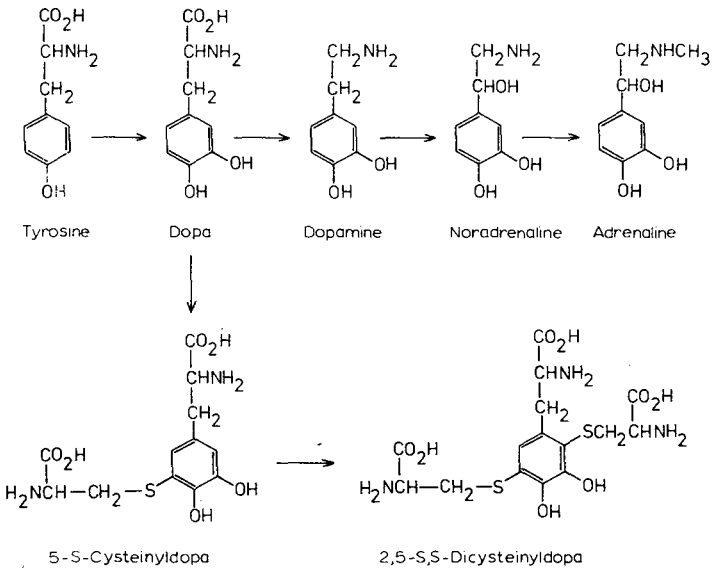


Fig. 1. The biochemical pathways for the formation of cysteinyldopas and catecholamines from tyrosine.

As the concentration of the actual catecholic compounds is generally low in biological fluids, detection is often a problem. In many instances neither UV nor fluorescence detectors can be used, and although increased sensitivity can be obtained by derivatization [10], the method is often less attractive due to incomplete reaction. In this situation the electrochemical detector developed

by Kissinger [3], which, as already mentioned, was used in our previous work on 5-S-cysteinyldopa and dopa, has satisfied a long-felt need in the case of easily oxidizable substances.

5-S-Cysteinyldopa is the major cysteinyldopa in serum and urine. In addition to this compound, three other cysteinyldopas, namely 2-S- and 6-S-cysteinyldopa and 2,5-S,S-dicysteinyldopa, have been identified in urine [11, 12]. The concentrations of all these amino acids in biological fluids are of great interest, since they reflect the oxidation status in the melanocyte. In this work, the separation and sensitivity of determination of cysteinyldopa and other tyrosine metabolites such as dopa, dopamine, adrenaline and noradrenaline in serum and urine have been studied in order to achieve the simultaneous analysis of these compounds (see Fig. 1). To this end, a thorough study of the utility of various commercial reversed-phase packing materials of the C_{18} type was made and the properties of the mobile phase changed by varying its pH and ionic strength. The effect of adding anionic modifiers was also investigated. Electrochemical detection was used throughout, except in the study of the effect of adding sodium sulphate to the mobile phase, where a UV detector was used.

The variation of sensitivity with the working potential of the electrochemical detector was studied for some of the compounds. The compatibility of the detector with the various mobile phases was also examined. A column packing technique especially suited to reversed-phase packing material is described in detail. As in our previous investigation, alumina was used to prepurify samples of urine and serum. The recovery of catecholic amino acids after elution with acids of different strengths was established. The present study is mainly concerned with the development of proper separation conditions for the compounds of interest.

EXPERIMENTAL

Apparatus

A Varian Model 8500 (Varian, Palo Alto, Calif., U.S.A.) positive displacement pump was used. Samples were injected with a valve injector Rheodyne model 7120 (Rheodyne, Berkeley, Calif., U.S.A.).

In most cases a thin-layer amperometric detector, Model LC-10 (Bioanalytical Systems, West Lafayette, Ind., U.S.A.) was used. The electrode was in most cases operated at 0.75 V vs. an Ag—AgCl reference electrode. The graphite paste was CPO.

A variable wavelength UV detector, Varian Vari-Chrom liquid chromatography detector, was used for studying the effect of adding sodium sulphate to the mobile phase.

Column packing material

Four different commercially available reversed-phase packing materials were used. The mean particle diameter was 10 μm unless otherwise stated: Nucleosil C_{18} (5 or 10 μm) (Macherey, Nagel & Co., Düren, G.F.R.), Spherisorb ODS (Phase Separations, Queensferry, Great Britain), LiChrosorb RP-18 (E. Merck, Darmstadt, G.F.R.), Partisil ODS (Whatman, Clifton, N.J., U.S.A.).

Column tubing and fittings

The columns consisted of 200 × 6.35 mm O.D. × 5 mm I.D. stainless-steel 316 tubing with either a polished or unpolished inner surface. They were all equipped with modified Swagelok or Parker-Hannifin compression fittings. Very thin stainless-steel mesh discs were placed at both ends of the column (part No. 206, hetp, Macclesfield, Great Britain). The valve injector was connected to the column via 1/16 in. O.D. (0.15 mm I.D.) stainless-steel tubing and the detector via 1/16 in. O.D. (0.15 mm I.D.) PTFE tubing (see Fig. 2).

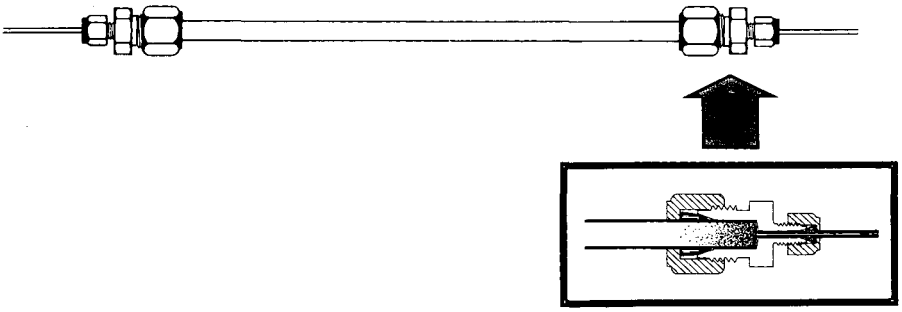


Fig. 2. Column and design of column head and bottom for valve injection.

Column packing technique

Columns were packed according to the upward slurry packing technique [13]. For reversed-phase packing materials we have found that both acetone and chloroform are suitable.

A Haskel pneumatic amplifier pump Model DST-150 (Haskel, Burbank, Calif., U.S.A.) was pressurized with solvent to 150 or 400 atm with a Whitey valve Model NB-SS-3NBF4 (Whitey, Oakland, Calif., U.S.A.), closed on the outlet side. In certain cases of packing 10 μm material at 150 atm, peak tailing was obtained. However, this could be eliminated by packing at 400 atm instead. For 5-mm I.D. columns about 2.9 g of packing material was slurried in 70 ml of solvent in an ultrasonic bath for 5 min. The slurry reservoir, Crawford Type 304-HDF4-75 (part of a slurry packing kit, part No. 316; hetp) was filled and the column mounted pointing upwards. Solvent was filled to the top of the column. The end fitting was connected and the valve was opened. About 250 ml of solvent were passed, and the column was turned pointing downwards. The valve was closed, and after 5 min the column was disassembled, washed with methanol and tested. This technique is applicable for packing both 5 and 10 μm ODS material, as well as other reversed-phase materials like LiChrosorb RP-2 and RP-8 [14]. The technique is also well suited for packing silica with methanol as slurrying medium.

Chemicals

Acetone, chloroform, phosphoric acid (85%), sodium metabisulphite, perchloric acid (75%), and sodium hydroxide were all of analytical grade (Merck).

Sodium *n*-octyl sulphate (for tenside tests, Merck) methanesulphonic acid (for synthesis, Merck), methanol (analytical reagent grade, May & Baker, Dagenham, Great Britain). 2-S-, 5-S- and 6-S-cysteinyldopa and 2,5-S,S-dicysteinyldopa were synthesized as described by Agrup et al. [15]. DL-α-3,4-Dihydroxyphenylalanine (DL-dopa), DL-1-(3,4-dihydroxyphenyl)-2-aminoethanol hydrochloride (noradrenaline), 3,4-dihydroxyphenyl-2-methylaminoethanol (adrenaline) were all obtained from Sigma, St. Louis, Mo., U.S.A. Aluminium oxide (Merck, nach Brockmann) was prepared by the method of Anton and Sayre [16].

All the above standards were stored as such or in solution in a refrigerator. 2-S-, 5-S-, 6-S-cysteinyldopa, and 2,5-S,S-dicysteinyldopa were obtained in 0.1 *M* HCl solution and stored as such. Dopa and noradrenaline were dissolved and stored in the mobile phase. Before injections were made all standards were dissolved in the mobile phase.

Chromatographic conditions

All chromatographic experiments were performed at ambient temperature. The flow-rate was 100 ml/h unless otherwise stated. No extra precautions were necessary in order to isolate the electrochemical detector electrically.

Procedures

Before any measurements were made, a standard solution containing dopa and cysteinyldopas was injected repeatedly to establish steady-state conditions. Capacity factors* were calculated as the mean from at least two injections. The column void volume was estimated by the injection of sodium nitrate when using the UV-detector and taken as the first base-line disturbance when using the electrochemical detector.

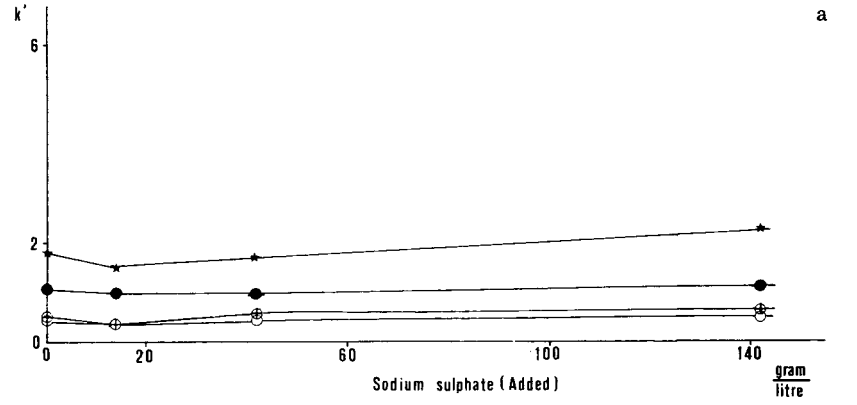
Effect of ionic strength on retention. This was investigated for four commercially available 10-μm reversed-phase packing materials by adding sodium sulphate to an aqueous mobile phase containing 2.9 g of phosphoric acid per litre (see also Fig. 3).

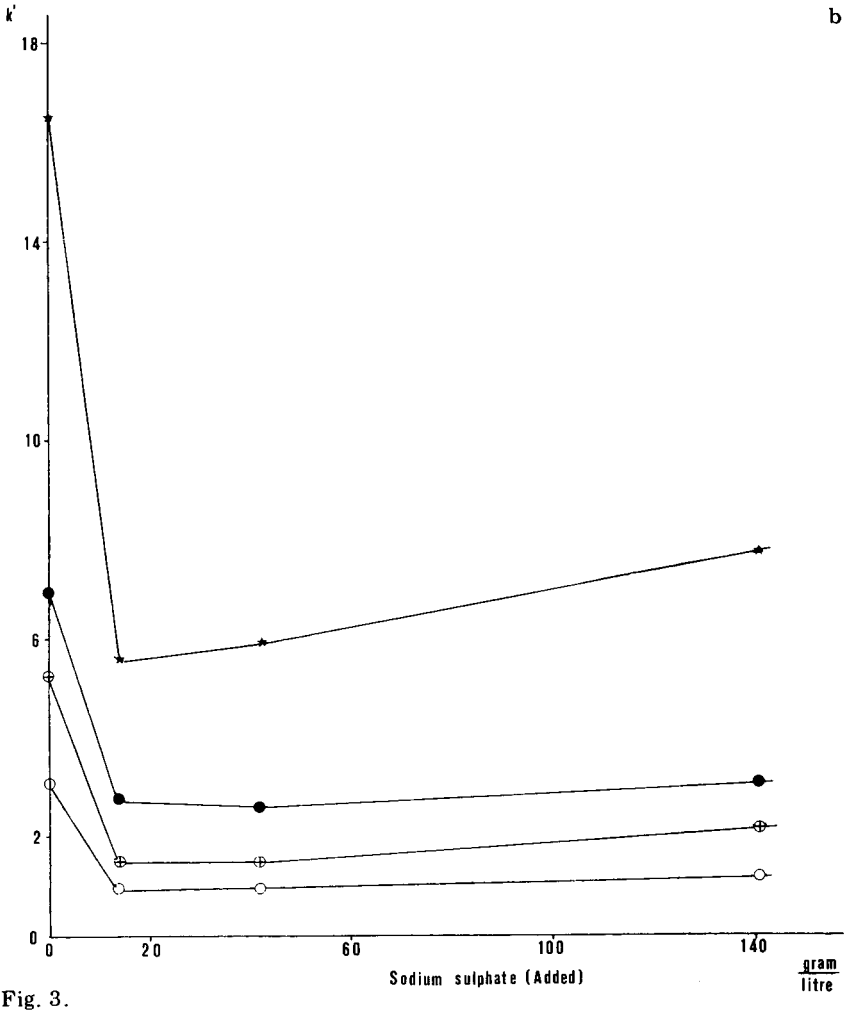
Effect of pH on retention. This was studied for 5 μm Nucleosil C_{18} by adjusting the pH of the aqueous mobile phase containing either sodium sulphate or the modifier methanesulphonic acid. In the first case, the mobile phase contained 14 g of sodium sulphate per litre, and the pH was adjusted by adding phosphoric acid and 5 *M* sodium hydroxide to this solution. In the second case, the mobile phase contained 0.48 g of methanesulphonic acid and 2.9 g of phosphoric acid per litre, and the pH was adjusted with 5 *M* sodium hydroxide (see Figs. 4 and 5).

Effect of the amount of modifier on retention. This was examined for 5 μm Nucleosil C_{18} by varying the methanesulphonic acid content of the aqueous mobile phase containing 2.9 g of phosphoric acid per litre. The pH was adjusted to 1.75 with 5 *M* sodium hydroxide (see Fig. 6).

Recovery from alumina adsorption. This was investigated by treating standards according to the procedure for serum and urine [2].

*Capacity factor $k' = \frac{V_R - V_0}{V_0}$; V_R = retention volume, V_0 = void volume.


a
k'
6
2
0
0
20
60
100
140
Sodium sulphate (Added)
gram
litre


b
k'
18
14
10
6
2
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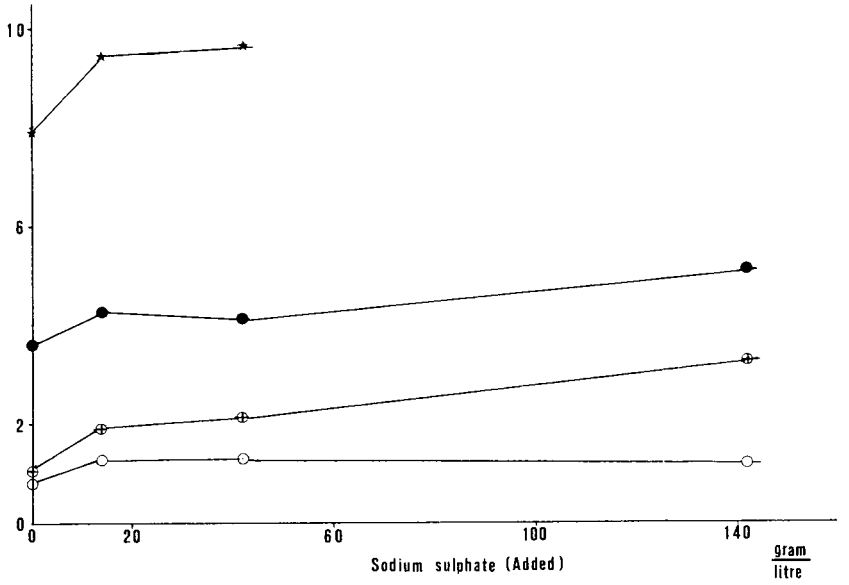
0
20
60
100
140
Sodium sulphate (Added)
gram
litre

Fig. 3.



d

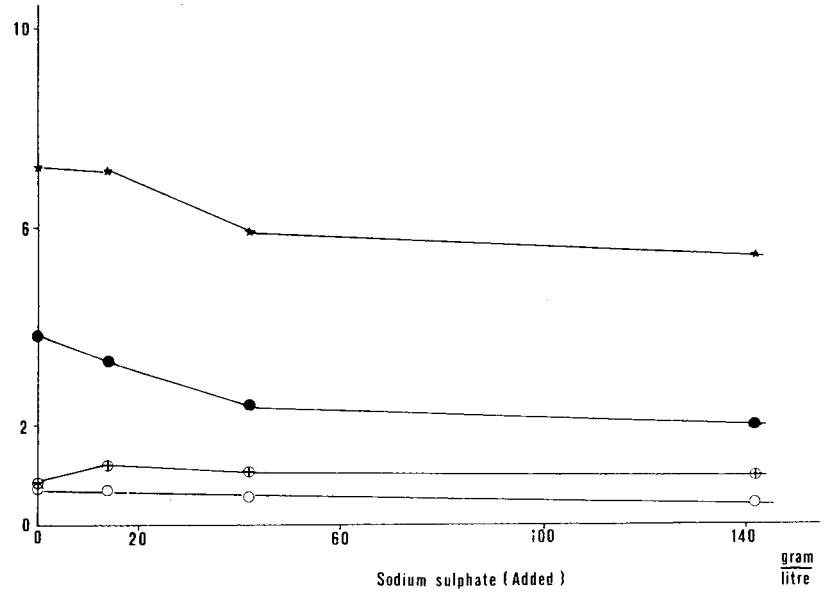


Fig. 3. Relationship between capacity factor k', and amount of sodium sulphate added to the mobile phase. Column: reversed-phase ODS materials (10 μm), 200 × 5 mm. Eluent: water, 2.9 g phosphoric acid per litre, with the addition of sodium sulphate. Flow-rate: 100 ml/h. ⋆, 5-S-Cysteinyldopa; •, dopa; ○, 2-S-cysteinyldopa; ⊕, 2,5-S,S-dicysteinyldopa. (a) Partisil ODS; (b) Spherisorb ODS; (c) Nucleosil C_{18}; (d) LiChrosorb RP-18.

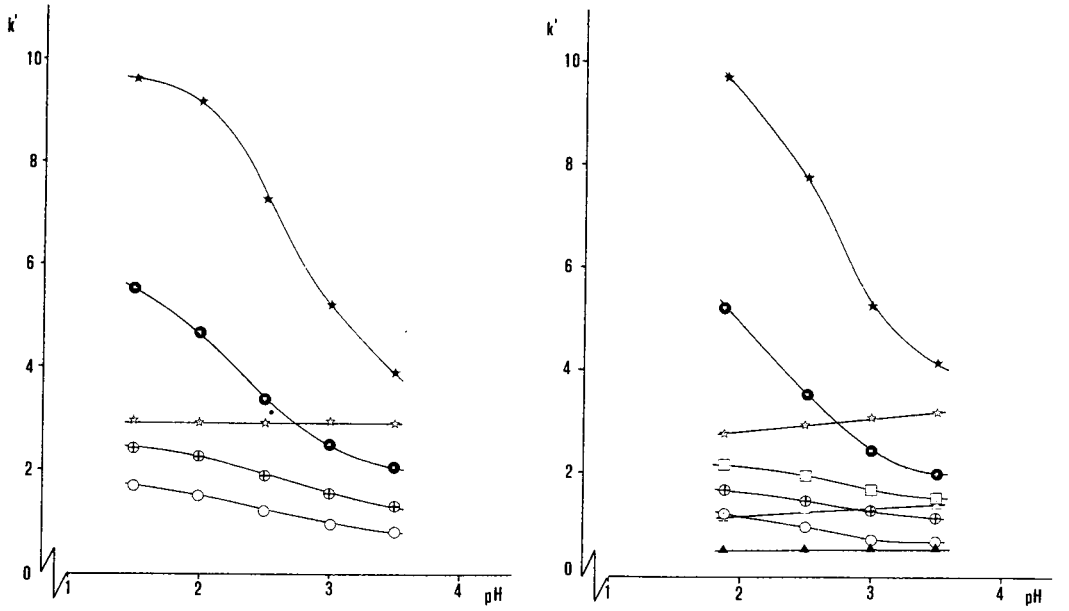


Fig. 4. Relationship between capacity factor k', and pH for cysteinyldopas, dopa and dopamine. Column: Nucleosil C_{18} (5 μm), 200 × 5 mm. Eluent: water, 14 g sodium sulphate per litre, pH was adjusted by the addition of phosphoric acid and 5 *M* sodium hydroxide. Flow-rate: 100 ml/h. Pressure: 168 atm. ★, 5-S-Cysteinyldopa; ●, dopa; ☆, dopamine; ⊕, 2,5-S,S-dicysteinyldopa; ○, 2-S-cysteinyldopa.

Fig. 5. Relationship between capacity factor k', and pH of cysteinyldopas, dopa, dopamine, adrenaline and noradrenaline. Column: Nucleosil C_{18} (5 μm), 200 × 5 mm. Eluent: water, 0.48 g methanesulphonic acid and 2.9 g phosphoric acid per litre. pH was adjusted with 5 *M* sodium hydroxide. Flow-rate: 100 ml/h. Pressure: 168 atm. ★, 5-S-Cysteinyldopa; ●, dopa; ☆, dopamine; □, 6-S-cysteinyldopa; ⊕, 2,5-S,S-dicysteinyldopa; ○, 2-S-cysteinyldopa; △, adrenaline; ▲, noradrenaline.

Separation of 2-S, 5-S, 6-S-cysteinyldopa, and 2,5-S,S-dicysteinyldopa, dopa, dopamine, noradrenaline, and adrenaline. Serum and urine samples were treated as described elsewhere [2] with the exception that 1 *M* perchloric acid was used for elution from alumina instead of 0.1 *M* in order to improve recovery, especially for 2,5-S,S-dicysteinyldopa. The separation was performed on 5 μm Nucleosil C_{18} using an aqueous mobile phase with 2.9 g of phosphoric acid and 6 g of methanesulphonic acid per litre. The pH was adjusted to 1.75 with 5 *M* sodium hydroxide (see Fig. 7).

Characterization of reversed-phase packing materials. The carbon content of the packing material was determined in duplicate on a Model 1102 Carlo Erba elemental analyzer. For estimation of silanol interaction the columns were eluted with hexane and the capacity factors for nitrobenzene and benzene were determined [17].

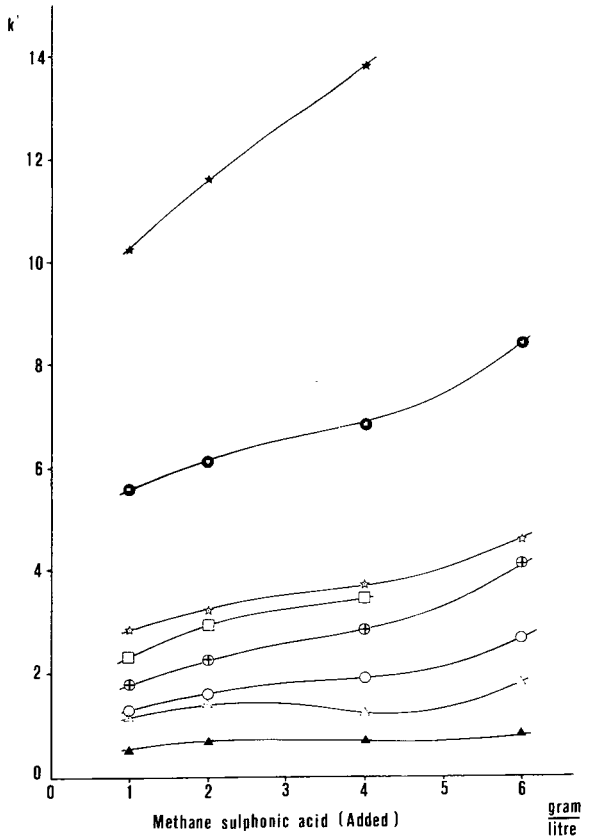


Fig. 6. Relationship between capacity factor, k', and added amounts of methanesulphonic acid to the mobile phase for cysteinyldopas, dopa, dopamine, adrenaline and noradrenaline. Column: Nucleosil C_{18} (5 μm), 200 × 5 mm. Eluent: water, 2.9 g phosphoric acid per litre. pH was adjusted to 1.75 with 5 *M* sodium hydroxide. Flow-rate: 100 ml/h. Pressure: 168 atm. ★, 5-S-cysteinyldopa; ●, dopa; ☆, dopamine; □, 6-S-cysteinyldopa; ⊕, 2,5-S,S-dicysteinyldopa; ○, 2-S-cysteinyldopa; △, adrenaline; ▲, noradrenaline.

RESULTS AND DISCUSSION

Comparison of reversed-phase packing materials and effect of ionic strength on retention

The effect of ionic strength on the retention of 2-S- and 5-S-cysteinyldopa and 2,5-S,S-dicysteinyldopa and dopa was studied for four 10-μm commercial reversed-phase packing materials by adding sodium sulphate to the acidified aqueous mobile phase. The results are presented in Fig. 3 a—d. The names and some properties of the packing materials are given in Table I.

As can be seen from Fig. 3 a—d there are considerable differences in the k' values of the test substances on the four packing materials. As they all have octadecyl groups chemically bonded to silica this differences must be connected with the properties of the silica matrix and with the method of fixing the

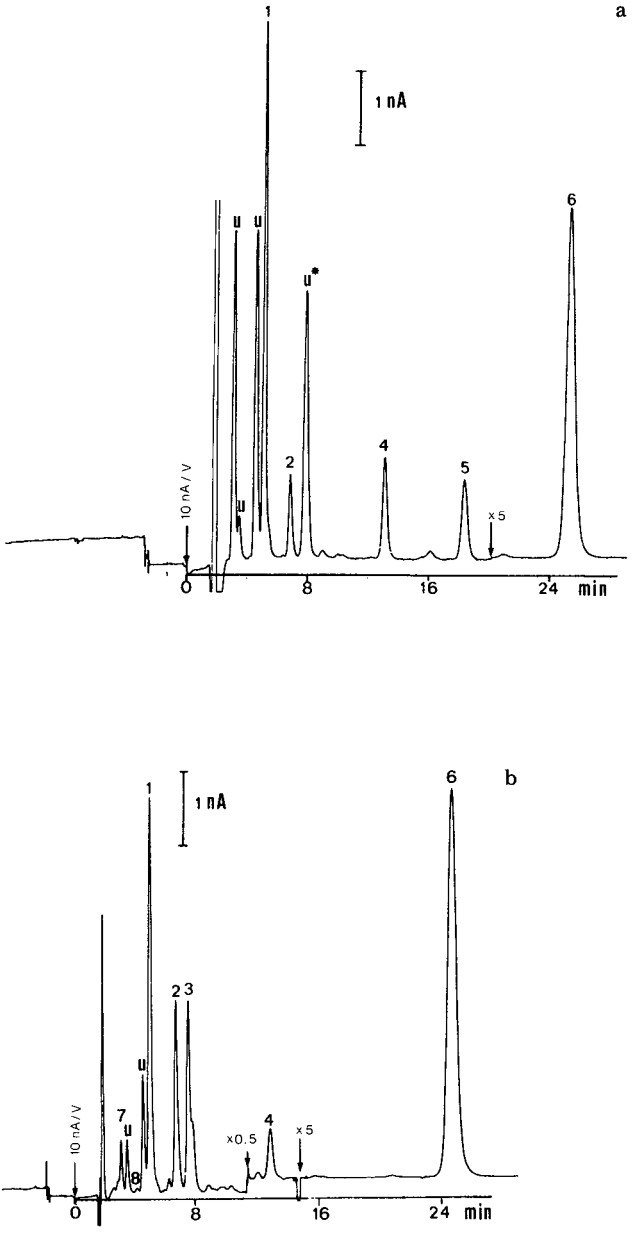


Fig. 7. Chromatograms of purified serum (a) and urine (b) obtained from patients with melanoma metastasis. Eluent: water, 2.9 g phosphoric acid and 6 g methanesulphonic acid per litre. pH was adjusted to 1.75 with 5 *M* sodium hydroxide. Column: Nucleosil C_{18} (5 μm), 200 × 5 mm. Flow-rate: 102 ml/h. Peaks: 1 = 2-S-cysteinyldopa; 2 = 2,5-S,S-dicysteinyldopa; 3 = dopamine; 4 = dopa; 5 = isoprenaline (internal standard); 6 = 5-S-cysteinyldopa; 7 = noradrenaline; 8 = adrenaline; u = unidentified. 6-S-cysteinyldopa is not detected, retention time between peaks 2 and 3. In contrast to peak 3 in Fig. 7b, u* does not correspond to dopamine which was shown by chromatography with other mobile phases.

TABLE I

CARBON CONTENT, SURFACE AREA AND RETENTION CHARACTERISTICS FOR DIFFERENT CHEMICALLY BONDED REVERSED-PHASE MATERIALS

Column packing material (10 μm)	Carbon content (%)	Surface area* (m^2/g)	k' with hexane as eluent	
			Benzene	Nitrobenzene
Partisil ODS	4.00	300	0.15	0.80
Spherisorb ODS	6.67	220	0.14	0.58
Nucleosil C_{18}	18.6	300	0.20	0.45
LiChrosorb RP-18	19.8	400	<0.1	0.21

*According to the manufacturer.

octadecyl groups to the matrix. This latter method determines the structure of the hydrocarbon part of the packing material, which is either monomeric or polymeric [18].

It is argued that under otherwise fixed conditions the intrinsic retentive capability is higher for packing materials with higher carbon content [19]. This is not verified by the results obtained here with salt-free systems, since the highest k' values are observed for the low-carbon phase Spherisorb ODS. The considerable difference between Partisil ODS and Spherisorb ODS is also noteworthy. Although their carbon contents are of the same magnitude the latter phase gives considerably higher k' values. However, Partisil ODS, with the lowest carbon content of the phases tested, was also least retentive.

Unreacted silanol groups on the silica surface can play a role, but their effect on retention is not clear, Karch et al. [17] argued that the values of the capacity factors for benzene and nitrobenzene using nonpolar mobile phases like hexane reflect the degree of interaction with unreacted silanol groups. However, there is no correlation between the k' values in Table I and the retentions observed for the test substances on the four packing materials.

On increasing the ionic strength by addition of sodium sulphate a decrease or increase in retention results. A great decrease takes place for Spherisorb ODS while for other packing materials the change is only moderate. In general the effect on retention for the four test substances is similar on the same phase. The change in retention observed for Spherisorb ODS and to a much smaller extent for Partisil ODS is in accordance with the theory of Horváth et al. [20]. However, for Nucleosil C_{18}, retention increases with an increase in ionic strength, which is contrary to the theory. On LiChrosorb RP-18 an increase of the k' values took place for some compounds and a decrease for others. The retention differences observed for the four packing materials indicate that the nature of the reversed-phase packing material plays an important role in the retention mechanism. Horváth and Melander [19] have pointed out some properties of the packing material which can be responsible for the observed differences.

In the absence of sodium sulphate, complete separation of the test mixture was obtained for Spherisorb ODS only; for the other packing materials separation was achieved upon addition of sodium sulphate. However, the necessary amount of salt differs. Thus, on Partisil ODS 5—10 times more salt had to be added in order to accomplish separation, cf. Fig. 3 a—d. It should be noted that the test mixture contains only four of the eight compounds present in the final mixture. Of the missing compounds, adrenaline and noradrenaline do not separate but travel with the front.

The total plate number of the columns was poor in the above systems, 500—2000 theoretical plates being obtained for the test compounds at 100 ml/h, using a valve injector with a 10-μl loop. The efficiency was least for Spherisorb ODS, the number of theoretical plates only amounting to 500—800. Although the chemical nature of the packing materials is unknown, the very low efficiency for Spherisorb ODS might indicate a polymeric type of chemically bonded phase. It also has the lowest surface area among the packing materials investigated. The average low efficiency obtained could also partly be due to poor wettability of the packing material with concomitant increase in resistance to mass transfer between the phases [5].

Effect of pH on retention

The effect of pH on retention was studied either with a mobile phase having approximately constant ionic strength or with a mobile phase containing methanesulphonic acid as modifier. In both cases the packing material was 5 μm Nucleosil C_{18}. The results are presented in Figs. 4 and 5. In both cases the test mixture was complete, containing all eight compounds to be analyzed in serum and urine, except for 6-S-cysteinyldopa in the first case. Adrenaline and noradrenaline are not included in Fig. 4, as they did not separate and moved with the front in the pH range investigated.

As shown by the figures, the influence of pH on retention is considerable for dopa and 5-S-cysteinyldopa, but only moderate for the remaining amino acids in the actual pH-range. The decrease in retention is due to zwitterion formation as the isoelectric point is approached, the solubility of an amino acid going through a minimum at this point. There is no obvious explanation for the difference in pH-sensitivity between dopa and 5-S-cysteinyldopa on the one hand and the remaining amino acids on the other. However, it appears that the pH-sensitivity of dopa is greatly reduced by introducing cysteinyl groups at the 2- or 6-position. For the catecholamines dopamine, adrenaline and noradrenaline, there is a slight increase in k' values with increasing pH, reflecting the gradual transformation of substituted ammonium cations into neutral compounds. At higher pH values than those studied here, this transformation causes a steep rise in the curve due to decreasing solubility, as shown by Molnár and Horváth [7].

For these systems the efficiency was better, amounting 6000—7000 theoretical plates for the systems in Fig. 4 and about 8000 theoretical plates for those in Fig. 5. This can partly be ascribed to the fact that 5 μm particles were used. For the systems containing methanesulphonic acid, improved mass transfer between the phases also contributes to increasing the number of theoretical plates obtained.

Effect of addition of modifiers on retention and separation of cysteinyldopas, dopa, dopamine, adrenaline and noradrenaline in serum and urine

Sufficient resolution could not be attained for the separation of all eight compounds involved by merely varying ionic strength and pH. That an enhancement of retention of adrenaline and noradrenaline could be achieved by adding an organic modifier like methanesulphonic acid was already shown in Fig. 5. On that basis we wished to study in some detail the effect of adding anionic modifiers to the mobile phase, using 5 μm Nucleosil C_{18} as packing material.

Original attempts to use sodium *n*-octyl sulphate ($\sim$ 10 mg/l) as modifier were not quite successful. Although good separations of all compounds were obtained, steady-state conditions were achieved very slowly, indicating a successive adsorption of detergent on the surface of the packing material [6]. In addition, the peak for 2,5-S,S-dicysteinyldopa was, for unknown reasons, seriously broadened.

By using methanesulphonic acid instead, steady-state conditions were achieved much faster. Fig. 6 presents relations between k' values and concentration of methanesulphonic acid in the mobile phase for all compounds of interest. As can be seen, for most of the compounds there is an increase in retention with an increase in concentration of modifier.

On the basis of the previous experiments, conditions were chosen which permitted the separation of all catecholic amino acids studied, as well as dopamine, noradrenaline and adrenaline on 5 μm Nucleosil C_{18}. Fig. 7 a and b shows chromatograms obtained for serum and urine, respectively, from patients with malignant melanoma. All compounds except 6-S-cysteinyldopa, which was estimated to be present in too low concentration to be detected, have been identified on the basis of retention data of standards. The concentrations of dopamine, noradrenaline and adrenaline in serum are estimated to be low. Furthermore, unidentified compounds elute together with dopamine and noradrenaline. Consequently, these compounds are not visible in Fig. 7a. However, their detection in urine poses no problem [21], although a somewhat more concentrated urine sample than that represented in Fig. 7b is desirable. Additional work on the quantitative analysis of the above compound is in progress. Further improvement in resolution is necessary in order to achieve quantitative determination of all compounds involved in one run.

Recovery after adsorption on alumina

The recovery of four catecholic amino acids after adsorption on alumina was studied using three acidic eluents, and the results are given in Table II. Although 0.1 *M* perchloric acid was used in our previous determination of dopa and 5-S-cysteinyldopa [2], 1 *M* perchloric acid was chosen for the analysis of serum and urine samples, because of the higher average recovery. Use of alumina might lead to the appearance of fines in the eluate and thus filtration might be necessary. Otherwise the column can be blocked.

Electrochemical detection

The electrochemical detection of catechols is advantageous due to their low oxidation potential. We have found that the LC-10 detector is compatible

TABLE II

RECOVERY OF CATECHOLIC AMINO ACIDS AFTER ADSORPTION ONTO ALUMINA AND ELUTION WITH ACID

Compound	Amount added (ng)	Recovery (%)		
		0.1 *M* $HClO_4$	1 *M* $HClO_4$	35% HBF_4
Dopa	330	71	76	71
5-S-Cysteinyldopa	927	31	56	62
2-S-Cysteinyldopa	480	38	62	72
2,5-S,S-Dicysteinyldopa	330	11	55	47

TABLE III

EFFECT OF CHANGING WORKING POTENTIAL ON SENSITIVITY

Mobile phase: 3% (v/v) methanol in water, 2.9 g H_3PO_4 per litre. Injected amount: 5—10 ng each.

Compound	Percentage increase in peak height when changing the working potential from 0.72 V to 0.90 V
5-S-Cysteinyldopa	25
Dopa	38
Dopamine	49
2,5-S,S-Dicysteinyldopa	36

with all the chromatographic systems studied here. The combination of this detector with highly efficient columns packed with microparticulate reversed-phase material has made it possible to detect and accurately determine 25 pg of 5-S-cysteinyldopa [2].

The sensitivity of the detector can be increased by increasing the working potential. This is shown in Table III. Although sensitivity is markedly increased for the compounds investigated, selectivity will decrease with higher oxidizing potential, non-catecholic substances will also be oxidized. The sensitivity was found to differ slightly even on packing the electrode with graphite paste from the same lot. Sensitivity was also found to be higher for the first two or three injections. It thus seems advisable to make several injections on a fresh electrode in order to avoid differences in sensitivity before quantitative analysis is carried out. Due to these differences in sensitivity, an internal standard is advantageous.

CONCLUSIONS

High-performance liquid chromatography (HPLC) with a chemically bonded reversed-phase packing material combined with electrochemical detection has

been used to separate and detect the catecholic amino acids dopa, 2-S- and 5-S-cysteinyldopa and 2,5-S,S-dicysteinyldopa in serum from patients with malignant melanoma. In urine the catecholamines dopamine, noradrenaline and adrenaline were also separated, together with the above-mentioned compounds, in a single chromatographic run. Separation is achieved with the addition of methanesulphonic acid to an acidified aqueous mobile phase. By changing the pH of the mobile phase and the concentration of methanesulphonic acid, the retention can be varied, thus allowing for the optimization of the separation conditions. Modifiers with a longer lipophilic group could also be valuable in cases where a further enhancement of retention is desired, as shown by Horváth et al. [22].

A comparison of the performance of four commercial reversed-phase packing materials containing chemically bonded octadecyl groups using a standard mixture of catecholic amino acids shows that there is a considerable difference between packing materials from different manufacturers. This was also evident from a study of the variation of retention of the test mixture when the ionic strength of the mobile phase was changed by the addition of sodium sulphate.

Efficiency is markedly increased using 5 μm packing material. The addition of an organic modifier also improves efficiency, presumably due to an enhancement of mass transfer between the phases.

Systems involving combination of reversed-phase microparticulate packing materials with acidified aqueous mobile phases containing salts or organic modifiers are well suited to the analysis of catecholic compounds with electrochemical detection. It is possible to detect very small amounts of catecholic amino acids and amines, e.g. 25 pg of 5-S-cysteinyldopa. Essential for this result is the selective adsorption of catecholic compounds on alumina prior to separation by HPLC. By this method the overall sensitivity and selectivity is considerably improved.

ACKNOWLEDGEMENTS

This investigation was supported by grants from the Swedish Cancer Society (No. 626-B77-06XC and No. 626-B77-01P) and from the Swedish Medical Research Council (No. B78-04X-56-14B).

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QUANTITATIVE ANALYSIS OF METHYLGUANIDINE AND GUANIDINE IN PHYSIOLOGIC FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY—FLUORESCENCE DETECTION METHOD

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(First received March 9th, 1978; revised manuscript received July 2nd, 1978)

SUMMARY

A rapid and sensitive high-performance liquid chromatographic method has been developed for the quantitative analysis of methylguanidine and guanidine in physiological fluids. These guanidino compounds are separated on a 6 × 0.23 cm cation-exchange column with 0.5 *M* sodium hydroxide solution. The guanidino compounds are detected with a fluorometer, which monitors the fluorescent guanidine derivatives produced by the reaction of the eluted constituents with 9,10-phenanthrenequinone. Sensitivity to sub-nanomole levels of methylguanidine and guanidine is demonstrated. The method was successfully applied to physiological fluids such as serum and cerebrospinal fluid from uremic patients.

INTRODUCTION

Methylguanidine levels have been demonstrated to be elevated significantly in sera of uremic patients [1—5], suggesting it is one of the toxic substances in uremia [6]. Therefore, a routine method for the quantitative analysis of methylguanidine in physiological fluids is strongly desired for clinical purposes.

The conventional column chromatographic method for the analysis of methylguanidine is time-consuming and needs a large blood sample [1—6]. Furthermore, this method does not resolve methylguanidine from guanidine, which leads to errors in the estimation of methylguanidine. Recently, all guanidino compounds in physiological serum, including methylguanidine, were analyzed using a modified automatic amino-acid analyzer [7,8]. However, methylguanidine and guanidine contents were not measured with accuracy, even for the uremic serum because of the low sensitivity of the method.

The purpose of this study was to develop a rapid and sensitive method for

the quantitative analysis of methylguanidine and guanidine. This paper describes the development of a high-performance liquid chromatographic (HPLC) procedure for separating guanidine, methylguanidine and other guanidino compounds, along with a means of detecting these guanidino compounds fluorometrically by utilizing the fluorophor produced by the reaction of the guanidines with 9,10-phenanthrenequinone. 9,10-Phenanthrenequinone has been reported to form a highly fluorescent product with guanidino compounds in alkaline solution [9].

EXPERIMENTAL

Chemicals

Methylguanidine hydrochloride and guanidine hydrochloride were obtained from Sigma (St. Louis, Mo., U.S.A.). Other guanidino compounds were purchased from Sigma and Pierce (Rockford, Ill., U.S.A.). 9,10-Phenanthrenequinone was obtained from Tokyo Chem. Co. (Tokyo, Japan). N,N-dimethylformamide was purchased from Nakarai Chem. Co. (Kyoto, Japan). Durrum DC-4A cation-exchange resin was obtained from Durrum (Palo Alto, Calif., U.S.A.).

Chromatographic system

Fig. 1 is a schematic presentation of the high-performance liquid chromatograph which was used in these experiments. Two minipumps (Milton-Roy) served to pump the column eluent and the reagent solution through the system. The column eluent was pumped through a 6-port sample injection valve supplied with a 100-μl sample loop. A jacketed, stainless-steel column, 6 × 0.23 cm

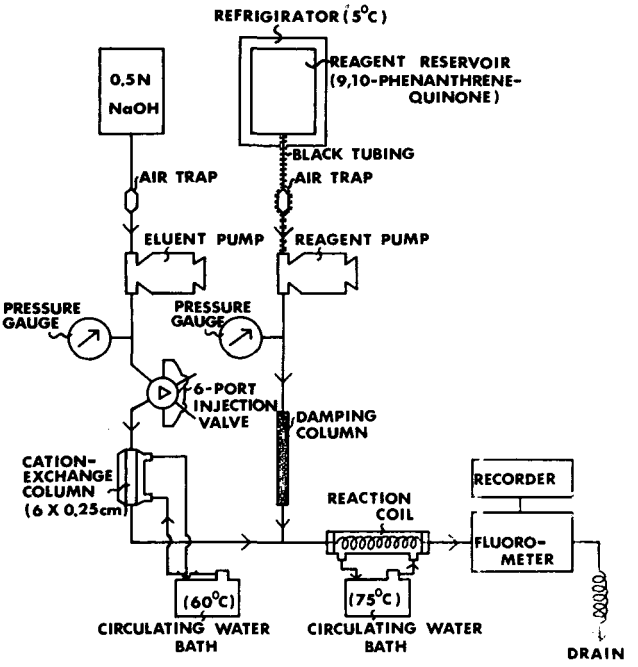

Fig. 1. Schematic diagram of high-performance liquid chromatograph for the separation and fluorescence detection of guanidine and methylguanidine.

I.D., was used in this investigation. The column was slurry-packed with Durrum DC-4A resin (8 ± 2 μm), which was supported by a 1/8- to 1/16-in. reducer union containing 1 μm stainless-steel frit. The column was operated at 60° utilizing a Haacke constant-temperature circulator. The reservoir of 9,10-phenanthrenequinone reagent solution was stored in a refrigerator at 5° to keep the reagent from degradation. The PTFE tubing connecting the reservoir to the reagent pump was covered with black tubing to prevent the exposure of the reagent to light. A pulse-damping device, a 30 × 0.23 cm I.D. column packed with Zipax (Du Pont), was inserted between the reagent pump and the mixing tee. The eluent from the chromatographic column was mixed with a stream of 9,10-phenanthrenequinone in a heated stainless-steel reaction coil, 153 × 0.05 cm I.D. At a total flow-rate of 18 ml/h, dwell time within the reaction coil was ca. 1 min. A JASCO FP-100 FluoroMonitor (Japan Spectroscopic Co., Tokyo, Japan) was used to detect the fluorescent guanidine derivatives produced by the reaction of 9,10-phenanthrenequinone with the constituents from the column. The FluoroMonitor was equipped with a high-pressure mercury lamp which emits strong 365 nm light and with primary and secondary filters to detect visible light beyond 460 nm emitted from the fluorescent compounds in the flow cell.

Operation of the chromatographic system

Methylguanidine, guanidine and other guanidino compounds were separated on cation-exchange resin using a 0.5 *M* sodium hydroxide solution. A 100-μl sample was introduced into the column using a 6-port sample injection valve. The column was operated at a flow-rate of 12 ml/h and a column inlet pressure of 10 kg/cm^2. A 2.5 m*M* solution of 9,10-phenanthrenequinone was prepared in the reagent reservoir by dissolving the reagent in dimethylformamide. The reagent solution was pumped into the reaction coil at a flow-rate of 6 ml/h. As mentioned later, the reaction coil was heated up to 75° to attain the highest detectability.

Preparation of physiologic fluid samples

Serum and cerebrospinal fluid samples from uremic patients were used for the analysis of guanidine and methylguanidine. A 1-ml aliquot from each serum sample was centrifuged at 80 *g* for 2 h in a CF-25 Centriflo® membrane [10] (Amicon, Lexington, Mass., U.S.A.), yielding approximately 0.5 ml of ultrafiltrate. A 0.1-ml sample of the deproteinized ultrafiltrate was applied to the column of the chromatographic system. Cerebrospinal fluid sample was used directly for the analysis without any deproteinization treatment.

RESULTS

Effect of alkalinity of mobile phase on retention time and resolution

Because of the strong basicity of guanidine and methylguanidine they are not eluted with basic buffers but with strong alkaline solutions. The effect of the alkalinity of the mobile phase on the retention time was tested in the range of 0.1—1.0 *M* NaOH. A plot of retention times and resolution ratios against the concentration of sodium hydroxide is shown in Fig. 2. Here, the resolution

ratio is the standard quantity R_s. Increasing alkalinity tends to produce a faster chromatogram of guanidine and methylguanidine. On the other hand, the resolution ratio increased with increasing alkaline concentration up to a maximum around 0.4 *M*, followed by a gradual decrease at higher alkaline concentration.

Using these data, it was determined that 0.5 *M* sodium hydroxide solution should be used as a column eluent to obtain optimum separation results (resolution and analysis time).

Fluorescence properties of 9,10-phenanthrenequinone derivatives of guanidine and methylguanidine

9,10-Phenanthrenequinone derivatives of guanidine and methylguanidine show fluorescence maxima at 525 nm and 510 nm, respectively. Excitation maxima are observed at 386 nm and 298 nm for guanidine derivative, and at 378 nm and 298 nm for methylguanidine derivative.

The effect of temperature on the reaction of the guanidines with 9,10-phenanthrenequinone was studied in the range of 30—95°. Fig. 3 shows a plot of the fluorescence intensity as a function of reaction coil temperatures after passing through the coil for 1 min. It can be seen that the degree of conversion to fluorescent derivatives shows a maximum at 75° for methylguanidine and at 80° for guanidine and that the response decreases at higher temperatures. This is presumably due to a breakdown of the reagent or the product at the higher temperatures. The fluorescence intensity of guanidine and methylguanidine derivatives obtained at 75° is 7—8 times greater than that at room temperature.

Accordingly, the reaction coil temperature utilized in the chromatographic system for the analysis was 75°.

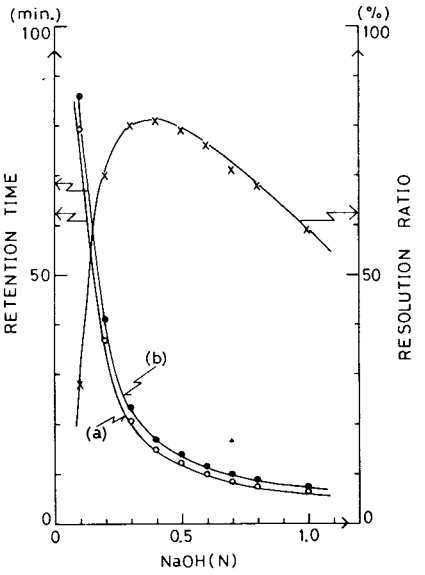

Fig. 2. Effect of alkaline concentration of mobile phase on retention time and resolution of guanidine and methylguanidine. (a) Guanidine, (b) methylguanidine.

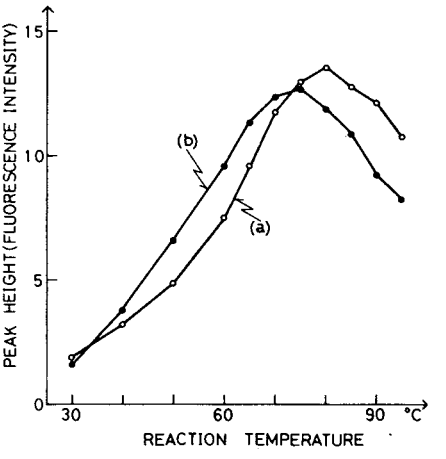

Fig. 3. Effect of reaction coil temperature on the reaction of conversion to fluorescent guanidine derivatives. (a) Guanidine, (b) methylguanidine.

Chromatographic separation and quantitative response

Fig. 4 shows a typical separation of a standard mixture of guanidine, methylguanidine and other guanidino compounds, each at a concentration of 1 nmole per 100 μl. All guanidino compounds except for guanidine and methylguanidine are eluted as a group in the first 7 min. Guanidine and methylguanidine are eluted at 12.4 min. and 14.1 min., respectively, showing satisfactory resolution. The entire analysis can be completed in less than 15 min.

The linear range of the analytical system was evaluated from 0.025 nmoles to 2.5 nmoles of guanidine and methylguanidine, and the relative weight response of each compound remained essentially constant, indicating good linearity. The minimum detectable quantity for these compounds was found to be 0.49 ng for guanidine and 0.63 ng for methylguanidine. In order to evaluate the precision and accuracy of the analytical method a recovery study was done using 20 serum samples from uremic patients. The recoveries of guanidine and methylguanidine are 98.5 ± 2.3% and 97.3 ± 3.2%, respectively and seem to be essentially quantitative.

Analysis of physiologic fluids

Several serum and cerebrospinal fluid samples were analyzed for guanidine and methylguanidine. Typical results for samples from uremic patients are shown in Fig. 5. The chromatogram in Fig. 5A represents a serum sample from a uremic patient. The major peak, eluting early in the chromatogram, contains other less basic guanidino compounds such as guanidinosuccinic acid, guanidinoacetic acid, creatinine and arginine. The labelled peaks were identified from the retention times as compared to those of a standard mixtures and estimated to be 0.47 nmoles for guanidine and 1.18 nmoles for methylguanidine. The contents of guanidine and methylguanidine were determined as 28.1 μg/dl and 86.5 μg/dl, respectively. The chromatogram in Fig. 5B shows a cerebrospinal fluid sample from a uremic patient. The labelled peaks of guanidine and methylguanidine were estimated to be 0.108 nmoles and 0.228 nmoles, respectively. The contents of guanidine and methylguanidine in this cerebrospinal fluid sample were determined to be 6.40 μg/dl and 16.7 μg/dl, respectively.

DISCUSSION

The conventional method for the analysis of methylguanidine is based on the colorimetric procedure after the isolation of methylguanidine by means of adsorption of charcoal [2] or ion-exchange column [3—6]. This method requires large sample amounts (usually 5—20 ml), and prior to the colorimetric measurement the fractionated sample is concentrated by the evaporation procedure. A recent analytical method using a modified automatic amino-acid analyzer [8,9] also requires concentration techniques such as lyophilization prior to application to the chromatographic column. Nevertheless, methylguanidine is sometimes not determined even for the uremic serum because of the lower sensitivity of this method. In experiments, not less than 1 ml of serum was passed through an ultrafiltration membrane and then 0.1 ml of the deproteinized sample was directly applied to the analytical column. Our new sensitive analytical method is advantageous particularly when only a limited sample is

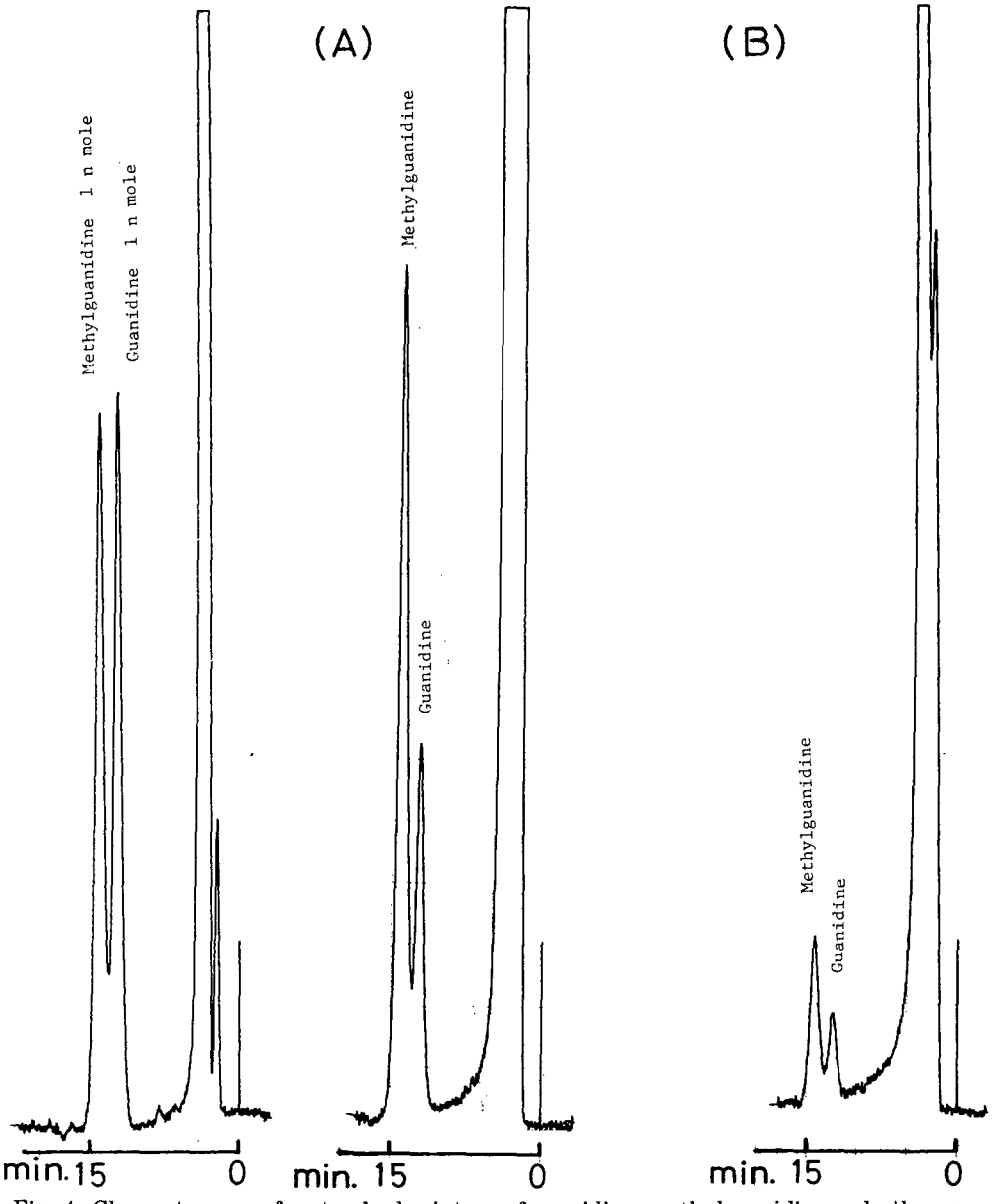

Fig. 4. Chromatogram of a standard mixture of guanidine, methylguanidine and other guanidine compounds, each at a concentration of 1 nmole/100 μl.
Fig. 5. (A) Analysis of a serum sample from a uremic patient. (B) Analysis of a cerebrospinal fluid sample from a uremic patient.

available, i.e. sampling of the cerebrospinal fluids or repetitive sampling in patients or in small animals. In the case of cerebrospinal fluid samples, only 0.2 ml of sample is required for analysis because deproteinization treatment is not required. Use of such small samples is possible because our fluorometric detection method is two orders of magnitude more sensitive than the conventional colorimetric procedure [1—9].

The conventional method did not separate methylguanidine from guanidine. Therefore, the results obtained showed artificially high values of methylguanidine. The use of an automatic amino-acid analyzer for the analysis of guanidino compounds [8,9] enabled the separation of most of the guanidino compounds in physiological fluids. Shainkin et al. [9] reported that guanidine and methylguanidine were found in small amounts only in uremic plasma. However, the amount of these guanidino compounds was not determined with accuracy because of the limitation in sensitivity of their method. With our analytical method, the cerebrospinal as well as serum levels of guanidine and methylguanidine were easily determined for all samples from uremic patients.

A large number of uremic serum and cerebrospinal fluid samples are currently being analyzed, and the results obtained from our analytical method will be the subject of a later report.

CONCLUSION

A simple and direct HPLC system has been described for the quantitative analysis of guanidine and methylguanidine in physiological fluids.

Our new analytical method has several distinct advantages:

(a) only a small amount of physiological fluid sample, i.e. no more than 1 ml of serum or 0.2 ml of cerebrospinal fluid, is needed for analysis.

(b) the method is so sensitive that no concentration techniques such as lyophilization or evaporation are required.

(c) the separation of guanidine and methylguanidine is satisfactory and the simultaneous quantitative analysis is easily performed.

(d) the entire analysis can be completed in less than 15 min, so that a large number of samples can be analyzed in reasonable time.

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CHROMBIO. 228

QUANTITATIVE ANALYSIS OF AMINEPTINE (S-1694) IN BIOLOGICAL SAMPLES BY GAS CHROMATOGRAPHY—MASS FRAGMENTOGRAPHY

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(First received March 13th, 1978; revised manuscript received July 19th, 1978)

SUMMARY

A sensitive, specific method for the quantitative analysis of amineptine in rat blood and brain is described. After extraction and purification amineptine is detected by mass fragmentography, monitoring the fragment ion at *m/e* 192. The method allows the quantitative analysis of as little as 50 ng of amineptine per ml blood and 200 ng per g for brain.

INTRODUCTION

Amineptine (S-1694), [dihydro-10,11-dibenzo(*a*,*d*)cycloheptenyl-5-amino]-7-heptanoic acid, is a new stimulant of the central nervous system whose biochemical and pharmacological effects are different from amphetamine: in rats amineptine causes increases in locomotor activity, stereotyped movements and hyperthermia, like amphetamine, whereas it produces little or no anorectic effect in this animal species [1,2]. Moreover, while amphetamine affects both dopaminergic and noradrenergic systems [3—5], amineptine appears to act preferentially on the dopaminergic system [2].

As a basis for future work in exploring the possibility of a relationship between the pharmacological effect of amineptine and its concentration in different brain areas, a specific, sensitive method was developed for measuring amineptine levels in biological materials.

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MATERIALS AND METHODS

Standard and reagents

Amineptine was obtained from Servier Labs. (Paris, France). The following reagents were used: methanol, ethyl acetate, *n*-hexane (R.P.E.; Carlo Erba, Milan, Italy). Trimethylanilinium hydroxide (TMHA) was synthesized in our laboratories [6].

Apparatus

An LKB Model 9000 gas chromatograph—mass spectrometer equipped with an accelerating voltage alternator was used. The gas chromatographic (GC) conditions were as follows: the chromatographic column was a glass tube (2 m long and 2 mm I.D.) packed with 1% OV-17 on Gas-Chrom Q, 60—80 mesh (Applied Science Labs., State College, Pa., U.S.A.); the column and injector temperature was 290° and the carrier gas (helium) flow-rate was 25 ml/min.

The mass spectrometer was operated under the following conditions: molecular separator temperature, 290°; trap current, 60 μA; electron energy, 70 eV; accelerating voltage, 3.5 kV; filters, 20 Hz.

The mass spectrometer was focused on the ion at *m/e* 192, characteristic of the tricyclic nucleus, allowing continuous monitoring of amineptine and its internal standard, opipramol, in the effluent of the GC column. Fig. 1 shows a typical mass fragmentogram of amineptine and its internal standard.

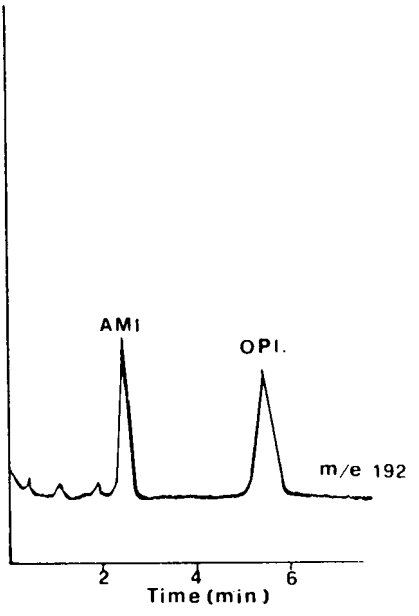

Fig. 1. Mass fragmentogram of amineptine (AMI) and its internal standard opipramol (OPI) obtained focusing at *m/e* 192.

Construction of the calibration graphs and quantitative analysis of amineptine

For calibration and quantitative analysis, the internal standard technique was used. Amineptine was dissolved in methanol in concentrations from 250 to 2000 ng/ml; 1 ml of each solution was evaporated and the residue was dissolved in 0.1 ml of methanol containing 100 ng/ml of opipramol. An aliquot of these solutions (1—3 μl) was injected into the GC column together with 1 μl of 0.2 *M* TMAH in methanol. Amineptine can be quantitated when the relative peak height is used as an index of concentration, as a linear relationship exists between relative peak heights of amineptine in the range 2.5—20 ng.

Extraction from blood

Two ml of heparin-treated rat blood were made alkaline by adding 5 ml of phosphate buffer (pH 8.5) and extracted twice with 10 ml of ethyl acetate by shaking for 10 min on an automatic shaker. After centrifugation the combined organic phases were evaporated to dryness. The residue was dissolved in 5 ml of 0.1 *M* HCl and washed twice with 5 ml of *n*-hexane. After washing, the acid phase was adjusted to pH 8.5 by adding 5 ml of 0.2 *M* phosphate buffer (pH 8.5) and extracted twice with 10 ml of ethyl acetate. The combined organic phases were evaporated to dryness in a rotating evaporator. The residue was dissolved in 0.1 ml of methanol containing a fixed amount of the internal standard (opiramol), and submitted to mass fragmentographic analysis.

Extraction from brain

Rat brain was homogenized in a glass Potter apparatus with absolute ethanol (1:10, w/v). After centrifugation the alcoholic phase was separated from the precipitate and evaporated to dryness. The residue was dissolved in 5 ml of 0.1 *M* HCl and the acid phase was submitted to the clean-up procedure described above for blood.

Recovery studies

The addition of amineptine, in amounts ranging from 100 to 2000 ng, to drug-free blood and brain samples resulted in over-all recoveries of 91.7 ± 5.2% for blood and 83.1 ± 4.8% for brain. The minimum detectable amount of amineptine under these experimental conditions was 50 ng/ml for blood and 200 ng/g for brain.

Animal studies

To check the sensitivity and specificity of the method for pharmacokinetic studies in vivo, adult male CD-COBS rats (Charles River, Calco, Italy) weighing 230—250 g were used. The animals were maintained on a standard diet and in standardized environmental conditions, fasted overnight before experiments and treated intraperironeally with amineptine at a dose of 20 mg/kg. At various times after drug administration, groups of 4 animals were killed and their blood and brains were collected and analysed as described above.

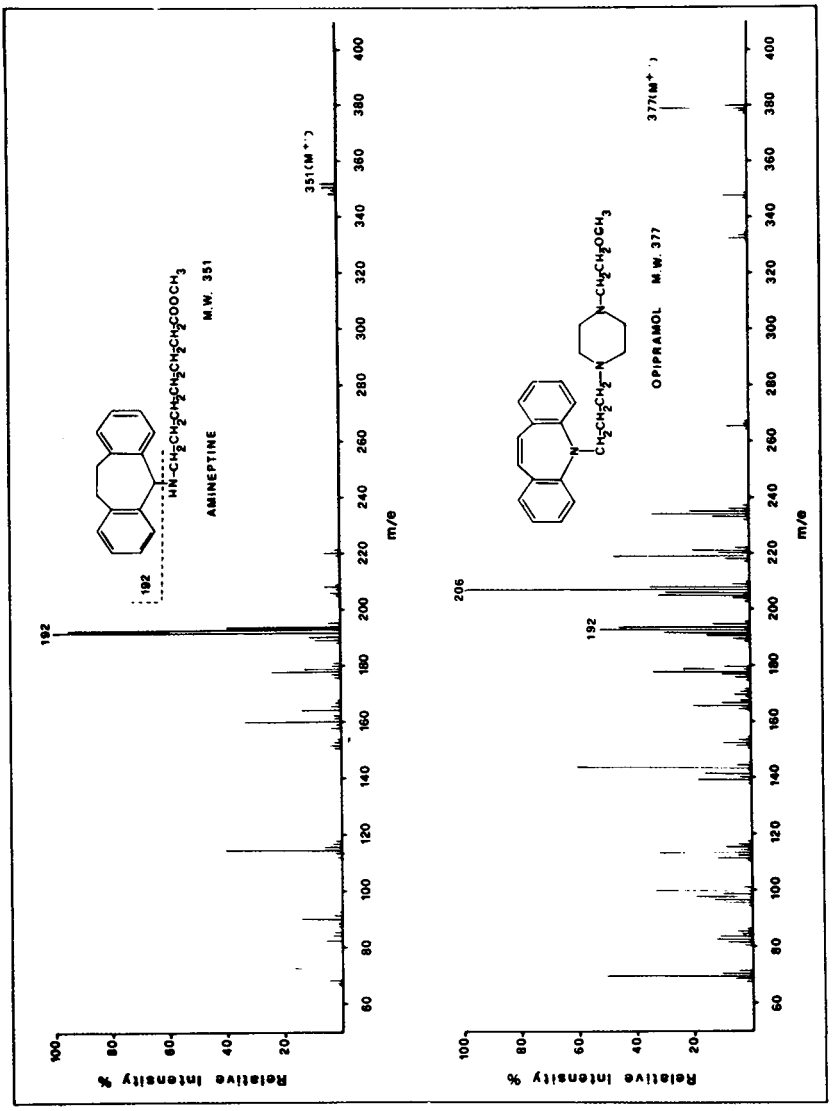

Fig. 2. Mass spectra of amineptine and opipramol after reaction with TMAH.

RESULTS AND DISCUSSION

The mass spectra of amineptine and its internal standard opipramol, after methylation with TMAH, are shown in Fig. 2. Since no stable isotope-labelled amineptine was available, the internal standard was added at the end of the extraction procedure in order to correct possible errors due to variability of injected volumes or changes in instrumental conditions. The variability of the recovery efficiency was taken into account by analysing, together with each set of samples, control samples enriched with a known amount of amineptine. Opipramol was chosen as an internal standard because of its suitable retention time and because, like amineptine, it gives an intense ion at *m/e* 192, characteristic of the tricyclic nucleus, allowing the detection of both amineptine and opipramol by single-ion monitoring.

Typical mass fragmentograms obtained from a blood sample of a control rat treated with 20 mg/kg (i.p.) of amineptine (B) are shown in Fig. 3.

A peak (C) with a retention time shorter than that of amineptine was found in all the blood and brain samples from treated rats but not in samples from control animals, suggesting the formation of a metabolic product of amineptine. Like amineptine, the peak corresponding to the unknown compound was not eluted from the column unless the sample was injected by the on-column methylation technique using TMAH; this suggests there may be a polar group in the molecule.

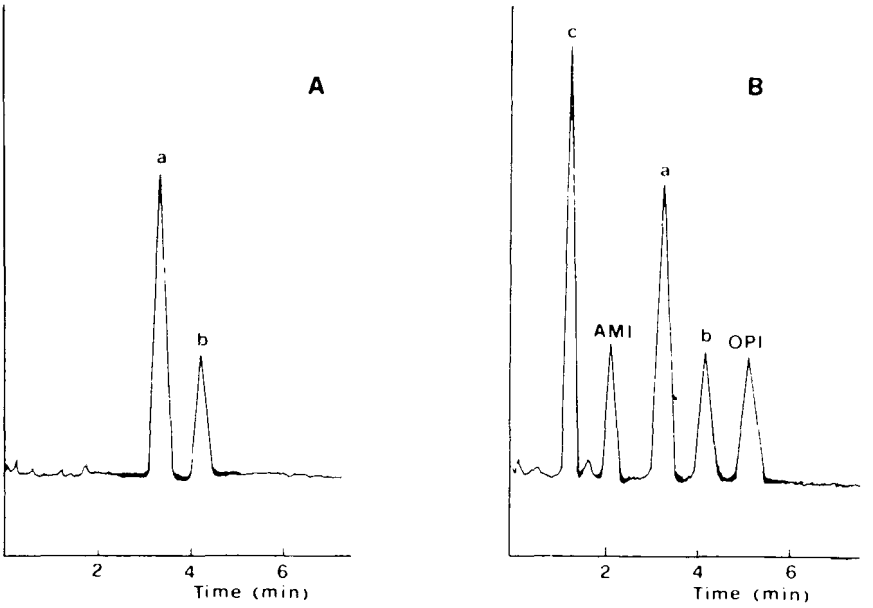

Fig. 3. Mass fragmentograms obtained from rat blood. A, control animal; B, 15 min after treatment with amineptine (AMI) (20 mg/kg, i.p.) with the addition of the internal standard opipramol (OPI). a and b, endogenous substances; c, amineptine metabolite.

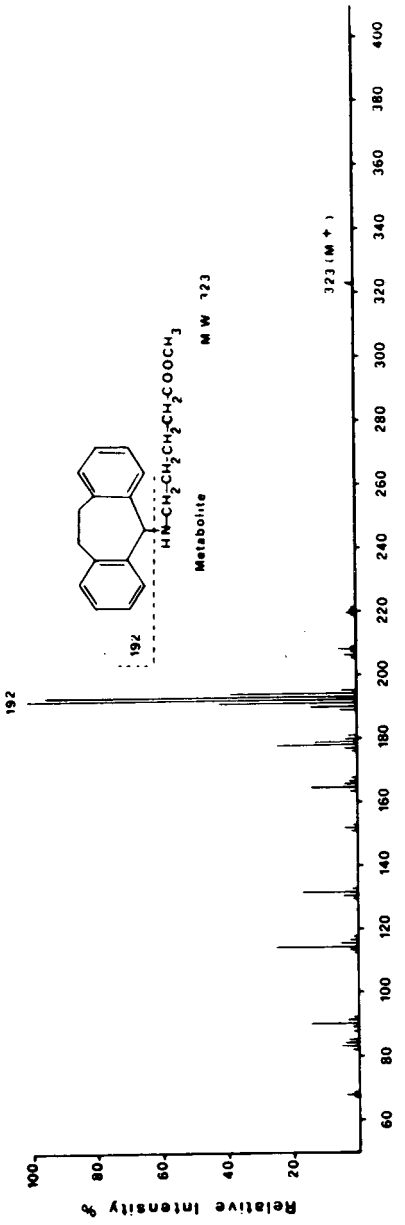

Fig. 4. Mass spectrum of amineptine metabolite after methylation using TMAH.

The mass spectrum corresponding to the unknown compound is given in Fig. 4. There is a base peak at *m/e* 192, suggesting that the tricyclic nucleus is not modified, and an apparent molecular ion at *m/e* 323, suggesting that the aliphatic chain in the amineptine molecule might be biotransformed with the loss of 2 CH_2 units. The structure of the metabolite was also confirmed by reaction of the biological extract with diazomethane and BSTFA in pyridine. The reaction with diazomethane gave a peak with the same retention time and the same mass spectrum of the TMAH product, while the reaction with BSTFA gave a peak with a longer retention time and with a mass spectrum retaining the base peak at *m/e* 192 and an apparent molecular ion at *m/e* 381.

Blood and brain levels at various times after amineptine treatment are shown in Fig. 5. Amineptine brain levels decrease rapidly and are not detectable 120 min after treatment, while in blood amineptine is still measurable 240 min after treatment.

The results indicate that this method is suitable for measuring amineptine in blood and brain of animals and may possibly be useful for the determination of amineptine biotransformation products.

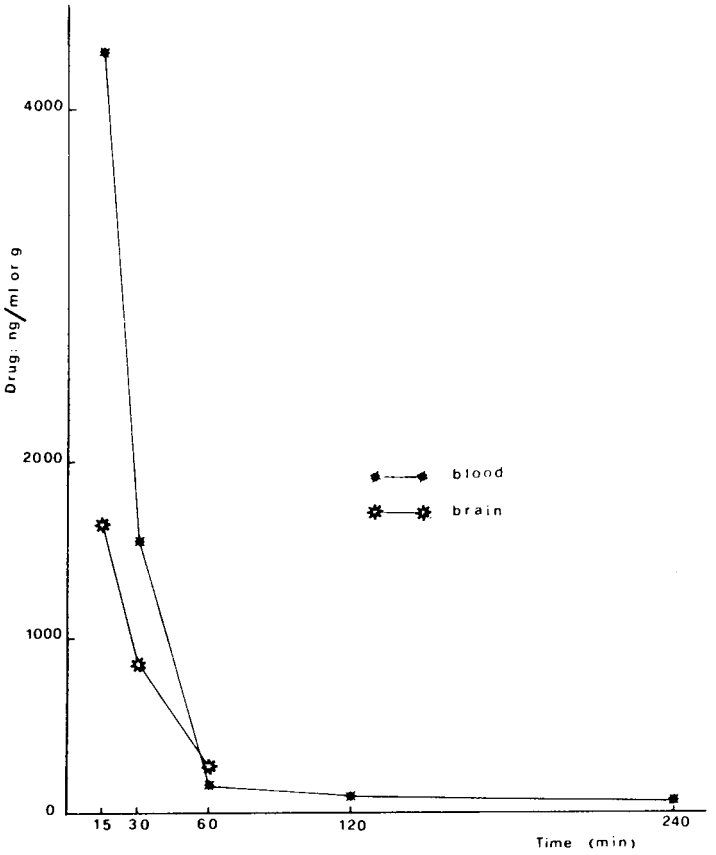

Fig. 5. Blood and brain levels of amineptine in rats treated at a dose of 20 mg/kg (i.p.).

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Journal of Chromatography, 162 (1979) 39—45
Biomedical Applications

CHROMBIO. 238

RAPID, SENSITIVE AND SPECIFIC ELECTRON CAPTURE—GAS CHROMATOGRAPHIC METHOD FOR THE QUANTITATION OF 6-CHLORO-2-(1-PIPERAZINYL)PYRAZINE IN BIOLOGICAL FLUIDS

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(First received February 3rd, 1978; revised manuscript received July 25th, 1978)

SUMMARY

A highly specific and sensitive gas chromatographic method for the determination of 6-chloro-2-(1-piperazinyl)pyrazine (MK-212), a central serotonin-like agent, in biological fluids is described. MK-212 and a related internal standard are extracted into benzene from an alkaline solution, back-extracted into acid and then re-extracted into benzene at an alkaline pH. The amines are converted to the trifluoroacetyl derivatives (characterized by gas—liquid chromatography—mass spectrometry), chromatographed and detected with a ^{63}Ni electron capture detector. The sensitivity of the method is such that 10 ng of drug can be measured per aliquot of biological fluid. The precision and accuracy of the method are well within acceptable limits. Specificity of analysis was established by gas—liquid chromatography—mass spectrometry techniques.

INTRODUCTION

Recently, MK-212 was shown to exhibit central serotonin-like activity. The compound, when administered systemically, was shown to elicit four distinct responses characteristic of serotonin-receptor activation in the nervous system [1]. Complete abolition of these responses to MK-212 was achieved by pretreatment of the animals with a centrally acting indoleamine antagonist, whereas a peripherally acting antagonist was ineffective [1]. The anorexigenic and ancillary actions of MK-212 have also been described [2]. The synthesis of a series of 2-(1-piperazinyl)pyrazines, including MK-212, has recently been reported [3]. These authors also discussed the use of molecular orbital calculations and computer graphics to gain insight into the structural features of MK-212 which might interact with serotonic receptors.

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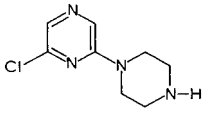
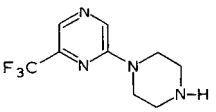
This report describes a highly specific and sensitive gas—liquid chromatographic (GLC) method for the determination of MK-212 using the internal standard, 6-trifluoromethyl-2-(1-piperazinyl)pyrazine (I), derivatization with trifluoroacetic anhydride, and detection using a ^{63}Ni electron capture detector.



MK-212



I

Analysis of biological specimens from rats, dogs and monkeys were utilized to demonstrate the applicability of the assay procedure following pharmacological doses of MK-212.

EXPERIMENTAL

Reagents and Chemicals

The reagents and chemicals used were: 6-chloro-2-(1-piperazinyl)pyrazine (MK-212), the internal standard 6-trifluoromethyl-2-(1-piperazinyl)pyrazine (I), pesticide grade benzene, 2.5% solution of trifluoroacetic anhydride (Aldrich, Milwaukee, Wisc., U.S.A.) in benzene, 0.2 *M* phosphate—NaOH buffer (pH 9) and 5% aqueous ammonium hydroxide. The amines were supplied as the hydrochloride salts; however, all concentrations are expressed in terms of the free bases. Standard solutions of MK-212 were prepared in water and were diluted to final concentrations of 0.125—2 μg/ml. The internal standard solution was diluted to a final concentration of 5 μg/ml.

Instrumentation

GLC. Analyses during the past year were performed on either a Packard gas chromatograph (Model 7400) equipped with a ^{63}Ni electron capture detector (ECD) and a glass column (1.2 m × 4 mm I.D.) packed with 3% OV-210 on Gas-Chrom Q (100—120 mesh) or a Hewlett-Packard gas chromatograph (Model 5830A) with a ^{63}Ni detector and a similar column. The instruments were operated isothermally with a carrier gas (helium, Packard; argon—methane, Hewlett-Packard) flow-rate of 50 ml/min. The injection port and detector temperatures were 20—30° higher than the column temperature (190—205°).

GLC—mass spectrometry (MS). All mass spectra were obtained on an LKB-9000S mass spectrometer using a GLC inlet system. A 1.2 m × 3 mm I.D. glass column packed with 3% OV-210 was used. The gas chromatograph was operated isothermally at 200° with a helium flow-rate of 30 ml/min. The mass spectrometer ionizing and accelerating potentials were 70 eV and 3.5 KV, respectively. The source, separator and injection port temperatures were 270°, 250° and 245°, respectively.

Measurement of MK-212 in biological samples

The concentration of MK-212 in biological fluids is determined as follows. To an appropriate aliquot of plasma, urine or brain homogenate in a 50-ml glass-stoppered centrifuge tube are added 100 μl of I (500 ng), 1 ml of 0.2 *M*

buffer (pH 9) and 15 ml of benzene. The tube is shaken for 10 min, centrifuged and the organic phase transferred to a clean 25-ml glass-stoppered tube containing 1 ml of trifluoroacetic anhydride (TFAA) reagent. The contents of the tube are heated for 30 min at 65° in a water bath. The tube is cooled and the contents are shaken with 1 ml of water (vortex, 0.1 min) and 1 ml of 5% NH_4-OH. Following centrifugation, the benzene phase is transferred to a clean test tube and the contents evaporated under a gentle stream of nitrogen to about 1 ml. An appropriate aliquot (usually 2 or 5 μl) is injected into the gas chromatograph. The retention times of MK-212 and I as the trifluoroacetyl derivatives were 4.4 and 2.4 min, respectively.

Plasma, urine or brain homogenates spiked with known quantities of MK-212 (12.5—2000 ng) and I (500 ng) are analyzed concurrently with each set of unknown samples. A standard curve is prepared for each series of analyses by plotting the peak height ratio (MK-212/I) vs. the weight ratios of MK-212 to I. Concentrations of MK-212 in the unknown samples are obtained by reference of the particular peak height ratio obtained to the standard curve.

Biological studies

Three rhesus monkeys (3.0—4.4 kg) were administered MK-212 intravenously at a dose of 2.5 mg/kg, and three additional rhesus monkeys (4.0— 5.2 kg) were dosed orally (2.5 mg/kg). Male Sprague-Dawley rats received the drug (2.5 mg/kg) either orally (21 rats) or intravenously (24 rats). Six beagle dogs received the drug at the same dose. The animals were fasted overnight prior to MK-212 administration. Blood specimens were collected in heparinized tubes, plasma was separated by centrifugation and appropriate aliquots removed for analysis. Urine specimens were immediately frozen upon collection and remained frozen until analyzed.

RESULTS AND DISCUSSION

During the development of MK-212 for potential use as a serotonin-like agent, a method was needed to determine blood and urinary levels of the compound for absorption, excretion and metabolism studies. Preliminary data in rats using radiolabeled material indicated that low levels (ng range) of MK-212 would be encountered following a pharmacological dose. Since electron-capture techniques have been utilized by a number of investigators [4—8] to enhance the sensitivity of detection, this approach was explored for the analysis of MK-212. The use of trifluoroacetic anhydride as the derivatizing agent provided the required sensitivity. The derivatization procedure is similar to that reported previously [4]. A number of related 2-(1-piperazinyl)pyrazines were examined for possible use as an internal standard. The choice of I was based upon its chromatographic behavior (as the trifluoroacetyl derivative) relative to the MK-212 derivative. The trifluoroacetyl derivatives provided good chromatographic properties on the OV-210 column for these compounds. The formation of each trifluoroacetyl derivative was confirmed by combined GLC—MS.

Fig. 1 presents gas chromatograms of the following samples: (a) control dog plasma, (b) control dog plasma to which 500 ng of MK-212 and 500 ng of I were added, and (c) dog plasma (0.5 ml) obtained 30 min after oral dosing (I was added as described in method). Fig. 2 presents gas chromatograms of: (a)

control dog urine to which 500 ng of MK-212 and I was added and carried through the method, and (b) 0—24 hr dog urine to which 500 ng of I was added. All samples were carried through the described procedure. As stated previously, the trifluoroacetyl derivatives of MK-212 and I exhibited retention times of 4.4 and 2.4 min, respectively. No interfering peaks were obtained with control plasma or urine.

A summary of the recovery results obtained following analysis of various added amounts of MK-212 to control plasma and urine from rat, dog or monkey is presented in Table I. All analyses were performed using the aforementioned instruments with electron-capture detectors over 1 year. In the 12.5—2000 ng range, the mean recovery of MK-212 from control plasma was 99.6%; the recovery from urine was 99.6%. The standard deviations within each set of analysis are listed in Table I. As is evident, the GLC method for MK-212 in

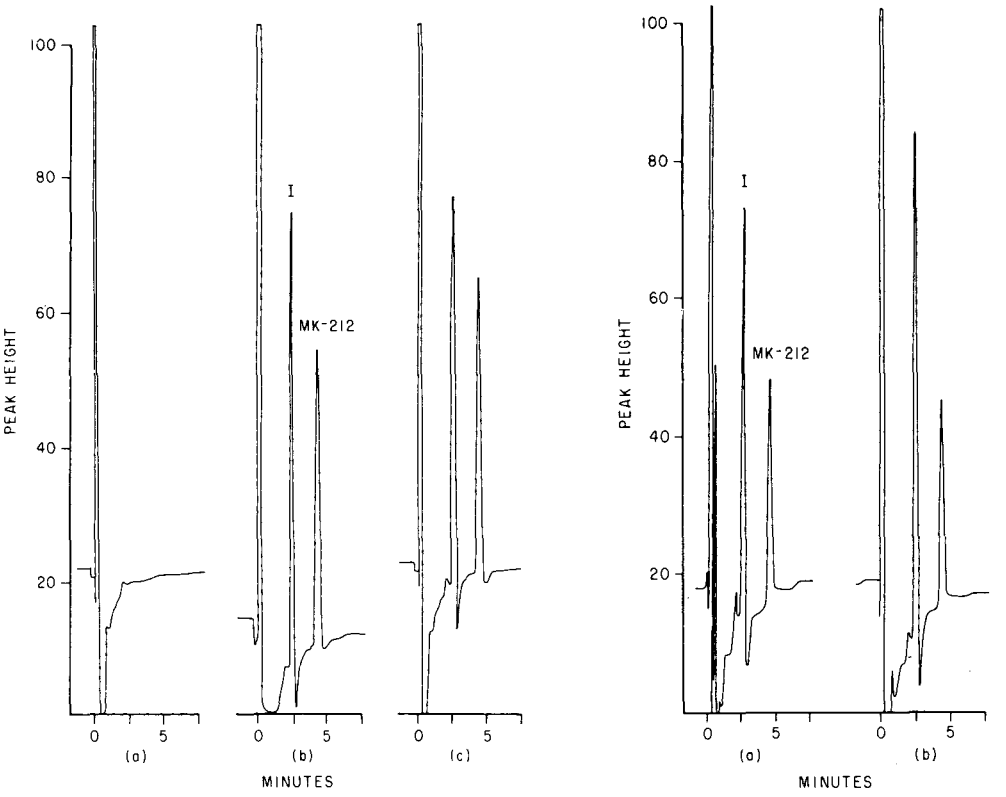


Fig. 1. Gas chromatograms of: (a) control dog plasma carried through extraction procedure, (b) 500 ng each of MK-212 and I added to control dog plasma and carried through procedure, and (c) material obtained from dog plasma following administration of MK-212.

Fig. 2. Gas chromatograms of: (a) control dog urine ti which 500 ng of MK-212 and I was added prior to extraction procedure and (b) material isolated from 0-24 h dog urine following administration of MK-212 (500 ng of I added to urine prior to analysis).

biological fluids is extremely sensitive and the accuracy and precision of the method are well within acceptable limits. Typical levels of MK-212 obtained following the administration of MK-212 to rats and monkeys are presented in Tables II and III, respectively.

TABLE I

RECOVERY OF MK-212 FROM PLASMA AND URINE USING ELECTRON-CAPTURE DETECTION

MK-212 added, (ng)		Amount Recovered* Plasma *n*	Mean ± S.D.	Urine *n*	Mean ± S.D.
2000	A	16	1983 ± 76.8	7	1997 ± 268.1
	B		99 ± 3.8		100 ± 13.4
1000	A	20	982 ± 71.6	15	982 ± 110.4
	B		98 ± 7.2		98 ± 10.6
500	A	24	487 ± 43.6	16	487 ± 65.9
	B		97 ± 8.7		97 ± 13.2
250	A	20	246 ± 14.7	16	240 ± 39.0
	B		99 ± 5.9		98 ± 10.7
100	A	21	98 ± 12.1	13	105 ± 13.3
	B		98 ± 12.1		105 ± 13.3
50	A	19	51 ± 9.2	8	52 ± 7.4
	B		103 ± 18.4		104 ± 14.8
25	A	16	26 ± 5.6	5	27 ± 7.6
	B		104 ± 22.4		107 ± 30.6
12.5	A	12	13 ± 3.1	0	
	B		101 ± 24.8		
12.5—2000	B	148	99.6± 13.6	80	99.6 ± 13.1

* Values in A rows represent nanograms recovered; values in B rows represent percent recovery.

TABLE II

MK-212 PLASMA LEVELS FOLLOWING ADMINISTRATION TO RATS (2.5 mg/kg)

Three rats (A, B, C) were sacrificed at each time period. A, B and C do not represent the same rats for all time periods.

Time (h)	MK-212 (ng/ml) I.V. A	B	C	Average	P.O. A	B	C	Average
0.08	664	591	591	615	*	*	*	—
0.5	306	327	425	353	123	73	160	119
1.0	275	237	344	285	122	93	144	120
2.0	294	231	208	244	45	73	58	59
4.0	368	150	180	233	5	4	*	3

*Less than 5 ng/ml.

Confirmation of specificity of analysis was obtained when representative, unknown, biological specimens from dogs were analyzed by combined GLC—MS. Fig. 3 presents a comparison of the mass spectrum of authentic MK-212 carried through the analytical method with the material isolated from urine and plasma. As observed, the mass spectra obtained from the analysis of the GLC peak

TABLE III

MK-212 PLASMA LEVELS FOLLOWING ADMINISTRATION OF MK-212 TO MONKEYS (2.5 mg/kg)

Time (h)	MK-212 (ng/ml) I.V.				P.O.			
	1	2	3	Average	4	5	6	Average
0.033	1725	1430	1780	1645	—	—	—	—
0.17	1065	1510	1255	1276	*	5	*	2
0.5	1045	1070	995	1036	5	21	*	9
1	710	790	505	668	10	242	6	86
2	500	373	322	398	22	123	21	55
4	328	315	88	243	20	37	36	31
6	196	165	26	129	15	18	20	18
24	8	6	*	5	*	*	*	0

*Less than 5 ng/ml.

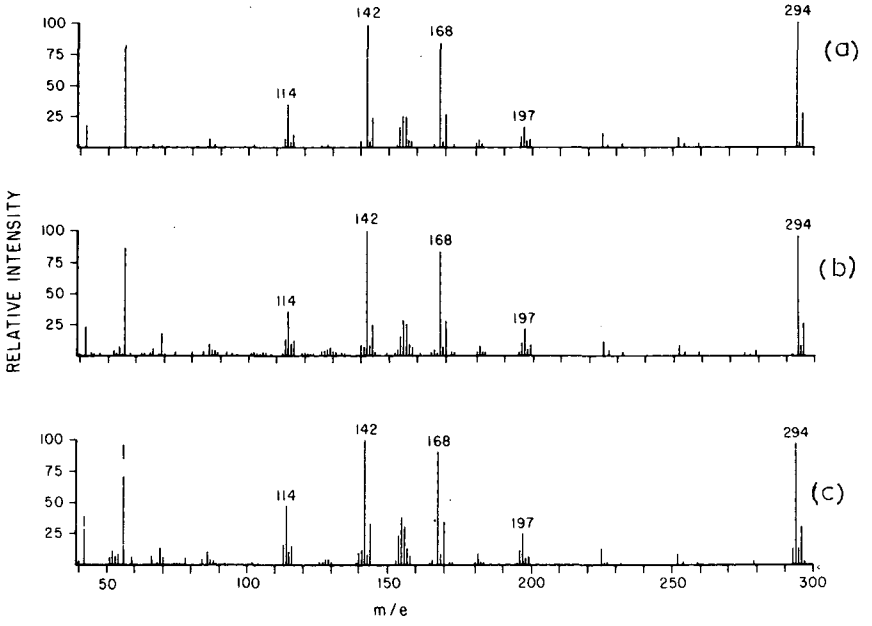


Fig. 3. Mass spectra of: (a) authentic MK-212 following addition (20 μg) to control dog urine, (b) material isolated from the urine of a dog treated with MK-212 and (c) material isolated from the plasma of a dog treated with MK-212.

corresponding to MK-212 (as the trifluoroacetyl derivative) were identical to that obtained when authentic MK-212 was carried through the entire procedure.

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DETERMINATION OF PROPRANOLOL AND SIX METABOLITES IN HUMAN URINE BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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(First received March 23rd, 1978; revised manuscript received July 17th, 1978)

SUMMARY

A method for the determination of propranolol and six of its metabolites, as well as their glucuronide and/or aryl sulfate conjugates in human urine is described. Propranolol and its basic and neutral metabolites are extracted into ether at pH 9.8, evaporated to dryness, reconstituted, separated on a reversed-phase, high-pressure liquid chromatographic system and quantitated using fluorescence detection. The aqueous urine aliquot is then made acidic and the acid metabolites extracted and measured using similar methods. The presence of 2% sodium metabisulfite in all urines collected is essential to ensure the stability of 4-hydroxypropranolol during collection and storage. Preliminary data is presented from 24-h urine samples collected from three patients chronically receiving propranolol.

INTRODUCTION

The widely used β-adrenergic blocking drug, propranolol, has been shown to be more than 95% metabolized by the liver in rat, dog and man [1,2]. Many of the metabolites of propranolol have been identified in human urine by sensitive gas chromatographic—mass spectrometric techniques [3] (Fig. 1). Initial reports suggested that 4-hydroxypropranolol and naphthoxylactic acid were two major propranolol metabolites excreted by man [4]. 4-Hydroxypropranolol was subsequently shown to have β-adrenergic blocking properties equivalent to those of propranolol [5]. This metabolite has been implicated in contributing to the β-adrenergic blockade observed following oral doses of propranolol [6—8] but the quantitative formation and excretion of this metabolite relative to propranolol dose has not been clearly defined. Many of the other metabolites have pharmacological properties of their own which may contribute to some of the actions of propranolol [3].

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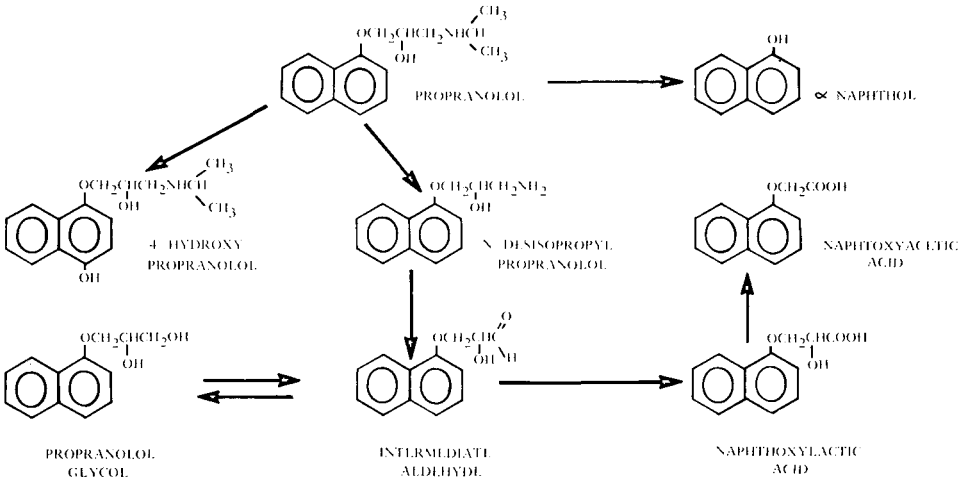

Fig. 1. The proposed metabolism of propranolol to the compounds measured by the HPLC assay procedure. Further metabolism of all the metabolites except α-naphthol has been postulated [3].

Studies in man using radiolabelled propranolol established that greater than 85—95% of the administered radioactivity appeared in the urine of the subjects studied [2]. Thus, the metabolites of propranolol are largely excreted via renal elimination. Therefore the urinary excretion profile of propranolol metabolites should be a reflection of the hepatic metabolism of this drug. Measurement of urinary propranolol metabolites would allow studies to be conducted concerning the effect of dose, routes of administration and disease states on the hepatic metabolism of propranolol. In view of the above considerations, a sensitive assay for determining the urinary concentrations of propranolol and six of its metabolites (Fig. 1) was developed.

EXPERIMENTAL

Standards and reagents

Propranolol HCl was obtained from Ayerst Laboratories, New York, N.Y., U.S.A. Naphthoxylactic acid and 4-hydroxypropranolol HCl were kindly supplied by I.C.I., Macclesfield, Great Britain. α-Naphthol and 2-naphthoxyacetic acid were obtained from Aldrich, Milwaukee, Wisc., U.S.A. α-Naphthoxyacetic acid and procainamide hydrochloride were both supplied by K&K Labs., Plainview, N.Y., U.S.A.

Propranolol glycol and N-desisopropylpropranolol were synthesized by previously described methods [3]. The melting points for propranolol glycol (95.5—96.5°, uncorrected) and N-desisopropylpropranolol (152.5—154.5°, uncorrected) were identical to those previously reported [3].

Reagent grade absolute ether was obtained from Matheson, Coleman & Bell, East Rutherford, N.J., U.S.A. All other reagents, including high-pressure liquid chromatography (HPLC) grade methanol and acetonitrile, were supplied by Fisher Scientific, Pittsburgh, Pa., U.S.A. Aqueous reagents were prepared using

high purity water obtained from a water purification system (Hydro Service and Supply, Durham, N.C., U.S.A.).

An aqueous internal standard solution was prepared containing 50 μg/ml procainamide hydrochloride (internal standard for the assay of propranolol and its basic metabolites) and 50 μg/ml 2-naphthoxyacetic acid (internal standard for the quantitation of the acidic metabolites of propranolol).

All centrifuge tubes and volumetric flasks were silanized and washed as previously described [9].

Instrumentation

Reversed-phase HPLC was performed utilizing a Model M-6000A solvent delivery system, a U6K Universal Injector, and a C_{18} μBondapak column (30 × 4mm I.D.; 10μm average particle size; Waters Assoc., Milford, Mass., U.S.A.). The fluorescent intensity of the column eluent was measured continuously utilizing an Aminco Fluoromonitor equipped with a germicidal lamp source and a primary UV interference filter (295 nm; American Instrument Co., Silver Springs, Md., U.S.A.). The absorbance of the eluent was determined using a variable wavelength UV absorption detector set at 295 nm (Model SF770; Schoeffel, Westwood, N.J., U.S.A.). Both relative fluorescence and absorbance were recorded on a dual channel OmniScribe recorder (Houston Instruments, Austin, Texas, U.S.A.) with an input range of 1—10 mV.

Assay procedure for the measurement of unconjugated propranolol and its metabolites in human urine

Both the acidic and basic metabolites of propranolol were extracted from the same urine aliquot. Urine (1.0 ml) was added to a 15-ml screw-capped centrifuge tube containing 100 μl of 20% sodium metabisulfite, 100 μl of internal standard solution and 1.0 ml of 1 *M* carbonate buffer, pH 10.2. The buffered urine was extracted with 8 ml of anhydrous ether for 10 min using an Eberbach shaker at high speed. The organic and aqueous layers were then separated by centrifugation at 300 *g* for 5 min at 4°. A 2-ml aliquot of the ether layer was transferred to a 10-ml screw-capped centrifuge tube and evaporated to dryness under air at 50°. This constituted the basic extract. The remaining ether in the original extraction tube was carefully removed with a Pasteur pipette and discarded. Sulfuric acid (5 *M*, 0.5 ml) was added to the urine aqueous phase. This acidified urine layer was extracted with 8 ml of anhydrous ether and centrifuged as described above for the basic extract. A 2-ml aliquot of the ether layer was transferred to a 10-ml centrifuge tube and evaporated to dryness yielding the acid extract. Both the acidic and basic extracts were reconstituted in mobile phase (100 μl) prior to injection of a 20-μl aliquot into the high-pressure liquid chromatograph.

For the basic extracts an acetonitrile—methanol—acetic acid—water (35:5:1:59) mobile phase was prepared and degassed for 2 h before use. A mobile phase consisting of acetonitrile—acetic acid—water (36:1:63) was prepared, degassed and employed in the analysis of all acid extracts. Flow-rate for both separations was 2.0 ml/min with the fluorescence and absorbance detector responses being recorded simultaneously on a dual channel strip chart recorder.

Deconjugation of urinary propranolol metabolites

To 1.0 ml of urine contained in a 15-ml screw-capped centrifuge tube was added 0.2 ml of 1 *M* acetate buffer (pH 4.7), 100 μl of 20% sodium metabisulfite, and 30 μl of a deconjugating enzyme solution yielding a final concentration of 0.20 I.U./ml β-glucuronidase and 0.25 I.U./ml aryl sulfatase (*Helix promatia;* Calbiochem, La Jolla, Calif., U.S.A.) The reaction mixture was capped and placed in a water bath at 45° for 4 h. Following the incubation, the acetate buffer was neutralized by the addition of 0.2 ml of 1 *M* NaOH. The deconjugated urine sample was then subjected to the assay procedure described above.

Preparation of standard curves

A stock solution of standards in methanol was prepared containing 53 μg/ml propranolol glycol, 60 μg/ml N-desisopropylpropranolol, 80 μg/ml α-naphthol, 50 μg/ml naphthoxyacetic acid, 520 μg/ml naphthoxylactic acid, and 492 μg/ml propranolol hydrochloride. A separate stock solution containing 471 μg/ml 4-hydroxypropranolol hydrochloride was also prepared. Standard solutions were prepared from each stock solution as follows: standard No. 1, 1:20 dilution of stock; standard No. 2, 1:10 dilution; standard No. 3, 1:4 dilution; standard No 4, 1:2 dilution; standard No. 5, straight stock solution. Standard curves were prepared by addition of 100 μl of each standard solution to the appropriate 15-ml screw-capped centrifuge tube and evaporating the methanol to dryness. One ml of drug-free urine was then added to each tube and the standards extracted and assayed as described above. Equations for the standard curves were calculated from the data by simple linear regression. All standard solutions in methanol were stored at 4° between use. The 4-hydroxypropranolol hydrochloride standards were freshly made up each week. New standard solutions containing propranolol hydrochloride and the rest of the metabolites were prepared monthly.

Stability of 4-hydroxypropranolol

In order to assess the stability of the 4-hydroxypropranolol standard solutions in methanol, four injections (10 μl) of a fresh solution of 4-hydroxypropranolol in methanol (15.2 μg/ml) were made into the chromatograph and the resultant peak heights recorded. This procedure was repeated 1,2,5, and 8 days later using the same solution of 4-hydroxypropranolol. Between injections the 4-hydroxypropranolol solution in methanol was stored at 4°.

Experiments were performed to assess the effect of various anti-oxidants on the stability of 4-hydroxypropranolol in urine. A 50-ml volume of freshly collected urine (drug free) was spiked with 168 μg of 4-hydroxypropranolol hydrochloride and 47 μg of propranolol hydrochloride. Sets of 8, 15-ml screw-capped centrifuge tubes, each containing 1 ml of the spiked urine, were treated by addition of one of the following reducing agents; no reducing agent; 100 μl, 20% sodium metabisulfite; 100 μl, 20% sodium bisulfite; 100 μl, 20% sodium dithionite and 50 μl, 10% sodium ascorbate. Four of the tubes in each group were stored for seven days at room temperature under nitrogen; the remaining four tubes were stored under air. All samples were then extracted, assayed and the peak height ratio of 4-hydroxypropranolol vs. propranolol recorded. These

ratios were then compared with the ratios obtained from freshly extracted spiked urine samples. Propranolol is stable under these conditions.

Stability of propranolol metabolites in frozen urine

Drug-free urine (100 ml) was spiked with the following compounds: 94 μg propranolol HCl; 98 μg N-desisopropylpropranolol; 152 μg 4-hydroxypropranolol HCl; 79 μg propranolol glycol; 78 μg naphthoxyacetic acid; and 228 μg naphthoxylactic acid. Groups of 6 screw-capped centrifuge tubes, each containing 1 ml of spiked urine, were frozen and assayed after 0,1,5, or 8 days of storage. Three of the 6 samples in each group also contained 100 μl of 20% sodium metabisulfite during storage. The peak height ratios of 4-hydroxypropranolol, propranolol glycol and N-desisopropylpropranolol to propranolol were determined at each time period. In addition, the peak height ratio of naphthoxylactic acid vs. naphthoxyacetic acid as assayed from the acid extracts were recorded.

Stability of propranolol and metabolites following extraction

Experiments were conducted in which the stability of 4-hydroxypropranolol and other metabolites were assessed following three post-extraction storage treatments. The first treatment involved dissolving the evaporated ether extract in mobile phase and storing it overnight at room temperature prior to injection into the chromatograph. The second storage procedure consisted of leaving the samples in the evaporated state at room temperature overnight prior to reconstitution in mobile phase and injection onto the chromatograph. A third treatment involved dissolution of evaporated extracts in methanol, storage overnight at 4°, evaporation to dryness under nitrogen and subsequent reconstitution in mobile phase and injection onto the chromatograph. These storage procedures were performed on 5—10 urine samples spiked with standard No. 3. The concentration of propranolol and the metabolites measured following each pretreatment was determined and compared to the values obtained from freshly extracted samples.

Stability of propranolol and its metabolites during deconjugation

Six 1-ml aliquots of urine, spiked with standard No. 3, were subjected to the deconjugation described above. Following 4 h of incubation, the concentrations of propranolol and metabolites were determined and compared to the initial amounts present prior to incubation.

RESULTS AND DISCUSSION

The HPLC separation of basic extracts from urine containing propranolol, 4-hydroxypropranolol, N-desisopropylpropranolol, α-naphthol, propranolol glycol and the internal standard, procainamide, is visualized in the absorbance and fluorescence chromatograms of Fig. 2. A mobile phase consisting of acetonitrile—methanol—acetic acid (35:5:1) was found to provide a good separation of propranolol and its metabolites without interference by other urinary constituents. Although fluorescence detection adds greater sensitivity to the analysis, it can be seen that absorbance responses at 295 nm would also provide

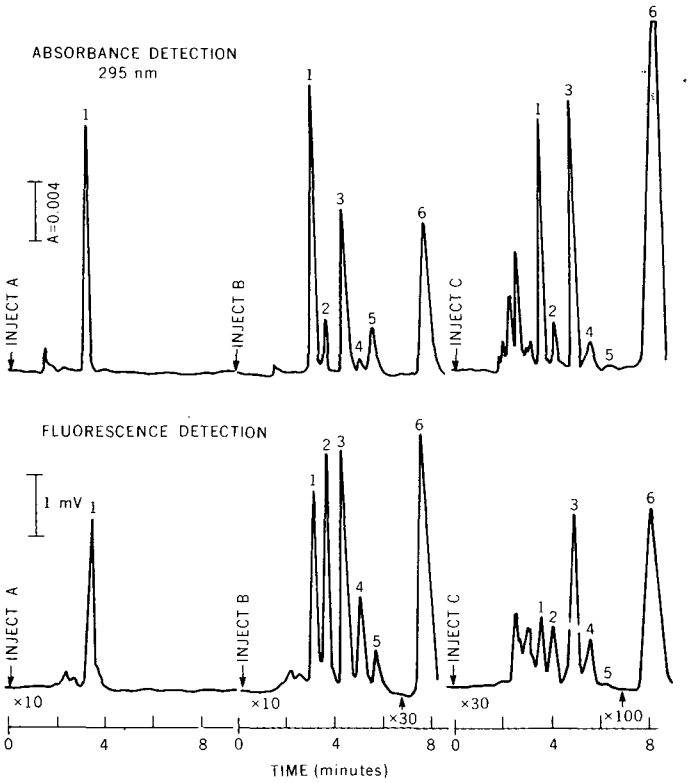

Fig. 2. The absorbance and fluorescence chromatograms of basic extracts from human urine. 1=Procainamide; 2=propranolol glycol; 3=4-hydroxypropranolol; 4=N-desisopropylpropranolol; 5=α-naphthol; 6=propranolol. A = Drug free urine; B = spiked urine, standard No. 3; C = deconjugated urine from a patient treated with propranolol, orally.

an adequate assay for many of these compounds in human urine, particularly propranolol and 4-hydroxypropranolol. A major advantage of recording both fluorescence and absorbance responses simultaneously is that, should unexpected background peaks appear in the fluorescence chromatograms of patient urine samples, the absorbance response often remains unchanged and thus can be used as an alternative method of analysis.

Of many compounds tested, procainamide emerged as the most suitable candidate as an internal standard. It is efficiently extracted under the basic conditions of the assay, has fluorescence and absorbance qualities comparable to propranolol and its metabolites, and is adequately resolved from background urine peaks and those of propranolol and its metabolites under the chromatographic conditions employed. However, urines from patients receiving both propranolol and procainamide cannot be analyzed by this methodology utilizing procainamide as the internal standard. The search continues for non-drug internal standards which may be applied to this analysis.

Fig. 3 depicts the absorbance and fluorescence chromatograms of the acid extracts. In this case fluorescence detection provides a much cleaner as well as a more sensitive method of detection for naphthoxylactic and naphthoxyacetic

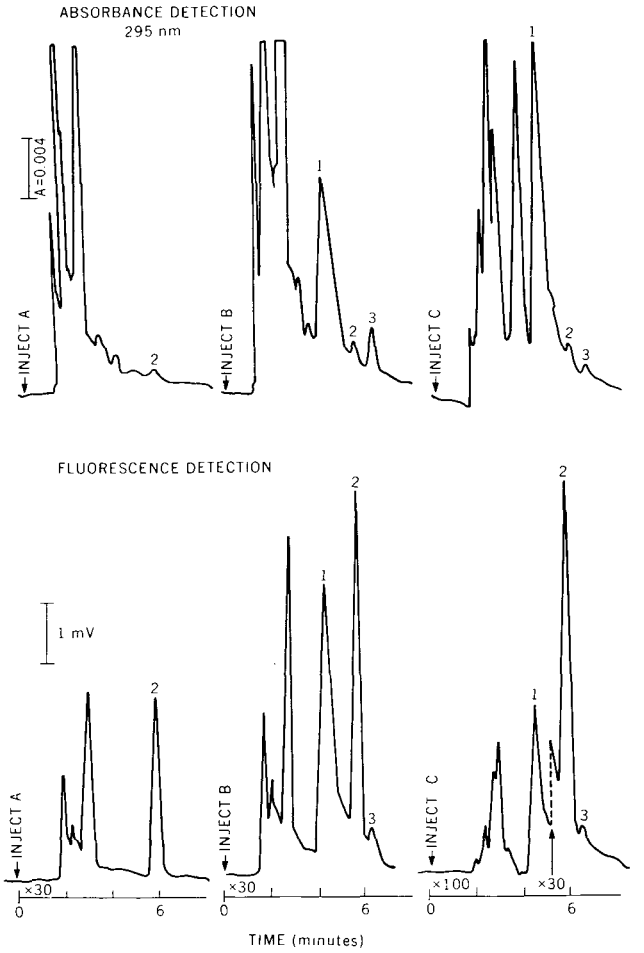

Fig. 3. The absorbance and fluorescence chromatograms of acidic extracts from human urine. 1=1-naphthoxylactic acid, 2=2-naphthoxyacetic acid, 3=1-naphthoxyacetic acid. A = Drug free urine; B = spiked urine, standard No. 3; C = urine from patient treated with propranolol orally.

acids than absorbance monitoring. 2-Naphthoxyacetic acid, a structural isomer of the metabolite, 1-naphthoxyacetic acid, had excellent fluorescent properties and proved to be a suitable internal standard.

Extraction of the urines with ether yielded much cleaner extracts than solvents such as ethyl acetate and benzene which have been employed in previous methods for the analysis of propranolol and/or its metabolites [9—11]. A pH between 9.5 and 10.0 was essential to ensure efficient extraction of 4-hydroxypropranolol [10]. Addition of 100 μl of the antioxidant, 20% sodium metabisulfite, reduced the urine pH by about 0.4 units in the presence of 1 *M* carbonate buffer. Therefore, it was necessary to employ a carbonate buffer whose pH was slightly greater than 10 (10.2) to yield a final extraction pH of between 9.5 and 10. Under acidic conditions, significant amounts of propranolol and the other basic metabolites may be extracted and interfere with the analysis of

the acid metabolites [3]. Therefore, it was necessary to perform the basic extraction step first. Under the conditions of the basic extraction, none of the acidic metabolites were extracted.

Excellent linearity ($r > 0.990$) was observed for the standard curves in all cases with the exception of α-naphthol. The coefficient of variation (C.V.) for α-naphthol was 14.7% (Table I). Since α-naphthol was found to be a minor urinary metabolite of propranolol (Table IV) further efforts to enhance the precision of the α-naphthol measurements were not pursued. The coefficients of variation for propranolol and the other basic metabolites were all less than 5% whereas naphthoxylactic acid and naphthoxyacetic acid yielded C.V.'s of 6.3 and 6.0% respectively (Table I).

TABLE I

INTRA-ASSAY VARIATION OF PROPRANOLOL AND ITS METABOLITES

C.V.'s were determined from the assay of 10 urine samples spiked with the concentrations of each compound indicated.

	Concentration (μg/ml)	C.V.
Propranolol	12.3	2.3
4-Hydroxypropranolol	11.8	5.0
N-Desisopropylpropranolol	1.5	3.6
Propranolol glycol	1.3	1.7
α-Naphthol	2.0	14.7
Naphthoxylactic acid	13.0	6.3
Naphthoxyacetic acid	1.25	6.0

Daily variations in the slope of the standard curves do occur making it necessary to run standards with each set of unknown samples. In a three week period during which five, five-point standard curves were constructed, the slopes ranged for each compound as follows: propranolol glycol, 0.870 to 0.959; 4-hydroxypropranolol hydrochloride, 0.0759 to 0.0992; N-desisopropylpropranolol, 0.265 to 0.322; α-naphthol, 0.090 to 0.145; propranolol hydrochloride, 0.301 to 0.344; naphthoxylactic acid, 0.0305 to 0.0359; and naphthoxyacetic acid, 0.0216 to 0.0274.

Recently Nation et al. [12] have described a HPLC method for the analysis of propranolol and 4-hydroxypropranolol in plasma. A comparison with our analysis for these compounds from urine reveals similar assay conditions. We have selected ether rather than ethyl acetate as the extracting solvent since in our experience less contamination by undesirable urinary compounds has been obtained. The excitation wavelength employed in the urine assay was 295 nm instead of 205 nm used by the above workers in plasma. Krol et al. [13] have demonstrated that the relative fluorescence of propranolol and 4-hydroxypropranolol is greater at excitation wavelengths less than 250 nm as compared to wavelengths greater than 250 nm. However, the sensitivity achieved with the 295 nm excitation wavelength is more than adequate to quantitate free and

conjugated forms of propranolol and its other basic metabolites in human urine.

Previous attempts to measure the 4-hydroxylated metabolite of propranolol in plasma have been hampered by the instability of this molecule. Walle et al. [10] stated that 4-hydroxypropranolol was stable for 7 h in methanol. In the present study, 1 day of storage in methanol at 4° yielded 104±3% of the peak height of 4-hydroxypropranolol when freshly prepared; 2 days storage, 97±1%; 5 days storage, 99±1%; and 8 days storage, 92±1%. Therefore, 4-hydroxypropranolol standard solutions in methanol were stable for up to one week if stored refrigerated between use.

Previous studies stated that 4-hydroxypropranolol was stable in plasma if a 2% solution of the antioxidant, sodium bisulfite, was present [10]. Experiments were conducted to establish the relative stability of 4-hydroxypropranolol in urine in the presence of various antioxidants (Table II). When no reducing agent was employed, virtually all the 4-hydroxypropranolol initially present in the urine samples was lost after 7 days of storage at room temperature. A 2% solution of sodium metabisulfite or sodium bisulfite completely protected 4-hydroxypropranolol from oxidation. Sodium dithionite and sodium ascorbate were less effective antioxidants. A nitrogen atmosphere yielded little added stability. In view of the efficacy of sodium metabisulfite in preserving 4-hydroxypropranolol in urine it was selected as the antioxidant for the assay procedure.

Freezing of fresh urine samples and storage for up to 8 days, either with or without 2% sodium metabisulfite, resulted in no significant changes in the levels of 4-hydroxypropranolol, N-desisopropylpropranolol, and propranolol glycol present relative to propranolol which itself is stable under such conditions. Naphthoxylactic acid levels in the urine were also stable over the 8 day study period when frozen upon collection. These results agree with previous studies [10] which reported that 4-hydroxypropranolol was stable in plasma for up to 1 week if frozen.

The stability of propranolol and its metabolites after extraction was also

TABLE II

STABILITY OF 4-HYDROXYPROPRANOLOL IN THE PRESENCE OF VARIOUS ANTIOXIDANTS

Samples were stored at room temperature for 7 days either directly exposed to air or capped under nitrogen. Results are expressed as the percentage of 4-hydroxypropranolol peak height ratio vs. propranolol relative to that measured in fresh extracts (100±3). Values represent the mean ± S.E. for 4 determinations. Initial 4-hydroxypropranolol concentration was 8.2 μg/ml urine.

	Air	Nitrogen
No reducing agent	3 ± 1	21 ± 17
2% Sodium metabisulfite	97 ± 3	95 ± 6
2% Sodium bisulfite	96 ± 3	96 ± 5
2% Sodium dithionite	65 ± 14	91 ± 3
0.5% Sodium ascorbate	72 ± 8	60 ± 7

assessed. 4-Hydroxypropranolol was unstable if left overnight in mobile phase (45±5% of initial concentration) or in the evaporated state (89±2%). In addition, only 76±3% and 80±3% of the added naphthoxylactic and naphthoxyacetic acid was recovered following storage overnight after evaporation. Prolonged exposure of the acid metabolites to the 5 *M* sulfuric acid (required for efficient extraction) may result in significant hydrolysis of these compounds. Thus, the shaking time should not exceed 15 min and storage in methanol following extraction should not exceed 2 h. Stability of all of the other metabolites with the exception of α-naphthol (45±9% reduction in activity) was preserved if the samples were reconstituted in methanol and stored at 4°. However, best results were obtained if: (1) samples were extracted and injected onto the chromatograph the same day; (2) samples were not left reconstituted in mobile phase any longer than 2 h prior to analysis.

Previous studies have suggested that propranolol and its basic metabolites are excreted in the urine predominantly as their glucuronide and/or sulfate conjugates [3,4,14]. Preliminary studies on hypertensive patients receiving chronic propranolol therapy appear to substantiate these observations (Table IV). Therefore, if estimates of total amounts of propranolol and metabolites (free plus conjugated) are desired it is necessary to deconjugate urine samples prior to analysis. Under the conditions of the deconjugation reaction, maximum release of conjugated metabolites was achieved following 4 h of incubation. All the basic metabolites of propranolol with the exception of α-naphthol were stable during deconjugation in the presence of 2% sodium metabisulfite (Table III). The reducing agent had no effect on the deconjugating enzyme preparation. The acidic metabolites were also stable under these conditions.

Propranolol and its metabolites were determined in 24-h urine collections from 3 patients chronically treated with 40 mg propranolol, 4 times daily (Table IV). All the basic compounds existed predominantly as O-glucuronides

TABLE III

STABILITY OF PROPRANOLOL AND ITS METABOLITES DURING THE DECONJUGATION REACTION

Results are expressed as the percentage of the initial concentration remaining after 4 h of incubation at 37° with β-glucuronidase—aryl sulfatase at pH 4.7. Values represent the mean ± S.E. of 6 determinations. The incubation was carried out in the presence of 2% sodium metabisulfite.

	Initial concentration (μg/ml)	Percent recovery after incubation
Propranolol	12.3	99 ± 2
4-Hydroxypropranolol	11.8	98 ± 2
N-Desisopropylpropranolol	1.5	99 ± 5
Propranolol glycol	1.3	102 ± 3
α-Naphthol	2.0	66 ± 11
Naphthoxylactic acid	13.0	112 ± 2
Naphthoxyacetic acid	1.25	110 ± 4

TABLE IV

24-H URINARY EXCRETION DATA FROM THREE PATIENTS CHRONICALLY TREATED WITH PROPRANOLOL

Each patient was receiving 40 mg propranolol hydrochloride, 4 times daily (542 μmoles). Sodium metabisulfite (20 g) was added to each urine bottle prior to collection. 1,2,3 = Patient numbers.

	Total μmoles excreted			Percent of dose			Percent conjugated		
	1	2	3	1	2	3	1	2	3
Propranolol	87.5	58.4	44.4	16.1	10.8	8.2	91	98	82
4-Hydroxypropranolol	13.6	25.4	27.7	2.5	4.7	5.1	93	87	88
Naphthoxylactic acid	90.1	152.6	106.9	16.6	28.2	19.7	0	0	0
N-Desisopropylpropranolol	2.8	0.9	7.9	0.5	0.2	1.5	92	76	100
Propranolol glycol	6.9	7.9	6.5	1.3	1.5	1.2	100	100	100
α-Naphthol	6.1	13.5	2.7	1.1	2.5	0.5	100	100	100
Naphthoxyacetic acid	nil	nil	nil	nil	nil	nil	–	–	–
Total	207.1	258.7	196.1	38.1	47.9	36.2			

or sulfated conjugates which could be liberated by the β-glucuronidase—aryl sulfatase enzyme preparation. Attempts were made to free any N-glucuronide conjugates present through acid hydrolysis [15] of patients urines in 0.2 *M* HCl for 15 min at 25°. No significant levels of propranolol and its metabolites were recorded above the concentrations of free propranolol and metabolites already present in the urines suggesting that N-glucuronidation does not play a significant role in the metabolic disposition of propranolol or the metabolites studied.

The major recoverable urinary excretion products were 4-hydroxypropranolol, naphthoxylactic acid and propranolol. These compounds have previously been identified as major urinary excretion products of propranolol in man [2—4] although the quantitative contribution of each compound to the metabolic disposition of propranolol is unknown. Early studies using ^{14}C-labelled propranolol [2] indicated that 85—95% of a single 40 mg dose in man was eventually excreted in the urine. Of interest was the finding in the present study that 60% of the administered dose remained unaccounted for in the patients studied suggesting that other unmeasured metabolites are present in these urines. Further studies are in progress to define the quantitative profile of propranolol metabolism in man.

ADDENDUM

Further experience with the method described has shown that: (1) different columns might have different chromatographic properties. Minor adjustment in the composition of the mobile phase can correct for this discrepancy and allow for appropriate resolution of the compounds measured; (2) 1-naphthaleneethanol (Aldrich) has been found to be a suitable non-drug internal standard.

A mobile phase acetonitrile—methanol—acetic acid—water (35:20:0.075:45), pH 3.4, has been used to obtain an adequate separation of propranolol and its metabolites using this internal standard; (3) absorbance detection at 310 nm has been found to provide sensitive measurement of 4-hydroxypropranolol free of any background which may be present in deconjugated urine.

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Journal of Chromatography, 162 (1979) 59—70
Biomedical Applications

CHROMBIO. 220

DETERMINATION OF QUINIDINE AND ITS MAJOR METABOLITES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(Received March 20th, 1978)

SUMMARY

A specific and precise assay, capable of quantitating in human plasma simultaneously but separately quinidine, dihydroquinidine and the quinidine metabolites 2'-quinidinone, 3-OH-quinidine and a third metabolite found — tentatively identified as the product formed by rearrangement of quinidine-N-oxide — is reported. The assay uses a normal phase high-performance liquid chromatographic (HPLC) system with a variable-wavelength UV detector at 235 nm and has a limit of sensitivity at approximately 20 ng/ml. The mobile phase consists of hexanes—ethanol—ethanolamine (91.5:8.47:0.03). A 2-ml plasma sample is worked up by adding primaquine base as an internal standard and extracting with ether—dichloromethane—isopropanol (6:4:1). The organic extract is evaporated and the residue reconstituted in 100—600 μl of mobile phase and an aliquot injected onto the column.

Comparison of this procedure with the Edgar and Sokolow (dichloroethane) extraction—fluorescence procedure and with the Cramer and Isaksson (benzene) double extraction—fluorescence assay indicates that both fluorescence procedures give quinidine concentrations up to 2.3 times those determined by HPLC. These discrepancies were shown to be due to carry-over of metabolites and some extraneous background fluorescence.

INTRODUCTION

Although alternative therapy is becoming available, quinidine is still among the major compounds used in the treatment of life threatening arrhythmias. Since its introduction as a cardiac depressant by Frey [1] in 1918, much effort has been expended in developing procedures to monitor its therapeutic activity in an attempt to minimize untoward reactions. Quinidine is known to have a narrow therapeutic index [2]. The definition of this range depends,

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to some extent, on the assay used to measure the drug in the blood [3, 4]. The most extensive study of clinical response vs. plasma levels is the investigation of Sokolow and co-workers [2, 5], who recommended dose adjustment until the plasma quinidine levels were greater than 2 μg/ml and less than 6 μg/ml of apparent quinidine as measured by their assay [5]. The assay involves extracting the alkalinized plasma with dichloroethane followed by measuring the fluorescence after acidification and addition of ethanol. In addition to quinidine, this method extracts dihydroquinidine and various amounts of metabolites [6].

The measurement of plasma quinidine concentration after the administration of quinidine to humans is obviously dependent on the specificity of the assay. But it is further complicated by an impurity in the commercially available quinidine. As a consequence of its method of manufacture by the isomerization of quinine, dihydroquinine is converted to dihydroquinidine. The resultant material of commerce is usually contaminated with from 5—30% of dihydroquinidine [3, 7—9]. The amount of dihydroquinidine in the commercial samples used by Sokolow and many other workers is unknown.

Dihydroquinidine has been reported to have antiarrhythmic activity similar to quinidine [3, 10], but the two compounds have not been compared as to activity in man. Both quinidine and dihydroquinidine are extensively metabolized in man via oxidative pathways. These lead to a series of hydroxylated compounds which accumulate to varying degrees in blood. The quinidine metabolites include 2'-quinidinone [11], 3-OH-quinidine [12] and O-desmethylquinidine [13]. During this study we have isolated another metabolite, tentatively identified as the rearrangement product formed from an intermediate N-oxide. Although incompletely investigated, the metabolites possess some degree of cardiovascular activity in animal studies [13, 14]. Since these metabolites might also possess some activity in man, it is far more logical to quantitate them, rather than attempt to remove them as is done in almost all presently available assays. This would be particularly important in cases of renal insufficiency where the metabolites will accrue in the plasma.

At least twenty different assay procedures have been reported in the literature. These include a titrimetric method with bromine [15], a nephelometric method using a precipitant [16], colorimetric methods using ion-pair extraction with a colored anion [17—19] and many different types of fluorimetric assays [6, 20, 21]. The method of Brodie and Udenfriend [20] is still very commonly used in clinical laboratories. It involves precipitation of the plasma proteins in a diluted plasma sample using metaphosphoric acid and measuring the fluorescence of the resultant supernatant. Several modifications have been reported using different precipitating agents [9, 22—24] and extraction solvents. Kelsey and Geiling [25] used ether. Edgar and Sokolow [5, 21] used dichloroethane for the extraction, then added trichloroacetic acid and ethanol prior to determining the fluorescence of the fluids. This method has been referred to in the literature as the single extraction method. More recently Cramer and Isaksson [6] used benzene as the extraction solvent and re-extracted the basic compounds into sulfuric acid prior to fluorescence determination (double extraction method). They claimed this solvent markedly reduced the amounts of metabolites extracted. Kessler et al. [4] added 1% amyl alcohol

whereas Greenblatt et al. [26] used 1% amyl alcohol in toluene as their extraction medium. Armand and Badinand [27] published a modification involving an additional clean up step using an intermediate alkali wash. It will be shown below that even though these methods claim improved specificity, they apparently include fluorescent contributions from metabolites as well as background fluorescence from unknown constituents of the plasma. They therefore lead to spurious estimates of the quinidine concentration.

Härtel and Korhonen [28] and Ueda et al. [29] used a thin-layer procedure to separate quinidine from other fluorescent constituents. They then extracted the quinidine band and assayed the resultant fluorescence. Depending on the solvent system used [30] such a thin-layer chromatographic (TLC) method can be made very specific and allows the simultaneous determination of metabolites and parent drug, but it is tedious, time-consuming and difficult to accomplish when large numbers of samples have to be processed within a short period of time. Direct fluorimetric determination on the TLC plate has also been described [31—33]. These methods however, require a densitometer or TLC scanning devices on the fluorimeter.

Midha and Charette [34] reported on a gas—liquid chromatographic (GLC) assay that resulted in rather poor resolution of the compounds. Although these authors indicate retention times for metabolites in the system used, they do not include these substances in their assay. Other GLC assays were reported by Valentine et al. [35] and Moulin and Kinsun [36]. A comparison of gas chromatographic and two different fluorescence methods was made by Huffman and Hignite [37]. Garland et al. [38] used a GLC method coupled to a mass spectrometer. This requires, however, a stable-isotope-tagged compound to establish quantitation. The most recently published methods utilize high-performance liquid chromatography (HPLC). Conrad et al. [39] used a 100-cm reversed-phase phenyl column, solvent programming, a fluorescence detector and a heated column. Powers and Sadee [40] recommend a specific assay using an alkylphenyl column and direct sample injection after protein precipitation. Similarly to Drayer et al. [41], Crouthamel et al. [42] utilized a reversed-phase HPLC method with separation conditions of pH 2.6. Similar to quinidine and dihydroquinidine, all of the reported metabolites possess two nitrogens which will be protonated at this pH. These polar compounds may not separate from the parent compound under the conditions of the assay. They report that their assay is in good agreement with the Cramer and Isaksson method [6]. The present investigation will show that this so-called double extraction method still includes quinidine metabolites and leads to spurious quinidine levels.

Two facts speak against the use of present methods of assay of quinidine. Most of them lack specificity due to partial co-extraction of metabolites. Secondly, since the metabolites may be contributing to the therapeutic response [13, 14], it would be advantageous to attempt to quantitate not only the parent compound, but its metabolites as well.

This paper includes a new HPLC assay which measures quinidine, dihydroquinidine and their major metabolites in plasma samples after extraction. The assay will be compared with several of the commonly used fluorescence assays.

EXPERIMENTAL

Reagents and chemicals

The solvents used were hexanes (HPLC grade; Fisher, Pittsburgh, Pa., U.S.A. or UV grade; Burdick & Jackson Labs., Muskegon, Mich., U.S.A.), ethanol 200 proof (Commercial Solvents, San Jose, Calif., U.S.A.) (stored over Na_2SO_4 and filtered before use), ethanolamine (Aldrich, Milwaukee, Wisc., U.S.A.), stored over molecular sieve 3Å, benzene (nanograde) and ethyl ether (anhydrous and peroxide-free; Mallinckrodt, St. Louis, Mo., U.S.A.); all other solvents were analytical grade (Mallinckrodt).

The quinidine primary standard used was prepared from commercially available quinidine (J.T. Baker, Phillipsburg, N.J., U.S.A.) by removing dihydroquinidine according to the method of Thron and Dirscherl [43]. The dihydroquinidine-free quinidine was crystallized from anhydrous ethanol, dried in the high vacuum for 1 h. The elemental analysis showed that the crystals contained 1 mole of ethanol. A quinidine standard solution was prepared containing 1 μg of pure quinidine base per ml of methanol and kept under refrigeration with no detectable decomposition. The internal standard used, primaquine, was obtained as primaquine diphosphate (Aldrich). The base was liberated from its salt with 2 *N* NaOH and extracted with dichloromethane. The organic extracts were washed twice with water, dried over anhydrous Na_2SO_4, evaporated under reduced pressure and dried in the high vacuum. A standard solution with 10 μg primaquine base per ml methanol was prepared and kept at 4°. Dihydroquinidine was obtained as hydrochloride (ACF Chemiefarma, Maarssen, The Netherlands) and the free base prepared in the same way as primaquine (see above). The quinidine metabolites 2′-quinidinone and 3-OH-quinidine were kindly supplied by Dr. Irwin Carroll, Triangle Research Institute*.

Instruments and chromatographic conditions

A Varian Model 8500 high-performance liquid chromatograph was used, equipped with a Varian UV detector with variable wavelength, set at λ = 235 nm, and a Varian MicroPak-Si 10 LSC column, 25 × 0.21 cm I.D. (¼ in. O.D.).

The solvent mixture used, hexanes—ethanol—ethanolamine (91.5:8.47:0.03), at a flow-rate of 60—70 ml/h gave good results and yielded a back-pressure of approx. 750 p.s.i.

Injections were made with Hamilton syringes (10—20 μl) through various high-performance injector devices (Varian Model 8500 stop-flow injector, Valco CV-6-UHPa sweep-flow injector).

Areas under the chromatogram peaks were measured by a Hewlett-Packard Model 3380 A integrator.

PROCEDURES

Sample preparation and interpretation of chromatograms

A 1.0-ml volume of methanolic primaquine standard solution containing

*Prepared under Contract PH-43 NIGMS-65 1057.

10 μg/ml (internal standard) is measured into a test tube (18 × 150 mm, PTFE-lined screw cap) and evaporated to dryness at 35° under a stream of nitrogen. A 2.0-ml quantity of the plasma to be assayed is added and vortexed for a few seconds. The plasma is subsequently extracted with 10 ml of a mixture of ether—dichloromethane—isopropanol (6:4:1) by vortexing for 30 sec. After centrifugation of the sample for 5 min at 540—1200 *g* the aqueous layer is frozen in a dry-ice—acetone mixture and the organic phase decanted into another test tube. The organic layer is once more centrifuged and separated from an aqueous residue after freezing and then evaporated to dryness at 35° under a stream of nitrogen. The residue is reconstituted in 100—600 μl of elution medium and an appropriate aliquot injected onto the column.

Standard curves were prepared daily by spiking 2.0 ml of blank, drug-free plasma with 10 μg primaquine and 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 μg, respectively of quinidine (expected range of plasma concentrations in our studies), extracting them as described above.

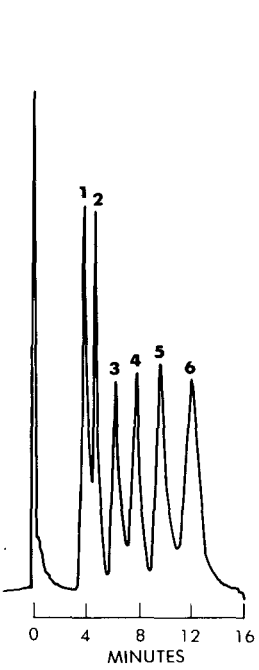
RESULTS AND DISCUSSION

Quinidine has two basic functions with pk_a of 8.4 and 4.0. Even at the pH of plasma (7.4), quinidine is reproducibly extracted with our solvent system (see below). Two authentic samples of quinidine metabolites were made available to us, namely 2′-quinidinone and 3-OH-quinidine. A third metabolite was isolated from urine specimens during our investigations and tentatively identified as a product formed by rearrangement of an intermediate N-oxide [44]. This newly isolated metabolite will be referred to below as quinidine—oxide-R. Although O-desmethyl-quinidine has been reported [13] and would be expected to be extracted in our solvent system, we did not detect any other quinidine metabolite in our single dose bioavailability studies, either by HPLC or by TLC.

Using the chromatographic conditions described under Experimental, quinidine, dihydroquinidine, internal standard and three quinidine metabolites could be separated as shown in Fig. 1, where a test mixture has been injected. The complex mixture can be separated within 20 min from plasma samples (Fig. 2).

Of the solvent systems tested, the one described above gave best results in respect of relative retention times of the different compounds. According to the status of the column, slight adjustments in the ethanol content might be necessary to yield the separation shown in Figs. 1 and 2. In addition, the ethanolamine concentration can be varied to get different retention times; however, long equilibration times (1—2 h) are needed to achieve stable conditions. Ethanolamine is added because it apparently suppresses ionization, reduces the retention times and eliminates tailing. It yields symmetrical peaks with maximal theoretical plates. Ethanolamine is apparently superior to ammonia because the former provides a more stable solvent mixture, a prerequisite for automation of sample injection, such as was used in our routine analyses.

The HPLC method described in this paper has been used to quantitate



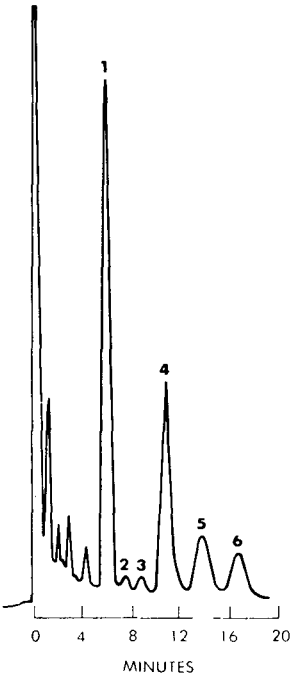


Fig. 1. HPLC separation of quinidine and metabolites in a test mixture. Column: Varian MicroPak-Si 10, 25 × 0.21 cm I.D.; mobile phase: hexanes—ethanol—ethanolamine (91.5:8.47:0.03); flow-rate: 1 ml/min; UV detection at 235 nm; peaks: 1 = quinidine, 2 = dihydroquinidine, 3 = 2′-quinidinone, 4 = primaquine, 5 = quinidine—oxide-R, 6 = 3-OH-quinidine.

Fig. 2. HPLC separation of quinidine and metabolites in patient plasma. Column: Varian MicroPak-Si 10, 25 × 0.21 I.D.; mobile phase: hexanes—ethanol—ethanolamine (92.97:7.0:0.03); flow-rate: 1 ml/min; UV detection at 235 nm; peaks: 1 = quinidine, 2 = dihydroquinidine, 3 = 2′-quinidinone, 4 = primaquine, 5 = quinidine—oxide-R, 6 = 3-OH-quinidine.

quinidine and metabolites in more than 5000 samples of blood or saliva. The limit of its sensitivity is at 20 ng/ml plasma. For plasma levels expected in the range of 20—100 ng/ml use of less internal standard is recommended.

Quantitation of quinidine was achieved on the basis of a standard curve, where known concentrations of drug have been plotted against the peak height ratio quinidine—primaquine. From these values a least square unweighted regression line was calculated.

To accommodate small variations in the chromatographic system which may occur from one day to another, standard curves were prepared daily. Due to insufficient amounts, no standard curve for 3-OH-quinidine, or quinidine—oxide-R could be prepared. Similarity of their extinction coefficients with that of quinidine was verified

Recovery and reproducibility of the HPLC method

In order to check the recovery and reproducibility of quinidine in the

extraction procedure with the solvent mixture ether—dichloromethane—isopropanol (6:4:1) plasma samples spiked with 0.1, 0.25, 0.5, 1.0, 1.5 and 2.0 μg/ml, respectively have been extracted according to the extraction procedure described above. The peak heights measured after extraction were compared with peak heights obtained after injecting exact volumes of a quinidine solution of known concentration onto the HPLC column by means of an injector loop. The result shows that quinidine is extracted over the range of 0.1—2.0 μg/ml by 91.5—93.6% with coefficients of variation (C.V.) between 0.9—3.8% and that 91.5% of primaquine internal standard is extracted at a concentration of 5.0 μg/ml (C.V. = 5%). The metabolites were shown to be extracted reproducibly with a C.V. of approx. 5%.

The slopes and intercepts of eight standard curves obtained over a period of about 3 months were compared in an analysis of variance [45] in order to assess their variation with time. As can be seen from Table I, the slopes but not the intercepts of the standard curves are found to vary with time ($p \leqslant 0.05$). Such changes are not unusual considering gradual changes in column performance.

TABLE I

ANALYSIS OF VARIANCE ON SLOPES AND INTERCEPTS DERIVED FROM THE STANDARD CURVES FOR QUINIDINE IN PLASMA

F Statistic (slope) (7, 32) = 3.370 S ($P \leqslant 0.05$); F statistic (intercept) (7, 32) = 2.026 NS ($P > 0.05$).

Curve No.	r^2	Slope	S.E. of slope	S.E. of slope (%)	Intercept	S.E. of intercept	S.E. of intercept (%)
1	0.9991	0.8379	0.0124	1.48	0.0312	0.0139	44.63
2	0.9991	0.8514	0.0131	1.54	—0.0025	0.0147	578.35
3	0.9977	0.8713	0.0209	2.40	0.0198	0.0235	118.69
4	0.9995	0.8982	0.0100	1.11	—0.0026	0.0112	432.05
5	0.9962	0.9591	0.0298	3.10	—0.0236	0.0334	141.85
6	0.9918	0.9350	0.0426	4.55	—0.0270	0.0478	177.04
7	0.9986	0.8739	0.0161	1.84	0.0141	0.0181	128.37
8	0.9987	0.9095	0.0162	1.78	—0.0110	0.0182	165.91

Comparison with other quinidine determination methods

Quinidine levels in patient blood samples, obtained by HPLC, were compared to results obtained by the single extraction method according to Edgar and Sokolow (Table II) and to the so-called double extraction method of Cramer and Isaksson (Table III)*.

Both of these methods yield higher results when compared to the results of the specific HPLC assay. The two sets of plasma samples were obtained from single oral doses of 400 mg quinidine sulfate administered to healthy adults. The only detectable metabolite at these concentrations was the quinidine—oxide-R. The values listed in column 4 of Tables II and III are the sums of the metabolite plus the quinidine concentration as measured by HPLC.

*The Edgar and Sokolow assay [5] will be referred to as the ES assay and the Cramer and Isaksson assay [6] as the CI assay.

TABLE II

COMPARISON OF QUINIDINE LEVELS MEASURED BY HPLC METHOD AND BY ES FLUORESCENCE ASSAY

Time after administration of quinidine (min)	Quinidine by HPLC (μg/ml)	Quinidine—oxide-R* by HPLC (μg/ml)	Quinidine + quinidine—oxide-R by HPLC (μg/ml)	Quinidine by ES (μg/ml)
0	0	0	0	0
5	0	0	0	0
10	0.07	0.04	0.11	0.15
15	0.19	0.07	0.26	0.39
25	0.54	0.14	0.68	0.98
30	0.67	0.15	0.82	1.12
40	0.84	0.14	0.98	2.36
46	0.91	0.14	1.05	1.46
51	0.92	0.20	1.12	1.88
75	0.69	0.14	0.83	1.32
91	0.69	0.17	0.86	1.02
105	0.66	0.17	0.83	0.88
122	0.53	0.14	0.67	0.74
181	0.46	0.14	0.60	0.82
242	0.41	0.08	0.49	0.63
307	0.41	0.09	0.50	0.50
360	0.32	0.06	0.38	0.51
421	0.26	0.07	0.33	0.43
480	0.24	0.07	0.31	0.37

*Quinidine—oxide-R refers to a newly isolated quinidine metabolite formed by rearrangement of an intermediate N-oxide.

Column 5 includes the results obtained directly using the ES or CI fluorescence assay. The quinidine values obtained by the ES assay (Table II, column 5) exceed the HPLC quinidine values (column 2) often by more than 100%. They usually exceed the combined quinidine plus metabolite concentrations, possibly indicating a changing blank value. On the other hand, the CI assay values (Table III, column 5) are reasonably explained in this set of samples by the sum of quinidine plus metabolite concentration (column 4). Both fluorescence assays therefore overestimate the intact quinidine.

In order to evaluate the degree of metabolite carryover, several sets of plasma samples were assayed by HPLC and fluorimetrically according to the CI method. The CI extracts were re-extracted with ether—dichloromethane—isopropanol (6:4:1) after alkalinizing to pH 9 with 3% NH_4OH. The content of quinidine and its metabolites in the CI extracts were then determined according to the HPLC method as described in the experimental section. Due to the instability of the internal standard in this procedure, amounts of quinidine, dihydroquinidine and metabolites in the samples were quantitated by comparison of their relative area under the chromatogram peaks to the areas obtained from injection of absolute amounts of quinidine.

Tables IV and V include data from one subject at steady state. Tables IV and VII include data from repeated assay of pooled patient plasma samples.

TABLE III

COMPARISON OF QUINIDINE LEVELS MEASURED BY HPLC METHOD AND BY CI FLUORESCENCE ASSAY

Time after administration of quinidine (min)	Quinidine by HPLC (μg/ml)	Quinidine—oxide-R by HPLC (μg/ml)	Quinidine + quinidine—oxide-R by HPLC (μg/ml)	Quinidine by CI (μg/ml)
0	0			0
6	0			0
15	0.37	0.11	0.48	0.47
19	0.65	0.12	0.77	0.76
28	0.95	0.18	1.13	1.24
34	1.14	0.21	1.35	1.48
43	1.28	0.24	1.52	1.54
48	1.27	0.29	1.56	1.74
54	1.24	0.22	1.46	1.57
61	1.21	0.21	1.42	1.50
76	1.28	0.31	1.59	1.49
92	1.20	0.26	1.46	1.49
106	1.16	0.22	1.38	1.31
124	1.09	0.24	1.33	1.01
183	0.87	0.19	1.06	1.06
242	0.84	0.21	1.05	0.88
306	0.73	0.08	0.81	0.78
363	0.60	0.12	0.72	0.82
426	0.46	0.10	0.56	0.55
487	0.47	0.09	0.56	0.47
860	0.24			
1390	0.10			

The direct HPLC determination indicated that the two sets of samples differed in the amounts and ratios of the constituents. The CI fluorescence assay in each instance overestimates the quinidine concentration. In Table IV the values range from 194 to 228% of the quinidine content measured by direct

TABLE IV

COMPARISON OF QUINIDINE LEVELS IN A PATIENT AT STEADY STATE, MEASURED BY HPLC METHOD AND BY CI METHOD

Plasma sample (time after last dose, min)	Plasma levels by HPLC method (μg/ml)			Plasma levels by CI method (μg/ml)
	Quinidine	Quinidine—oxide-R	3-OH-quinidine	
29	0.60	0.11	0.35	1.37
46	0.66	0.11	0.33	1.46
60	0.69	0.11	0.39	1.56
120	0.80	0.13	0.39	1.57
184	0.69	0.10	0.41	1.34

TABLE V

IDENTIFICATION AND QUANTITATION OF COMPOUNDS EXTRACTED BY CI ASSAY

Plasma sample (time after last dose, min)	Plasma levels by CI method (μg/ml)	Quantitation of quinidine and metabolites in CI samples by HPLC (μg/ml)		
		Quinidine	Quinidine—oxide-R	3-OH-quinidine
29	1.37	0.63		0.21
46	1.46	0.60		0.17
60	1.56	0.69	*	0.20
120	1.57	0.99		0.24
184	1.34	0.62		0.16

*Insufficient amounts for quantitation.

TABLE VI

COMPARISON OF QUINIDINE LEVELS IN POOLED PATIENT PLASMA, MEASURED BY HPLC METHOD AND BY CI METHOD

Sample	Plasma levels by HPLC method (μg/ml)					Plasma levels by CI method (μg/ml)
	Quinidine	Dihydro-quinidine	2'-Quini-dinone	Quinidine—oxide-R	3-OH-quinidine	
1	1.51	0.08	0.07	0.30	0.34	2.13
2	1.45	0.08	0.08	0.35	0.28	2.17
3	1.57	0.08	0.09	0.38	0.28	2.17

TABLE VII

IDENTIFICATION AND QUANTITATION OF COMPOUNDS EXTRACTED BY CI ASSAY

Sample	Plasma levels by CI method (μg/ml)	Quantitation of quinidine and analogues in CI samples by HPLC (μg/ml)				
		Quinidine	Dihydro-quinidine	2'-Quini-dinone	Quinidine—oxide-R	3-OH-quinidine
1	2.13	1.39	0.11		0.03	0.18
2	2.17	1.51	0.11	*	0.03	0.19
3	2.17	1.44	0.09		0.04	0.17

*Insufficient amounts for quantitation.

HPLC. In Table VI the corresponding values average at 143%. Apparently, virtually all of the quinidine is extracted by the benzene extraction as shown by the subsequent HPLC determination (see quinidine concentration in Tables IV + V and VI + VII, respectively). This is probably also true for dihydroquinidine. In addition, the studies indicate that the metabolites also are partly

extracted by benzene, 3-OH-quinidine to 50—60% and quinidine-oxide-R to 10%. The extraction efficiency of benzene for 3-OH-quinidine of 50—60% correlates well with the observation of Drayer et al. [41]. Insufficient concentrations of 2'-quinidinone were present to permit estimation of the benzene extraction efficiency for this metabolite. Since the concentrations of the metabolites vary from patient to patient, the error introduced by use of the CI assay is unpredictable and no correction factor can be applied. This is particularly important in patients with renal insufficiency.

Although limited studies were done, the method of Armand and Badinand [27] as applied by Huynh-Ngoc and Sirois [3] was investigated in the same fashion as with the CI method. It was noted that the repeated extraction of the samples with the 0.1 *N* NaOH led to large discrepancies in the fluorescence assay as shown in Table VIII.

TABLE VIII

COMPARISON OF QUINIDINE LEVELS IN A PATIENT AT STEADY STATE, MEASURED BY HPLC METHOD AND BY ARMAND—BADINAND METHOD

NA = Not available.

Specification of plasma sample (time after last dose, min)	Plasma levels by HPLC method (μg/ml)			Plasma levels by Armand—Badinand method (μg/ml)
	Quinidine	Quinidine—oxide-R	3-OH-quinidine	
301	0.72	NA	NA	1.16
471	0.51	0.05	0.34	0.74
0	0.56	0.07	0.36	0.88
38	0.74	0.13	0.44	1.19

From these experiments it can be concluded that in spite of its superiority over protein precipitation methods and single extraction methods the double extraction CI method still lacks specificity for quinidine even when an additional clean-up step with alkaline washings is introduced. It is obviously impossible to predict a priori quinidine and metabolite ratios in humans. Therefore, no correction factor can be devised to estimate true quinidine levels from data obtained by unspecific methods as has been suggested for data obtained from precipitation methods [37]. In order to attempt to assess the specificity, several new assays [35, 37, 42, 46] have been compared to the CI method or its modification according to Armand et al. If a good correlation between the two assays in question was found, the authors assumed them to be specific and reliable. The results of the present study, however, show that their standard of comparison is nonspecific and unreliable. Therefore these new assays must be re-assessed as to their claims of specificity.

ACKNOWLEDGEMENTS

This work was supported by funds from Food and Drug Administration Contract No. 223-74-3145 and by a program projects grant from the National

Institute of General Medical Sciences (GM-16496-08/09). T.W.G. acknowledges support from Swiss National Science Foundation.

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Note

Thin-layer chromatographic separation of keto derivatives of free bile acids

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(Received May 26th, 1978)

Chavez and Krone [1] reported that the 3-keto derivatives of cholic and lithocholic acids can be separated by thin-layer chromatography (TLC) on pre-coated silica-gel plates using Petcoff's developing solution (hexane—methyl-ethylketone—glacial acetic acid, 56:36:8, v/v). Reported here is the verification of this observation and in addition the separation of the 3-keto derivatives of deoxycholic and chenodeoxycholic acids.

METHODS

Experimental conditions were identical to those previously reported [1]. The 3-keto derivatives of cholic, chenodeoxycholic, deoxycholic and lithocholic acids (Supelco, Bellefonte, Pa., U.S.A.) were prepared by exposure of these bile acids separately to the 3-α-hydroxy steroid dehydrogenase (STDHP; Worthington Biochemicals, Freebold, N.J., U.S.A.) enzymatic mixture under the conditions described by Iwata and Yamasaki [2] and MacDonald [3]. The bile acids were allowed to react for 15 min and then acidified with 5 *N* hydrochloric acid. Then the bile acids and their derivatives were extracted with diethyl ether, the ether phase washed three times with distilled water, exposed to anhydrous sodium sulfate and then filtered. The ether phase was evaporated under a gentle stream of air in a warm water bath. The remaining material was re-dissolved in methanol and subjected to TLC (plates, EM 5763 from EM Labs., Elmesford, N.Y., U.S.A.) in Petcoff's solution along with the parent free bile acids. Under these conditions each 3-keto derivative appears as a major band though additional minor bands are seen. All bands were detected with a fine water spray [4] and the major bands were marked, scraped and eluted with ether, filtered and then evaporated to dryness. All the eluted material was

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re-dissolved in methanol and re-chromatographed. This procedure was performed twice with each band to assure purity. The presence of 3-keto derivatives was confirmed by phenylhydrazone formation when reacted with 2,4 dinitrophenol hydrazine, keto absorption in infra-red spectroscopy and failure to produce reduced diphosphopyridine nucleotide (DPNH) upon re-exposure to the 3-α-hydroxy steroid dehydrogenase enzymatic mixture. In addition, standards of 3-keto derivatives of cholic and lithocholic acid obtained commercially (Steraloids, Wilton, N.H., U.S.A.) chromatographed at the position identical with those prepared as mentioned.

The 3-keto derivatives of chenodeoxycholic and deoxycholic acids when chromatographed with Petcoff's solution migrated at the same R_F. Separation of these two keto derivatives was accomplished by using a second developing solution of 1% glacial acetic acid in diethyl ether. A sample of human duodenal fluid was deconjugated according to Nair and co-workers [5—7] and chromatographed with the two solutions.

RESULTS

Fig. 1 shows the separation of the four free bile acids found in human bile and their respective 3-keto derivatives achieved with Petcoff's solution. Fig. 2* is a chromatogram of deconjugated bile acids from intestinal fluid along with appropriate standards developed in Petcoff's solution. Although the 3-keto

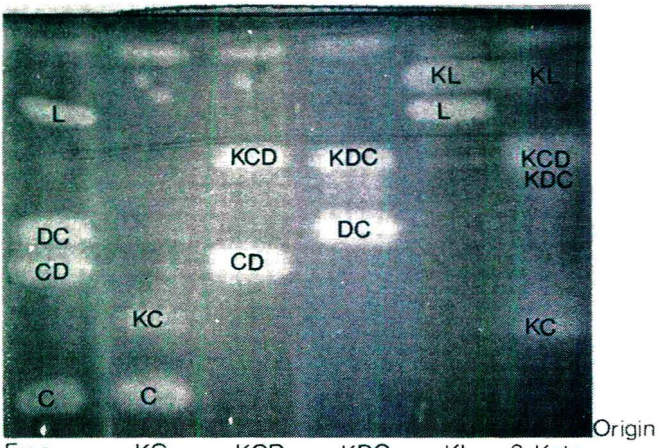

Fig. 1. Separation of four free bile acids and their respective 3-keto derivatives in Petcoff's solution. From left to right: Free mix, top to bottom, lithocholic acid (L), deoxycholic acid (DC), chenodeoxycholic acid (CD), and cholic acid (C); 3-keto cholic acid (KC) and cholic acid; 3-keto chenodeoxycholic acid (KCD) and chenodeoxycholic acid; 3-keto deoxycholic acid (KDC) and deoxycholic acid; 3-keto lithocholic acid (KL) and lithocholic acid and 3-keto bile acid mix, top to bottom, 3-keto lithocholic, 3-keto deoxycholic with 3-keto chenodeoxycholic and 3-keto cholic acids.

*Figs. 2 and 3 are both composite photographs of separate TLC plates to illustrate more clearly the TLC separation of the compounds.

Fig. 2. Separation of four free bile acids, 3-keto derivatives of these four bile acids and hydrolyzed intestinal fluids in Petcoff's solution. From left to right: Free mix and 3-keto derivatives same as Fig. 1 and hydrolyzed intestinal fluid. Minor bands are unknown compounds.

derivatives of cholic and lithocholic acid separate well from each other and their parent compounds, the 3-keto derivatives of chenodeoxycholic and deoxycholic acids migrate as one band.

Figs. 3* and 4 show the separation of these compounds in 1% glacial acetic acid in ether. Although this developing solvent clearly separates the 3-keto derivatives of cholic, chenodeoxycholic, deoxycholic and lithocholic acids from each other, it fails to separate chenodeoxycholic and deoxycholic acids. The trailing seen on Figs. 2 and 4 in the intestinal fluid sample can, in actual prac-

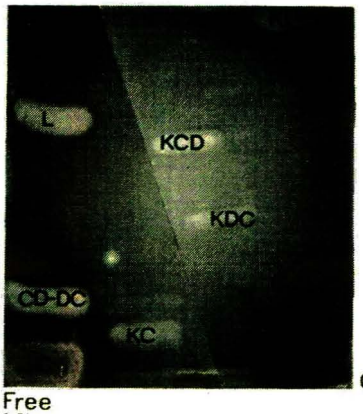

Fig. 3. Separation of four free bile acids and the 3-keto derivatives of the four free bile acids in 1% glacial acetic acid in diethyl ether. Free bile acids mix, from top to bottom: Lithocholic acid (L), chenodeoxycholic acid (CD) with deoxycholic acid (DC); and cholic acid (C); 3-keto derivatives, from left to right: KC, KCD, KDC and KL.

*See footnote on p. 72.

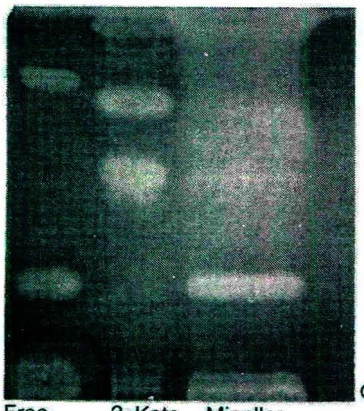

Fig. 4. Separation of four free bile acids, 3-keto derivatives and hydrolyzed intestinal fluid in 1% glacial acetic acid in diethyl ether. Free mix and 3-keto mix same as Fig. 3 (3-keto cholic acid is not seen because of very low concentration). Hydrolyzed intestinal fluid same as Fig. 2.

tice, be avoided by applying it as a longer streak to the plate. Obviously, the intestinal fluid contains only traces of the keto bile acids.

Table I shows the approximate R_F values for the compounds in the solvent systems reported here. Standards should always be included in the chromatograph as these values vary somewhat with each run.

TABLE I

R_F VALUES FOR FREE BILE ACIDS AND THEIR 3-KETO DERIVATIVES.

Solvent 1 = **Hexane**—methylethylketone—glacial acetic acid (56:36:8, v/v).
Solvent 2 = Glacial acetic acid—diethyl ether (1:99, v/v).

	Solvent 1 (Petcoff's Solution)	Solvent 2 R_F 1.00 = 14.0 cm
Free bile acids		
Cholic acid	0.11	0.04
Chenodeoxycholic acid	0.46	0.20
Deoxycholic acid	0.52	0.20
Lithocholic acid	0.77	0.68
3-Keto derivatives of:		
Cholic acid	0.31	0.11
Chenodeoxycholic acid	0.68	0.61
Deoxycholic acid	0.68	0.41
Lithocholic acid	0.99	0.96

DISCUSSION

Depending on the individual investigator's interest, one may use one or both developing systems to separate the four basic human bile acids and their respective 3-keto derivatives. If quantitation is desired the plates must be prewashed with the developing solution, otherwise a faint yellow band will separate along or near the lithocholic acid band. This band will be eluted with ether along with lithocholic acid and turns into a deeper yellow color at pH 9.5, increasing the spectrometric quantitative readings of the DPNH. This may be the same interfering compound discussed by Sandberg, et al. [8] and explains the unaccountably high recovery of lithocholic acid reported by Bruusgaard [9]. Quantification of the 3-keto compounds of cholic and chenodeoxycholic acids might be achieved by using the enzymatic mixture of 7-α-hydroxy steroid dehydrogenase as suggested by Hazelwood et al. [10] and MacDonald et al. [11]. The derivatives of deoxycholic might be determined by using the 12-α-hydroxy steroid dehydrogenase enzyme if it becomes available. In certain situations the preliminary separation by TLC may make the quantification by gas–liquid chromatography easier and more accurate.

ACKNOWLEDGEMENTS

This work was supported by the Medical Research Service of the Veterans Administration. Technical assistance of Mr. Aloysius Lorenc and Jim Murillo is acknowledged.

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Note

Demonstration of soluble immune complexes by analytical isotachophoresis

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(First received February 10th, 1978; revised manuscript received July 1st, 1978)

The basic principle of isotachophoresis whereby ions are separated on the basis of net mobility has been known for many years and been described by several authors [1, 2]. However, it was only in 1970 that Arlinger and Routs [3] and Svendsen and Rose [4] applied the technique to the fractionation of protein mixtures. The present report is the first to describe detection of soluble immune complexes by means of analytical isotachophoresis. Advantages of the technique include rapidity of analysis, i.e., approximately 30-min running time, and small sample size, as little as 2—10 μl of sample containing microgram concentrations of protein.

EXPERIMENTAL

Chemicals

Tris (hydroxymethyl) aminomethane (tris), $Ba(OH)_2$ and HCl were obtained from Fisher Scientific (Pittsburgh, Pa., U.S.A.). Insolubilized protein A (protein A Sepharose CL-4B) was obtained from Pharmacia (Uppsala, Sweden). 2-Amino-2-methyl 1,3-propanediol (ammediol) was purchased from Eastman Kodak (Rochester, N.Y., U.S.A.). Hydroxypropylmethycellulose methyocel A 4M premium was a gift of Dow Chemical (Midland, Mich., U.S.A.).

Methods

A standard quantitative precipitin test similar to that of Heidelberger and Kendall [5] was performed. Rabbit immunoglobulin G (IgG) was prepared by passing rabbit antiserum to bovine serum albumin (BSA) over insolubilized

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**The views of the author do not purport to reflect the positions of the Department of the Army or the Department of Defense.

protein A which binds to IgG through its F(c) portion and then eluting the absorbed IgG with 0.02 *M* glycine—HCl, pH 2.5. After the pH of IgG was readjusted to 7.3, increasing microgram amounts of BSA were mixed with a fixed concentration of rabbit anti-BSA IgG. The antibody—antigen mixtures were incubated at 37° for 1 h and at 4° for 6 days. The incubation tubes were then centrifuged at 3000 *g* for 15 min and the precipitates and supernatants were collected for study.

Precipitates were washed three times with cold 0.05 *M* phosphate buffered saline (PBS), pH 7.3, and the pellets were redissolved in 0.1 *N* sodium hydroxide for spectrophotometric examination at 280 nm. Identical mixtures of BSA and rabbit IgG, which did not contain antibodies to BSA were incubated together and processed in the same fashion. These served as the negative controls in the isotachophoretic analysis of the supernatants above the precipitated immune complexes.

Analytical isotachophoresis studies were performed with an LKB 2127 Tachophor apparatus. The separation chamber consisted of a 23-cm PTFE capillary tube (0.5 mm I.D.) maintained at a constant temperature of 20°. The apparatus was equipped with a thermal detector and with a UV detector set at 280 nm. The leading electrolyte, 0.005 *M* HCl—ammediol, pH 8.5 was supplemented with 0.04% A-4 methyl cellulose (Dow Chemical). The terminal buffer was 0.01 *M* ϵ-amino caproic acid (EACA) adjusted to pH 9.5 with $Ba(OH)_2$. A 0.01% solution of ampholine pH 3.5—10 (LKB) was used as a non-UV absorbing spacer. Analyses of the individual supernatants were performed at a constant current of 75 μA; the voltage increased from 3 to 20 kV during the running time of approx. 25 min per sample.

RESULTS AND DISCUSSION

A graph plotting optical density of the precipitate against the amount of antigen added is shown in Fig. 1. The equivalence point was established be-

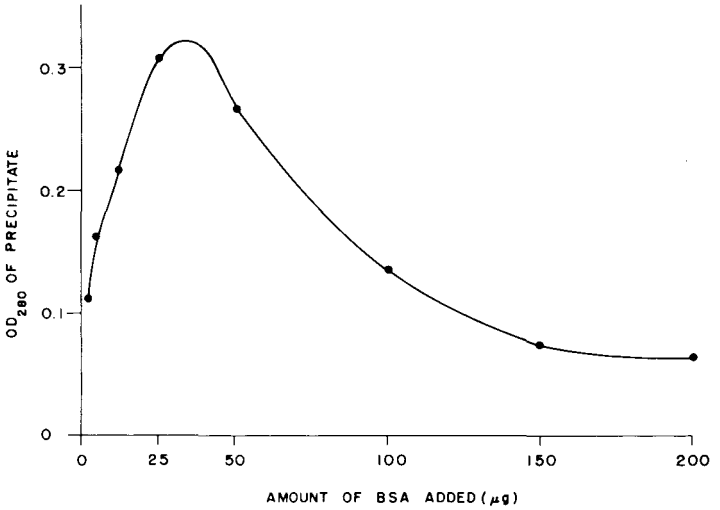


Fig. 1. Precipitin curve of bovine serum albumin (BSA) with IgG anti-BSA.

tween 30 and 35 μg of BSA. Supernatant material taken from the area of marked antigen excess provided a source of soluble immune complexes [5].

In those situations where rabbit IgG without antibody activity to BSA was mixed with increasing concentrations of BSA (Fig. 2, 1—5), analytical isotachophoresis demonstrated in these negatives controls an unchanging level of IgG as denoted by the areas under the black bar, and a progressive increase in the concentration of BSA, noted by the area under the open bar. In contrast in the isotachophoretic analyses shown in Fig. 2, 6—10, of the supernatants above the immune complex precipitates it can be seen that at point 7 of Fig. 2 there has been a reduction in the total amount of IgG. At point 8 in Fig. 2 a soluble complex appears as noted by the striped bar. The size of this complex increases as antigen excess increases and this is most evident in point 10, Fig. 2.

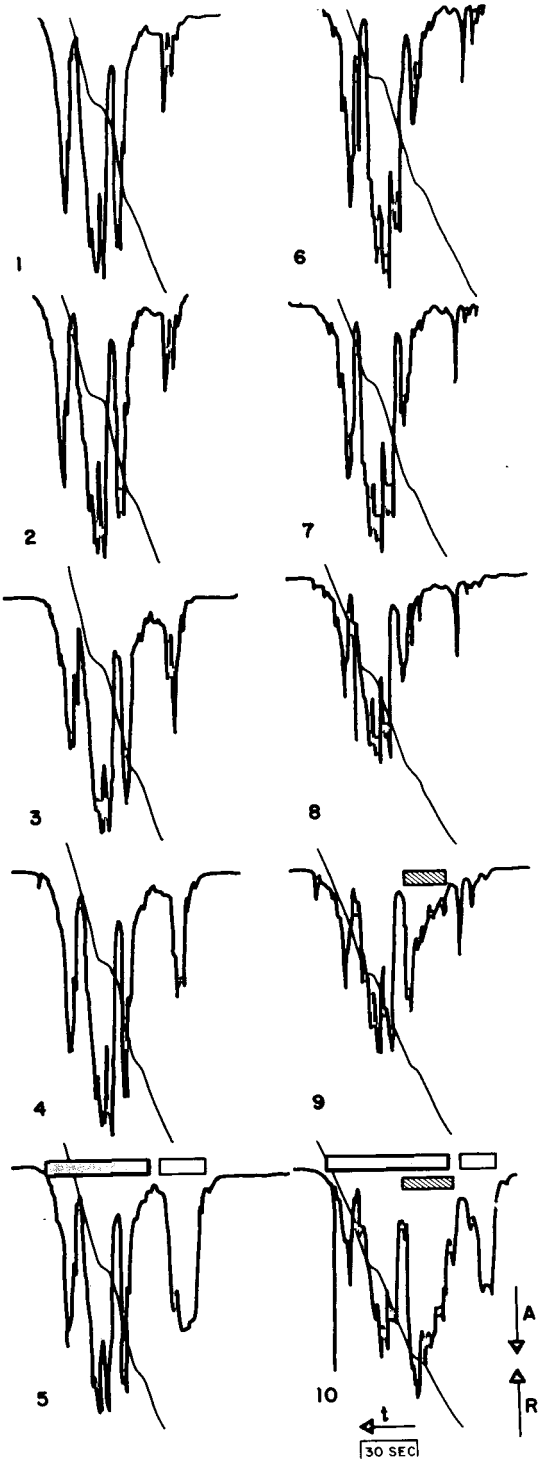
In Fig. 3 (1) the striped area represents the bulk of the IgG. Fig. 3 (2) shows the supernatant at equivalence; the total amount of IgG is reduced following its loss in the insoluble precipitate and a soluble immune complex appears with a net mobility that is intermediate between that of IgG and BSA. Passage of supernatant material over insolubilized protein A removed both the original IgG peak and the middle peak which as a soluble immune complex would also contain IgG. This is shown in Fig. 3 (3).

Serological activity of samples analyzed by isotachophoresis is presented in Table I and supports the hypothesis that the newly formed intermediary peak was indeed an immune complex. Samples containing soluble immune complexes had complement fixing activity that could be removed by absorption with protein A confirming the presence of IgG-containing components. A similar mixture of BSA and rabbit IgG which did not have anti-BSA specificity did not result in the formation of an immune complex as measured by complement fixing activity or isotachophoretic analysis (see Fig. 2, 2—5).

In summary the combination of increasing amounts of antigen with a fixed amount of homologous antibody results in the formation of both insoluble and soluble immune complexes. If one studies the supernantants above the removed insoluble immune complexes soluble complexes can be identified by complement fixing activities and by the formation of a unique intermediate peak identified by means of analytical isotachophoresis. If one adsorbs the IgG components from the supernatants by means of protein A both complement fixing activities and the appropriate IgG and IgG-containing components seen by isotachophoresis are removed. Neither complement fixing activity nor inter-

Fig. 2. Isotachopherograms of supernatants obtained from various points of the precipitin curve in Fig. 1. 1—5 represent the preimmunization rabbit IgG controls; 6—10 represent the points to which 0, 12.5, 25, 100 and 200 μg of BSA were added to a fixed concentration of antibody. Solid black bar, IgG; open bar, free BSA and striped bar, immune complex. A 5-μl volume of each sample was injected with 1 μl of a 0.01% solution of ampholine, pH 3.5—10. A = increasing UV absorption; R = increasing resistance; t = time.

Fig. 3. Isotachopherograms of supernatants obtained from various points of precipitin curves. 1 = IgG alone; 2 = sample of supernatant taken at a point where the amount of antigen was in excess of the amount of antibody directed against it. This shows a reduction in the free IgG and the formation of a soluble immune complex (IC); 3 = sample of supernatant identical to 2 after absorption on protein A, IgG and IgG-containing complexes have been removed. A 5-μl volume of each sample was injected with 1 μl of a 0.01% solution of ampholine, pH 3.5—10. A = increasing UV absorption; R = increasing resistance; t = time.


1
6
2
7
3
8
4
9
5
10
A
R
t
30 SEC

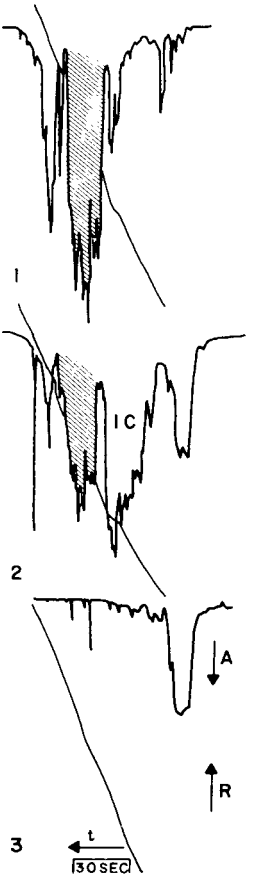

1
IC
2
A
R
3
t
30 SEC

TABLE I

COMPLEMENT FIXING ABILITY OF SOLUBLE SUPERNATANT SPECIMENS

Supernatant specimen*	BSA added (μg)	Complement fixation titer
6	0	0
7	12.5	0
8	25	0
9	100	1:16
10	200	1:32
10**	200	0

*Specimens correspond to those described in Fig. 2.

**Supernatant after adsorption of protein A (corresponding to Fig. 3 (3).

mediate peaks are demonstrated by simply mixing BSA and IgG without BSA antibody specificity.

This is the first reported demonstration of the identification of soluble immune complexes by means of analytical isotachophoresis. It is hoped that the technique can be extended to the study of a wide variety of antigen and antibody systems to exploit the speed and convenience allowed by analytical isotachophoresis.

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Note

Gas chromatographic determination of nifedipine and one of its metabolites using electron capture detection

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(Received April 24th, 1978)

Nifedipine, 4-(2′-nitrophenyl)-2,6-dimethyl-3,5-dicarbomethoxy-1,4-dihydropyridine, BAY a 1040, is a calcium antagonistic drug with a marked effect on the excitation—contraction coupling in different types of smooth muscle and myocardium [1,2].

Clinical studies have demonstrated that nifedipine is an effective drug in the treatment of coronary heart disease, and preliminary results in the treatment of arterial hypertension have been promising [3].

The finding of a close correlation between the clinical effects and the plasma concentrations of nifedipine [3], measured by a relatively troublesome and time consuming fluorimetric method [4], has called for the development of a sensitive and specific gas chromatographic (GC) method for determination of nifedipine in blood plasma.

EXPERIMENTAL

Standards and reagents

Nifedipine as a pure crystalline compound and in solution for injection (0.1 mg/ml) was kindly supplied by Bayer, Leverkusen, G.F.R. Standard solutions of nifedipine were protected from light and stored at 4°.

Toluene (analytical reagent) and tritisol buffer (pH 9) were obtained from Merck, Darmstadt, G.F.R.

The internal standard solution was 1 μg/ml of diazepam in water.

Preparation of the metabolite

A 1-ml sample of nifedipine (0.1 mg/ml) was oxidized at 50° for 1 h with 2 ml 0.01 *M* potassium permanganate at pH 9. After cooling to room tempera-

ture the aqueous solution was extracted three times with 10 ml of diethyl ether. After evaporation at 30° with a gentle stream of nitrogen, the residue was dissolved in a suitable amount of ethanol. A standard solution of oxidized nifedipine corresponding to 10 μg/ml of nifedipine in ethanol was prepared.

Apparatus

A Varian 2100 gas chromatograph equipped with a ^{63}Ni electron capture detector (DC mode) was used. The GC column was glass (180 cm × 2 mm I.D.) filled with 2% OV-17 (Pierce, Rockford, Ill., U.S.A.) on Gas Chrom Q, 80—100 mesh, conditioned for 24 h at 275° with a nitrogen flow-rate of 25 ml/min. The operating conditions were: column temperature 240°, injector temperature 240°, detector temperature 290° and carrier gas (nitrogen) flow-rate 25 ml/min. Under these conditions the metabolite, the internal standard (diazepam) and nifedipine had retention times of 1.3, 2.4 and 4.0 min respectively.

For the mass spectrometric work a Jeol D-100 mass spectrometer connected to a gas chromatograph was used. Conditions: ionization voltage 25 eV, ionization current 300 μA. The separator (double stage jet) was heated to 260°. The GC column was a 1 m, 3% OV-17 on Gas-Chrom Q, 80—100 mesh., column temperature 220° and carrier gas (helium) 20 ml/min.

Procedure

To a 10-ml glass-stoppered centrifuge tube containing 1 ml of tritisol buffer (pH 9), 250 μl of the internal standard solution (1 μg/ml diazepam in water)

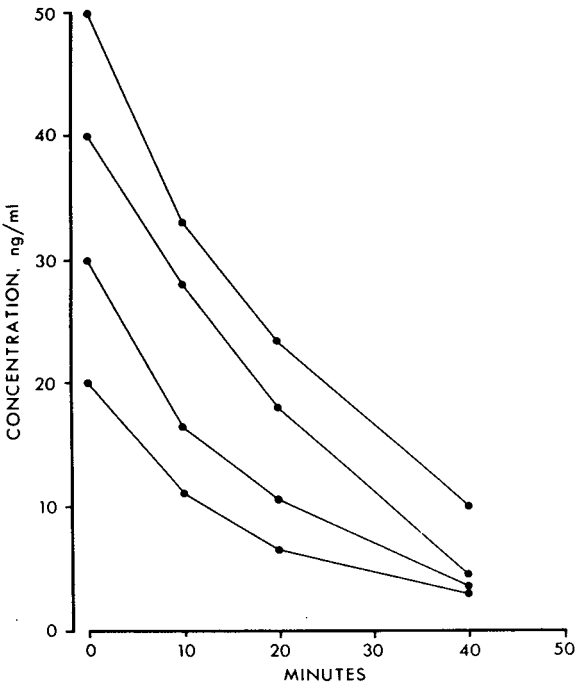

Fig. 1. Degradation of nifedipine in 4 different concentrations after exposure to normal laboratory light. Solvent: toluene.

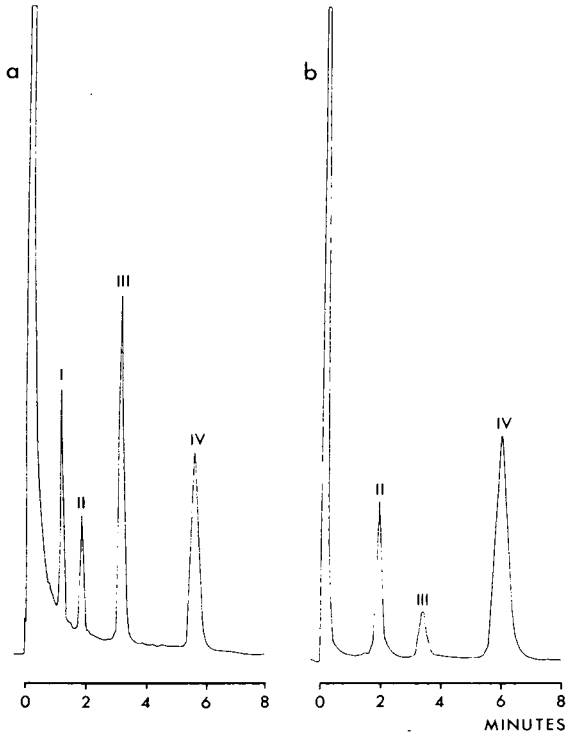

Fig. 2. Gas chromatograms of the light-degradation product (I), the oxidation product (II), diazepam (III) and nifedipine (IV). (a) Injection of approx. 100 ng of each compound using flame ionization detection; solvent: chloroform; (b) injection of approx. 100 pg of each compound using electron capture detection; solvent: toluene. Column temperature: 220°.

and 1 ml of toluene was added 0.5 ml of a plasma sample. The tube was shaken vigorously on a Whirly mixer for 15 sec. After centrifugation (3000 rpm, 10 min) 2 μl of the organic phase was injected onto the gas chromatograph. During the whole procedure the centrifuge tube was protected from light.

Preparation of standard curves

Known amounts of nifedipine and the synthetized metabolite were added to human plasma. Samples of 0.5 ml were treated as described above. The standard curves were constructed by plotting the ratio of the peak height of nifedipine or metabolite and that of the internal standard (diazepam) against the concentration of nifedipine or metabolite.

RESULTS AND DISCUSSION

Nifedipine is sensitive to light. Fig. 1 shows the degradation of nifedipine in toluene solution at different concentrations after exposure to normal laboratory light. Logarithmic transformation of the data revealed a first order kinetics with a rate constant of 0.05 min^{-1} for the degradation. In the dark nifedipine is stable in toluene for at least one day. Consequently care was taken to protect the samples from day light. Heparinized plasma samples were stored in brown

centrifuge tubes and during the extraction procedure the samples were kept in cardboard containers.

Oxidation of nifedipine under mild conditions gives a product which has excellent GC and electron capturing properties. This oxidation product is resistant to light exposure; therefore conversion of nifedipine by oxidation might seem to be a possible method for determination of nifedipine. However, plasma samples from individuals receiving the drug show a GC peak with exactly the same retention time as that of the oxidation product. This finding suggests that the oxidized drug is a metabolite of nifedipine.

While nifedipine and its oxidized derivative have excellent electron capturing abilities the opposite is found for the light-degradation product. Fig. 2 shows gas chromatograms of the three compounds and the internal standard using flame ionization and electron capture detection.

In order to obtain optimal extraction conditions 0.5 ml amounts of plasma samples containing 100 ng/ml of nifedipine were adjusted to different pH values. 100% extraction was accomplished at pH values between 3 and 13 with equal amounts of organic and aqueous phase using an extraction time of 15 sec.

Fig. 3 shows gas chromatograms of plasma samples after extraction. As shown in Fig. 4 the standard curves for nifedipine and the oxidation product were linear between 0 and 100 ng/ml with regression coefficients of 0.9983

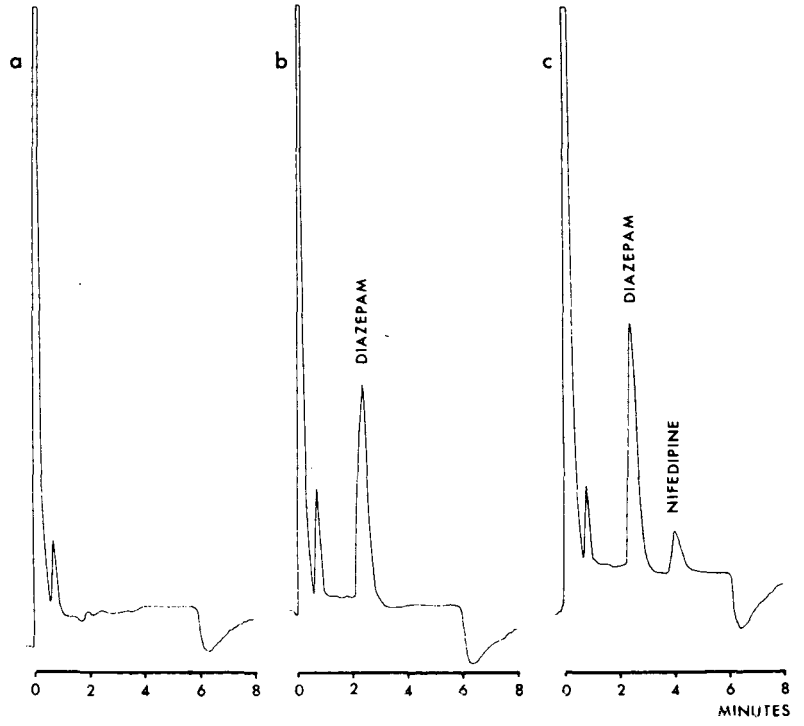

Fig. 3. Gas chromatograms of human plasma samples after toluene extraction. (a) Plasma blank; (b) after addition of diazepam, 500 ng/ml; (c) after addition of diazepam, 500 ng/ml, and nifedipine, 25 ng/ml. Column temperature: 240°.

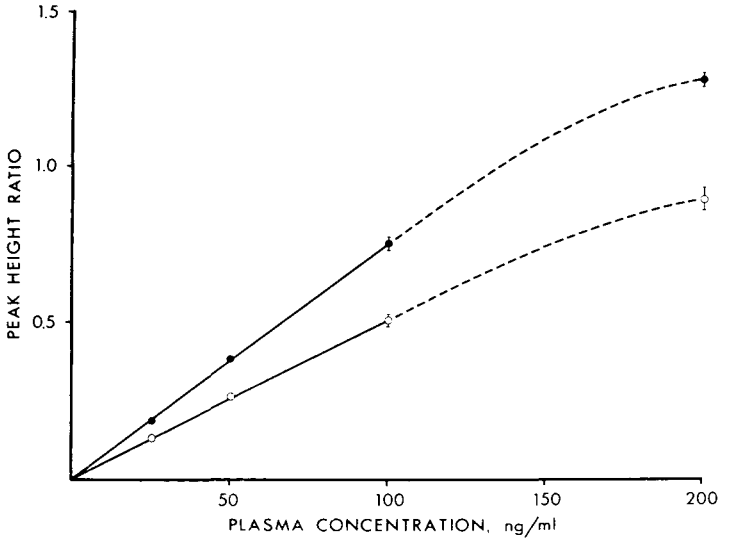

Fig. 4. Standard curves for determination of plasma concentrations of nifedipine, • (r = 0.9983, 0—100 ng/ml) and the metabolite, ○ (r = 0.9976, 0—100 ng/ml). Each point is the mean(± S.D.) of the results from 6 determinations. The ordinate refers to the ratio between the peak height of nifedipine or metabolite and the peak height of diazepam.

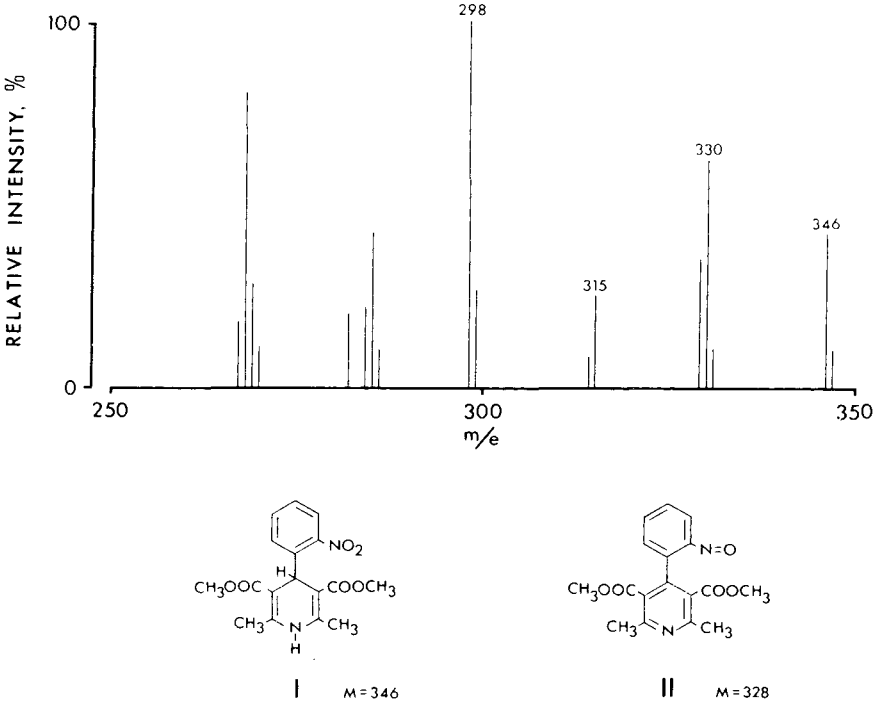

Fig. 5. Mass spectrum of nifedipine and structural formulae of nifedipine (I) and the light-degradation product (II).

for nifedipine and 0.9976 for the oxidation product. The minimum detectable concentration was found to be about 1 ng/ml for both nifedipine and the metabolite.

Within-run precision was determined from 6 separate extractions of plasma samples containing 50 and 100 ng of nifedipine or metabolite per ml of plasma. The within-run coefficient of variation was 2.1% for 50 ng/ml and 2.8% for 100 ng/ml with respect to nifedipine. The coefficient of variation for determination of the metabolite was 3.5% for 50 ng/ml and the same for 100 ng/ml.

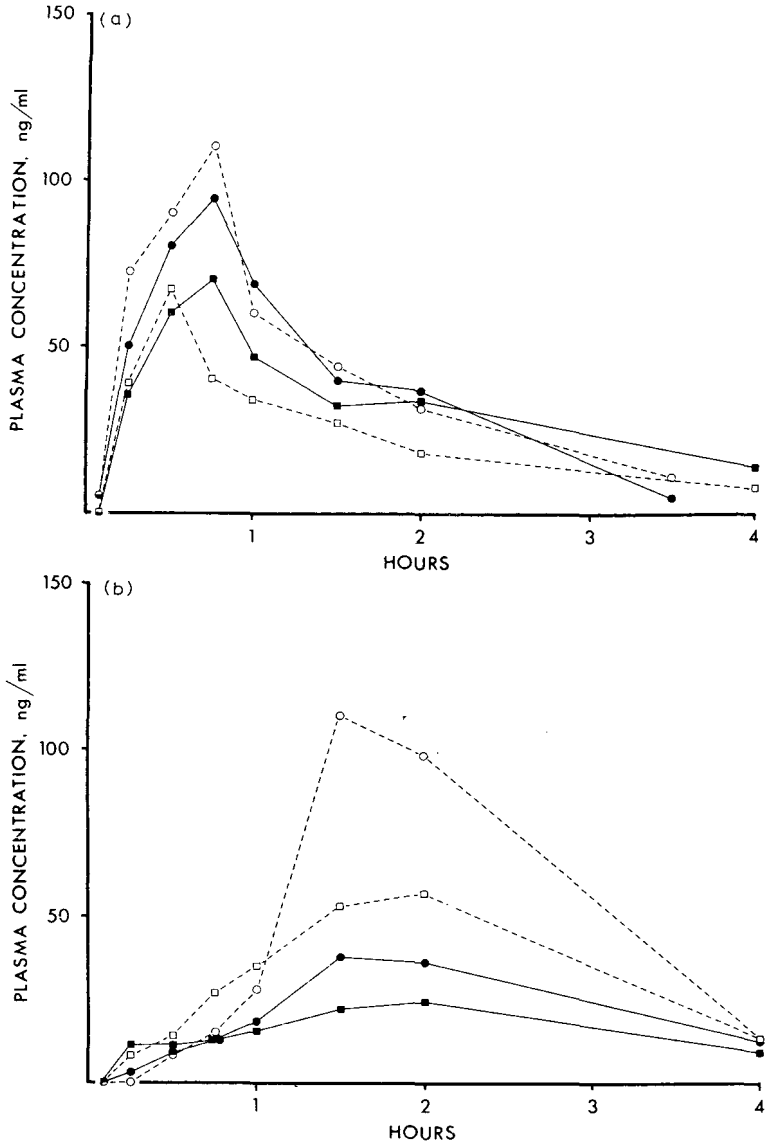

Fig. 6. Plasma concentrations of nifedipine (solid line) and the metabolite (broken line) as a function of time in four healthy persons after sublingual administration of 10 mg of nifedipine. (a) Two persons showing fast absorption; (b) two persons showing slow absorption.

Combined GC—mass spectrometry (GC—MS) confirms, that nifedipinè is stable on the GC column. Fig. 5 shows the GC—MS of nifedipine (I). The GC—MS of the light-degradation product showed a molecular ion 18 mass units smaller than that of nifedipine, consistent with formula II. The GC—MS of the oxidation product showed an extremely large base peak at *m/e* 298 and a small ion at *m/e* 313. It is likely that nifedipine is easily oxidized in the dihydropyridine ring to the pyridine derivative. Using the fluorimetric method [4] both compounds are determined as nifedipine. In preparations of isolated human veins this metabolite is ca. one hundred times less potent than nifedipine in inhibiting the potassium- and noradrenaline-induced contraction. The structure of the metabolite has not yet been confirmed.

APPLICATION

Sublingual administration of 10 mg of nifedipine was performed in four healthy volunteers. They were instructed to bite the capsule apart and keep the substance in the mouth for 5 min before swallowing. Blood samples were drawn 0, 5, 15, 30, 45, 60, 120, 240 and 480 min after administration. After immediate centrifugation the plasma was stored at - 20° until analysis was carried out. Plasma concentrations of nifedipine as well as the metabolite were determined. Two types of absorption curves were found. Fig. 6 shows the plasma concentration curves from the four persons. Two persons had a fast absorption with maximum concentrations of nifedipine of 70—100 ng/ml reached within 30—60 min after administration (Fig. 6a) and two persons had a slower absorption with maximum concentrations of 20—40 ng/ml reached within 90—120 min after administration (Fig. 6b).

Further studies on the pharmacokinetics and metabolism of nifedipine are in progress.

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Note

Quantitative analysis of furosemide in micro plasma volumes by high-performance liquid column chromatography

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(Received March 26th, 1978)

Furosemide is a potent diuretic agent which is widely used for the treatment of various conditions in adult patients. Several reports have appeared in the literature relating to the disposition of furosemide in adults with [1—3] or without [1, 4, 5] renal disease, and in adult patients with heart failure [5]. The analytical method used to measure furosemide in serum or plasma in the majority of those studies [1, 2, 4, 5] was the spectrofluorometric assay of Haussler and Hajdu [6], or one of the modifications of it [7, 8]. Rose et al. [3] used radio-labeled furosemide in their investigation. The spectrofluorometric methods [6—8] use 1 ml of serum or plasma for analysis and suffer from a number of disadvantages, some of which have been discussed elsewhere [5, 8].

In addition to its use in adult patients, furosemide may also be required for administration to infants [9, 10]. One brief report [11] of some pharmacokinetic aspects of the drug in infants has been published. In that study furosemide in plasma was measured by gas chromatography, but the details of the analytical method were not reported. The present authors decided to initiate further pharmacokinetic studies in infants. In reviewing potential methods for the analysis of furosemide in plasma (or serum) it soon became obvious that the spectrofluorometric procedures [6—8] would not be suitable [5, 8]; not the least objection to their use is the relatively large volume of plasma (1 ml) which would need to be collected from small infants. At least one gas chromatographic technique [12] and four methods based on high-performance liquid chromatography (HPLC) [13—16], have been re-

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ported. Four of these five methods [12—14, 16] each require the use of 1 to 2 ml of plasma to achieve quantitation of furosemide in plasma with a lower limit between 0.1 and 1 μg/ml.

Blair et al. [15] have reported an HPLC method for the determination of furosemide in small volumes of serum. In that procedure 5-μl aliquots of serum were injected directly into the high-performance liquid chromatograph. A cation-exchange column maintained at 74° and eluted with buffer was used to achieve chromatographic separation, and furosemide was detected by fluorescence monitoring. The lower limit of quantitation was not stated explicitly, but appeared to be in the range of 0.5 to 1 μg/ml. A precolumn in the chromatographic system was necessary, apparently to avoid plugging the main HPLC column with the proteins contained in the serum. Also, furosemide was not completely resolved from 4-chloro-5-sulfamoylanthranilic acid which is the major breakdown product of furosemide [15] and also is a metabolite of furosemide [6].

The purpose of this paper is to report a more sensitive HPLC assay for furosemide in plasma which utilizes small sample volumes, is specific, and requires only short sample preparation procedures prior to chromatography.

EXPERIMENTAL

Materials

Furosemide was kindly supplied by Hoechst-Roussel (Sommerville, N.J., U.S.A.). 4-Chloro-5-sulfamoylanthranilic acid was purchased from U.S. Pharmacopeia (Rockville, Md., U.S.A.). Glass-distilled acetonitrile was purchased from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.), and phosphoric acid was obtained from Fisher Scientific (Fair Lawn, N.J., U.S.A.). Other drug substances which were tested for potential interference of the assay had, in most cases, been donated by pharmaceutical manufacturing companies.

Apparatus

A Model M-6000A pump was used to deliver the mobile phase to a Model U6K injection loop and a μBondapak C_{18} reversed-phase column (30 cm × 3.9 mm I.D., 10 μm particle size), all of which had been supplied by Waters Assoc. (Milford, Mass., U.S.A.). The chromatography was carried out at ambient temperature (approximately 24°). A Model FS970 fluorescent detector obtained from Schoeffel Instruments (Westwood, N.H., U.S.A.) was used to monitor the column effluent; the excitation wavelength of the detector was set at 225 nm, and an emission filter (KV 389) was used to select the fluorescence emission for detection. The output from the detector was connected to a 10-mV potentiometric 25.4-cm recorder (Houston Instrument, Austin, Texas, U.S.A.). The HPLC mobile phase was acetonitrile—0.05% phosphoric acid (30:70). This was pumped through the HPLC system at a rate of 2 ml/min, and the resulting pressure was approximately 1500 p.s.i.

Procedure

Aliquots of 100 μl of plasma were pipetted into 13 × 100 mm culture tubes followed by the addition of 250 μl of acetonitrile. After sealing with

a PTFE-lined screw cap each tube was vortexed for 10 sec and this was followed by centrifugation at 800 *g* for approximately 1 min. Subsequently, most of the clear supernatant solution was poured into another culture tube, and 10 μl of this solution was then injected into the high-performance liquid chromatograph for analysis. Standard curves were developed by supplementing blank human plasma with known concentrations of furosemide. This was achieved by placing 5- to 10-μl aliquots of methanolic solutions of furosemide in containers and subsequently adding 1-ml aliquots of blank human plasma to each tube. Aliquots of 100 μl of each spiked plasma sample were carried through the procedure described above for the analysis of furosemide in plasma. Peak height measurements were used for quantitation.

RESULTS AND DISCUSSION

The HPLC conditions were selected after appropriate preliminary investigations with a number of different mobile phase compositions and detector conditions (excitation and emission wavelengths).

Chromatograms resulting from the analysis of plasma samples collected from a 2-month old infant who was receiving furosemide therapy are shown in Fig. 1. This infant was receiving theophylline, phenobarbital, and vitamins in addition to the furosemide. Also shown in Fig. 1 is the chromatogram resulting from the analysis of a plasma sample which was collected from a 7-week-old infant who was not receiving furosemide but who was being administered theophylline, phenobarbital, digoxin and vitamins. The small volumes (200—300 μl) of capillary blood samples collected from the infants were obtained by heel-stick. The total analysis time per sample was approx. 10—12 min; furosemide eluted from the HPLC system with a retention time of 8.0 min. No endogenous fluorescent substances with a retention time similar to furosemide were observed during the analysis of plasma collected from infants (Fig. 1) or adults who were not receiving furosemide. The major breakdown product [15] and metabolite [6] of furosemide, namely 4-chloro-5-sulfamoylanthranilic acid, does not interfere with the analysis of furosemide since it has a retention time of approx. 2 min under the conditions of analysis described here for furosemide.

The relationship between the furosemide peak height and furosemide concentration in plasma (Table I) was linear over the concentration range studied as indicated by the high value of the coefficient of determination for the linear regression (r^2 = 0.9999), and the constancy of the response factor (peak height divided by concentration). The maximum sensitivity for furosemide detection was 0.1 μg/ml of plasma since at that concentration the signal-to-noise ratio was ca. 4 or 5 : 1.

The precision of the method was acceptable over the entire concentration range investigated. The coefficient of variation on a given day ranged from a low of 1.03% at 10 μg/ml up to 6.22% at the 0.1 μg/ml concentration. These reproducibility data compare favorably with those of the earlier reported methods of analysis [12—16]. The coefficient of variation for the analysis of the same spiked plasma sample (5 μg/ml) on three days over a period of two weeks was 7.97%. The inclusion of an internal standard in the procedure

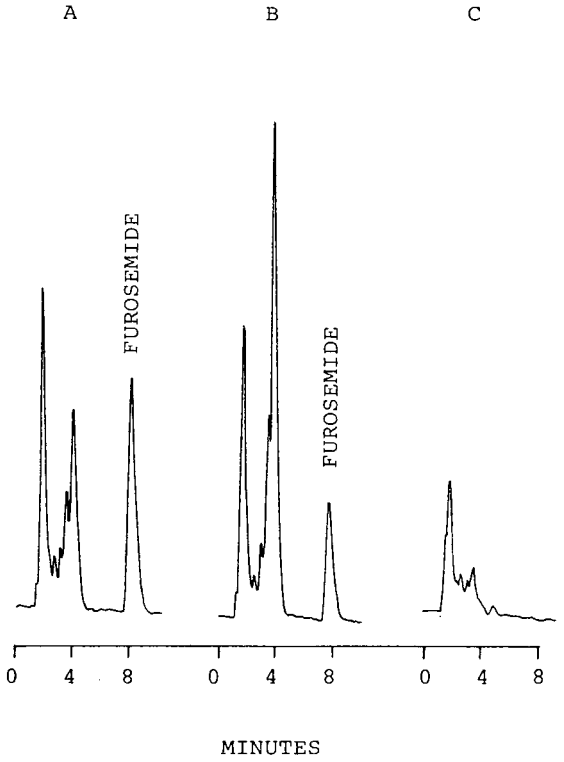

Fig. 1. Chromatograms A and B resulted from the analysis of plasma collected from a 2-month-old female infant (body weight 1.04 kg) who received a 2-mg oral dose of furosemide followed 12 h later by a similar dose. The patient was also being administered theophylline, phenobarbital and vitamins. Sample A, which was collected 2.25 h after the first dose, had a furosemide concentration of 2.51 μg/ml and sample B, which was collected 10.5 h after the second furosemide dose, had a concentration of 1.27 μg/ml. Chromatogram C resulted from the analysis of plasma collected from a 7-week-old male infant (1.36 kg) who was not receiving furosemide but who was being administered theophylline, phenobarbital, digoxin and vitamins. Detector range setting was 0.2 μA for all chromatograms and photomultiplier voltage was 820 V.

may improve the reproducibility on a day-to-day basis. If an internal standard was chosen which had a retention time of approx. 5—7 min, then such an assay based on internal standardization would not be useful for patients receiving salicylates, since salicylic acid elutes in that retention window. On the other hand, an internal standard such as the methyl ester of furosemide would elute after the drug using reversed-phase chromatography [16], hence increasing analysis time. In using the present method based on external standardization it is suggested that one or two standard samples be included on days when patient samples are to be analyzed.

The recovery of furosemide following the deproteinization of plasma with acetonitrile was essentially complete. The mean (± S.D.) peak heights (detector range 0.1 μA), for furosemide obtained by carrying 1 μg/ml aqueous and plasma solutions through the analytical procedure were 3.65 ± 0.097 cm and

TABLE I

STANDARD CURVE DATA FOR FUROSEMIDE IN PLASMA

Linear least squares regression equation for the data is: $y = 4.24 \times -0.018$ ($r^2 = 0.9999$).

Concentration of furosemide in plasma (μg/ml)	Furosemide peak height ± SD (coefficient of variation, %)*	Mean response factor**
0.1	0.45 ± 0.03 (6.22)	4.50
0.2	0.90 ± 0.06 (6.22)	4.48
0.5	2.14 ± 0.08 (3.93)	4.28
1	4.22 ± 0.07 (1.75)	4.22
2	8.47 ± 0.13 (1.54)	4.23
5	20.91 ± 0.27 (1.29)	4.18
10	42.55 ± 0.44 (1.03)	4.26

*Furosemide peak height in cm when detector range setting was 0.1 μA. Five determinations were performed at each concentration, all on the same day. Photomultiplier voltage was 820 V.

**Peak height divided by furosemide concentration.

3.55 ± 0.069 cm, respectively, when each type of solution was assayed in five determinations. It should be noted that plasma sample volumes as small as 10 or 25 μl may be prepared for analysis by adding 2.5 volumes of acetonitrile for the purpose of deproteinization [17—19].

It is important to establish the specificity of any analytical method which is designed for measurement of drug concentrations in biological fluids, because it is very common for patients to receive other drugs concurrently. The other drugs which are quite commonly administered to infants (particularly in an intensive care setting), include phenobarbital, phenytoin, theophylline, digoxin and ampicillin. None of these drugs interfere with the analysis of furosemide. In addition, ephedrine, caffeine, theobromine, acetominophen, phenacetin, tetracycline and salicylic acid were shown not to interfere.

The method described is rapid and suitable for the quantitative determination of furosemide in small volumes of plasma collected from infants. The limit of detection of furosemide is 0.1 μg/ml of plasma and the reproducibility is satisfactory. The method is specific for furosemide; the main breakdown product and metabolite of furosemide, and a number of other commonly used drugs do not interfere with the analysis.

ACKNOWLEDGEMENT

The authors wish to thank Michael Reed of the Department of Pharmacy Practice, College of Pharmacy, University of Illinois at the Medical Center for supplying the plasma samples.

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Note

High-performance liquid chromatographic assay for allopurinol and oxipurinol in human plasma

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(Received March 22nd, 1978)

Allopurinol (4-hydroxy-3,4-*d*-pyrazolopyrimidine) is a potent xanthine oxidase inhibitor used in the treatment of hyperuricemia. As part of an investigation into the pharmacokinetics of allopurinol absorption from a variety of dosage forms, it was necessary to develop an assay capable of detecting the drug and its primary, active metabolite, oxipurinol (3,4-dihydroxy-3,4-*d*-pyrazolopyrimidine), in plasma. Although two high-performance liquid chromatographic (HPLC) methods have been recently published [1,2], we wish to report an HPLC procedure that is equally sensitive but is greatly simplified and requires considerably smaller volumes of plasma.

EXPERIMENTAL

Apparatus

A Glenco System I high-performance liquid chromatograph (Glenco Scientific, Houston, Texas, U.S.A.) equipped with a 254-nm UV detector and a reversed-phase column (Spherisorb ODS 5 μm, 25 cm × 4.1 mm; Laboratory Data Control, Riviera Beach, Fla., U.S.A.) was used for all assays. The detector was connected to an electronic integrator (Autolab Minigrator, Spectra Physics, Santa Clara, Calif., U.S.A.) and chromatograms were recorded on a chart recorder (Linear Instruments, Irvine, Calif., U.S.A.).

Mobile phase

A phosphate buffer (pH 6.0, 0.05 *M*) was prepared by adding 0.508 g of dibasic potassium phosphate and 6.41 g of monobasic potassium phosphate per l of glass-distilled water and adjusting pH to 6.0 with HCl or NaOH. All

solutions were passed through an 0.22-μm filter (Millipore Filter, Bedford, Mass., U.S.A.) and degassed by stirring with a magnetic stirrer before use.

Drug standards

Allopurinol and oxipurinol in pure form were gifts of Dr. George Reddin, Burroughs Wellcome, Research Triangle Park, N.C., U.S.A. Acetaminophen (4'-hydroxyacetanilide), the internal standard, was purchased from Eastman Organic Chemicals, Rochester, N.Y., U.S.A.

Standard solutions

An aqueous solution of allopurinol and oxipurinol, 100 μg/ml of each, was prepared in glass-distilled water. Appropriate volumes were then diluted to 10 ml with human plasma to yield standard solutions of 0.5, 1.0, 2.0, 5.0, 10.0 and 20.0 μg/ml.

The internal standard, acetaminophen, was prepared at a concentration of 100 μg/ml in glass-distilled water.

Assay procedure

To 0.5 ml of plasma were added 0.1 ml internal standard and 0.2 ml 20% trichloroacetic acid. Following mixing (Vortex Genie; Scientific Products, Houston, Texas, U.S.A.) the sample was centrifuged at 8700 *g* (Microfuge B; Beckman, Palo Alto, Calif., U.S.A.) and a 50-μl aliquot injected onto the column. Solvent flow-rate was 2 ml/min (1500 p.s.i.) and the detector was maintained at 0.1 a.u.f.s. The areas under the peaks of interest were calculated by the integrator and standard curves constructed by plotting the ratio of the area under either the allopurinol peak or oxipurinol peak to that of acetaminophen against the concentration of the respective compound.

RESULTS

A chromatogram obtained from blank plasma is shown in Fig. 1A. A chromatogram from standard plasma (allopurinol and oxipurinol, 10 μg/ml of each) in Fig. 1B. Fig. 1C is a chromatogram of a representative sample from a volunteer subject following the oral ingestion of a 300 mg allopurinol tablet (Zyloprim; Burroughs Wellcome).

As can be seen by comparison of Figs. 1A and B, there are no interfering peaks in the areas where allopurinol, oxipurinol and acetaminophen elute. Each of the three peaks is sharp, symmetrical and well defined with respect to the base line. Calibration curves for both compounds were linear from 0 to 20 μg/ml. The lower limit of the assays has been found to be approximately 0.1 μg/ml for both compounds. Recovery over the range 0.5 to 20 μg/ml averaged 98.2 ± 1.75% for oxipurinol and 99.3 ± 0.93% for allopurinol.

The retention times of oxipurinol, allopurinol and acetaminophen were 9.8, 13.0 and 26.0 min, respectively. The following related compounds were found not to interfere in the assay: xanthine, hypoxanthine, 5-fluorouracil, caffeine, theobromine, theophylline, uric acid and 6-thiouric acid. However, 6-mercaptopurine has the same retention time as allopurinol under the conditions used.

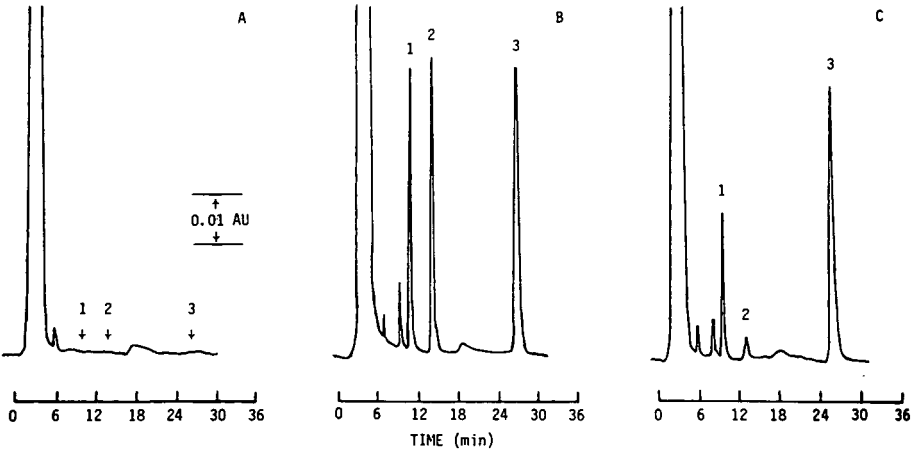

Fig. 1. Chromatograms of (A) blank plasma, (B) standard plasma containing allopurinol and oxipurinol, 10 μg/ml of each and (C) plasma sample from a volunteer subject 4 h after the ingestion of a 300-mg tablet of allopurinol. 1=Oxipurinol, 2=allopurinol; 3=acetaminophen (internal standard).

DISCUSSION

The assay method described in this paper is rapid, sensitive and specific. A representative plot of plasma allopurinol and oxipurinol concentrations vs. time for a volunteer subject following a 300-mg oral dose is shown in Fig. 2 and demonstrates the utility of the method.

Endele and Lettenbauer [1] and Brown and Bye [2] have reported HPLC assays for allopurinol and oxipurinol utilizing anion exchange columns. In each case, 1 ml of plasma was required and samples were subjected either

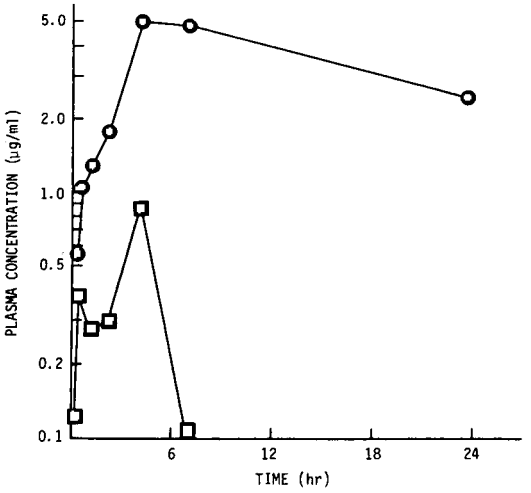

Fig. 2. Plasma allopurinol (▪) and oxipurinol (•) concentration—time curves obtained in a volunteer subject following the ingestion of a 300-mg tablet of allopurinol.

to solvent extraction [1] or column [2] clean-up procedures before chromatography. Our procedure requires no extraction and therefore has fewer steps and requires less time. Further, by use of the appropriate volumes of internal standard and trichloroacetic acid, the assay may be run using as little as 100 μl of plasma, making it appropriate for clinical use even in pediatric patients.

Although the injection of the supernate obtained from trichloroacetic acid-precipitated plasma can potentially clog or contaminate the column, we have found that the use of a 2-μm inline filter (Model 7302, Column Inlet Filter, Rheodyne, Berkeley, Calif., U.S.A.) prevents column damage as evidenced by consistent peak shapes and retention times. Filters are replaced periodically when back pressure increases above normal. In addition, due to the force generated by centrifugation at 8700 *g*, essentially no particulate matter may be detected in the supernate.

A reversed-phase HPLC assay for allopurinol and its metabolite, oxipurinol, in human plasma, has been described. The method may be applied to samples as small as 0.1 ml, has a lower limit of sensitivity of 0.1 μg/ml of either compound, and is suitable for pharmacokinetic and clinical studies.

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Note

Specific determination of dipyridamole in serum by high-performance liquid chromatography

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(Received May 23rd, 1978)

During the last ten years a number of investigators have reported a beneficial effect of dipyridamole treatment of patients suffering from various thromboembolic diseases [1—4]. In some investigations the time lapse of serum concentrations of dipyridamole has been followed using spectrophotofluorimetric methods as described by Beizenhertz et al. [5], Zak et al. [6] and Mellinger et al. [7]. These methods are rather time-consuming, however, and, furthermore, a number of drugs with fluorescence potential may interfere with the analysis. The present paper describes a specific, sensitive and rapid method for the determination of dipyridamole in serum and other biological fluids by means of high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Apparatus

The HPLC system used was a Waters Model 600 liquid chromatograph equipped with a U6K injector, a μBondapak C_{18} column (30 × 0.39 cm I.D.; particle size 10 μm) and a Model 440 dual channel filter absorbance detector in conjunction with a Tarkan W + W 600 recorder.

Reagents

Dipyridamole was purchased from Böhringer-Ingelheim (Copenhagen, Denmark). All other reagents (analytical grade) were obtained from Merck (Darmstadt, G.F.R.). The eluting solvent was a mixture (75:25) of methanol and a 0.02 *M* solution of sodium acetate in water adjusted to pH 4 with acetic acid. Stock solutions of dipyridamole and indomethacin, 5 mg/ml in ethanol, could be stored at - 20° in darkness for up to six months. Two buffer solutions of 1 *M*

tris(hydroxymethyl)aminomethane, one adjusted to pH 8.6 with hydrochloric acid, were used in the extraction procedures. Ethanol (96%, w/v) was used for the protein precipitation of serum samples.

HPLC conditions

A solvent flow-rate of 2 ml/min producing a pressure of approximately 2000 p.s.i. was used in the experiments. The absorbance spectrum of a solution of dipyridamole in the solvent mixture showed a maximum at about 280 nm and consequently filters for this nominal wavelength in conjunction with instrumental absorbance settings of 0.05—0.005 a.u. were used for continuous detection of the compound. A volume of 25 μl of the samples in ethanol or buffered water was injected into the column.

Standard solutions

Dilute standard solutions in ethanol, which were made from the stock solutions, could be kept stable in darkness at 5° for several weeks. In order to estimate the stability in daylight of standards and samples prepared from serum, the decline in concentration of dipyridamole in freshly prepared solutions in 1 *M* Tris buffer (pH 8.6), in ethanol and in ethanol-precipitated serum was followed as a function of time in different types of reagent tubes (polyethylene, polypropylene and ordinary glass).

Sample preparation

Two preparation procedures were investigated.

Procedure A. To 100 μl serum were added 200 μl ethanol and after gentle mixing the sample was allowed to stand for 15 min for protein to precipitate. After further centrifugation at 600 *g* for 5 min, 25 μl of the clear deproteinized supernatant were injected directly onto the column.

Procedure B. To 1 ml of serum was added 1 ml of 1 *M* Tris buffer (pH 8.6) and the mixture was extracted twice with 8 ml diethyl ether. The combined ether extracts were then evaporated under nitrogen to about 2 ml and re-extracted with 230 μl of 0.1 *N* hydrochloric acid. The ether phase was removed and the acid phase was neutralized with 20 μl 1 *M* Tris. Samples of 25 μl were used for injection.

A few experiments on the isolation and determination of dipyridamole glucuronide as described by Zak et al. [6] were also performed. This procedure involves treatment of the serum residual from procedure B with β-glucuronidase and subsequent sample treatment according to procedure B.

In some experiments indomethacin was added to the ethanol phase (A) or to the 0.1 *N* hydrochloric acid phase (B) and used as an internal standard for control of the injection volume, column efficiency and detector response.

Quantitation

Determination of the serum concentration of dipyridamole was carried out as follows. The peak height of the serum sample (P_{se}) was compared to the peak height of a standard (P_{st}) of 1 μg/ml. The calculations related to the sample preparation procedures, A and B, respectively, were as follows:

$$\frac{P_{se} \times 3 \times 0.936}{P_{st}} = \text{serum dipyridamole } (\mu g/ml)$$

and

$$\frac{P_{se}}{4 \times P_{st}} = \text{serum dipyridamole } (\mu g/ml)$$

RESULTS AND DISCUSSION

Sample preparation procedures

One of the main advantages of HPLC in comparison to other chromatographic procedures is the possibility of a simple and quick sample-preparation procedure. Precipitation of serum proteins with ethanol or trichloroacetic acid has previously been found to be an adequate sample preparation procedure, which allows direct injection of the deproteinized serum into the chromatograph without any damaging effect on the column [8]. The ethanol precipitation procedure affords complete recovery of the dipyridamole in serum, contrary to the inconsistent recoveries found by the use of trichloroacetic acid precipitation. However, the precipitation technique involves sample dilution and this method only allows determination of serum concentrations above approximately 0.1 μg/ml. The quantitative extraction procedure used by other investigators [6, 9] was consequently slightly modified by adapting the extraction volumes in a way that allowed determination of much smaller concentrations. The experiments with the isolated dipyridamole glucuronide metabolite showed that the present method can easily be adapted to specific determination of this metabolite. This is of interest in pharmacokinetic investigations.

Chromatographic conditions

Reversed-phase packing compounds have been used in the HPLC determination of a number of different drugs [10], and the μBondapak C_{18} packing compound was chosen for this analysis. The pH and composition of the eluting fluid are important factors in the determination of retention volume and magnitude of detector signal. In the present analysis, the optimum detector signal was obtained with a pH between 4 and 7, and based on its excellent column compatibility a 0.02 *M* acetate buffer adjusted to pH 4 was chosen. Satisfactory retention volumes of 5—15 ml were obtained using 65—75% methanol in the buffer as eluent, and the retention volumes were not influenced by changes in pH. When indomethacin was used as internal standard it was necessary to lower the methanol content of the eluent to obtain complete analytical separation. Fig. 1 shows a chromatogram, developed with 65% methanol in acetate buffer at pH 4, using a flow-rate of 2 ml/min.

Linearity and sensitivity

A standard curve of dipyridamole in the concentration range 0.1—10 μg/ml showed a linear relationship between concentration and peak height (absorbance units) with a slope of 3.319×10^{-3} a.u. per μg, y intercept = 2.4×10^{-5} a.u., and $r = 0.9999$. The coefficient of variation in duplicate serum analyses

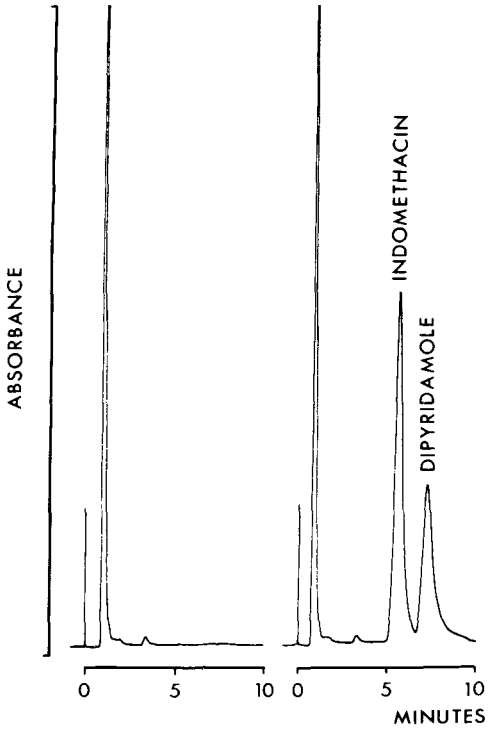

Fig. 1. Left: chromatogram from a normal serum sample treated as described in text (procedure A). Right: chromatogram from a person who had ingested dipyridamole. Indomethacin was added as internal standard (procedure A). Chromatographic conditions as described in the text.

was always below 2%. The detection limit in serum, defined on the basis of the amount of compound injected that caused an absorption of two times the standard deviation of baseline noise, was found to be 0.8 ng of injected compound, corresponding to about 100 ng/ml in procedure A and about 8 ng/ml in procedure B. The analytical sensitivity of the photometric method, expressed as the amount of drug giving a detector response of 0.008 a.u., was calculated to be about 0.016 μg.

Interference and recovery

Interference from naturally occurring substances or other drugs was not found in analyses of serum samples from a number of patients under treatment with dipyridamole. In particular, no interference was found from drugs used in anti-thrombotic therapy, including aspirin, sulfinpyrazone and coumarin anticoagulants. No interference from the dipyridamole glucuronide was found in either of the sample preparation procedures.

Experiments with the addition of known amounts of dipyridamole to serum with subsequent sample preparation according to procedure A or B resulted in complete recovery of the drug. However, the concentration of dipyridamole in the supernatant of deproteinized serum or plasma was constantly found to be 6.8% ± 1.1 S.E. higher than the expected value; this is probably due to dis-

placement of dipyridamole from the denaturated serum protein particles. The recovery of dipyridamole from extracted serum samples was 100.4% ± 0.4 S.E.

Stability

The stability of freshly prepared standard solutions exposed to daylight showed considerable variation. The rate of degradation of dipyridamole seemed to follow a first-order function, and the rate constant was obviously dependent on the light intensity in the laboratory. The maximum degradation rate was found in the solutions with Tris-buffered water, in which the half-time of dipyridamole varied from about 3 to 30 h. The corresponding half-time in ethanol was 12—240 h. Samples prepared with deproteinized serum were stable for several days. No difference between the reagent tubes was found, and there was no evidence of adsorption on the glassware or plastic tubes. These findings strongly indicate that all samples and standards should be protected from light.

Applications

In a number of clinical and experimental studies it has been shown that dipyridamole either alone or in combination with other drugs can be used as an anti-platelet agent in the prevention of thrombo-embolic disease [1—4, 11]. Although dipyridamole is an inhibitor of platelet phosphodiesterase, inhibition of platelet aggregation in vitro has only been demonstrated in concentrations about 10 times the maximum concentration obtained during continuous therapy. This is in contradiction to the strikingly beneficial effect of dipyridamole in the treatment of thrombo-embolic disorders characterized by increased platelet turnover. Recently, Summers et al. [12] have studied the effect of

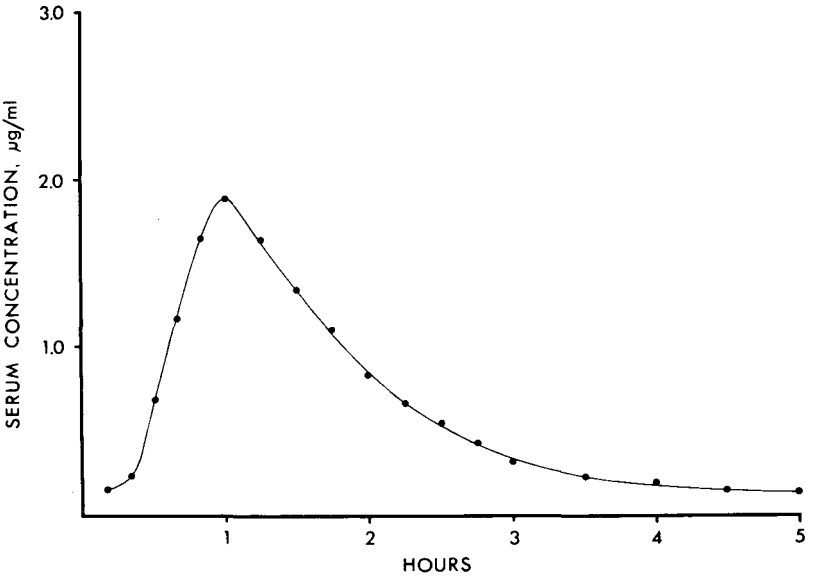

Fig. 2. Serum concentration curve following oral administration of dipyridamole (1.25 mg/kg) to a normal man. Determinations were carried out as described in the text (procedure A).

dipyridamole on [^{14}C]adenosine uptake in human platelets ex vivo, and the results showed a significant inhibition of adenosine uptake which was strongly correlated to the plasma concentration of dipyridamole. Another report [9] has stated a correlation between the dipyridamole plasma level and inhibition of platelet aggregation in patients after isolated mitral valve replacement. But these results seem to be based on an insufficiently described technique and remain to be verified. However, these investigations call for a more detailed description of dipyridamole pharmacokinetics in man. Until now, only inadequate spectrofluorimetric assay methods have been available for the study of dipyridamole plasma concentrations.

The present HPLC method was developed in order to reduce the time required for sample treatment and in order to increase precision and specificity. Fig. 2 shows the serum concentration curve after oral administration of 1.25 mg/kg to a human volunteer. Pharmacokinetic analyses of a number of such experiments [13] have revealed that the drug exhibits first-order kinetics with regard to absorption, distribution and elimination and fits the model of an open two-compartment system.

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Journal of Chromatography, 162 (1979) 104—109
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Note

Simultaneous determination of phenytoin, phenobarbital and their para-hydroxylated metabolites in urine by reversed-phase high-performance liquid chromatography

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(First received March 17th, 1978; revised manuscript received July 20th, 1978)

Phenytoin (DPH) and phenobarbital (PB), alone or in combination, remain the drugs of choice in epilepsy characterized by general convulsive seizures [1]. Both DPH and PB are para-hydroxylated by hepatic smooth endoplasmic reticular enzymes to inactive metabolites which are subsequently conjugated with glucuronic acid and excreted in the urine. Of daily doses, approximately 60—70% of DPH and 25—80% of PB appear in the urine as glucuronidated 5-(*p*-hydroxyphenyl) 5-phenylhydantoin (HPPH) and 5 ethyl 5-(*p*-hydroxyphenyl) barbituric acid (pHPB) respectively [2,3]. Less than 4% of DPH and 30% of PB is excreted unchanged in the urine.

Urinary excretion data provide a non-invasive method of assessing the overall elimination constant for loss of drug from the body, as well as the rate constants for the production of metabolites [4]. Analytical systems applicable to the measurement of urinary DPH and PB should permit simultaneous quantitation of both unchanged drugs and metabolites. Gas—liquid chromatographic (GLC) techniques for the analysis of PB are characterized by poor reproducibility and non-linear calibration curves due to peak tailing as a result of irreversible or reversible adsorption to column packing. Attempts to circumvent these problems by derivatization have resulted in some improvement, though derivative formation may be incomplete and column degradation occurs with methylating agents [5]. The analysis of HPPH and pHPB by GLC has also been reported and could be subjected to the same criticism [6,7]. Radio-immunoassay methods have been utilized for DPH and PB determinations but recently pHPB has been shown to interfere substantially with the quantitation of PB in one

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such system [8]. High-performance liquid chromatography (HPLC) circumvents many of the problems associated with GLC determinations, such as thermal degradation, prerequisite derivatization and laborious extractions. DPH, PB and HPPH have been successfully quantitated in urine by normal as well as reversed-phase methods [9—11]. The simultaneous determination of DPH, PB and HPPH in serum has recently been reported but PB and HPPH were poorly resolved [12]. This lack of resolution could invalidate quantitation of HPPH in urine where PB levels may greatly exceed HPPH and the degree of interference would depend on the amount of PB present. Residues of pHPB have not been evaluated in these or other HPLC systems and the simultaneous quantitation of pHPB, PB, HPPH and DPH by HPLC has not been reported. A reversed-phase HPLC technique has been developed which allows the simultaneous determination of pHPB, PH, HPPH and DPH in biological fluids by use of an inexpensive fixed wavelength liquid chromatograph.

MATERIALS AND METHODS

Apparatus

Chromatography was performed on a component system constructed from a Model 110 solvent-metering pump (Altex Scientific, Berkeley, Calif., U.S.A.), a Spectra Physics Model 3100 liquid chromatograph (Chromatronix, Berkeley, Calif., U.S.A.) equipped with a Model 230 dual channel UV absorbance detector and a 20-μl loop sample injection valve. The column, 300 × 3.9mm I.D., was packed with 10 μm μBondapak C_{18} (Waters Assoc., Milford, Mass., U.S.A.) The mobile phase was a 40:60 (v/v) mixture of methanol—NaH_2PO_4 (0.025 *M*) adjusted to pH 8. The column effluent was monitored at 0.01 and 0.04 a.u.f.s. at 254 nm and the flow-rate was 1.0 ml/min.

Reagents

Methanol and ethyl acetate, redistilled ACS reagent grade, were obtained from Caledon (Georgetown, Canada). All other chemicals were reagent grade.

Standards

Phenacetin (acetophenetidine), the internal standard, and phenytoin (sodium 5-5 diphenylhydantoin) were purchased from Sigma (St. Louis, Mo., U.S.A.), HPPH (5-(*p*-hydroxyphenyl) 5-phenylhydantoin) from Aldrich (Milwaukee, Wisc., U.S.A.) and phenobarbital from Allen Hanbury (Toronto, Canada). The pHPB (5-ethyl 5-(*p*-hydroxyphenyl) barbituric acid) was a generous gift from Hoffman-La Roche (Montreal, Canada). Stock solutions (10 mg/ml) of DPH, PB, HPPH and pHPB were prepared by dissolving each in 10 ml of methanol. Standard spiking solutions containing 100, 200, 400, 600 and 1000 μg/ml of PB, DPH, HPPH and pHPB were prepared by diluting aliquots of the stock solutions to 10 ml with methanol. A stock solution of phenacetin (4mg/ml), the internal standard, was prepared in methanol and diluted 1:10 for extractions. Calibration standards were prepared from 1:10 dilutions of the spiking solutions and 1:100 dilutions of the stock internal standard solution. These standard solutions were stable for at least 1 month at 4°.

Procedure

Aliquots of the total collection period urine volume were stored at -20° or analyzed directly as follows. Samples (2ml) of urine and 2.0 ml of 12 *M* HCl were heated in loosely-capped round-bottomed centrifuge tubes for 120 min at 90°. After cooling to room temperature, 2.0 ml of 12 *M* NaOH were added and the acidic urine mixture adjusted to pH 7 with 20% NaOH. Following the addition of 0.2 ml of internal standard and 2.0 ml of 0.10 *M* phosphate buffer (pH 6.3), the parent drugs and metabolites were extracted with 10 ml of ethyl acetate on a serological shaker rotating at 40 rpm for 15 min. After centrifugation at 600 *g* for 10 min, the organic layer was transferred to a graduated conical drying tube. The ethyl acetate extraction was repeated and the combined organic levels evaporated to dryness under a stream of air in a Multi-Bloc heater at 50°. The extracted residue was redissolved in 2.0 ml of methanol and 20 μl was injected into the liquid chromatograph. The peak height ratios of pHPB:internal standard, PB:internal standard, HPPH:internal standard and DPH:internal standard were calculated.

Calibration and recovery

A standard curve was constructed by adding known amounts of pHPB (10—60 μg/ml) to drug-free urine. Extraction efficiency was determined by adding the internal standard to ethyl acetate layer residues from urine containing known amounts of pHPB, PB, HPPH and DPH. Efficiency was calculated by comparing the ratios of pHPB:internal standard added to ethyl acetate layer residues to ratios of pHPB:internal standard obtained from calibration solutions. Similarly, PB, HPPH and DPH extraction efficiencies were determined.

RESULTS AND DISCUSSION

Optimum separation was dependent upon achieving adequate retention of pHPB, minimizing the retention of DPH and resolving PB and HPPH. Retention times for pHPB, HPPH, and DPH were found to be mainly influenced by the methanol content of the eluent, while PB retention was more sensitive to changes in pH. From plots of retention time versus eluent methanol concentration, a 40:60 (v/v) mixture of methanol—NaH_2PO_4 (0.025 *M*) buffer was selected. The retention times for pHPB, PB, HPPH, S (phenacetin) and DPH were 5.3, 9.2, 11.4, 14.2 and 28.5 min respectively (Fig. 1A). Resolution of PB and HPPH was only achieved at pH 8 or above. Since silica dissolves at about pH 8, leading to column degradation with loss of the stationary liquid phase, and resolution and PB and HPPH are not sufficiently resolved below pH 8 maintenance of a pH of 8 is imperative. Using a potassium phosphate—acetonitrile eluent at pH 8, Soldin and Hill [13] reported the separation of parent anticonvulsants extracted from serum but not their metabolites. We have not found column deterioration at pH 8 during 3 months of continual use, results in agreement with Soldin and Hill [13]. The separation factor for PB and HPPH with the methanol—NaH_2PO_4 (0.025 *M*) eluent was found to be 1.35 compared to 1.14 previously reported for a 17:83 mixture of acetonitrile and water [12]. This difference may seem insignificant until the band size ratio of PB and HPPH at equal concentrations is considered. Under these conditions

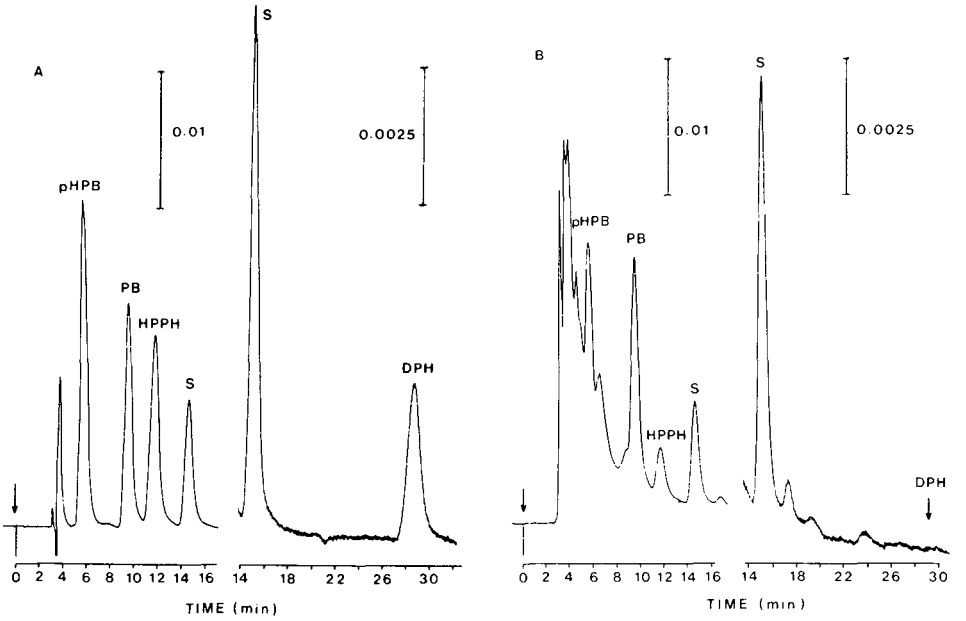

Fig. 1. High-performance liquid chromatogram of phenobarbital (PB), diphenylhydantoin (DPH) and the para-hydroxylated metabolites, hydroxyphenobarbital (pHPB) and 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (HPPH). The internal standard (S) was phenacetin. Trace A shows a typical chromatogram for the reference standard containing 40 μg/ml of pHPB, PB, HPPH and DPH measured at 0.04 or at 0.01 a.u.f.s. due to the low sensitivity of DPH. Trace B shows a chromatogram of a urine sample from a female patient containing, on analysis, 19 μg/ml pHPB, 59.5 μg/ml PB and 14.8 μg/ml HPPH. DPH was below the limit of detection at 0.01 a.u.f.s.

we found a band size ratio of 1:1—2:1 and a resolution of approx. 1.1—1.25 compared with 3:1—4:1 and an estimated resolution of 0.8—1.0 from the previous method [12]. Since a resolution of 1.0 is required for accurate peak height measurements, PB could interfere with the quantitation of serum HPPH in the acetonitrile—water system where HPPH levels are lower than PB as well as in urine where variable relative proportions of PB and HPPH may exist.

The response of the detector at 254 nm to DPH was much less than for pHPB, PB and HPPH but dual channel capability permits the simultaneous monitoring at 0.01 a.u.f.s. of DPH and at 0.04 a.u.f.s. of pHPB, PB and HPPH at levels found in clinical samples of serum and urine. The internal standard concentration of phenacetin (4 μg/ml of urine) was selected to retain detector response within scale at each of the detector settings, enabling peak height ratio quantitation of each of the compounds simultaneously. The detection limits for pHPB, PB, HPPH and DPH were 1.5 μg/ml, 2.0 μg/ml, 2.0 μg/ml and 5 μg/ml respectively. The sensitivity of the detector was not sufficient to measure free DPH in urine but would be suitable for determinations in serum. The 254 nm detection wavelength was selected because it is usually available in low cost detectors and is more selective than the 195 nm wavelength used in a previous study [12].

TABLE I

LINEAR REGRESSION PARAMETERS FOR STANDARD CALIBRATION CURVES

n=20.

Drug	Range (μg/ml)	Slope	Intercept	Correlation coeff.
pHPB	10— 60	0.067	-0.08	0.9987
PB	10—100	0.039	-0.03	0.9945
HPPH	10—100	0.036	-0.02	0.9995
DPH	10—100	0.007	0.00	0.9979

Standards in methanol were analyzed and from these data standard curves were constructed by plotting peak height ratio (drug:internal standard, y axis) versus drug concentration (μg/ml). Each point on the curve was the mean of at least 5 determinations. The relationships were linear for all compounds as summarized in Table I and standards prepared in urine gave similar results.

Extraction efficiencies of urine solutions containing pHPB, PB, HPPH and DPH were 90% (coefficient of variation, C.V., 3.5), 81% (C.V. 8.2), 83% (C.V. 3.8) and 102% (C.V. 6.0) respectively. Ethyl acetate solvent extraction of urine is convenient because of its relative density and volatility. Occasionally in samples of male urine, a small peak was observed to immediately precede the elution of PB, but no substantial interference was found.

A diol metabolite of DPH, 5-(3,4-dihydroxycyclohexa-1, 5-dienyl)5-phenylhydantoin occurs to the extent of about 10—20% in the urine of male volunteers under steady conditions of DPH [14]. Acid hydrolysis of the glucuronide of this diol has been shown to produce dehydration of the metabolite to give equal amounts of the 30H and 40H isomers of HPPH and the latter would interfere with the assay [15]. Procedures involving pre-extraction with isoamyl alcohol to remove this metabolite prior to hydrolysis have been described, but since pHPB and HPPH are known to be conjugated with glucuronic acid, enzymatic cleavage with β-glucuronidase would seem to be more appropriate [16]. Since our primary objective was to describe a chromatographic system capable of the simultaneous separation of both the parent drugs and their major metabolites, our extraction procedure did not include provision for removal of the diol metabolite.

Fig. 1B depicts a chromatogram of a patient's urine that contained, per ml, 19 μg of pHPB, 59.5 μg of PB and 14.8 μg of HPPH. DPH, if present, was below the detection limits of the assay. Day-to-day precision calculated from assays of a single sample containing 40 μg/ml of each compound on 10 consecutive days was for pHPB, PB, HPPH and DPH; 39.0 ± 1.18 μg/ml (C.V. 3.0), 41.5 ± 4.7 μg/ml (C.V. 11.4), 40.7 ± 1.39 μg/ml (C.V. 3.4) and 42.8 ± 0.48 μg/ml (C.V. 1.1) respectively.

The effects of concomitant administration of PB and DPH on drug metabolism are variable. Clinical studies have shown that PB increased plasma DPH levels by interfering with the formation of HPPH [17]. Conversely in other studies, DPH levels were found to rise following cessation of PB [18]. Investi-

gations utilizing the simultaneous quantitation of these compounds in urine and serum should permit evaluation of the factors affecting the dose-dependent kinetics of DPH, genetic and environmental contributions to hydroxylation, as well as the equivocal effects of simultaneous DPH and PB administration. The sensitivity of this method should permit investigation of factors affecting the hydroxylation of PB and DPH through the convenient and reproducible measurement of the parent drugs and their para-hydroxylated metabolites in biological fluids at levels encountered in clinical samples.

ACKNOWLEDGEMENTS

We thank Dr. J. Gray, Dalhousie University, Halifax, N.S. and D. Shaw, McGill University for providing urine samples from hospitalized patients. This work was supported by Grant MA-3611 from the Medical Research Council of Canada.

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Note

Determination of nitrofurantoin (Furadantine®) and hydroxymethylnitrofurantoin (Urfadyn®) in plasma and urine of man by means of high-performance liquid chromatography

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(Received April 4th, 1978)

Nitrofuran derivatives are highly effective chemotherapeutic drugs in the treatment of chronic pyelonephritis and other infections of the urinary tract [1]. The effectiveness of the treatment is influenced by the urinary pH, the concentration of the drug in the urine and the nature and concentration of the bacteria [1—3]. The widely used colorimetric method of Conklin and Hollifield has a limit of sensitivity of 2 μg/ml [4,5]. This does not allow accurate measurement of plasma concentrations, and also lacks specificity with regard to possible metabolites [6]. For the purpose of the study of bioavailability of nitrofuran derivatives from their pharmaceutical preparations, a high-performance liquid chromatography (HPLC) method was developed. This method enables the determination of plasma concentrations as low as 0.02 μg/ml and therefore can be used for study of the bioavailability of these drugs and their rate of dissolution as a function of their pharmaceutical formulation. Pharmacokinetic parameters of these drugs such as half-lives, renal clearance constants and other distribution parameters may be obtained in this way.

MATERIALS AND METHODS

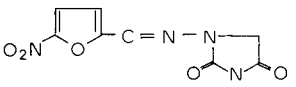
connected to a 1 mV recorder (BD7; Kipp & Zonen, Delft, The Netherlands). A stainless-steel column, 15 cm × 4.6 mm I.D. packed with LiChrosorb RP-8, particle size 5 μm, was used. An injection loop of 100 μl was used. The detection of the nitrofurantoin and its derivatives was performed at 370 nm, the detection limit being 0.02 μg/ml.

Solvent

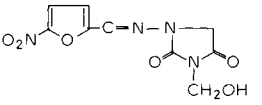
The solvent was a mixture of water with 5% ethanol. The solvent flow accounted for 1.6 ml/min at a pressure of 174 atm.

Drugs

Nitrofurantoin, nitrofurazolidone and nitrofural were from Norwich Benelux (Utrecht, The Netherlands), hydroxymethylnitrofurantoin was from Inpharzam (Amsterdam, The Netherlands) and metronidazole was obtained from Specia (Amstelveen, The Netherlands).



NITROFURANTOIN



HYDROXYMETHYLNITROFURANTOIN

Subjects

Six healthy volunteers, all employees of the Department of Clinical Pharmacy participated in this study. Nitrofurantoin was administered in a dose of 100 mg (0.42 mole) as Furadantine® tablets. Hydroxymethylnitrofurantoin was administered in a dose of 120 mg (0.45 mole) Urfadyn® tablets. The drug was taken orally in the morning, 1.5 h after a standard breakfast of two slices of bread and a cup of tea [6,7].

Blood samples of 0.2 ml were collected at scheduled time intervals by fingertip puncture (Microlance No. 433, Becton-Dickinson). Spontaneously voided urine was collected over 15 h. The pH of the urine was maintained alkaline (pH 7—8) in the volunteers by the regular intake of 10 g of sodium bicarbonate per day. An acidic urine pH was reached by the intake of 8 g of ammonium chloride per day (pH 5—6) [8,9].

Sample preparation

Plasma. A 10-μl volume of human plasma is mixed thoroughly on a Vortex mixer with 0.5 ml of perchloric acid (0.33 *N*). After centrifugation at 2600 *g* for 5 min (Heraeus Christ centrifuge), 100 μl of the supernatant is injected onto the column. A calibration curve is produced by adding known concentrations of either nitrofurantoin or hydroxymethylnitrofurantoin to blank human plasma.

Urine. A 10-μl volume of human urine is mixed on a Vortex mixer with 0.5 ml of perchloric acid (0.33 *N*). A 100-μl aliquot of the solution is injected onto the column.

Sample stability

An aqueous solution of nitrofurantoin appeared to be unstable on standing at room temperature. Fig. 1 shows the degradation of nitrofurantoin in a urine sample, diluted with water or with perchloric acid. After 4 h of standing at room temperature only 50% of the peak height related to a freshly extracted sample could be measured, also the peak shape of nitrofurantoin was altered (peak broadening). This phenomenon may be due to photochemical degradation of the compound in solution at room temperature, which leads to compounds with only a slightly altered retention time, but with a reduced molar extinction. Therefore all blood and urine samples were processed upon receipt and within 5 min of preparation of the samples for injection. Blood and urine samples remained stable when stored at - 20° [10].

Recovery

Recovery of nitrofurantoin and hydroxymethylnitrofurantoin added to human plasma in the concentration range of 0.02—10 μg/ml was found to be 92 ± 4 % S.D. The recovery of nitrofurantoin and hydroxymethylnitrofurantoin added to urine was 100 ± 2 % S.D.

RESULTS

Nitrofuran derivatives are well separated from each other and from the structurally related metronidazole (Flagyl®), as can be seen from Fig. 2 and Table I. Chromatograms of blanks did not show any interfering substances from plasma

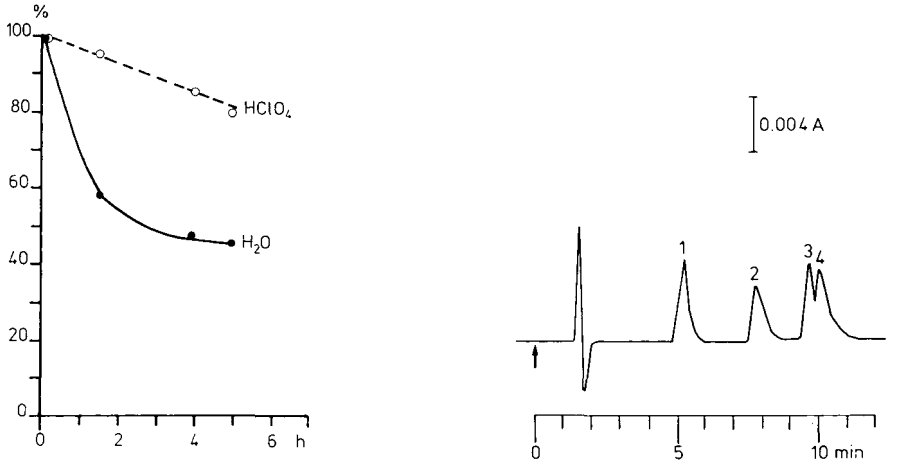


Fig. 1. Instability of nitrofurantoin solutions in water or perchloric acid upon standing at room temperature. The peak height of the nitrofurantoin in the chromatogram obtained after standing is expressed as a percentage of the peak height of a freshly extracted sample.

Fig. 2. High-performance liquid chromatogram of a mixture of nitrofuran derivatives. Column, LiChrosorb 5 RP-8; solvent, 5% ethanol in water; solvent flow, 1.6 ml/min, 1 = metronidazole, 2 = nitrofurantoin and hydroxymethylnitrofurantoin, 3 = nitrofural, and 4 = nitrofurazolidone.

and urine at the wavelength of detection, 370 nm. Within the indicated concentration ranges for plasma and for urine a linearity between peak height ratio and concentration could be established (r = 0.99).

The pharmacokinetic parameters of nitrofurantoin and hydroxymethyl-

TABLE I

RELATIVE RETENTION TIMES OF NITROFURANTOIN, HYDROXYMETHYLNITROFURANTOIN AND SOME RELATED COMPOUNDS

Column, LiChrosorb 5 RP-8; solvent, water + 5% ethanol; solvent flow, 1.6 ml/min.

Compound	Retention time relative to the unretained compound (K')
Metronidazole (Flagyl®)	3.21
Nitrofurantoin (Furadantine®)	4.72
Hydroxymethylnitrofurantoin (Urfadyn®)	4.72
Nitrofural (Nitrofurazone®, Furacine®)	5.88
Nitrofurazolidone (Furoxone®)	6.12

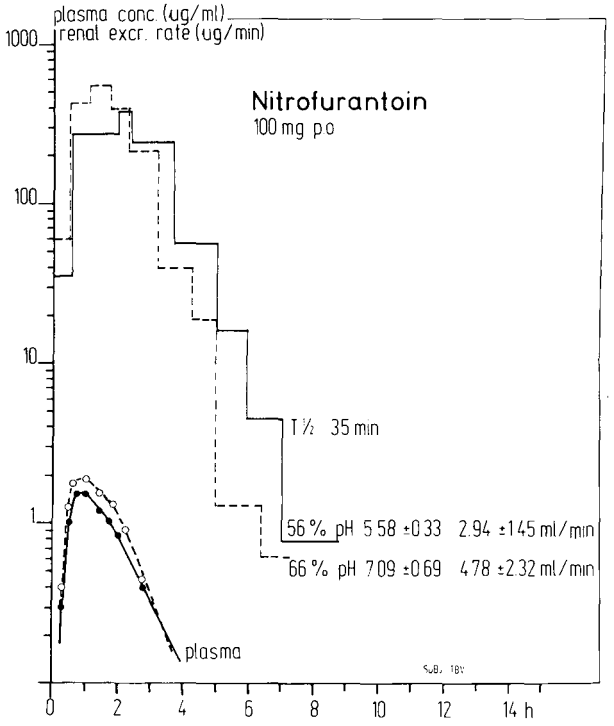


Fig. 3. Plasma concentration (μg/ml) and renal excretion rate (μg/min) of nitrofurantoin in man after administration of 100 mg (Furadantine orally. Note that in the same subject under alkaline urinary conditions the absorption and renal excretion rate are somewhat higher than under acidic urinary conditions (Table II).

TABLE II

SOME PHARMACOKINETIC PARAMETERS OF NITROFURANTOIN AND HYDROXYMETHYLNITROFURANTOIN IN MAN

Compound	Subjects (*n*)	Urine pH	Bodyweight (kg)	Dose (mg)	Amount excreted unchanged (%)	Plasma t½ (min)	Maximum plasma concentration (μg/ml)	Maximum renal excretion rate (μg/min)
Nitrofurantoin	6	5—7	63.7 ± 4.5	100	70.4 ± 20.8	45 ± 15	2.33 ± 0.42	466 ± 106
Hydroxymethyl-nitrofurantoin	6	5—7	63.7 ± 4.5	120	65.4 ± 15.6	45 ± 10	1.20 ± 0.30	360 ± 55

nitrofurantoin in man under either alkaline or acidic urinary pH conditions reveal only small differences. Fig. 3 shows that nitrofurantoin in one volunteer is eliminated with the same half-life of 35 min under alkaline as well as acidic urinary pH conditions. The total amount excreted under alkaline conditions is 66% while in the same subject this figure under acidic urinary conditions was 56%.

Hydroxymethylnitrofurantoin is excreted with almost the same half-life ($t_{1/2}$ = 30 min) as nitrofurantoin (Fig. 4). The amount of hydroxymethylnitrofurantoin excreted unchanged under alkaline conditions was somewhat less (54%) than under acidic conditions (60%). These differences are within the variation when the amounts totally excreted in the whole group of volunteers (Table II) are considered. The renal clearance (K_r) of both compounds is not dependent on the urinary pH and the urine flow, and shows a linear relationship between the renal excretion rate and plasma concentration, indicating that the excretion process is a linear one.

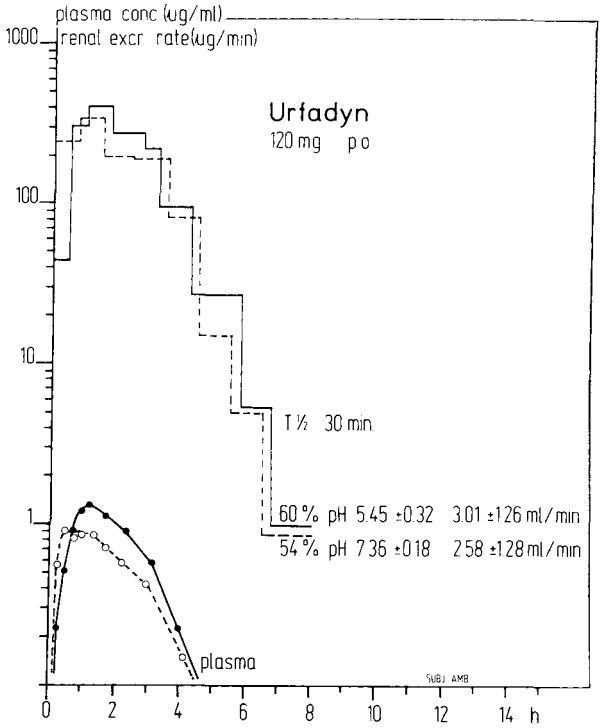


Fig. 4. Plasma concentration (μg/ml) and renal excretion rate (μg/min) of hydroxymethylnitrofurantoin in man after administration of 120 mg Urfadyn orally. Note that in the same subject under acidic urinary conditions the absorption and renal excretion are slightly higher than under alkaline urinary conditions (Table II).

DISCUSSION

The HPLC method of determining the nitrofuran derivatives has the advantages over the colorimetric method of higher sensitivity, specificity and rapidity, allowing the measurement of plasma concentration in the therapeutic range. A limit of sensitivity of 0.2 μg/ml was already reached with the improved spectrophotometric Hyamine 10 X method [11], and a recently published alternative HPLC method [10] had reached the limit of 0.02 μg/ml. However, this method [10] required 0.2 ml of biological fluid, which can only be obtained by venipuncture. The present method needs only 0.01 ml of plasma allowing fingertip puncture. The advantage of the present method over the method of Aufrère et al. [10] furthermore is its rapidity, its ease of handling during sample preparation and also the possibility of determining, with no modification, the structurally-related drug metronidazole (Flagyl).

The simplicity of the method makes it useful for studies on the bioavailability of the drug [12], for routine monitoring, e.g., for drug compliance studies, and of course for the determination of pharmacokinetic parameters. Influence of renal impairment on plasma concentration and renal excretion rate can be studied in this way. The difference in chemotherapeutic behaviour with changing pH of the urine [2,3,9] is not related to differences in pharmacokinetic behaviour as renal excretion rates and half-lives are almost identical under alkaline and acidic urinary conditions. Derivatives and pharmaceutical formulations have been produced with the prospect of reduced side-effects, especially in the stomach.

The clinically observed differences in tolerance and therapeutic efficiency of the various marketed products have stimulated the investigation of the relative bioavailabilities of hydroxymethylnitrofurantoin and nitrofurantoin from their pharmaceutical formulation. This method is appropriate to such pharmacokinetic bioavailability studies of different nitrofuran-derivatives and pharmaceutical formulations, and the results of the study will be published elsewhere, together with its clinical implications.

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Note

Determination of metronidazole and tinidazole in plasma and feces by high-performance liquid chromatography

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(Received May 11th, 1978)

Metronidazole (Fig. 1a) is an antimicrobial agent used for treatment of some

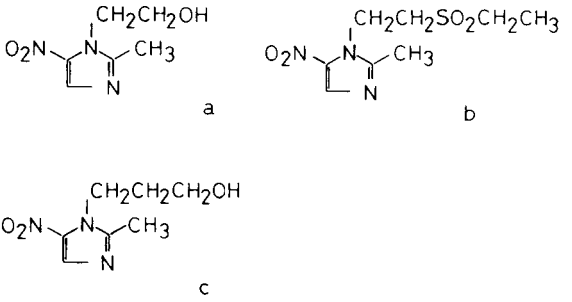

Fig. 1. Structural formulae: a = metronidazole; b = tinidazole; c = 1-(3-hydroxypropyl)-2-methyl-5-nitroimidazole, internal standard.

infectious diseases, mainly infections caused by *Trichomonas vaginalis*, *Endamoeba histolytica* and *Giardia lamblia*. The drug tinidazole (Fig. 1b) has similar indications. These two drugs have been assayed by biological polarographic [1, 2] and colorimetric [3,4] methods. For metronidazole, gas chromatographic methods have been described [5,6]. The liquid chromatographic (LC) method described here was developed in connection with a clinical trial on Crohn's disease with metronidazole. The reason was that the biological assay tended to give lower values after storage of frozen samples and was not sensitive enough for many of the samples. The polarographic method was too unspecific. The

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gas chromatographic methods were considered too tedious, because relatively large solvent volumes had to be handled, and too insensitive due to background interference.

The LC method is based on a reversed-phase system and UV detection. Both metronidazole and tinidazole are analysed on the same system and either may be used as internal standard for the other. In the present method 1-(3-hydroxypropyl)-2-methyl-5-nitroimidazole (Fig. 1c) was also used as internal standard for metronidazole.

During the preparation of this paper an assay for tinidazole in plasma was published using high-performance liquid chromatography (HPLC) [7]. This assay uses no internal standard and the sensitivity is about twenty times less than in the method described here. One reason for the difference in sensitivity may be that the chromatographic system is not very suitable for tinidazole which is indicated by the broad tailing peak in the chromatogram published.

EXPERIMENTAL

System

The liquid chromatograph used consisted of a pump Model M 600, a U6K injector from Waters Assoc. (Milford, Mass., U.S.A.) and a UV detector Model 770 from Spectra-Physics. The column (0.15 m × 4.6 mm I.D., stainless steel) was slurry-packed with Spherisorb S5 ODS (particle size 5 μm). The mobile phase consisted of 0.01 *M* phosphate buffer (pH 5.5) mixed with 15% acetonitrile operated at a flow-rate of 1 ml/min at room temperature (ca. 20—22°). The detector wavelength setting was 320 nm.

Extraction

Plasma. To 100 μl of plasma in a 10-ml screw-capped tube were added 0.5 ml 0.1 *M* carbonate buffer, pH 9, and 100 μl internal standard solution. The internal standard solution contained 10 μg/ml of 1-(3-hydroxypropyl)-2-methyl-5-nitroimidazole or 15 μg/ml of tinidazole when metronidazole was analysed and 10 μg/ml of metronidazole when tinidazole was analysed. The extraction took place on a shake board (15 min) with ether—methylene chloride (1.5:1, 2 × 3 ml) followed by centrifugation (500 *g*, 10 min). The extracts were combined and evaporated in a stream of nitrogen. The residue was dissolved in 250 μl of the eluent mixture. After filtration through a pasteur pipette containing fine glass wool, 20 μl was injected into the liquid chromatograph.

Feces. To 20 mg of freeze-dried feces was added 0.5 ml 0.1 *M* carbonate buffer, pH 9, and the mixture was exposed to ultrasonic treatment for 5 min. Extraction was carried out as above except that an exact volume of the organic phase was evaporated. The residue obtained after evaporation of the solvents was dissolved in 250 μl of the eluent mixture. The solution was filtered through a pasteur pipette packed in the following order: fine glass wool, Celite 10 mg (Johns-Manville, Lompoc, Calif., U.S.A.) and glass wool in the narrow part of the pipette. 40 μl were injected into the liquid chromatograph.

Preparation of 1-(3-hydroxypropyl)-2-methyl-5-nitroimidazole internal standard (IS)

1,3-Propane-diol was monobenzylated and reacted with *p*-toluene sulfonyl-chloride [8]. The resulting benzyloxypropyl *p*-toluene sulfonate was reacted with 2-methyl-5-nitroimidazole [9]. After removal of the benzyl group, the hydrochloride of the IS was precipitated in ethanol with ether—HCl and filtered off.

RESULTS

The recovery of 5 μg/ml of metronidazole, tinidazole or the IS after extraction from plasma was slightly above 90% for metronidazole and almost quantitative for tinidazole and IS.

The reversed-phase system used gave a low background chromatogram with no interfering peaks. Fig. 2a shows the chromatogram obtained from the analysis of a plasma sample containing 5 μg/ml of metronidazole and IS. Fig. 2b shows the chromatogram obtained from the analysis of a plasma sample containing 5 μg/ml of tinidazole. Fig. 2c shows the analysis of a blank plasma sample. IS was preferred as internal standard in the analysis of metronidazole because of its structural similarity and close retention time which made the analysis relatively fast.

Since the output of the UV detector is linear in absorbance units, the area under the peak is proportional to the concentration. Two calibration graphs were constructed for metronidazole by analysing samples to which 0—16 μg/ml of metronidazole had been added and plotting the peak area ratios (metronidazole:internal standard) versus the concentration. For one graph IS was used as internal standard and for the other tinidazole. Both graphs were linear and passed through the origin. The standard deviation was determined by analysing 8 samples to which 7.5 μg/ml of metronidazole had been added and was found to be about 1.5% with either of the internal standards.

Plasma samples were prepared in order to evaluate the lower limit for quantification of metronidazole and it was found to be about 25 ng/ml provided 50%

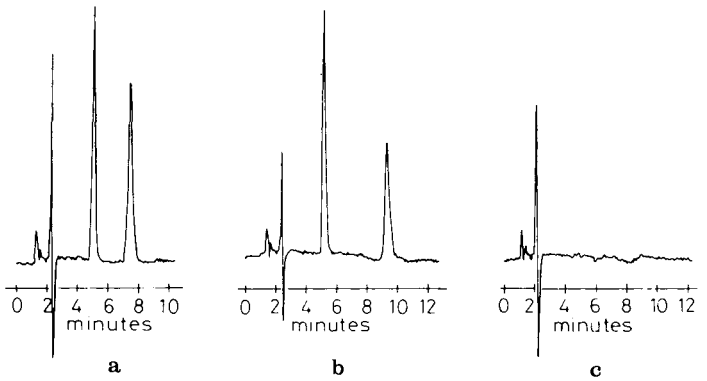

Fig. 2. Chromatograms showing; (a) plasma sample containing metronidazole and IS, (b) plasma sample containing metronidazole and tinidazole, (c) blank plasma sample.

of the extracted amount was injected into the liquid chromatograph.

A calibration graph for the plasma determination of tinidazole was prepared by the same procedure as that for metronidazole. The standard deviation was 3% (5 samples, concn. = 7.5 μg/ml). The response of the detector to tinidazole was about half that of metronidazole.

The determination in feces was only carried out with metronidazole and no internal standard was used. The chromatograms in Fig. 3a and b result from the analysis of a feces sample containing 49 ng/mg metronidazole and a blank feces sample respectively (aliquot of sample injected in Fig 3b, 2.7 times larger than that in 3a). The chromatograms of feces samples were surprisingly free of background peaks. Extra peaks sometimes appeared but in about 50 different analyses none have interfered with the metronidazole peak. The calibration graph for feces determinations was constructed by adding 0—500 ng in 500 μl water to 20-mg samples of freeze-dried feces and exposing them to ultrasonic treatment for 10 min. They were then allowed to stand for 20 min before the analysis described above. The peak areas obtained were plotted against the concentration. The standard deviation was determined by analysing 6 samples containing 10 ng/mg of dry feces and was found to be 2.6%.

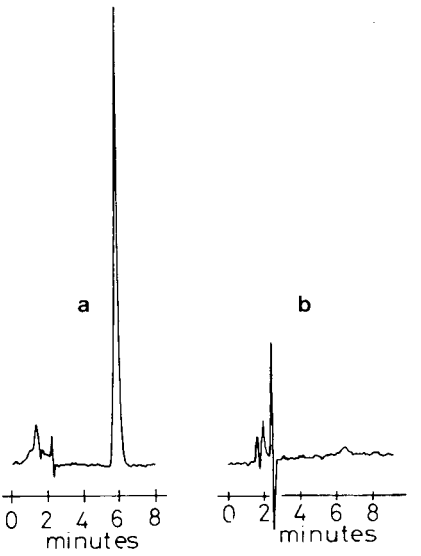

Fig. 3. Chromatograms showing; (a) feces sample containing metronidazole, (b) blank feces sample.

ACKNOWLEDGEMENT

The authors wish to express their appreciation to Mr Sigurd Eriksson for his skillful drawing of the figures.

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Note

Determination of ketoprofen in plasma and urine by high-performance liquid chromatography

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(Received April 17th, 1978)

The determination of the non-steroidal anti-inflammatory agent ketoprofen, 2-(3-benzoylphenyl)propionic acid, in plasma and urine is necessary in order to study its absorption and excretion in patients with rheumatoid arthritis. Previously described methods for the analysis of ketoprofen are time-consuming and lack sensitivity. They include gas—liquid chromatography (GLC) [1], thin-layer chromatography (TLC) plus GLC [2] and TLC plus UV spectrophotometry [3, 4]. We have found that high-performance liquid chromatography (HPLC) requires minimal sample preparation time and can determine a minimum quantity of 2.5 ng ketoprofen in an analytical time of 4 min.

EXPERIMENTAL

Extraction

Urine and plasma samples are acidified to pH 1 with hydrochloric acid (conc.) and stored at -11°. For total ketoprofen in urine, the ketoprofen glucuronide is hydrolysed at pH 1 at 98° for 30 min. It was found that hydrolysis is complete and the ketoprofen is stable under these conditions. The internal standard, oxyphenbutazone 500 μg, is added to 4 ml of the hydrolysed urine, and diluted to 10 ml with distilled water. For the free ketoprofen content of urine, the hydrolysis stage is omitted.

For total ketoprofen in plasma, a 2-ml sample plus 250 μg of oxyphenbutazone is made up to 5 ml with distilled water and extracted with 5 ml diethyl ether by vortex mixing for 1 min, followed by centrifugation.

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Chromatographic analysis

A constant pressure pump (Haskel DST-122) was used to deliver eluent to a column (50 × 4.6 mm I.D.) slurry-packed with Spherisorb-5 ODS (Particle size 5 μm; Phase Separations, Queensferry, Great Britain). An injector valve (Specac, Orpington, Great Britain) fitted with a 5-μl internal sample loop was used. Detection was at 260 nm using a variable wavelength UV monitor (CE-212, Cecil Instruments, Cambridge, Great Britain) at a range of 0.05 to 0.02 a.u. f.s. The eluent was 35% methanol in distilled water at pH 3.5 with acetic acid at a flow-rate of 2 ml/min. Ketoprofen and oxyphenbutazone gave capacity factors of 6 and 8, respectively. Calibration graphs of peak height ratio (ketoprofen to oxyphenbutazone) to ketoprofen concentration in the range 1.5 to 25 μg/ml were constructed, from 3 to 5 replicate injections of all solutions.

RESULTS AND DISCUSSION

The determination of ketoprofen has been studied when dissolved in the HPLC mobile phase, in diluted urine, in ether extract of diluted urine, in diluted plasma and in ether extract of diluted plasma (Fig. 1). All the calibrations were linear and statistically identical with correlation coefficients (r) between 0.995 and 0.999, so that experimental factors influence the analytical method chosen.

For urine samples, direct analysis of diluted urine is preferred since ether extraction is not necessary. All the components of urine are eluted before ketoprofen so that no residues accumulate on the column. The mean extraction recovery is 101.1% (n = 18) with a limit of error (at 95% degree of confidence) of ± 1.5%. It is a very rapid and simple technique capable of estimating both free drug and glucuronide levels. A rheumatoid patient on 300 mg ketoprofen daily excreted 29.1% free and 30.3% as the glucuronide over 24 h in the urine.

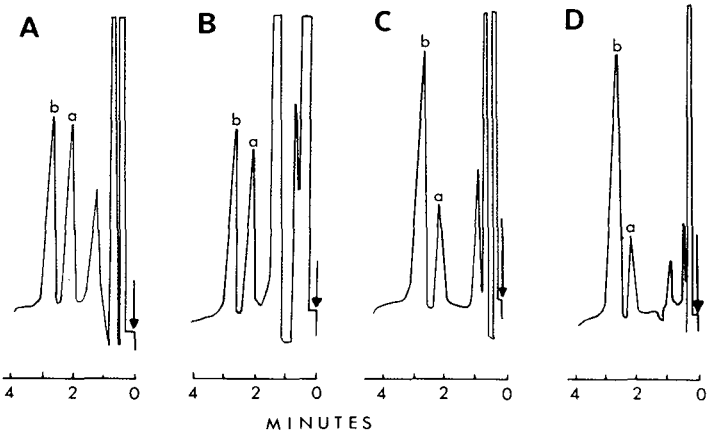

Fig. 1. HPLC of internal standard, oxyphenbutazone (a) and ketoprofen (b) contained in urine and plasma collected from rheumatoid patients. Eluent: 35% methanol, pH 3.5, flow-rate 2 ml/min. Column: 50 × 4.6 mm, Spherisorb-5 ODS. UV detection at 260 nm. A = diluted urine; B = ether extract of diluted urine; C = diluted plasma; D = ether extract of diluted plasma.

For plasma samples an ether extract is recommended in order to prevent the precipitation and accumulation of plasma proteins at the top of the column. Ether extraction increases the sample preparation time slightly, but results in a more accurate procedure, with a mean extraction recovery (n = 18) of 100.6 ± 2.1%. For both urine and plasma samples, the proposed method is accurate, rapid, produces excellent baseline stability and the columns may be used indefinitely.

ACKNOWLEDGEMENTS

We thank the Lagos State Government of Nigeria for the award of a research studentship to Mr. Thomas, Dr. J. Davies and Dr. N.D. Hall of the Royal National Hospital for Rheumatic Diseases for clinical samples of urine and plasma and May and Baker Ltd. for a sample of ketoprofen.

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Book Review

Analytische und präparative Methoden in der klinischen Biochemie, 2. (erweiterte) Aufl., by N. Rehfeld and D. Reichelt, Akademie-Verlag, Berlin, 1977, 496 pp., price M 62.00.

The requirements of clinical-chemical laboratories are doubling every 4—5 years. This means that the direct application of methods is necessary, especially with respect to the rapid development of appropriate analytical and separation techniques. Therefore the decision of the authors to revise the first edition (from 1971) is welcomed. The present edition is no "working-book" but gives a review of the principles of clinical-chemical analysis. The emphasis is laid heavily on the description of modern separation methods.

Among the chromatographic methods, paper chromatography, as opposed to thin-layer chromatography, is treated briefly, whilst there are also descriptions of gas chromatography and gel permeation chromatography as well as affinity chromatography. High-performance liquid chromatography and high-performance thin-layer chromatography are omitted although they already possess a broad spectrum of biochemical applications. The descriptions of individual methods are completed by few examples where perhaps a table of the most important applications would demonstrate better the developments of chromatographic methods and would provide several suggestions. As well as the classic electrophoretic methods, the different forms of electrofocusing are described. Besides centrifugation and dialysis the older methods of fractional precipitation and denaturation are also mentioned.

Among the analytical methods the revision was focused upon the enzymatic methods of analysis including immobilized enzymes, immunological, radiochemical and histochemical techniques and X-ray structural analysis. Mechanical techniques are placed beside approved manual techniques. A special chapter deals with the methods of elucidation of the structure of proteins. The book ends with the analysis of experimental results and with a supplement on the international system of units.

A comprehensive representation of the whole field would require the production of a large manual. It is to the credit of the authors that they have presented their material in a clear and articulate form and in such depth. The book is therefore directed not only at chemists and physicians, but also at medical students and graduates of medical schools.

Leipzig (G.D.R.) J. WAGNER

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Book Review

Analysis of drugs and metabolites by gas chromatography—mass spectrometry, Vol. 2, Hypnotics, anticonvulsants, and sedatives, by B. J. Gudzinowicz and M.J. Gudzinowicz, Marcel Dekker, New York, Basel, 1977, XI + 493 pp., price Sfr. 144.00, ISBN 0-8247-6585-0.

This book succeeds in offering its readers detailed information on the gas chromatographic (GC) analysis of hypnotics, anticonvulsants, sedatives and their metabolites. It is divided almost equally between barbiturates (Chapter 1) and non-barbiturates (Chapters 2 and 3). The authors have abstracted many articles, giving detailed information concerning isolation procedures and paths of metabolism as well as GC operating conditions and retention behaviours. Structural formulae are given for most of the compounds discussed, a desirable feature. Derivatization techniques, key steps in many GC methods, are well covered. As the majority of references date from before the explosive growth period of bioanalytical GC—mass spectrometry (MS) (for example, only 15% of the references in Chapter 1 are post 1973), the emphasis is on GC and not GC—MS methods. Further, reference is made too frequently to "unpublished results" or technical bulletins supplied by instrument manufacturers. Although valuable information can sometimes be found in the latter, such reports have not been scrutinized by a referee system and their use tends to have commercial overtones. Other minor criticisms of this volume include occasional over-emphasis of peripheral subject matter (e.g., pharmacokinetics) and the failure of the editorial staff always to insist on proper sentence structure. This reviewer also questions whether a book covering limited subject matter needs to comprise nearly 500 pages.

This is a book of methods (some proposed, some proven) rather than a book on methodology. As a compilation of GC and GC—MS methods, however, it will be helpful to analytical toxicologists, clinical chemists, forensic chemists, workers in drug metabolism, etc., especially those just entering their respective fields.

Rahway, N.J. (U.S.A.) W.J.A. VANDENHEUVEL

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Book Review

Analysis of drugs and metabolites by gas chromatography—mass spectrometry, Vol. 3, Antipsychotic, antiemetic, and antidepressant drugs, by Benjamin J. Gudzinowicz and Michael J. Gudzinowicz, Marcel Dekker, New York, Basel, 1977, X + 268 pp., price SFr. 92.00, ISBN 0-8247-6586-9.

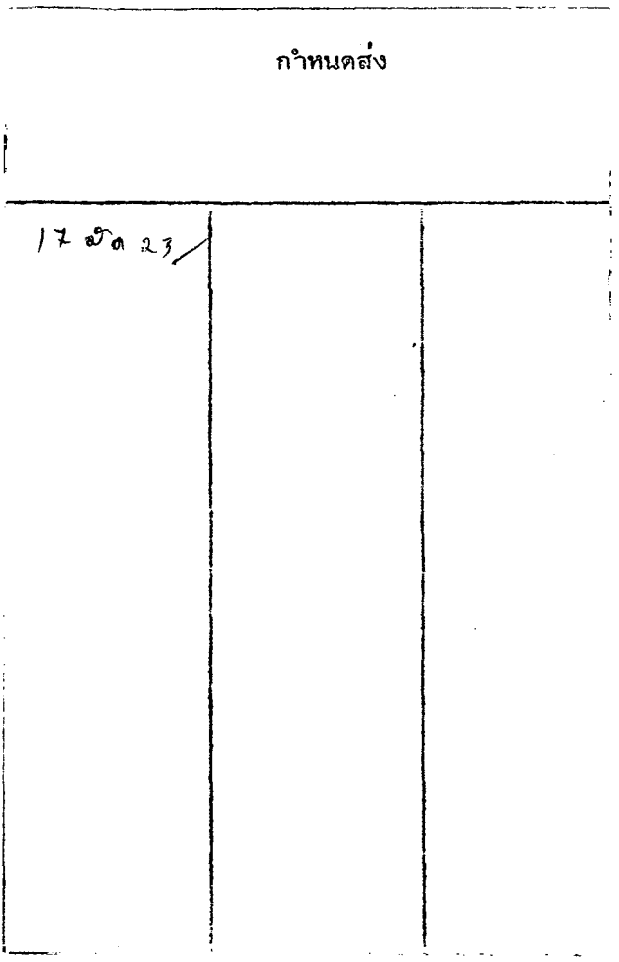
The lion's share of the first chapter in this volume is devoted to phenothiazines (especially chlorpromazine), with lesser coverage given to substituted butyrophenones and thioxanthene derivatives. Monoamine oxidase inhibitors and tricyclic antidepressants are discussed in Chapter 2. Although the subject matter is adequately covered (numerous examples of analyses, many references, plenty of structural formulae), this reviewer had hoped for a better presentation. One can overlook relatively minor points, such as an incorrect structural formula (p. 2), reference to loss of sample on the column as "absorption" rather than "adsorption" (p. 14), and occasional misspelling of words (pp. 108, 179). The too frequent reference to unpublished and not refereed advertising material from instrument manufacturers is particularly troublesome. In addition, the authors have the annoying habit of referring the reader to a previous volume of this series rather than presenting operating conditions or information in this volume. The inconsistency ("fish hooks" vs. "arrows") in the fragmentation mechanisms to explain the loss of $HN(CH_3)_2$ in the mass spectra of two drugs and the incorrect assignment of *m/e* values for observed metastable ions (m^*) are unfortunate (pp. 66, 193). The mass spectrometric behavior of the N-oxides and sulfoxides of several phenothiazines is presented on pp. 84 and 85, but we are not told whether these metabolites exhibit molecular ions; this is a point of critical importance.

My main concern about the value of this book arose from reading Chapter 2. Upon reading the second paragraph on p. 131 I had a feeling of *déjà vu*, and in going back through Chapter 1 I discovered that this paragraph had been lifted from p. 23. Chapter 2 covers tricyclic antidepressants and is not an appropriate place for more than a minimal discussion of electron capture detection. The authors present a 13-page table (2.12) listing the structures, names, etc. of 50 electron capturing pesticides, herbicides and related compounds. The structure of the sex attractant of the American cockroach and the analysis of lead alkyls in gasoline are also mentioned in this chapter. These topics are

are totally irrelevant and should have been deleted by the editorial staff. Equally frustating is the authors' tendency to use the terms "mass chromatogram" and "mass fragmentogram" interchangeably. The two techniques are entirely different.

Useful information can be found in Vol. 3 of the Gudzinowicz and Gudzinowicz series, but here it is so diluted that I cannot highly recommend the book. While gas chromatography—mass spectrometry is increasingly important to drug research, the need for a half-dozen volume series on this topic is dubious. Publishers must exercise restraint and good judgment in producing books on specialized topics.

Rahway, N.J. (U.S.A.) W.J.A. VANDENHEUVEL



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3 H. C. S. Wood and R. Wrigglesworth, in S. Coffey (Editor), *Rodd's Chemistry of Carbon Compounds, Vol. IV, Heterocyclic Compounds*, Part B, Elsevier, Amsterdam, Oxford, New York, 2nd ed., 1977, Ch. 11, p. 201.
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