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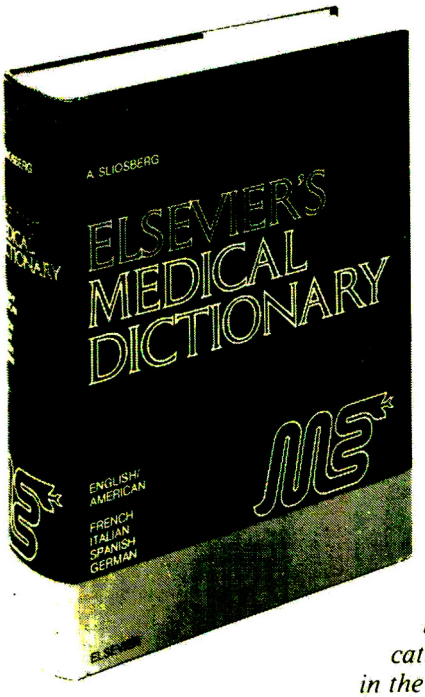

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CHROMBIO. 644

SIMULTANEOUS DETERMINATION OF HISTAMINE AND N^{τ}-METHYLHISTAMINE IN HUMAN PLASMA AND URINE BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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(Received March 4th, 1980)

SUMMARY

A new and sensitive method is described for the determination of histamine and N^{τ}-methylhistamine in human plasma and urine by gas chromatography—mass spectrometry. $^{15}N_2$-Labeled histamine and N^{τ}-[methyl-d_3]methylhistamine were used as internal standards. Histamine and N^{τ}-methylhistamine were converted to the derivatives N^{α}-heptafluorobutyryl-N^{τ}-ethoxycarbonylhistamine and N^{α}-heptafluorobutyryl-N^{τ}-methylhistamine, respectively. After these derivatives had been purified on a small column packed with CPG-10, the molecular ions were monitored during selected ion monitoring. Linear standard curves were obtained in the range of 0.5—10 ng/ml for both compounds. The reliability of the histamine analysis was demonstrated by using two different ion pairs, while a comparison with results from two different derivatizations on the same urine sample also established the specificity of the N^{τ}-methylhistamine analysis. An increase of 1 ng of histamine in the plasma could be precisely determined by the present method. The histamine content of plasma from five normal subjects was determined as 0.83 ± 0.37 (S.D.) ng/ml and the N^{τ}-methylhistamine content in most subjects was below the limits of this measurement. High excretion of histamine was noted in the urine collected in the early morning from a patient with nephritis.

INTRODUCTION

Histamine is released as a chemical mediator from mast cells and basophils during inflammatory and allergic reactions. The released histamine is metabolized via two enzymatic pathways. Histamine is deaminated by diamine oxidase (histaminase) to form imidazoleacetic acid. Alternatively, histamine is methylated to form N^{τ}-methylhistamine by the enzyme, histamine-N-methyltransferase. N^{τ}-Methylhistamine is subsequently deaminated by monoamine oxidase to form N^{τ}-methylimidazoleacetic acid. Previous studies [1—3] have

shown that ring N^{τ}-methylation represents the principal pathway of histamine metabolism in a variety of mammalian species and it is known that N^{τ}-methylhistamine, N^{τ}-methylimidazoleacetic acid and imidazoleacetic acid are the main metabolites of histamine in man [4, 5]. Since it has been suggested that histamine plays a role in the function of the central nervous system, the metabolism of histamine is of psychiatric interest. Determination of histamine and N^{τ}-methylhistamine by colorimetric methods has been reported by White [6] and Yamatodani et al. [7]. However, these methods raise some questions as regards specificity and, moreover, they lack the necessary sensitivity for low-level assays.

The present authors have reported a method for the quantitative determination of biological samples containing a relatively large amount of histamine by selected ion monitoring [8]. This paper describes a method for the simultaneous determination of histamine and N^{τ}-methylhistamine in human plasma and urine by gas chromatography—mass spectrometry.

EXPERIMENTAL

Materials

Histamine dihydrochloride was purchased from E. Merck (Darmstadt, G.F.R.) and N^{τ}-methylhistamine dihydrochloride was synthesized in our laboratory. Heptafluorobutyric anhydride (HFBAn) and pentafluoropropionic anhydride (PFPAn) were purchased from Wako (Osaka, Japan). Ethyl chloroformate was obtained from Tokyo Kasei Kogyo (Tokyo, Japan) and distilled before use. Ethyl acetate was stored over a molecular sieve type 3A. CPG-10 (120—200 mesh) was obtained from Electro-Nucleonics (Fairfield, NJ, U.S.A.). $^{15}N_2$-Labeled histamine dihydrochloride was synthesized and used as an internal standard [8]. Deuterated N^{τ}-methylhistamine dihydrochloride was synthesized using dimethyl-d_6 sulfate (99 atom% D; Merck Sharp & Dohme, Montreal, Canada) by the method of Rothschild and Shayer [9] as modified in our laboratory, and it was dissolved in 0.01 *N* hydrochloric acid solution to give a concentration of 800 ng/ml. [2,5-^{3}H]Histamine dihydrochloride was purchased from the Radiochemical Centre (Amersham, Great Britain). Synthesis of N^{τ}-[methyl-^{14}C]methylhistamine was examined using histamine, S-adenosyl-L-[methyl-^{14}C]methionine and the enzyme histamine-N-methyltransferase [10].

Analytical procedures

About 7 ng of N^{τ}-[methyl-Cd_3]methylhistamine and 10 ng of $^{15}N_2$-histamine were added as internal standards to 1 ml of plasma or 100 μl of urine. Protein was removed by centrifugation after the addition of 10 ml of ethanol. This deproteinization step can be omitted in the analysis of urine. After reducing the volume of the supernatant to 1 ml under reduced pressure, the concentrated supernatant was added to a mixture of 2 g of sodium chloride, 0.5 g of sodium carbonate and 2 ml of 0.25 *M* borate buffer (pH 10). The mixture was then extracted with 5 ml of butanol. The organic layer was transferred to a tube containing 7 ml of heptane and 1.5 ml of 1 *N* hydrochloric acid solution. After the histamine and N^{τ}-methylhistamine had been returned to the hydrochloric acid phase, the aqueous phase was removed and evaporated to dryness

under reduced pressure. The residue mixed with 50 μl of a mixture of HFBAn and ethyl acetate (1:1, v/v) was heated at 90°C for 30 min. After allowing the mixture to come to room temperature, the excess reagent was removed under a nitrogen stream. The residue was treated with 50 μl each of ethyl chloroformate and ethyl acetate at room temperature for 30 min to form N^{α}-heptafluorobutyryl-N^{τ}-ethoxycarbonylhistamine (HA-HFB-ETO), to which 1 ml of 10% sodium carbonate solution and 2 ml of dichloromethane were added. The mixture was shaken, centrifuged, and the aqueous phase was discarded. Following removal of the organic phase to dryness under a nitrogen stream, the residue re-dissolved in a small volume of dichloromethane was loaded onto a small column of CPG-10 (1.5 × 0.5 cm I.D., equilibrated with hexane) and 2 ml of hexane and benzene were successively passed through the column. HA-HFB-ETO was eluted with 2 ml of ethyl acetate—ether (1:1, v/v). The column was next washed with 2 ml of isopropanol, and N^{α}-heptafluorobutyryl-N^{τ}-methylhistamine (MEHA-HFB) was eluted from the column with 1.5 ml of methanol. After the ethyl acetate—ether fraction and the methanol fraction had been evaporated to dryness under a nitrogen stream, the residues re-dissolved in ethyl acetate were injected into a gas chromatograph—mass spectrometer. When plasma was analyzed, the re-dissolved residues could be mixed before measurement. In the case of urine samples, the measurement should be carried out without mixing of the residues.

Samples

Blood samples were collected from five healthy individuals who were members of the laboratory staff. Venous blood was drawn into a plastic syringe containing a small amount of heparin and centrifuged at room temperature. Urine was collected in the early morning. All samples were stored at −20°C until assay.

Gas chromatography—mass spectrometry

A Shimadzu LKB 2091 gas chromatograph—mass spectrometer and data processing system (Shimadzu PAC500FDG) connected to a minicomputer (Okitac-4300b; Oki Electric Industry, Tokyo, Japan) were used. The glass column (2 m × 2.4 mm I.D.) was packed with 5% SE-30 on Supelcoport (80—100 mesh; Supelco, Bellefonte, PA, U.S.A.). The flow-rate of carrier gas (helium) was about 12 ml/min, the trap current was 50 μA and the ionization energy was 20 eV. The temperatures employed were as follows: injection, 240°C; oven, 200°C; separator, 250°C; and ion source, 220°C.

RESULTS AND DISCUSSION

For quantification of histamine, HA-HFB-ETO was prepared and the molecular ions at *m/z* 379 and 381 were used for monitoring histamine and $^{15}N_2$-labeled histamine, respectively, while N^{τ}-methylhistamine was converted to MEHA-HFB. Fig. 1 shows a mass spectrum of the deuterated MEHA-HFB; the base peak at *m/z* 111 is formed by the elimination of $NHCOC_3F_7$ with hydrogen. The peak height ratio of *m/z* 321/324 was less than 0.0013 in deuterated MEHA-HFB. This product is a satisfactory internal standard, since

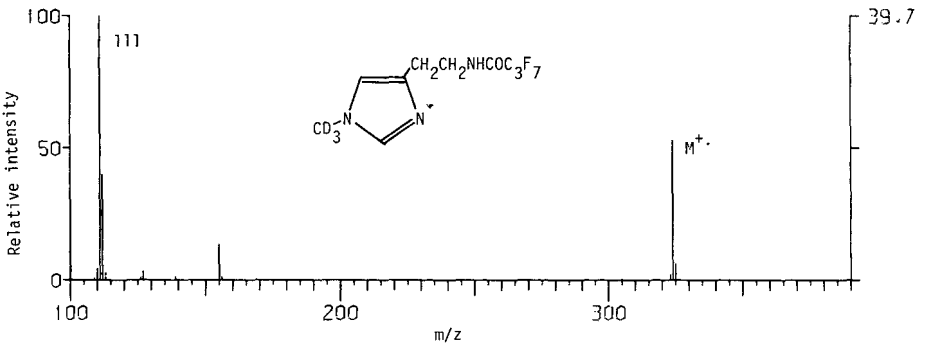

Fig. 1. Mass spectrum of the derivative of deuterated N^{τ}-methylhistamine.

a low background contribution from the internal standard is of value for analyses requiring high sensitivity. The quantification of N^{τ}-methylhistamine was performed by measuring the molecular ions at *m/z* 321 and 324 for the internal standard.

The histamine and N^{τ}-methylhistamine concentrations were determined from standard curves, constructed by plotting the peak height ratio of *m/z* 379/381 and *m/z* 321/324, respectively, against concentration. Linear standard curves were obtained in the concentration range 0.5—10 ng/ml for both compounds by adding known amounts to 1 ml of distilled water and treating the solutions as described above.

The detection limit was 20 pg of N^{τ}-methylhistamine, with a signal-to-noise ratio of 6:1. Higher sensitivity was found for N^{τ}-methylhistamine than for histamine.

The derivatives of histamine and N^{τ}-methylhistamine were purified by chromatography on CPG-10. Recently, Hashimoto and Miyazaki [11] reported a clean-up method for catecholamines with a CPG-10 column and stated that such CPG-10 column chromatography afforded some advantages over silica gel for purifying the biogenic amines. The CPG-10 column was also useful for the clean-up procedure of the derivatives of histamine and N^{τ}-methylhistamine. When the derivative of [^{3}H]histamine was chromatographed on CPG-10, 95.4 ± 8.2% (S.D.; n=5) of the radioactivity was eluted with ethyl acetate—ether. On the other hand, the recovery was 64.0 ± 13.0% (S.D.; n=5) by elution with methanol for ^{14}C-labeled MEHA-HFB. When the organic eluates from the CPG-10 column were combined and concentrated, a reduced sensitivity, which was considered to be due to decomposition of the derivative, was observed for the determination of histamine. Based on these results, after drying the solvents, each fraction re-dissolved in ethyl acetate was simultaneously drawn into a syringe and injected into the gas chromatograph—mass spectrometer. With the clean-up procedure described here, selected ion profiles free from any interfering peak were obtained for the plasma extracts. However, certain urine components which eluted with the ethyl acetate—ether from CPG-10 interfered with the determination of N^{τ}-methylhistamine. Thus, in the case of urine analysis, each residue must not be mixed before measurement. A selected-ion profile recorded during the analysis of a plasma extract is shown in Fig. 2.

The specificity of the proposed method was tested as follows. If compounds

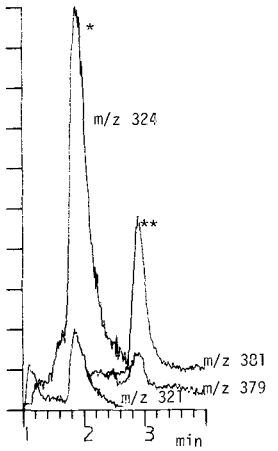

Fig. 2. Selected-ion profile obtained by analysis of a human plasma extract.
★ = N^{α}-Heptafluorobutyryl-N^{τ}-methylhistamine; ★★ = N^{α}-heptafluorobutyryl-N^{τ}-ethoxycarbonylhistamine.

other than histamine were present in the peak, the relative ion intensity would be different from the authentic histamine and the sample extracts. As shown in Table I, the intensity ratio at the molecular ion and the fragment ion resulting from elimination of the ethoxycarbonyl group in the authentic histamine was identical to that of histamine extracted from plasma and urine. This finding indicates that fragments from compounds other than histamine did not interfere with these ions, and so confirms the specificity of the determination of histamine. An attempt was made to assess the specificity for N^{τ}-methylhistamine determination in the same manner. However, since there are no proper fragment ions for this purpose in the higher mass region as shown in Fig. 1, the following experiment was performed. Since a very small amount of N^{τ}-methylhistamine was detected in plasma, 100 μl of urine were used instead of plasma as the sample for the experiment. An aliquot of the sample was analyzed by the procedure described under Experimental. A second aliquot of the same sample was extracted, derivatized with PFPAn in place of HFBAn, and the molecular ions at *m/z* 271 and 274 were monitored for unlabeled and labeled MEHA-PFP, respectively. If compounds other than N^{τ}-methylhistamine were not present in this peak, the values obtained by the two different methods would be consistent with each other. The measured values by derivatization with HFBAn were, in fact, consistent with those from the PFP-derivative (Table II), so the specificity of the determination of N^{τ}-methylhistamine was confirmed.

To estimate assay precision, histamine in amounts of 0.71 and 1.42 ng was

TABLE I

SPECIFICITY OF HISTAMINE DETERMINATION BY SELECTED ION MONITORING

Data are expressed as means ± S.D. (n=5).

	m/z 381/*m/z* 308	*m/z* 379/*m/z* 306
Authentic	1.17 ± 0.04	1.15 ± 0.03
Plasma	1.20 ± 0.11	1.22 ± 0.06
Urine	1.18 ± 0.15	1.16 ± 0.06

TABLE II

COMPARISON OF RESULTS WITH PENTAFLUOROPROPIONYL AND HEPTAFLUOROBUTYRYL DERIVATIVES OF N^{τ}-METHYLHISTAMINE FOR THE SAME URINE SAMPLE

The results, in ng/100 μl, were obtained by derivatization with heptafluorobutyric anhydride (for HFB) and pentafluoropropionic anhydride (for PFP).

Urine	HFB	PFP	HFB/PFP
A	10.6	11.4	0.93
B	23.1	24.0	0.96
C	22.3	22.1	1.01
			0.97 ± 0.04 (S.D.)

TABLE III

ACCURACY OF DETERMINATION OF HISTAMINE IN HUMAN PLASMA

Added (ng)	Found (ng/ml)		Recovery (%)
—	0.71 0.63 0.45	0.60 ± 0.13 (21.7)*	
0.71	1.34 1.16 1.34	1.28 ± 0.10 (7.8)*	95.8
1.42	2.05 2.32 2.40	2.26 ± 0.18 (8.0)*	116.9

*Coefficient of variation (%).

added to 1-ml aliquots of pooled plasma. The amount of histamine in the samples was measured by the present method. The results are shown in Table III. The histamine content of the pooled plasma measured in triplicate was 0.60 ± 0.13 ng/ml. The amounts of histamine added agreed well with the amounts of histamine measured. The recoveries of the 0.71 and 1.42 ng of histamine added to the plasma pool were found to be 95.8 and 116.9%, respectively. The precision of this method was within acceptable limits. To elucidate the pathogenesis of an asthmatic patient, it is important to trace the variance in the plasma histamine level. It was proved experimentally that an increase of 1 ng of histamine could be detected precisely by the present method.

Although values for the concentration of N^{τ}-methylhistamine in human urine have been reported by Fram and Green [12], no comparable report for the N^{τ}-methylhistamine levels in human plasma has yet appeared. Preliminary results for five normal plasma samples gave values of 0.83 ± 0.37 ng/ml for histamine. On the other hand, the N^{τ}-methylhistamine content was below the limits of this measurement in three of five subjects. The plasma of subjects A and E contained 1.41 and 0.59 ng/ml of N^{τ}-methylhistamine, respectively (Table IV).

Next, urine collected in the early morning was analyzed. It was found that the concentrations of histamine and N^{τ}-methylhistamine in the urine varied. A high

TABLE IV

CONTENT OF HISTAMINE AND N^{τ}-METHYLHISTAMINE IN HUMAN PLASMA AND URINE

Urine was collected in the early morning and the results are expressed in ng/ml.

Subject*	Sex	N^{τ}-Methylhistamine	Histamine
Plasma			
A	F	1.14	0.77
B	M	n.d.**	0.89
C	F	n.d.	1.43
D	F	n.d.	0.63
E	M	0.59	0.45
Urine			
E***	M	105.8	24.1
E***	M	222.9	42.0
F	M	230.8	48.4
G	F	68.9	22.5
H	M	99.0	733.0

*A—G, normal healthy subjects; H, patient with nephritis.
**n.d. = Not detectable.
***Collected on two consecutive days.

excretion of histamine, 733 ng/ml, was found in a patient with nephritis, but there was no concomitant increase in N^{τ}-methylhistamine.

The method described here has a high sensitivity, permits the determination of both histamine and N^{τ}-methylhistamine in the same sample, and is thus expected to facilitate the measurement of both amines in tissues.

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SUMMARY

The method presented for the simultaneous determination of xanthine and hypoxanthine, uses mass-fragmentography in the electron impact (EI) mode, after the gas chromatographic separation of butylated derivatives. Butylation, rather than methylation, is used in order to avoid interference coming from exogenous caffeine, which is frequently encountered. [7,9-^{15}N]Xanthine is used as the internal standard, and for each sample, a blank is obtained by xanthine oxidase reaction. In the biological fluids studied the sensitivity was about 50 ng/ml.

INTRODUCTION

Analytical methods for oxypurines determination have been restricted for a long time to an overall estimation of xanthine and hypoxanthine by UV absorption spectrophotometric methods [1, 2]. More recent reports, including enzymatic spectrophotometry [3], thin-layer chromatography combined with spectrophotometry [4], high-performance liquid chromatography (HPLC) [5, 6], or ion-exchange column chromatography [7] have allowed the simultaneous determination of xanthine and hypoxanthine. These methods, often requiring a large amount of sample [3], give no confirmation as to the identity of the substances quantitated, and also yield non-consistent data as Pfadenhauer [5] pointed out. Confirmation of identity was obtained by Snedden and Parker [8] who used high-resolution mass spectrometry with direct introduction into the mass spectrometer of lyophilized samples of blood and muscle. This method does not require previous separation of the different substances since it allows a selection of accurate mass peaks, but it involves tedious equipment calibration. Not having a high-resolution mass spectrometric

system at our disposal, we used a classical quadrupolar gas chromatographic—mass spectrometric (GC—MS) system, which allowed previous separation of the oxypurines.

MATERIALS AND METHODS

Chemicals

Standards. Xanthine and hypoxanthine (Merck, Darmstadt, G.F.R.), and [7,9-^{15}N]xanthine (C.E.A., Saclay, France) prepared in weak ammoniacal solutions were found to be stable for several months at 4°C.

0.5 M formiate buffer. Prepared by dissolving 23 g formic acid (Riedel de Haën, Hoescht, G.F.R.) in 1 l of distilled water, and adjusting to pH 3.65 with 5 *N* sodium hydroxide.

Solvents. n-Butanol obtained from Prolabo (Paris, France), and methanol GR obtained from Merck.

Reagents. N,N-Dimethylacetamide (Fluka, Buchs, Switzerland) was stored in the dark over anhydrous sodium sulfate. The tetrabutylammonium hydroxide, 0.1 *N* in methanol and butyl iodide were purchased from Merck. The Silyl-8 was from Pierce (Rockford, IL, U.S.A.).

Enzymatic preparations. Xanthine oxidase, pronase and subtilisin were purchased from Boehringer (Mannheim, G.F.R.).

GC—MS system

Experiments were performed by mass fragmentography, on a quadrupolar GC—MS Hewlett-Packard 5985 system, equipped with an electron impact—chemical ionization (EI—CI) source. Scanning, and selected ion monitoring were carried out under the software control of a computer (Hewlett-Packard 1000 integrated data system). Mass spectrometry operating conditions were set up as follows: electron impact, 70 eV and emission current, 300 μA. Helium was used as the carrier gas at a flow-rate of 20 ml/min, and gave a pressure of $2 \cdot 10^{-6}$ torr in the source. Methane was used as the reactant gas for chemical ionization.

The GC column was a 150 cm × 2 mm I.D. glass column filled with 3% OV 17 on Gas Chrom Q 100—120 mesh. The column was conditioned and then silylated at 150°C, by injecting 10 μl of Silyl-8 five times. The chromatograph was operated with temperature programming from 220°C to 260°C at 10°/min and the injection port was maintained at 250°C.

Glassware problem

During preliminary studies, the calibration curves obtained with extracts from standard solutions, did not go through zero, which indicated a possible adsorption on glass. This phenomenon was also observed by Driessen et al. [9] with 5-fluorouracil. The use of polyethylene vials for the different steps of the oxypurines evaluation eliminated this problem.

Ultrafiltration

Serum was deproteinized by ultrafiltration in Amicon CF 25 centriflo cones (Lexington, MA, U.S.A.) according to the methods of Lakings et al. [10]

or Yamamoto et al. [11]. Serum was introduced into a cone and centrifuged at 600 *g* for 15 min. The residue was then washed with distilled water and centrifuged again.

Procedure

Each serum and urine sample was analyzed according to the scheme described in Fig. 1.

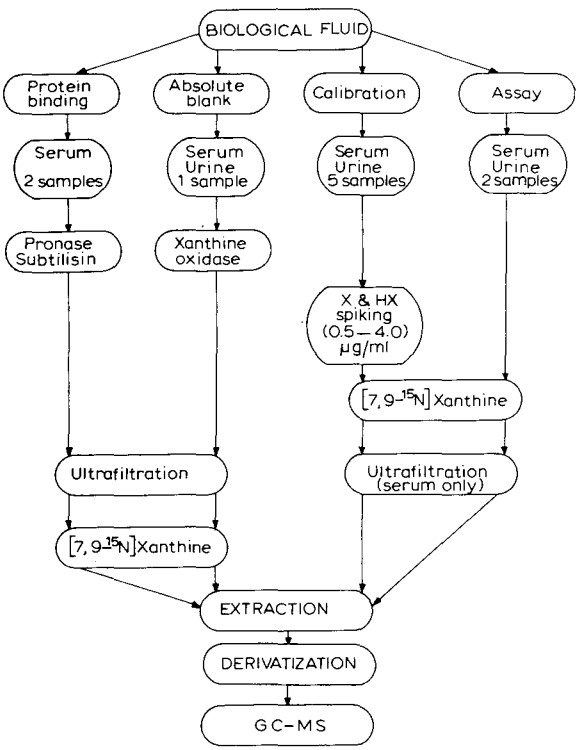

Fig. 1. Scheme for the analysis of xanthine and hypoxanthine in biological fluids.

Serum analysis

Each sample (0.5 ml) was spiked with [7,9-^{15}N]xanthine and then ultrafiltered. The ultrafiltrate was buffered to pH 4.20 with the formiate buffer solution (200 µl) and extracted by shaking for 1 min, on a vortex mixer, with *n*-butanol (2 ml) in 15-ml polyethylene vials.

Different solvents commonly used to extract other xanthines, such as theophylline [12, 13] or allopurinol [14], from biological fluids were also checked. However, it appeared that only *n*-butanol, used by Pantarotto et al. [15] to quantitate nucleoside analogues (5-fluorouracil, 6-mercaptopurine, 5-fluoro-2′-deoxyuridine) gave an acceptable recovery.

After centrifugation, the organic layer was evaporated to dryness, at 50°C under a stream of nitrogen. The residue was then dissolved in 120 µl of N,N-dimethylacetamide, and 50 µl of tetrabutylammonium hydroxide. After vortex

mixing for 10 sec, a 20-μl aliquot of butyl iodide was then added to the solution. The container was capped, then vortexed for 1 min and allowed to stand for 15 min at room temperature. After evaporation at 50°C under a stream of nitrogen, the residue was redissolved in 20 μl of methanol, and 2-μl aliquots were injected into the GC—MS system.

Urine analysis

Urine samples were diluted ten times and were then assayed as the serum samples, but without undergoing the ultrafiltration step.

RESULTS AND DISCUSSION

Preliminary assays to procedure

Protein binding. The ultrafiltration of the serum is reliable, because oxypurines are not bound to serum proteins. This was verified by reacting pronase an subtilisin with the serum, according to the method of Osselton et al. [16], before the ultrafiltration and extraction steps. The enzymatic hydrolysis performed at pH 8.0, for 60 min at 55°C (enzymatic preparation: 2 μg/ml of biological fluid) never generated a significant increase in the oxypurines recovery.

Absolute blanks. A blank was prepared by reacting each sample with xanthine oxidase at 37°C for 1 h, prior to the ultrafiltration, spiking of [7,9-^{15}N] xanthine, and extraction steps. The reaction was carried out by using 50 μl of enzymatic suspension for the serum and 10 μl for the urine, according to the mechanism given in Fig. 2.

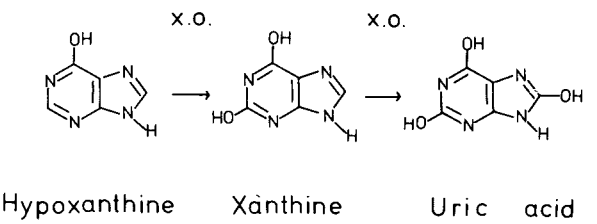

Fig. 2. Hypoxanthine oxidative transformation in uric acid by xanthine oxidase (X.O.).

Derivatization. As oxypurines are polar molecules, they must be derivatized before GC analysis. First derivatization attempts, by flash methylation with 0.2 *N* trimethylanilinium hydroxide in methanol, currently used with barbiturates [17] or purines [18], were not successful for two reasons:

(1) no chromatographic conditions could be found to separate the trimethylated derivative of xanthine (or caffeine) from endogenous stearic acid methyl ester and

(2) the preparation of blanks, by the reaction of xanthine oxidase with the biological fluids, revealed the presence of exogenous caffeine, leading therefore to an important error in the xanthine evaluation; caffeine and stearic acid were effectively identified by their mass spectra.

Derivatization by butylation, according to Greeley's method [19], modified by Pranskevich et al. [20], eliminated these interferences.

Mass spectra

Mass spectra obtained in the EI mode, showed that tributylated derivatives of xanthine and [7,9-^{15}N]xanthine gave molecular ions with *m/z* of 320 and 322 respectively. Because of their relative intensity of 60% and because of the absence of any interfering peak in this part of the spectrum, they were found to be suitable for the mass fragmentographic quantitation (Fig. 3).

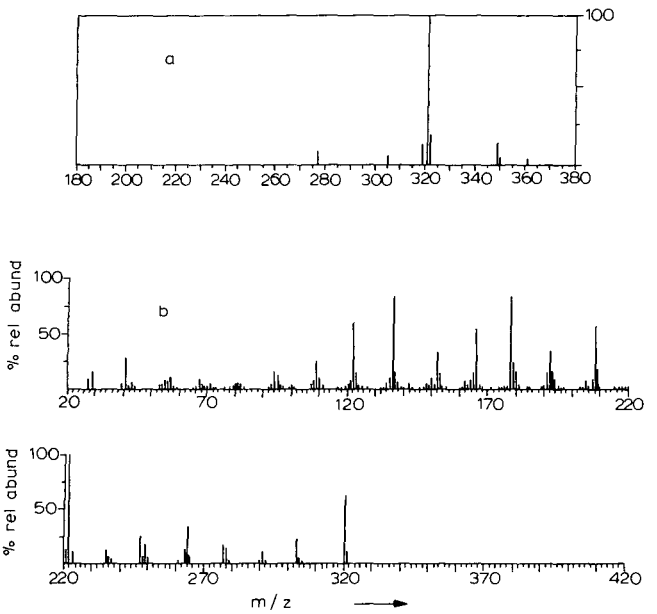

Fig. 3. Mass spectra of tributylated derivative of xanthine: (a) in CI mode and (b) in EI mode.

Hypoxanthine gave a dibutylated derivative whose molecular ion *m/z* 248 (Fig. 4) has a relative intensity of about 40%. Because this mass is subject to interference in some samples, we selected the ion *m/z* 231, whose relative intensity is about 60%, for the quantitation of hypoxanthine.

A correction was made to the [7,9-^{15}N]xanthine peak evaluation, to take into account the contribution of the xanthine peak at mass M + 2. (The M + 2 xanthine peak whose relative intensity 2.5% adds to the [7,9-^{15}N]xanthine peak).

Mass spectra obtained in the EI mode were confirmed in the CI mode (Figs. 3 and 4). Fig. 5 shows a selected ion monitoring (SIM) recording of xanthine, [7,9-^{15}N]xanthine, and hypoxanthine from a serum extract.

Recovery

The extraction recovery was determined from samples of serum spiked with [7,9-^{15}N]xanthine (1.25 μg/ml). In spite of an extraction recovery that appears rather low (55 ± 4%) the analytical method is still worthwhile for two reasons: [7,9-^{15}N]xanthine is practically the ideal internal standard, because its physico-chemical behaviour, in the extraction and derivatization steps,

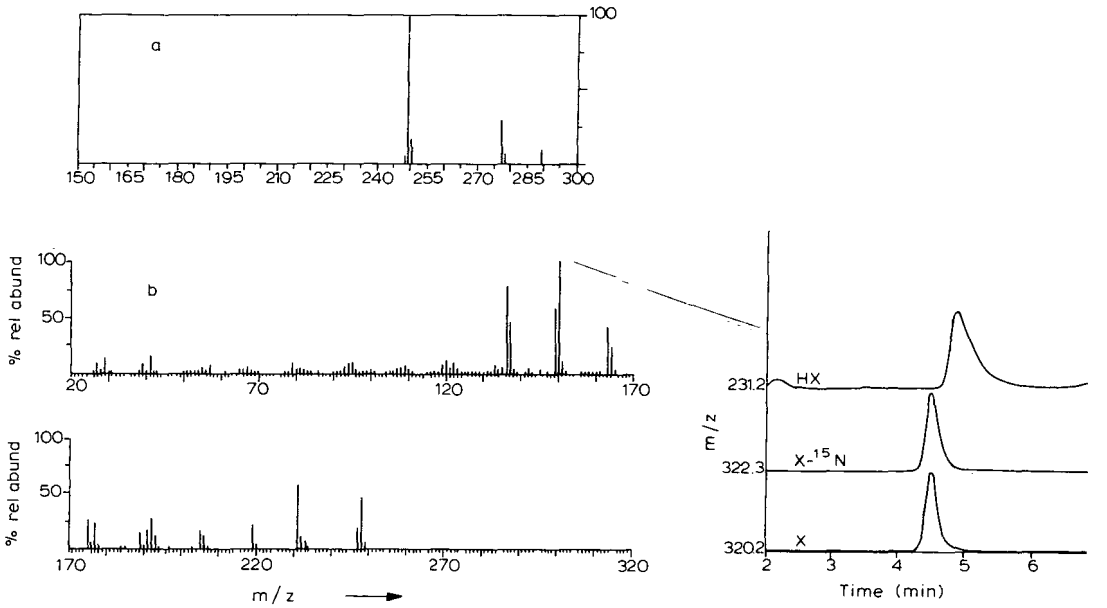

Fig. 4. Mass spectra of dibutylated hypoxanthine: (a) in CI mode and (b) in EI mode.

Fig. 5. SIM recording of xanthine (X), [7,9-^{15}N]xanthine (X-^{15}N) and hypoxanthine (HX) from serum extract.

is similar to that of the molecule quantitated. The lack of any significant protein binding for oxypurines, as was demonstrated by the action of proteolytic enzymes, such as pronase and subtilisin. The relatively low recovery of the extraction seems to be due only to an unfavourable partition coefficient of these substances between the fairly polar solvent used and water.

Sensitivity, reproducibility and calibration

The detection limit for oxypurines quantitation in biological fluids is 50 ng/ml, for 0.5 ml of serum and 0.1 ml of urine. The reproducibility determined on ten replicate analyses of serum and spiked serum (two concentrations) is given in Table I and the intra-assay coefficient of variation is about 5%.

TABLE I

REPRODUCIBILITY OF THE ASSAY OF XANTHINE (X) AND HYPOXANTHINE (HX) IN SERUM

Concentration (μg/ml) (n= 10)		Coefficient of variation (%)
X	0.5	5.0
X	1.0	5.2
HX	1.5	5.7
HX	2.0	6.8

Calibration curves, obtained for serum samples spiked with xanthine and hypoxanthine, were found to be linear for concentrations ranging from 0.5 to 4.0 μg/ml. Correlation coefficients are greater than 0.995 for the xanthine calibration, and greater than 0.970 for the hypoxanthine calibration. An example is given in Fig. 6.

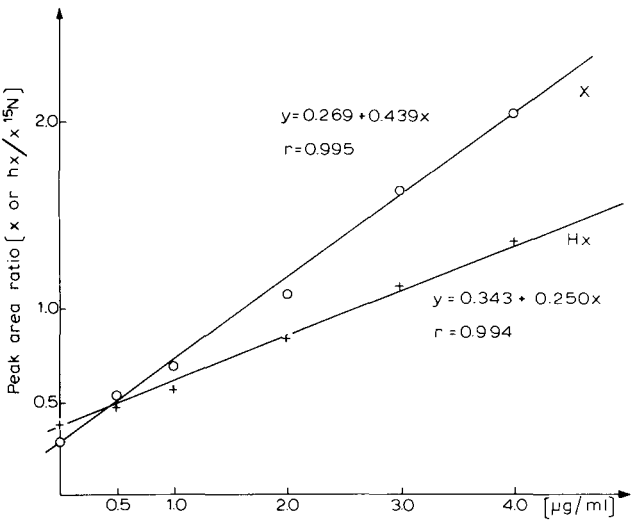

Fig. 6. Calibration curves of xanthine (X) and hypoxanthine (HX), an example given for subject No. 12.

TABLE II

SERUM CONCENTRATION AND URINARY ELIMINATION RATE OF XANTHINE (X) AND HYPOXANTHINE (HX) IN NORMAL SUBJECTS

Subject No.	Serum (μg/ml)		Urine (mg/24 h)	
	X	HX	X	HX
1	0.61	1.50	6.5	6.3
2	0.69	2.50	6.5	5.5
3	0.57	1.20	4.5	5.4
4	0.95	3.25	12.8	11.4
5	0.69	3.80	14.5	13.8
6	0.53	1.45	8.6	6.4
7	0.62	1.10	11.6	11.8
8	0.52	0.90	8.3	13.5
9	0.65	1.55	7.9	8.6
10	0.58	1.52	8.8	6.8
11	0.60	1.31	9.5	11.6
12	0.62	1.56	10.6	6.3
13	0.60	1.36	9.0	7.6
14	0.60	1.55	6.8	5.3
15	0.63	1.65	9.4	6.4
Mean	0.63	1.75	9.0	8.5
σ	0.10	0.80	2.6	3.1

Applications in humans

The study was conducted with 15 healthy volunteers, who were fasted overnight. Blood was collected by venipuncture (20 ml in dry tubes) and urine was collected over the following 24 h. Serum and urine samples were stored frozen (−20°C) until analysis. The results summarized in Table II are in good agreement with those obtained by HPLC [5]. In Table II, the serum concentrations are expressed in μg/ml, and urinary elimination is expressed in mg/24 h. It can be noted that the ratio of xanthine to hypoxanthine serum concentrations ranges from 0.25 to 0.50, and that the ratio of xanthine to hypoxanthine urinary elimination rates is about 1.0.

CONCLUSION

In comparison with the spectroenzymatic or the HPLC methods, the GC—MS method presented here offers the advantage of combining accuracy, sensitivity and specificity.

The different techniques used during the development of the procedure have eliminated any interference, and have also established the reliability of the method.

The preparation of absolute blanks was carried out by treating each urine and serum sample with xanthine oxidase. The action of pronase and subtilisin on the serum samples confirmed the absence of protein binding for these oxypurines and allowed the precise determination of the extraction recoveries. Finally, the use of [7,9-^{15}N]xanthine as the internal standard allowed us to obtain an optimal accuracy for the calibration curves. When first applied to normal subjects, this method allowed us to obtain accurate and relatively homogenous results, which were used to define a mean serum concentration and a mean daily urinary elimination rate for both xanthine and hypoxanthine. This method proved to be also extremely effective in following the development of oxypurines serum concentrations in hyperuricemic subjects, during their treatment with allopurinol, a xanthine oxidase inhibitor.

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STUDIES OF HYPERLIPIDEMIA IN DRUG-INDUCED DIABETIC RATS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A hyperlipidemic condition is often associated with diabetes. The possibility that specific serum lipids (i.e., individual triglycerides or cholesterol esters) may be altered in the diabetic state was investigated. Serum lipids from both controls and streptozotocin- and alloxan-treated rats were separated into approximately twenty chromatographic fractions by reversed-phase high-performance liquid chromatography; a number of individual triglycerides and cholesterol esters were identified. The methodology described allowed subtle changes in individual lipid components to be detected. Only minor variations in the cholesterol and cholesterol ester fractions were observed between the control and diabetic samples. While not uniform throughout, elevations in the triglyceride fractions occurred in the diabetics. Also, differences in triglyceride content were found to exist between the groups of streptozotocin- and alloxan-treated animals.

INTRODUCTION

Hyperlipidemic conditions which include complicated lipoprotein changes [1—3] and enhanced triglyceride levels [1, 4] are known to be implicated in human diabetes. Their potential role in cardiovascular disease is of some concern.

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Alterations in lipid metabolism can also be observed in certain animal diabetes models, such as streptozotocin- or alloxan-treated rats [5–7]. The mechanism of hypertriglyceridemia, although still largely unclear, has been extensively studied. While data of Bierman et al. [7] suggest that alloxan-treated rats derive their high triglyceride plasma levels from dietary fat, carbohydrates and proteins can also contribute to hypertriglyceridemia in the streptozotocin-treated animals [8]. According to Reaven and Reaven [6], high triglyceride levels in streptozotocin-induced diabetic rats are due to a defect in peripheral lipoprotein removal as well as an increased hepatic production. Transplantation of islets of Langerhans to diabetic rats can somewhat stabilize plasma glucose, insulin and glucagon levels, but a mild hypertriglyceridemia persists [9].

Different metabolic causes of hyperlipidemia undoubtedly exist [10], and various diabetic conditions could result in both qualitative and quantitative changes in lipid metabolism. While it is usual to determine lipids as "total triglycerides" and "total cholesterol esters", new analytical approaches now allow profiling the individual components. As shown recently by Skorepa et al. [11] such information is potentially important for both clinical distinction and an improved understanding of the related metabolic conditions.

The present study utilizes a recently developed methodology [12, 13] of reversed-phase chromatography to follow serum neutral lipid levels in rats with experimentally induced diabetes. As expected, certain lipid fractions are elevated in diabetic rats as compared to control animals. Treatment of serum samples with lipase readily distinguishes triglyceride peaks from those of cholesterol esters. Triglycerides were further characterized through hydrolysis of the fractions trapped after liquid chromatography and determination of their fatty acid content by gas chromatography.

Triglyceridemia of the diabetic rats appears to be of a heterogeneous nature, i.e., elevation is different for the individual fractions. In addition, differences exist between the groups of streptozotocin- and alloxan-treated animals.

EXPERIMENTAL AND RESULTS

Sprague-Dawley rats were used in the experiments and maintained on a diet of normal rat chow (Lab-blox from Wayne Feeds, Bloomington, IN, U.S.A.). The rats, all weighing between 180 and 200 g, were divided into three groups. Control rats were intravenously injected with 1 ml normal saline solution into the tail vein. A second group was given a single injection of 65 mg/kg streptozotocin (a gift from Upjohn, Kalamazoo, MI, U.S.A.) in saline solution. After a 24-h fast, a third group was intravenously injected with 40 mg/kg alloxan (alloxane monohydrate, a product of Sigma, St. Louis, MO, U.S.A.) in saline.

Blood glucose values, as measured with the Beckman glucose analyzer, ranged between 102—122 mg/100 ml for the controls. The blood glucose values were considerably higher for the diabetic animals. They ranged from 320—523 mg/100 ml and 484—545 mg/100 ml for the alloxan and streptozotocin animals, respectively.

The animals were sacrificed exactly two weeks following the injections.

Trunk blood was collected from decapitation of all experimental animals. The serum samples were stored frozen until analyzed. Cholesterol, cholesterol esters, and triglycerides as well as other lipids were quantitatively extracted from a 0.5-ml sample with 20 volumes of chloroform—methanol (1:1, v/v), according to a modified Folch procedure [14]. The extracts were concentrated to 100 μl prior to liquid chromatographic analysis through solvent evaporation. A 25 cm × 4.6 mm I.D. Zorbax ODS column, particle size 5 μm (DuPont, Wilmington, DE, U.S.A.) was used in all of the reversed-phase high-performance liquid chromatography (HPLC) separations. The mobile phase was isopropanol—acetonitrile (1:1, v/v or 5:2, v/v), delivered at 1.0 ml/min with a Waters 6000 reciprocating pump (Waters Assoc., Milford, MA, U.S.A.). Injection volumes were typically 10 μl. The lipids were detected with a variable-wavelength UV detector (Model LC-55, Perkin-Elmer, Norwalk, CN, U.S.A.) set at 215 nm. Typical liquid chromatographic profiles for a control rat and the two experimentally induced diabetic rats are shown in Fig. 1.

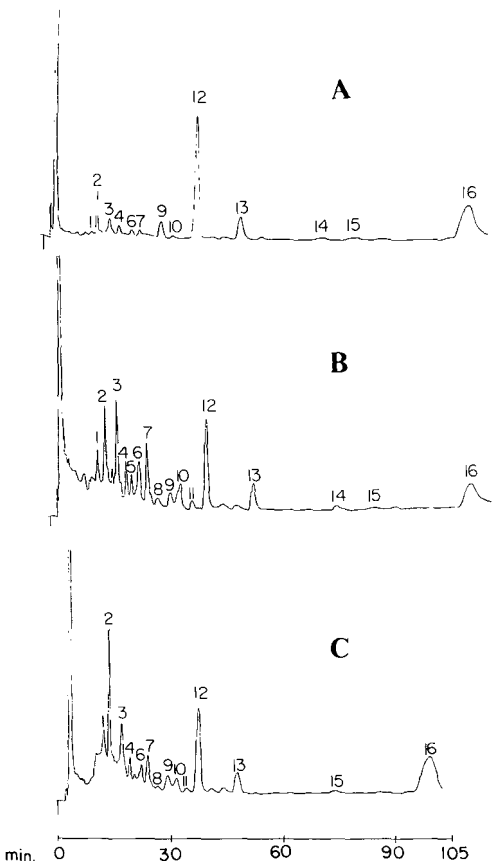

Fig. 1. Reversed-phase HPLC of blood lipids from (A) control rat; (B) streptozotocin diabetic rat; and (C) alloxan diabetic rat. Chromatographic conditions: mobile phase, isopropanol—acetonitrile (1:1, v/v) and mobile-phase flow-rate, 1.0 ml/min. Peak numbers correspond to Tables I and II.

With the given chromatographic conditions, there was some overlap of triglyceride and cholesterol ester fractions. To determine the extent of each, the triglycerides were hydrolyzed to mono- and diglycerides and the corresponding fatty acids with an initial treatment of the sample with pancreatic lipase (crude Steapsin, Sigma). The samples were incubated for 2 h at 37°C in the presence of calcium chloride according to the procedure of Mattson and Volpenhein [15]. The lipids were then extracted with chloroform—methanol as described above. The products of this enzymatic hydrolysis eluted from the column early and did not interfere with either cholesterol or cholesterol ester determinations. The blood lipid profiles of a streptozotocin diabetic rat are shown in Fig. 2 before and after treatment of the sample with lipase. Peaks 1, 3, 8, 10, and 11 appear to be triglycerides.

To further characterize these triglycerides, the fractions were collected and hydrolyzed with alcoholic potassium hydroxide. The liberated fatty acids were then methylated with hydrochloric acid—methanol [16] and chromatographed on a 30 m × 0.25 mm I.D. OV-101 glass capillary gas chromatographic column.

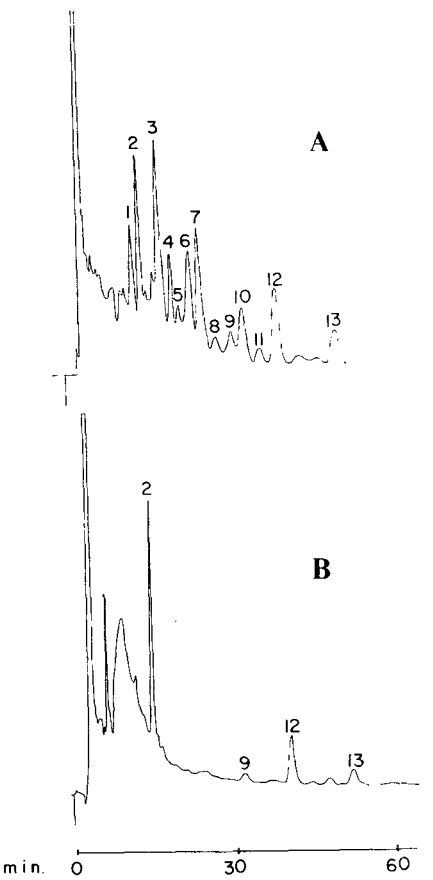

Fig. 2. Reversed-phase HPLC of blood lipids from (A) chloroform—methanol extract of streptozotocin diabetic rat and (B) chloroform—methanol extract of the same sample as A after treatment with pancreatic lipase. Chromatographic condition: same as for Fig. 1.

The column was temperature-programmed from 90°C to 230°C at 2°C/min. A modified Varian Model 1400 (Varian, Palo Alto, CA, U.S.A.) was used to record all fatty acid profiles. The methyl esters were identified by comparison of their retention times with those of standards. The distribution of fatty acids in the collected fraction, for individual triglycerides or a mixture of triglycerides, is given by the peak height ratios for the esters.

In addition to the information pertaining to fatty acid composition, the determination of the Integral Partition Numbers (IPN) [17] of the triglyceride fractions was considered in the structural assignments listed in Table I. The Integral Partition Number is calculated as the total number of carbon atoms in the fatty acid chains minus twice the total number of double bonds in the triglycerides. In the reversed-phase mode of HPLC, the lipids elute in the order of increasing partition numbers.

The partition numbers in Table I were established by chromatographing two triglycerides, trilinolein (IPN = 42) and triolein (INP = 48), and oils of known triglyceride composition. Because partial separation was achieved within a given IPN, partition numbers for all fractions could not be unequivocally established. Only trilinolein, peak number 3, was verified by co-chromatography with a standard triglyceride.

Cholesterol and the cholesterol esters were identified by their retention

TABLE I

PEAK RATIOS OF DIABETICS TO CONTROLS FOR THIRTEEN CHROMATOGRAPHIC FRACTIONS

Peak No.*	Integral Partition Number	Identification**	(Streptozotocin/ control)***	(Alloxan/ control)***
1	40	MMA, LLA, and/or MLA	24 (18 —32)	8.3 (4.0—14)
2		cholesterol	2.3 (1.4— 2.9)	1.7 (0.8— 3.3)
3	42	LLL	9.4 (6.5—12)	3.3 (1.8— 4.9)
4		an unknown triglyceride	8.9 (3.5—11)	3.2 (1.8— 5.0)
5		an unknown triglyceride	10.0 (3.6—16)	4.1 (0.5—12)
6	44	OLL	14 (7.6—20)	4.7 (2.2— 7.5)
7	44	PLL	13 (6.7—17)	4.7 (1.9— 7.2)
8		an unknown triglyceride	20 (5.0—60)	6.0 (1.0—20)
9	46	unidentified component, not a triglyceride	0.8 (0.7— 0.85)	0.6 (0.5— 0.9)
10	46	OOL, PPL, and/or POL	22 (6.2—24)	7.0 (3.5—12)
11		an unknown triglyceride	20 (3.0—70)	5.0 (10 —30)
12		cholesteryl arachidonate	0.5 (0.3— 0.7)	0.4 (0.2— 0.6)
13		cholesteryl linoleate	1.3 (0.9— 1.5)	1.16 (1.1— 1.2)
14		cholesteryl oleate		
15		cholesteryl palmitate		
16		I.S. (cholesteryl stearate)		

*Peak numbers correspond to Figs. 1 and 2.

**Triglyceride fatty acids: M = myristic; L = linoleic; O = oleic; P = palmitic; A = arachidonic.

***Average value based on four determinations each of control, streptozotocin diabetic and alloxan diabetic animals. Range of values in parentheses.

times. Because there was no detectable cholesteryl stearate in the samples, this ester was used as an internal standard in the quantitative studies. Cholesteryl linolenate and cholesteryl palmitoleate were also absent from the profiles; these esters would appear partially resolved from cholesteryl arachidonate and cholesteryl linoleate, respectively.

Reproducibility in the range of 4—8% (see Table II) was achieved for the extraction and liquid chromatographic analysis of four replicate determinations of a control blood sample. As indicated in Table I, the major quantitative differences between the control and diabetic samples occurred in the triglyceride fractions. This study further indicates that the elevation of individual triglyceride fractions is not uniform. In the streptozotocin diabetics, the triglyceride fractions are increased by a factor of 2.3 (peak 2 of Table II) to 24 (peak 1) over the control values. For alloxan diabetics, this increase ranges from 1.7 (peak 2) to 8.3 (peak 1). In addition, differences exist between the groups of streptozotocin- and alloxan-treated animals. For example, fraction 8 is increased by a factor of 20 in the streptozotocin diabetics, but only by a factor of 6 in the alloxan diabetics.

TABLE II

REPRODUCIBILITY DATA FOR THE CHLOROFORM—METHANOL EXTRACTIONS AND ANALYSIS BY REVERSED-PHASE LIQUID CHROMATOGRAPHY

Mobile phase: isopropanol—acetonitrile (5:2, v/v).

Peak No.*	Relative standard deviation (%)**
2	6.8
6, 7	4.4
9, 10	8.3
12	4.9
13	8.4

*Numbers correspond to Figs. 1 and 2 and Table I.
**Based on peak heights relative to cholesteryl stearate as internal standard for four determinations of a control blood sample.

DISCUSSION

Hyperlipidemic conditions are known to occur in rats with diabetes induced by either alloxan or streptozotocin. While the disordered lipid metabolism has now been shown in general terms in numerous studies, attempts for correlation with insulin, blood glucose levels, and type of diet still leave many unanswered questions. The methodology described in this article that allows both qualitative characterization and adequate quantitative measurements of neutral blood lipids is highly applicable to such studies.

While this work confirms the earlier observation that triglycerides are the major elevated fraction of blood neutral lipids in diabetic rats [5, 6, 8, 9] as compared to cholesterol and its esters, the measured increases are not uniform for the individual triglycerides. In fact, substantial differences exist in the

quantitative proportions of particular triglycerides as compared to control animals fed the same diet. Biochemical reasons for this "heterogenous triglyceridemia" are presently unknown. Further model metabolic studies involving diets of different composition should prove interesting. Also, duration of a given diabetic condition and its possible effect on the individual triglyceride fractions presents yet another unanswered question.

Both endogenous and exogenous factors seem to contribute to hypertriglyceridemia in diabetic humans and experimental animals [6, 8], including decreased clearance (peripheral removal) of plasma lipoproteins, reduced lipogenesis in adipose tissue, and "carbohydrate induced" hepatic turnover of different biochemicals [6]. Controversies exist concerning the role of low- or high-fat diet in such processes. The heterogeneous elevation of different triglycerides observed in this work appears to reflect the complexity of the above phenomena. However, a combination of isotopic studies with the methodology described here could be helpful in elucidation of different pathways.

This study also demonstrates certain differences in the triglyceride distribution between alloxan- and streptozotocin-treated rats. Earlier observations that the two drugs are not synonymous in their metabolic actions are those of Mansford and Opie [18] and Schein and Loftus [19] that relate to their hyperketonemic effect and the depression of pyridine nucleotides. Further biochemical studies will be needed to elucidate differences between the two important models of diabetes.

ACKNOWLEDGEMENT

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TERNARY SOLVENT MIXTURES FOR IMPROVED RESOLUTION OF HYDROXYLATED METABOLITES OF VITAMIN D_2 AND VITAMIN D_3 DURING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

This paper reports the development of three new ternary solvent mixtures for the liquid-chromatographic separation of metabolites of vitamin D on microparticulate silica. All solvent systems offer reduced peak tailing and improved resolution of vitamin D compounds, particularly of 24(R),25-$(OH)_2D_3$, when compared to the commonly used hexane—isopropanol mixture. The new mixtures can be substituted for hexane—isopropanol systems presently used for preparative liquid-chromatographic steps prior to radioimmunoassay or competitive protein-binding assay of 24,25-$(OH)_2D$ and 1,25-$(OH)_2D$ in human plasma. Hexane—isopropanol—methanol (87:10:3) mixtures are recommended where the lipid content of samples is high, whereas hexane—ethanol—chloroform (80:10:10) promises to be a useful mixture for differentiating vitamin D_3 metabolites from their vitamin D_2 analogs. A combination of the two solvent systems permits the separate assay of both 24(R),25-$(OH)_2D_3$ and 24(R),25-$(OH)_2D_2$ as well as 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_2$.

INTRODUCTION

High-performance liquid chromatography (HPLC) on microparticulate silica columns is becoming increasingly popular for the separation of metabolites of vitamin D during clinical assay [1, 2]. Though other solvent systems have been suggested [3, 4], a mixture of isopropanol and hexane (ca. 10:90) is the eluting solvent most frequently used [5], since it permits the partial resolution of most of the known metabolites of vitamins D_2 and D_3. Since the initial demonstration of the resolving power of this solvent system four years ago [5], we have

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applied HPLC using isopropanol—hexane to a number of problems including ultraviolet (254 nm) assay of 25-OH-D_2, 25-OH-D_3 and vitamin D in human serum [6], the separation of 24(R,S)$(OH)_2D_2$ stereoisomers [7] and the identification of products of 25-OH-D_3 in an isolated kidney perfusion system [8]. In the majority of cases, particularly where proportions of isopropanol are kept low, this solvent system provides adequate resolution. However, in some applications where higher solvent strengths are required and proportions of isopropanol must be raised above 10%, severe tailing occurs. This can also occur when anhydrous solvents are used and no attempt is made to equilibrate the solvent with water [9]. In applications where vitamin D metabolites are prepared for competitive protein assay [1, 2] or radioimmunoassay [10, 11], we perceived the need to develop a solvent system able to reduce tailing of one peak into another and to provide better resolution of vitamin D_3 metabolites from their vitamin D_2 analogs. In this paper we present three new alternative solvent mixtures and compare them to the standard isopropanol—hexane solvent system.

EXPERIMENTAL

Solvents

All solvents except ethanol were from Burdick & Jackson Labs., Muskegon, MI, U.S.A., "distilled-in-glass" spectroscopic grade. Ethanol was technical grade supplied by Consolidated Alcohols, Toronto, Canada.

Vitamin D metabolites

Crystalline 25-OH-D_2 and 25-OH-D_3 were generous gifts from Drs. J.A. Campbell, Jack Hinman and John Babcock of Upjohn, Kalamazoo, MI, U.S.A. Crystalline 24(R),25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ were kind gifts of Dr. M. Uskokovic of Hoffmann LaRoche, Nutley, NJ, U.S.A. 24(R),25-$(OH)_2D_2$ and 1,25-$(OH)_2D_2$ were synthesized respectively from stigmasterol by a chemical route [12] and 1,25-(OH)-D_2 by a biosynthetic method [13]. The latter product, 1,25-$(OH)_2D_2$, was purified by Sephadex LH-20 chromatography [14] and HPLC [15] prior to identification using mass spectrometry [16].

Concentrations of solutions of vitamin D metabolites were measured by a Model SP 1800 spectrophotometer (Pye-Unicam, Cambridge, Great Britain) assuming ϵ at 265 nm is 18 300.

High-performance liquid chromatography

The chromatograph used in these studies was a Model LC 204 fitted with a Model 6000A pumping system, U6K injection valve and a Model 440 ultraviolet fixed-wavelength (254 nm) detector (all from Waters Assoc., Milford, MA, U.S.A.). Stainless-steel columns (25 cm × 6.2 mm I.D.) prepacked with 6 μm diameter microparticulate silica (Zorbax-SIL) used in most of the experiments were purchased from Dupont Instruments, Wilmington, DE, U.S.A. For certain experiments indicated in the text, 25 cm × 4.6 mm I.D. columns of Zorbax-SIL (also from Dupont) or LiChrosorb SI-100, 10 μm (Brownlee Labs., Karlsruhe, G.F.R.) were used for the separation. A Sigma-10 chromatography Data System (Perkin-Elmer, Norwalk, CT, U.S.A.) was used to plot and

integrate the chromatograms.

Resolution, R_s, was calculated using the equation

$$R_s = \frac{1}{4}\left[\frac{\alpha-1}{\alpha}\right]\sqrt{\bar{N}}\left[\frac{K'}{K'+1}\right]$$

where $\bar{N}$ is the average number of theoretical plates based upon two peaks of the chromatogram and is calculated using the peak widths measured at the baseline (not from the peak width of a triangle drawn under the peak).

RESULTS

In extreme cases, chromatography of the principal metabolites of vitamin D_3 (25-OH-D_3, 24(R),25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$) on microparticulate silica (in this case Zorbax-SIL, 25 cm × 4.6 mm) with the solvent system hexane—isopropanol (85:15) can produce severe tailing effects (Fig. 1).

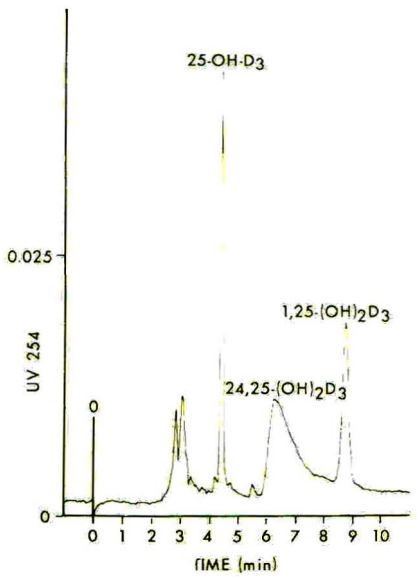

Fig. 1. Severe tailing during liquid chromatography of a mixture of synthetic metabolites of vitamin D_3. Chromatographic conditions: Zorbax-SIL, 25 cm × 4.6 mm; hexane—isopropanol (85:15); flow-rate 1.5 ml/min; 900 p.s.i.

Substitution of a new LiChrosorb SI-100 column (25 cm × 4.6 mm) for the Zorbax-SIL column in this situation produced no change in the degree of tailing under these particular conditions. It seems unlikely, therefore, that the tailing can be attributed to column voids but is solvent- or adsorbant-related. The possibility that 24,25-$(OH)_2D_3$ was contaminated with an impurity on the tail end of the peak was investigated by collecting subfractions of the broad peak and re-injecting them under identical conditions. The subfractions all gave a single peak which retained a tail and had a retention time identical to that of the parent peak. Extracolumn mixing, eddying and other chromatograph-related problems were ruled out when improvements were observed with

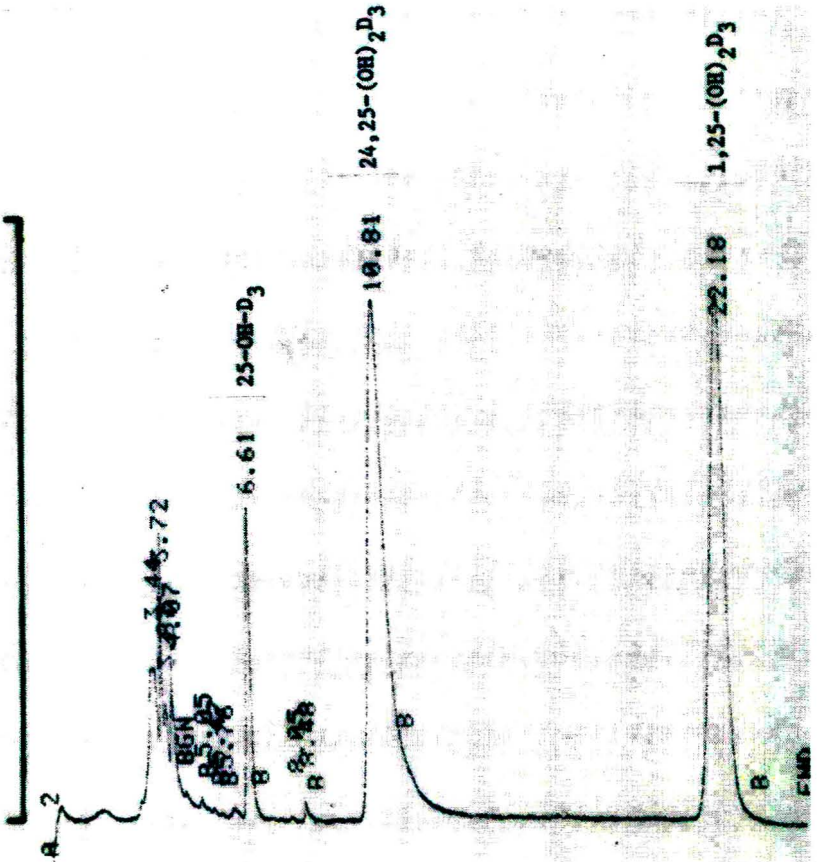

Fig. 2. Typical separation of 25-OH-D_3, 24(*R*),25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ on microparticulate silica using hexane—isopropanol (85:15) as solvent. Chromatographic conditions: Zorbax-SIL, 25 cm × 6.2 mm; flow-rate 1.5 ml/min; 600 p.s.i. Numbers above peaks are retention times in minutes. Bar on abscissa represents ultraviolet (254 nm) response of 0.006 absorbance units.

modified solvent mixtures. In fact, as we can see from a more typical run shown in Fig. 2 (Zorbax-SIL, 25 cm × 6.2 mm), other batches of the solvent hexane—isopropanol (85:15) produced less extensive tailing of the peaks. Nevertheless, as illustrated in Figs. 1 and 2, each metabolite was affected to a different degree by the tailing effect. 25-OH-D_3 and 1,25-$(OH)_2D_3$ were, at worst, only slightly broadened by hexane—isopropanol (85:15) whereas the other dihydroxylated metabolite of vitamin D_3, 24(*R*),25-$(OH)_2D_3$, was noticeably asymmetrical.

When ternary solvent systems based upon hexane—isopropanol—methanol (87:10:3), hexane—ethanol—chloroform (80:10:10), or hexane—methanol—methylene chloride (80:10:10) were used with the same 25 cm × 6.2 mm Zorbax-SIL column on the same day, significant reductions in tailing occurred and resolution of 25-OH-D_3, 24(*R*),25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ was greatly improved (Figs. 3—5 and Table I). This improvement was in the face of no change in flow-rate, column, primary solvent or approximate solvent strength.

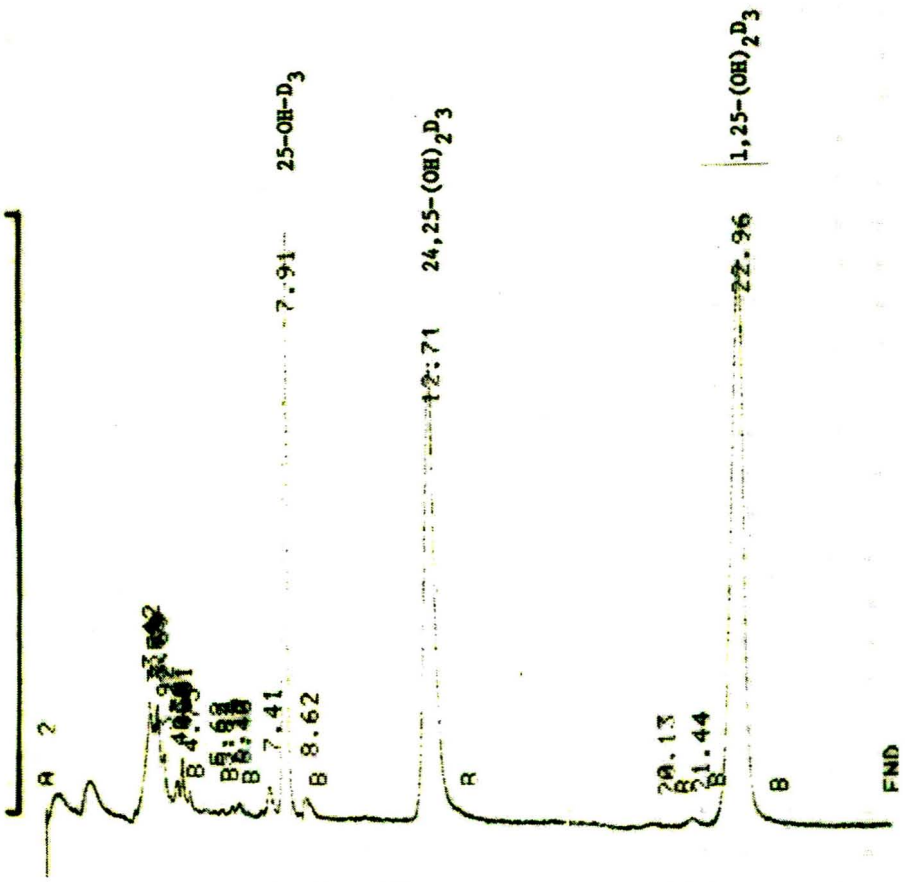

Fig. 3. Separation of 25-OH-D_3, 24(*R*),25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ on Zorbax-SIL using hexane—isopropanol—methanol (87:10:3) as solvent. Column and other chromatographic conditions as described in Fig. 2.

There was not only a marked reduction in the tailing of the 24(*R*),25-$(OH)_2D_3$ peak but also a sharpening of the 1,25-$(OH)_2D_3$ peak.

When we examined the separation of 1,25-$(OH)_2D_3$ from its vitamin D_2 analog, 1,25-$(OH)_2D_2$, using these three systems, we also observed improvement over the traditional hexane—isopropanol (85:15) mixture (Fig. 6). Whereas hexane—isopropanol (85:15) (Fig. 6A) gave only marginal separation of 1,25-$(OH)_2D_3$ (22.35 min) and 1,25-$(OH)_2D_2$ (20.89 min), this was slightly improved using hexane—isopropanol—methanol (87:10:3) (Fig. 6B) [1,25-$(OH)_2D_3$ (22.35 min); 1,25-$(OH)_2D_2$ (20.71 min)], and baseline resolved using hexane—ethanol—chloroform (80:10:10) (Fig. 6C) [1,25-$(OH)_2D_3$ (20.76 min); 1,25-$(OH)_2D_2$ (18.54 min)] or hexane—methanol—methylene chloride (80:10:10) (Fig. 5) [1,25-$(OH)_2D_3$ (13.0 min); 1,25-$(OH)_2D_2$ (11.8 min)].

Both solvent systems containing chlorinated hydrocarbons also provided the best separation of 24(*R*),25-$(OH)_2D_3$ from its vitamin D_2 analog, 24(*R*),25-$(OH)_2D_2$. Hexane—ethanol—chloroform (80:10:10) (not illustrated) gave retention times of 10.19 and 11.20 min for 24(*R*),25-$(OH)_2D_2$ and 24(*R*),25-$(OH)_2D_3$, respectively. Hexane—methanol—methylene chloride (80:10:10)

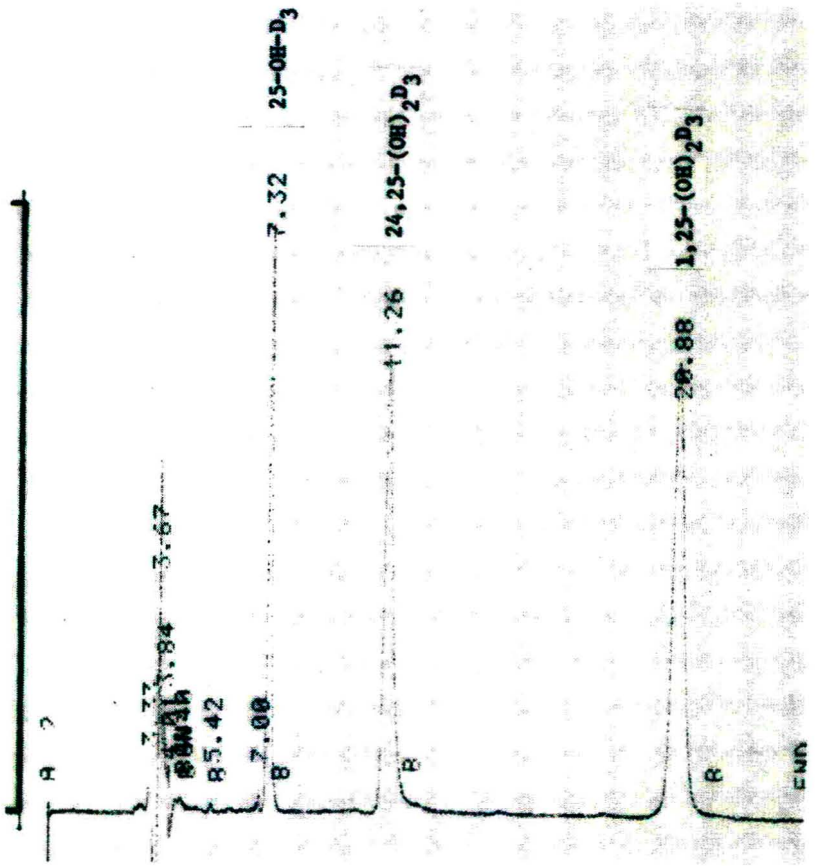

Fig. 4. Separation of 25-OH-D_3, 24(*R*),25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ on Zorbax-SIL using hexane—ethanol—chloroform (80:10:10) as solvent. Column and other chromatographic conditions as described in Fig. 2.

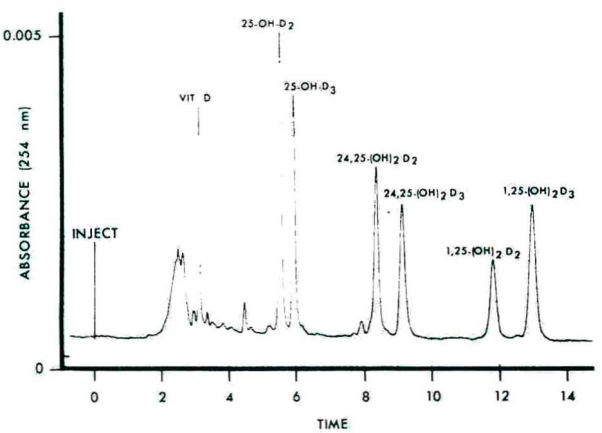

Fig. 5. Separation of the metabolites of vitamin D_2 and vitamin D_3 on Zorbax-SIL using hexane—methanol—methylene chloride (80:10:10) as solvent. Chromatographic conditions: Zorbax-SIL, 25 cm × 6.2 mm; flow-rate 2 ml/min; 900 p.s.i.

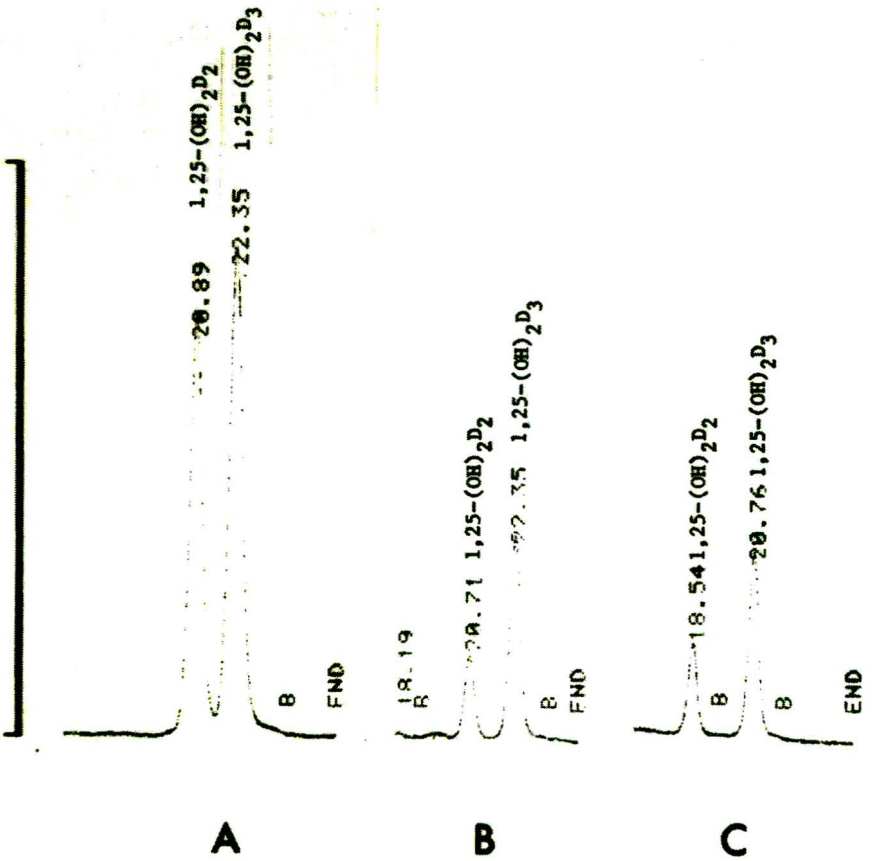

Fig. 6. Separation of 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_2$ and Zorbax-SIL using (A) hexane—isopropanol (85:15), (B) hexane—isopropanol—methanol (87:10:3), and (C) hexane—ethanol—chloroform (80:10:10) as solvent. Chromatographic conditions as in Fig. 2. Only the relevant region of each chromatogram is reproduced in the figure.

(Fig. 5) gave retention times of 8.6 and 9.1 min for these peaks with the same order of elution. The solvent system hexane—isopropanol (90:10) only partially separates 24(*R*),25-$(OH)_2D_2$ and 24(*R*),25-$(OH)_2D_3$ [5].

Tables I and II summarise the chromatographic data contained in Fig. 1—6 and provide resolution and theoretical plate counts for each of the solvent systems used. Though, as can be seen, the three new ternary solvent systems provided improved resolution of hydroxylated vitamin D_3 metabolites from each other and from their vitamin D_2 analogs, one major disadvantage was observed. The solubility of blood lipid in the solvents hexane—ethanol—chloroform (80:10:10) and hexane—methanol—methylene chloride (80:10:10) was much lower than for hexane—isopropanol—methanol (87:10:3) or hexane—isopropanol (85:15). This led to the formation of a two-phase system in the sample container whenever excessive lipid was dissolved in hexane—ethanol—chloroform (80:10:10) or in hexane—methanol—methylene chloride (80:10:10). If the sample was injected into the chromatograph in this form, peaks were invariably doublet in nature. The problem was not apparent with the new ternary system hexane—isopropanol—methanol (87:10:3).

TABLE I

CHROMATOGRAPHIC DATA FROM FIGS. 1—5

Fig. No.	Solvent system	K' 25-OH-D_3	K' 24,25-$(OH)_2D_3$	K' 1,25-$(OH)_2D_3$	$\bar{N}$*	R_s**
1	Hexane—isopropanol (85:15)***	0.48	1.21	2.03	707	1.81
2	Hexane—isopropanol (85:15)§	0.84	2.00	5.16	1061	4.17
3	Hexane—isopropanol—methanol (87:10:3)§	1.20	2.53	5.38	1764	4.69
4	Hexane—ethanol—chloroform (80:10:10)§	1.03	2.13	4.80	2010	5.16
5	Hexane—methanol—methylene chloride (80:10:10)§	1.20	2.39	3.81	2808	3.92

*$\bar{N}$, average number of theoretical plates based upon 24,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ peaks. Peak width measured at baseline.
**Resolution of 24,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ based upon the average number of theoretical plates, $\bar{N}$.
***Zorbax-SIL, 25 cm × 4.6 mm, 6 μm silica.
§ Zorbax-SIL, 25 cm × 6.2 mm, 6 μm silica.

TABLE II

CHROMATOGRAPHIC DATA FROM FIGS. 5 AND 6

Fig. No.	Solvent system	K' 1,25-$(OH)_2D_2$	K' 1,25-$(OH)_2D_3$	$\bar{N}$*	R_s**
6A	Hexane—isopropanol (85:15)§	4.80	5.21	2196	0.77
6B	Hexane—isopropanol—methanol (80:10:3)§	4.75	5.21	4059	1.17
6C	Hexane—ethanol—chloroform (80:10:10)§	4.15	4.77	3745	1.64
5	Hexane—methanol—methylene chloride (80:10:10)§	3.37	3.81	3538	1.37

*$\bar{N}$, average number of theoretical plates based upon 1,25-$(OH)_2D_2$ and 1,25-$(OH)_2D_3$ peaks. Peak width measured at baseline.
**Resolution of 1,25-$(OH)_2D_2$ and 1,25-$(OH)_2D_3$ based upon the average number of theoretical plates, $\bar{N}$.
§ Zorbax-SIL, 25 cm × 6.2 mm, 6 μm silica.

DISCUSSION

This paper reports three new ternary solvent mixtures for HPLC of the metabolites of vitamin D_3 and their vitamin D_2 analogs on microparticulate silica. These ternary solvent mixtures offer improved resolution and reduced peak tailing when compared to the hexane—isopropanol systems presently used for the separation of metabolites of vitamin D [5, 11, 17, 18]. These systems, particularly the solvent hexane—isopropanol—methanol (87:10:3), are well-suited for the preparation of purified fractions containing 24,25-$(OH)_2D$ and 1,25-$(OH)_2D$ during the assay of these compounds in human blood. Present methodology [10, 11] for the radioimmunoassay of 1,25-$(OH)_2D_3$ involves extensive purification of 1,25-$(OH)_2D_3$ fractions because of the almost equal affinity of antibodies for 1,25-$(OH)_2D_3$ and 24(R),25-$(OH)_2D_3$, and a 50-fold higher concentration of the 24(R) compound over the 1-hydroxylated compound in human plasma. Use of the new hexane—isopropanol—methanol (87:10:3) system ensures minimal tailing of the 24(R),25-$(OH)_2D_3$ peak into the 1,25-$(OH)_2D_3$ region of the chromatogram, thereby minimizing the possibility of 24(R),25-$(OH)_2D_3$ contamination of the 1,25-$(OH)_2D_3$ fraction. As antibodies with greater specificity for 1,25-$(OH)_2D_3$ become available, this improved resolution should lessen the need for pre-purification of plasma extracts prior to HPLC.

Peak tailing is a common problem in adsorption chromatography and is believed to be due to heterogeneity of the chromatographic surface due in turn to lack of total hydration of the active groups of the column [19]. It is not clear why the side-chain-dihydroxylated metabolite 24(R),25-$(OH)_2D_3$ is more severely affected than its A ring-dihydroxylated positional isomer, 1,25-$(OH)_2D_3$, but it is possibly related to the vicinal nature of the hydroxyl functions in the former compound and the interaction of these groups with the absorptive surface of the silica. Interestingly, the difference in the degree of tailing cannot be explained simply by an increase in the polarity of the molecule, since 1,25-$(OH)_2D_3$ is more strongly retained than 24(R),25-$(OH)_2D_3$ yet does not tail so severely. Peak tailing has been overcome in other situations by saturation of the eluting solvent with water [20], a modification not desirable here due to the labile nature of the solutes. Furthermore, because of the need to use any chromatographic development as a purification tool in the assay of 1,25-$(OH)_2D_3$, we avoided the use of agents, such as water, that would lengthen the time taken to evaporate solvent prior to radioimmunoassay. Thus, we used small percentages of methanol or ethanol [20] as a substitute for water and were able to significantly reduce tailing without the problems of using water referred to above. It is presumed that the addition of alcohol works by providing a more homogeneous hydration of the absorptive surface, thereby producing a more symmetrical peak.

Certain of the new solvent systems, particularly those containing a halogenated hydrocarbon (e.g., hexane—ethanol—chloroform, 80:10:10), offer improved resolution of the vitamin D_3 metabolites from their vitamin D_2 analogs. The baseline separation of 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_2$ or of 24(R),25-$(OH)_2D_3$ and 24(R),25-$(OH)_2D_2$ permits the separate assay of these metabolites in plasma extracts. However, in view of the poor solubility of plasma lipids in hexane—ethanol—chloroform (80:10:10), we suggest that this

is best achieved by rechromatography of fractions obtained after initial chromatography using silica and hexane—isopropanol—methanol (87:10:3). Development of separate assays for vitamin D_2 and vitamin D_3 analogs should open up the possibility of testing the validity of assays which purport to measure total 1,25-$(OH)_2$D and total 24(R),25-$(OH)_2$D in patients receiving large doses of vitamin D_2 for treatment of various mineral disturbances [21, 22]. Assays which claim to measure total 24,25-$(OH)_2$D or 1,25-$(OH)_2$D are under suspicion because of their differential sensitivity to vitamin D_2 analogs [23] or because of interference by 25,26-$(OH)_2D_2$ [24, 25], or calcidiol 26-23 lactone [26]. Methods described in this paper may help us to test these assays more rigorously and perhaps offer improvements.

The use of hexane—ethanol—chloroform in conjunction with (but after) hexane—isopropanol—methanol overcomes the only disadvantage noted for the halogenated solvent mixtures (that they provide poor lipid solubility) by minimizing the lipid content of the samples to be injected. Alternatively, the solvent mixtures hexane—ethanol—chloroform and hexane—methanol—methylene chloride may find their usefulness in the separation of closely similar compounds (e.g., isomers) and where exploitation of the solvent selectivity factor, α, is required. The importance of the factor α in the resolution equation (described in Experimental) is often overlooked since most separations are based upon increasing the theoretical plate count of the column or changing the nature of the chromatographic surface.

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DIRECT ASSAY FOR CREATINE KINASE BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A direct assay for creatine kinase (CK) activity was developed based on the separation and quantitation of adenosine triphosphate (ATP) by high-performance liquid chromatography. The total incubation time is 13 min and the elution time for ATP is 16 min. Using lyophilized CK as the sample, a sensitivity in the range of 8 U/l (units/liter) was obtained. The method presented also has clinical significance in that CK levels in serum can easily be determined with minimal sample preparation. Using serum samples from a healthy patient and a heart attack victim, activities of 26.6 U/l and 609.0 U/l, respectively, were obtained. Because of the direct measurement of ATP, this method eliminates the coupled reactions encountered in the common spectrophotometric and colorimetric methods of analysis resulting in a simpler and inexpensive assay.

INTRODUCTION

The assay of creatine kinase (ATP: creatine N-phosphotransferase; EC 2.7.3.2), hereafter referred to as CK, for the diagnosis of myocardial infarction and progressive muscular dystrophy has already been reported [1,2]. The activity of the BB isoenzyme of CK has also been shown to be of clinical importance in the presence of various neoplastic diseases [3—5].

The usual method for the assay of CK is basically the method of Oliver [6] which utilizes the more thermodynamically favored reverse reaction. It is known that the reverse CK catalyzed reaction proceeds approximately 6 times faster than the forward reaction. The entire enzymatically coupled assay is shown as follows:

$$\text{ADP + phosphocreatine} \xrightleftharpoons{\text{CK}} \text{ATP + creatine} \quad (1)$$

$$\text{ATP + D-glucose} \xrightleftharpoons{\text{HK}} \text{ADP + D-glucose-6-phosphate} \quad (2)$$

$$\text{D-glucose-6-phosphate + NAD}^+ \xrightleftharpoons{\text{G-6-PD}} \text{6-phosphogluconate + NADH} \quad (3)$$

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where ATP is adenosine triphosphate, ADP is adenosine diphosphate, HK is hexokinase, G-6-PD is glucose-6-phosphate dehydrogenase, and NAD^+ and NADH are the oxidized and reduced forms, respectively, of nicotinamide adenine dinucleotide. The increase in NADH is measured spectrophotometrically at 340 nm.

A problem that arises with this type of scheme is that the activity of CK is somewhat dependent on the coupled reactions chosen for the assay. The equilibrium constants for reactions 1, 2, and 3 have been reported [7] as 1.4×10^8, 1.55×10^2, and 6.0×10^{-7}, respectively. Since the assay utilizes the more favorable reverse reaction in the initial step, reaction 3, due to its low equilibrium constant, may not always reflect accurately the rate of formation of ATP in reaction 1.

The lack of agreement between optimal assay conditions has also been reported in the literature. Morin [8] has examined several sets of assay conditions in addition to the presentation of his own optimum conditions. The omission of reactions 2 and 3 in the coupled system would eliminate several of the differences in reactant concentrations by completely eliminating the reactants.

The presence of adenylate kinase, which catalyzes the conversion of two ADP molecules to 1 molecule of ATP and 1 molecule of AMP (adenosine monophosphate) presents a problem in real serum samples. Walter [9] has shown that when AMP is used to inhibit this competing enzyme, it also inhibits reaction 3 of the CK coupled system. Elimination of reaction 3 would thus enable the use of AMP as an inhibitor of adenylate kinase without affecting the assay for CK.

High-performance liquid chromatography (HPLC) has been utilized for the assay of several enzymes [10—12]. To facilitate the assay of CK, we have used HPLC for the rapid separation and quantitation of ATP produced in reaction 1. The amount of ATP can be directly related to the activity of CK. Besides eliminating the necessity of a coupled enzyme system, many of the problems stated previously can be avoided.

EXPERIMENTAL

Apparatus

An Altex Model 110A high-pressure pump equipped with an Altex Model 153 UV detector, constant wavelength (254 nm), (Altex, Berkeley, CA, U.S.A.) was used for the direct assay method. The injector used was a Rheodyne Model 7125 (Rheodyne, Berkeley, CA, U.S.A.). A 5 cm × 4.1 mm I.D. precolumn and 15 cm × 4.1 mm I.D. working column were used for the separations. The columns were packed with 10-μm RP-18 LiChrosorb (E. Merck, Darmstadt, G.F.R.) using a Micromeritics Model 705 column packer (Micromeritics, Norcross, GA, U.S.A.) and the Altex pump. Peaks were recorded by an Ommiscribe Model B-5000 recorder (Houston Instrument, Austin, TX, U.S.A.). The spectrophotometric assay was performed using a Cary Model 14 spectrophotometer (Cary Instrument, Monrovia, CA, U.S.A.) equipped with a Lauda K-2/R thermostated water bath (Brinkman Instrument Inc., Westbury, NY, U.S.A.).

Chemicals

All water used was triply distilled. Tetrabutylammonium hydrogen sulfate was supplied by either Sigma (St. Louis, MO, U.S.A.) or Aldrich (Milwaukee, WI, U.S.A.). Trizma Base [tris(hydroxymethyl)aminomethane], ATP (sodium salt), ADP (sodium salt or di-monocyclohexyl ammonium salt), AMP, phosphocreatine (disodium salt), β-NAD^+, α-D(±)glucose, monothioglycerol, CK (lyophilized from rabbit muscle), HK (crystalline suspension in 3.2 *M* ammonium sulfate), G-6-PD (lyophilized from *Leuconostoc mesenteroides*), and control serum (type 1-A, normal) were all obtained from Sigma. Potassium dihydrogen phosphate was obtained from Fisher Scientific Co. (Fair Lawn, NJ, U.S.A.) and NaOH was obtained from Matheson, Coleman, and Bell (Norwood, OH, U.S.A.). Methanol distilled in glass was obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Acetic acid was obtained from Scientific Products (McGaw Park, IL, U.S.A.), magnesium acetate from J.T. Baker (Phillipsburg, NJ, U.S.A.), and $KF \cdot 2H_2O$ from Mallinckrodt (St. Louis, MO, U.S.A.). All substrates and enzymes were stored refrigerated at 0—5°C or frozen in a desiccator as recommended. All chemicals were reagent grade or the highest purity available. Real serum samples, normal and elevated, were obtained from McCullough-Hyde Memorial Hospital (Oxford, OH, U.S.A.) and refrigerated at 0—5°C until use.

Procedures

CK timed reaction. All solutions were made or diluted with 0.1 *M* Tris buffer adjusted to pH 6.8 with acetic acid. A 0.5-ml aliquot of a reactant solution containing 6 m*M*/l ADP, 6 m*M*/l AMP, and 60 m*M*/l magnesium acetate was placed in a 75-mm glass test-tube. To this, 0.5 ml of a reactant solution containing 90 m*M*/l phosphocreatine and 18 m*M*/l $KF \cdot 2H_2O$ was added. Inhibition of adenylate kinase was performed by the addition of 2 m*M*/l AMP and 6 m*M*/l $KF \cdot 2H_2O$ (reaction concentrations) as recommended by Meiattini et al. [13]. In using the fluoride concentrations recommended by Morin [8], problems with the precipitation of MgF_2 occurred. The test-tube was then pre-incubated for 3 min at 30°C in a water bath equipped with a Polyscience Model 73 thermostat (Polyscience, Niles, IL, U.S.A.). A 0.5-ml aliquot of a pre-incubated (30°C) stock solution of 0.04 μg/ml CK solution containing 1.2 m*M*/l monothioglycerol was added to the test-tube. Reactions proceeded for 10, 20, and

TABLE I

REACTION CONDITIONS FOR THE HPLC AND THE SPECTROPHOTOMETRIC ASSAY OF CK

Reactant	Concentration (m*M*/l)	Reagents added additionally in the spectrophotometric assay	Concentration
ADP	2.0	NAD^+	2 m*M*/l
AMP	2.0	D-Glucose	15 m*M*/l
Magnesium acetate	20.0	HK	3000 NAD units/l
Phosphocreatine	30.0	G-6-PD	2500 NAD units/l
$KF \cdot 2H_2O$	6.0		

30 min and were terminated by placing the test-tubes in boiling water for approximately 75 sec. Final reaction concentrations are listed in Table I. Upon cooling, a 10-μl sample of the reaction mixture was injected into the chromatographic system. A solution of 0.40 μg/ml CK and 12 m*M*/l monothioglycerol was reacted in this manner.

Varying CK concentrations in control serum. All solutions were made or diluted with 0.1 *M* Tris buffer adjusted to pH 6.8 with acetic acid. A 0.5-ml aliquot of a reaction solution containing 10 m*M*/l ADP, 10 m*M*/l AMP, and 100 m*M*/l magnesium acetate was mixed with 0.5 ml of a reactant solution containing 150 m*M*/l phosphocreatine and 30 m*M*/l $KF \cdot 2H_2O$. Final reaction conditions are also listed in Table I. This ADP—phosphocreatine mixture was then pre-incubated at 30°C for 3 min. In a 75-mm glass test-tube, a 0.5-ml aliquot of a

TABLE II

ENZYME AND ACTIVATOR CONCENTRATIONS FOR THE HPLC ASSAY OF CK IN CONTROL SERUM

CK (μg/ml)	Monothioglycerol (m*M*/l)
0.08	2.4
0.20	6.0
0.40	12.0
0.60	18.0
0.80	24.0

CK—monothioglycerol solution of varying concentration (Table II) was added to 1.0 ml of control serum and pre-incubated at 30°C for 3 min. At time 0 min, the ADP—phosphocreatine mixture was added to the CK—serum mixture. The reaction proceeded for 10 min and was terminated by placing the test-tube in boiling water for approximately 75 sec. The test-tube was centrifuged at about 400 *g* for approximately 5 min on a semi-micro bench-top centrifuge to separate the coagulated protein from the liquid. A 10-μl volume of the decanted supernatant was then injected into the chromatographic system.

Real serum assay by HPLC. The procedure for real serum samples was essentially identical to that of the procedure for the CK time study. The CK solutions were replaced with the serum samples and only a reaction time of 10 min was used. For the normal serum, 0.5 ml of serum with 60 m*M*/l monothioglycerol (20 m*M*/l reaction concentration) was pre-incubated for 3 min at 30°C. To this, the ADP—phosphocreatine mixture was added and allowed to react for 10 min. The reaction was then terminated and treated in the same manner as the CK in the control serum procedure. For the elevated CK in serum, 0.5 ml of serum was diluted to 2.0 ml with 0.1 *M* Tris buffer (pH 6.8). The diluted serum solution also contained 60 m*M*/l (20 m*M*/l reaction concentration) of monothioglycerol. A 0.5-ml aliquot of this solution was then used as in the above procedure.

CK assay spectrophotometric method. For the spectrophotometric assay of CK, the method of Bowers and Kelley [14] was utilized with several minor adjustments. The blank used was 0.1 *M* Tris buffer (pH 6.8) with acetic acid. All solutions were made or diluted with the 0.1 *M* Tris buffer. The concentrations of the substrates, cofactors, activators, and inhibitors used in the HPLC assay are very similar to those recommended by Bowers and Kelley and were kept the same for the spectrophotometric method. Final reaction concentrations for glucose, NAD, HK, and G-6-PD are given in Table I. Serum volumes of 0.2 or 0.25 ml were used for each assay.

Calculations

Peak areas were calculated using a HP 3000 minicomputer equipped with a HP 7221A plotter (Hewlett Packard, Palo Alto, CA, U.S.A.). The activity in units/l were calculated from the area of the ATP peak and an ATP calibration curve. A constant injection volume of 10 μl was used to eliminate any necessary conversion factors. Activity was calculated by the following equation:

$$\text{Units/l} = \frac{[\text{PA} - \text{YI}] \times \text{TV}}{\text{SC} \times \text{T} \times \text{SV}}$$

where

PA = peak area of ATP produced (cm^2)
YI = *Y* intercept of the ATP calibration curve (cm^2)
TV = total reaction volume (μl)
SC = slope of the ATP calibration curve [$cm^2/(\mu M/\mu l)$]
T = reaction time (min)
SV = volume of sample (l)

The YI and SC terms must correspond to the same detector range as the PA term.

Activity in units/l for the spectrophotometric method were calculated in the typical manner [15].

RESULTS

In selecting a suitable mobile phase for the separation of AMP, ADP, and ATP, the method of Hoffman and Liao [16] was considered. Resolution using this method was good; with a slight adjustment in pH and composition percentage, a slightly better separation of the components was achieved. The mobile phase consisted of an 88% mixture of 0.1 *M* KH_2PO_4, 0.025 *M* butylammonium hydrogen sulfate, and 12% methanol. To this, enough 0.75 *N* NaOH was added to adjust the pH to 6.8. All three components could be resolved in 16 min as shown in the chromatograms.

For the assay proposed, the chromatographic unit must exhibit a linear response to the product being quantitated, ATP. Table III lists a series of various ATP concentrations that were injected into the system and detected at 0.04 absorbance units full scale (a.u.f.s.). Each concentration was injected three times. The correlation coefficient shows that linearity of the system to ATP

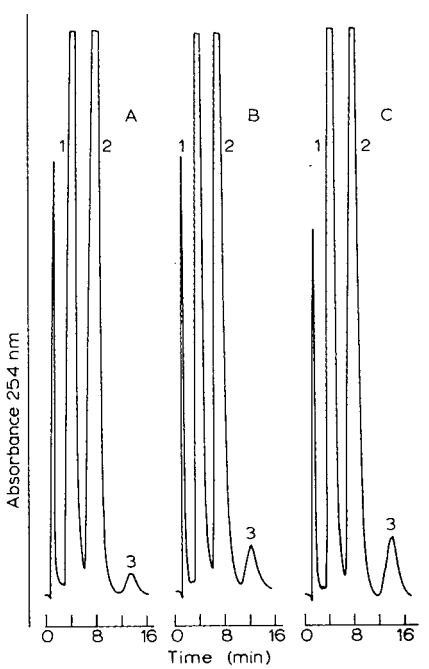
TABLE III

INSTRUMENT RESPONSE FOR ATP AT 0.04 a.u.f.s.

Slope = 5.96×10^3; intercept = 0.11; correlation coefficient = 0.9980.

ATP concentration $\times 10^{-4}$ (M)	Peak area (cm^2)	R.S.D. (%)
0.31	0.30 ± 0.05	16.67
1.24	0.84 ± 0.06	7.14
2.48	1.65 ± 0.18	10.91
3.73	2.22 ± 0.06	2.70
4.97	3.13 ± 0.21	6.71

was excellent. A similar response curve was obtained for ATP at 0.08 a.u.f.s. Six different concentrations of ATP ranging from $4.97 \times 10^{-4} M$ to $0.62 \times 10^{-4} M$ were injected into the system. Each concentration was injected three times with relative standard deviations of peak areas ranging from 1.19% at higher concentrations to 16.15% at lower concentrations. The response curve



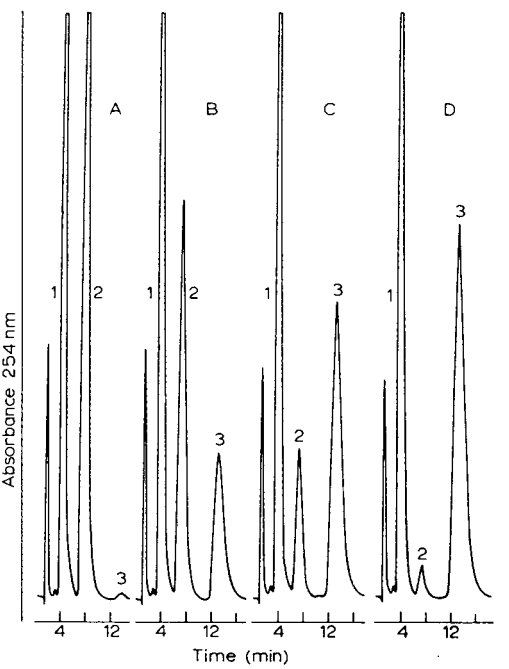


Fig. 1. Separation of AMP (1), ADP (2), and ATP (3) for reaction times of 10 min (A), 20 min (B), and 30 min (C) using 0.04 μg/ml CK. Chromatographic conditions: column, LiChrosorb C_{18}; eluent, see text; flow-rate, 2.0 ml/min; temperature, ambient; detection, UV 254 nm, 0.04 a.u.f.s.; injection volume, 10 μl.

Fig. 2. Separation of AMP (1), ADP (2), and ATP (3) for reaction times of 0 min (A), 10 min (B), 20 min (C), and 30 min (D) using 0.40 μg/ml CK. Chromatographic conditions: same as in Fig. 1 except detection range is 0.08 a.u.f.s. Chromatograms B, C, and D show the reduction of substrate ADP in addition to the leveling of the product ATP as time proceeds.

TABLE IV

LINEARITY OF CK ACTIVITY WITH RESPECT TO REACTION TIME FOR 0.04 μg/ml CK

Trial	Time (min)	Peak area (cm^2)	R.S.D. (%)	Slope	Intercept	Correlation coefficient
1	10	0.35 ± 0.02	5.7	0.046	−0.10	0.9999
	20	0.82 ± 0.11	13.4			
	30	1.27 ± 0.02	1.6			
2	10	0.34 ± 0.03	8.8	0.046	−0.13	0.9980
	20	0.75 ± 0.05	6.7			
	30	1.25 ± 0.06	4.8			
3	10	0.47 ± 0.07	14.9	0.042	−0.03	0.9519
	20	0.66 ± 0.06	9.1			
	30	1.32 ± 0.07	5.3			

for this range had a slope of 3.025×10^3, an intercept of 0.105, and a correlation coefficient of 0.996.

Once the linearity of the system to ATP was determined, the linearity of the reaction to time was investigated. Figs. 1 and 2 show the increase in the ATP peak area as time proceeds using CK concentrations of 0.04 μg/ml and 0.40 μg/ml. A visual inspection of the ATP peaks obtained using the CK concentration of 0.40 μg/ml (Fig. 2) is sufficient to demonstrate the leveling off the reaction between 20 and 30 min. Not only is the increase in the ATP indicative of this, but also the peak corresponding to ADP shows that the level of this substrate is insufficient to sustain the reaction at the initial rate.

Peak areas of ATP generated using a CK concentration of 0.04 μg/ml were quantitated and are listed in Table IV along with the corresponding reaction times. Slopes, intercepts, and correlation coefficients for each set of reaction times were calculated and are also listed in Table IV. As can be seen, linearity was achieved up to 30 min at this concentration of CK. All injections were performed in triplicate. For subsequent reactions, a reaction time of 10 min was adopted in that it seemed to be sufficiently long for low CK concentrations to be detected but not excessively long for high CK concentrations so that linearity was maintained. In addition to this, 10 min is not an excessive reaction time for the analysis of clinical samples.

Linearity of the assay with respect to CK concentration was determined. ATP peak areas obtained using the concentrations of CK in control serum in Table II were quantitated and used to calculate the respective units/l of CK. A plot of units/l of CK versus μg/ml of CK is shown in Fig. 3. Relative standard deviations for nine determinations are on the order of 3—10%. A slope of 671.3 was obtained with an intercept of −7.50.

Fig. 4 illustrates the difference in serum CK levels between a healthy patient and a heart attack victim. It is important to note that 0.5 ml of undiluted serum was used for the assay of the normal sample whereas 0.5 ml of a 2-ml

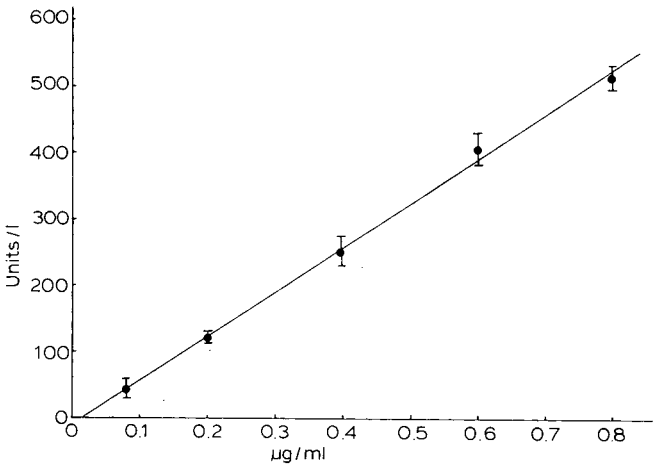


Fig. 3. Units/l of CK in control serum measured by the HPLC assay as a function of the CK concentrations listed in Table II. Conditions used are given in Table I. Each point represents the mean of nine determinations. Error bars indicate the standard deviation. Correlation coefficient = 0.9986.

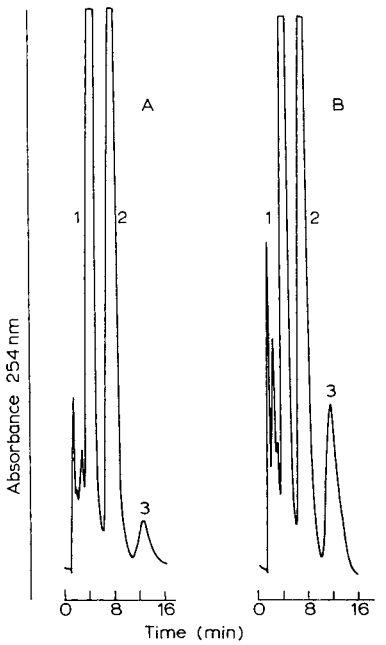


Fig. 4. Separation of AMP (1), ADP (2), and ATP (3) for CK assay of serum from a healthy patient (A) and a heart attack victim (B). Due to dilution of the elevated serum, the ATP peak area for (B) should be magnified 4 times when comparing to the ATP peak of (A). Chromatographic conditions are the same as those in Fig. 1.

solution containing 0.5 ml of serum was used for the assay of the elevated serum. Therefore, in order to give a proper perspective, the ATP peak in the elevated sample should be magnified four times due to the difference in sample volumes.

CK activities for real serum samples and standards determined using both the

TABLE V

COMPARISON OF THE HPLC AND THE SPECTROPHOTOMETRIC METHOD FOR THE ASSAY OF CK

CK source	HPLC method			Spectrophotometric method		
	Activity (U/l)	n^*	R.S.D. (%)	Activity (U/l)	n^*	R.S.D. (%)
0.04 μg/ml lyophilized CK	12.4 ± 4.5	3	36.3	10.0	1	—
0.40 μg/ml lyophilized CK	304.2 ± 26.2	4	8.6	87.3	1	—
Normal serum	26.6 ± 1.5	3	5.6	36.0 ± 1.0	3	2.8
Elevated serum	609.0 ± 28.3	2	4.6	256.0	1	—

*n = number of trials.

HPLC and spectrophotometric methods are shown in Table V. When serum samples were assayed by both methods, the normal sample produced similar values for the different assay procedures. However, when the elevated serum sample was assayed by both methods, a large difference in values was obtained. Possibly the serum matrix itself was a contributing factor to the difference in activities observed. To determine this, the ATP peaks corresponding to a reaction time of 10 min in the HPLC time studies were quantitated and converted to units/l. Both enzyme concentrations of 0.04 μg/ml CK and 0.40 μg/ml CK were used. These two solutions were then assayed by the spectrophotometric method. The activities obtained for the 0.04 μg/ml CK solution were similar for the two methods; however, the values obtained for the 0.40 μg/ml CK solution from the two methods differed considerably. This result, similar to that found for the serum samples, indicates there is some inhibitory factor in the coupled system and that the serum matrix was not a problem. Apparently, at high CK activities, the unfavorable equilibrium constant for the G-6-PD catalyzed reaction in the spectrophotometric method is a limiting factor. In addition, AMP inhibition of G-6-PD could also play a role. At low activities of CK, the G-6-PD catalyzed reaction is not a limitation and close CK activities for the two methods can be obtained.

DISCUSSION

The linearity of the system has been established for ATP over a concentration range of approximately one order of magnitude. Although the range is not extremely wide, it has proven to be sufficient for the purpose of this assay. For samples containing high levels of CK activity, simple dilutions will enable the ATP peak produced to be within this range. Although ATP concentrations above the reported upper limit were not tested, linearity would be expected to be maintained. At a.u.f.s. settings lower than 0.04, accurate quantitation of the ATP peak was difficult due to the inability of obtaining a stable baseline with the given mobile phase.

In performing this assay, the concern is mainly to determine whether or not

the CK levels in the serum are normal, borderline, or elevated. Bowers and Kelley [14] have reported the following ranges for CK activities in serum: normal 0—100 units/l, borderline 100—200 units/l, and elevated > 200 units/l. In using these ranges, the values obtained spectrophotometrically have shown that we have a serum sample with normal CK levels and also a serum sample with elevated CK levels. Before utilizing the HPLC procedure as a routine clinical assay, many more serum samples would need to be run to establish the normal, borderline, and elevated ranges for CK in serum. However, for these two serum samples, the HPLC method could clearly distinguish which is from a healthy patient and which is from a heart attack victim.

The direct assay for creatine kinase described offers several advantages over the usual enzymatically coupled spectrophotometric assays. The main advantage is that the measurement of the product is only dependent upon the rate of the CK reaction and not some function of a coupled reaction system. For the spectrophotometric method using NAD^+ as a substrate, G-6-PD must come from *L. mesenteroides*. This enzyme preparation is quite expensive, and the elimination of this enzyme would reduce analysis cost considerably. Analysis time is comparable; total analysis time, including incubation, is approximately 30 min. The separation is free from serum interferences and utilization of the substrate ADP could also be monitored if desired. Application of this assay for CK to a variety of other types of samples such as cerebrospinal fluid should be straightforward.

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THE EFFECT OF pH AND IONIC STRENGTH ON THE ELECTROPHORETIC SEPARATION OF ACIDIC GLYCOSAMINOGLYCANS

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SUMMARY

Using the electrophoretical methods applied to this study it is possible to determinate the dissociation constants (pK) of acid glycosaminoglycans containing a carboxylic group. The pK-values of the six acid glycosaminoglycans separated from animal connective tissues determined in this work were: hyaluronic acid (HA), pK = 3.0; chondroitin sulfate A (CS-A), pK = 2.8; chondroitin sulfate C (CS-C), pK = 3.3; dermatan sulfate (CS-B), pK = 3.3; heparatin sulfate (HeS), pK = 3.1 and heparin (HeP), pK = 2.4 and were measured at a constant ionic strength of I = 0.164 (NaCl) and at 10 ± 2°C.

Variation of ionic strength showed that physiological conditions seem to be most suitable for the electrophoretic separation of the glycosaminoglycans studied. A decrease of ionic strength causes increasing mobility but less accurate spots. In the case of increasing ionic strength the results are vice versa.

The second spot for HA very often appeared when pH values higher than 2 were used for electrophoresis. The spot had the same form as the original, high intensity, but an undecided migration in the pH range near the pK value of HA (3.0).

INTRODUCTION

Both one and two dimensional electrophoresis are widely used for the fractionation of acid glycosaminoglycans (GAG) [1—7]. To increase the fractionation effect the pH is often adjusted to a suitable range. Metal salt solutions are also used as buffer substances in the electrolytic chamber [6]. Other methods, such as column chromatography [7—9], thin-layer chromatography [10, 11] and gas chromatography [12, 13], also exhibit similarities in the fractionation of GAGs.

In the electrophoretic procedure the carrier solution is reduced in the anode and oxidized in the cathode chamber. Thus, to keep the pH constant, the electrolytic solution should have a good buffer capacity. Metal salt solutions such as $Cu(CH_3COO)_2$; $Ba(CH_3COO)_2$; $Zn(CH_3COO)_2$; $La(NO_3)_3$ and $Ca(CH_3COO)_2$ [6, 14] used in electrophoretical procedures have a very low

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buffer capacity and a pH change in the chambers cannot be avoided. Strong acids and some other good buffer substances are able to keep the pH in both chambers very stable.

The purpose of the present investigation was to study the migration speed of GAGs in an electric field, as a function of pH. The aim was also to test the influence of the ionic strength on the mobility, in both acidic and alkaline conditions, of GAGs. In some earlier studies these problems have been approached, but there are still many open questions [1, 15].

EXPERIMENTAL

The electrophoresis of the GAGs was performed using a cellulose acetate membrane (Beckman cellulose acetate strips) and Beckman Microzone R-200 equipment with a specially constructed water cooling system. The temperature was adjusted to 10 ± 2°C. A Microzone Duostat power supply model RD-2 was used as the power unit. The pH-measurements were carried out with a Radiometer pHM-26, and with a Beckman glass electrode type No. 41263. An immersion-type calomel electrode was used as a reference. A standard pH solution containing 0.01 *N* HCl in physiological NaCl was recommended. The pH of this solution was calculated to be 2.11 at 10°C [16]. The ionic strength was adjusted to $I \approx 0.164$ with NaCl (except when the effect of ionic strength was studied). The pH of the electrophoretic solution was checked both before and after the run from both the anode and cathode chamber.

The electrophoresis was performed using a 30-min migration time, 110-V power and 50-mA current. After electrophoresis the strips were stained with a 1% solution of Alcian blue (G.T. Gurr's) in 25% acetic acid, then rinsed with tap water and 10% acetic acid.

On the very alkaline side from pH 10 to 11.4 electrophoresis procedures were performed under a nitrogen gas stream in order to avoid contact with carbon dioxide.

Reagents

The reagents chosen for buffering the electrophoretic solutions were as similar as possible. They were the following: urocanic acid and histidine (Sigma, St. Louis, MO, U.S.A.); citric acid imidazole and barbituric acid buffers (E. Merck, Darmstadt, G.F.R.) and glycine (Eastman Organic Chemicals, Rochester, NY, U.S.A.). All buffers were adjusted to the desired pH range using 0.1 *N* HCl or 0.1 *N* NaOH solutions purchased from Merck.

Reference glycosaminoglycans, hyaluronic acid (HA); chondroitin sulfate A (CS-A); chondroitin sulfate C (CS-C); dermatan sulfate (CS-B); keratan sulfate-1 (KS-1); heparitin sulfate (HeS) and heparin (HeP), were gifts of Drs. M.B. Mathews, J.A. Cifonelli and L. Roden (Department of Pediatrics, The University of Chicago, Chicago, U.S.A.).

TABLE I

THE MIGRATION SPEED OF THE GAGs STUDIED IN THE ELECTRIC FIELD WITH VARIABLE pH VALUES FOR THE BUFFER USED

The desired pH range was produced by titrating the buffer with 0.1 *N* HCl or 0.1 *N* NaOH. Ionic strength was adjusted in each case, except for barbituric acid buffer, to $I = 0.164$ with NaCl. The initial pH and the pH after the run both in the cathode and anode were recorded.

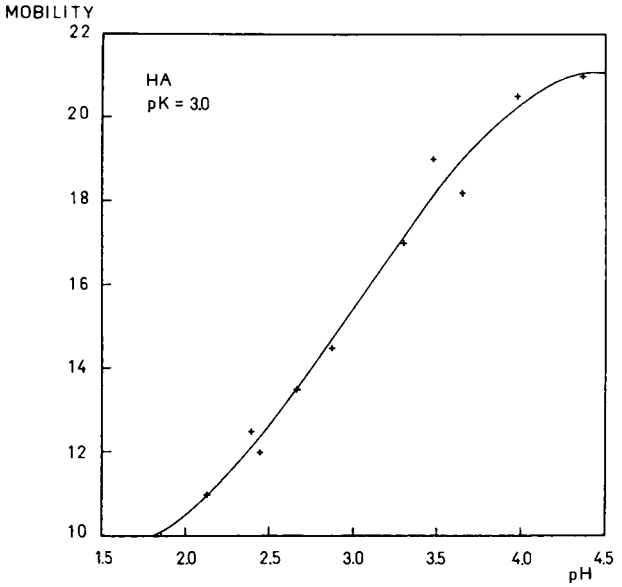
Buffer substance	Mobility (mm)							pH		
	HA	CS-A	CS-C	CS-B	KS-1	HeS	HeP	start	anode	cathode
0.1 *N* HCl	19.0	22.5	22.5	23.2	23.7	21.7	25.7	1.42	1.42	1.42
0.08 *N* HCl	18.0	21.0	21.0	21.0	22.0	20.0	25.0	1.44	1.44	1.44
0.06 *N* HCl	17.0	22.0	22.0	23.0	23.0	21.0	26.0	1.50	1.50	1.50
0.04 *N* HCl	13.5	21.2	21.5	22.7	23.7	20.5	28.0	1.61	1.61	1.61
0.02 *N* HCl	10.0	19.0	19.0	20.0	21.0	17.0	26.0	1.85	1.85	1.86
0.01 *N* HCl	11.0	21.5	22.5	22.7	24.0	19.0	29.5	2.12	2.11	2.12
0.02 *M* Histidine HCl	12.5	21.5	22.2	22.2	23.0	19.5	27.0	2.39	2.35	2.46
0.02 *M* Citric acid	12.0	22.0	22.7	22.7	23.0	19.5	28.7	2.44	2.44	2.50
0.02 *M* Histidine HCl	13.5	23.0	24.0	23.0	23.0	19.5	26.5	2.66	2.63	2.79
0.01 *M* Urocanic acid	14.5	23.5	23.5	23.5	22.5	20.5	28.0	2.87	2.74	3.04
0.02 *M* Citric acid	17.0	26.0	26.0	25.0	24.0	23.0	29.0	3.30	3.25	3.32
0.01 *M* Urocanic acid	19.0	28.5	28.5	27.0	25.2	25.2	31.5	3.48	3.37	3.70
0.02 *M* Citric acid	18.2	28.0	28.2	27.0	24.7	25.0	32.0	3.65	3.62	3.72
0.01 *M* Urocanic acid	20.5	28.5	29.5	28.5	24.0	25.0	30.7	3.98	3.79	4.17
0.01 *M* Urocanic acid	21.0	30.0	30.5	30.7	25.2	26.5	32.5	4.37	4.25	4.67
0.02 *M* Citric acid	20.5	28.0	28.5	29.0	23.5	25.2	30.5	4.83	4.79	4.90
0.02 *M* Citric acid	19.5	26.5	27.0	27.0	22.0	24.0	29.5	5.08	5.04	5.19
0.01 *M* Urocanic acid	21.0	28.7	30.0	30.2	24.0	25.7	30.5	5.48	5.35	5.61
0.01 *M* Urocanic acid	19.5	29.0	29.5	29.7	24.0	25.5	31.0	5.83	5.66	6.02
0.01 *M* Urocanic acid	21.0	29.0	30.5	30.7	25.5	26.5	31.5	6.20	6.07	6.36
0.02 *M* Imidazole	20.5	28.5	29.5	29.5	24.2	26.2	31.0	6.63	6.60	6.77
0.02 *M* Imidazole	21.0	30.2	31.0	31.0	25.5	27.0	32.0	7.10	7.06	7.12
0.02 *M* Imidazole	21.5	31.0	31.7	31.7	25.5	27.5	33.0	7.59	7.57	7.69
Barbituric acid buffer	20.0	31.5	31.5	31.5	23.5	26.5	32.5	8.50	8.36	8.51
0.02 *M* Histidine HCl	22.0	31.0	31.5	32.0	25.0	27.0	32.0	8.64	8.57	8.69
0.02 *M* Histidine HCl	22.5	31.0	31.5	31.5	25.5	26.5	32.5	9.08	9.07	9.26
0.02 *M* Histidine HCl	22.0	30.5	31.0	31.5	25.0	26.7	32.0	9.60	9.54	9.61
0.02 *M* Glycine	21.5	29.7	30.5	30.7	25.0	26.0	30.5	10.08	9.99	10.18
0.02 *M* Glycine	21.0	30.5	31.0	32.0	26.0	27.0	32.5	10.68	10.57	10.70
0.02 *M* Histidine	20.0	29.0	29.5	30.0	24.0	26.0	31.0	11.27	11.15	11.32
0.02 *M* Glycine	18.0	25.5	26.0	26.0	22.0	22.5	27.0	11.41	11.35	11.53

TABLE II

p*K* VALUES OF THE CARBOXYLIC GROUP OF ACIDIC GLYCOSAMINOGLYCANS

The values are based on electrophoretical measurements. Conditions: temperature, 10 ± 2°C; $I = 0.164$.

Substance	Abbreviation	p*K* (-COOH)
Hyaluronic acid	HA	3.0
Chondroitin sulfate A	CS-A	2.8
Chondroitin sulfate C	CS-C	3.3
Dermatan sulfate	CS-B	3.3
Keratan sulfate	KS-1	—
Heparitin sulfate	HeS	3.1
Heparin	HeP	2.4


MOBILITY
HA
pK = 3.0
22
20
18
16
14
12
10
1.5
2.0
2.5
3.0
3.5
4.0
4.5
pH

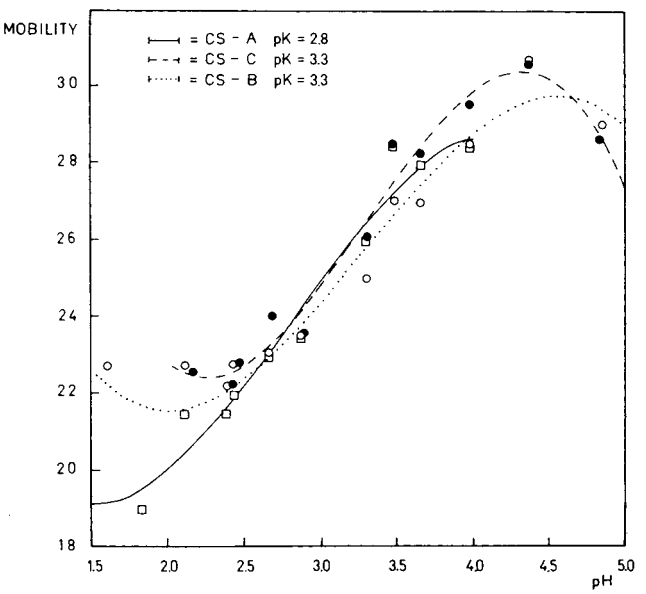

MOBILITY
= CS - A pK = 2.8
= CS - C pK = 3.3
= CS - B pK = 3.3
30
28
26
24
22
20
18
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
pH

Fig. 1.

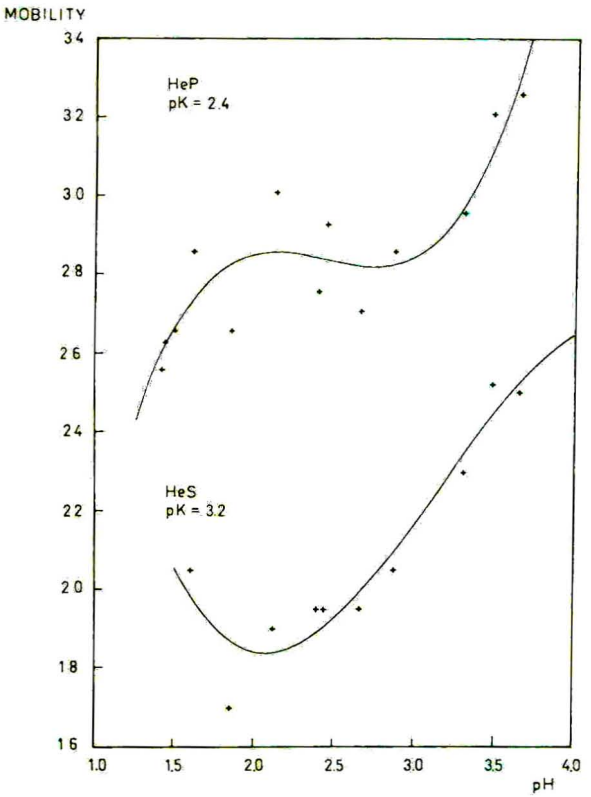


Fig. 1. The mobility of glycosaminoglycan in the electric field as a function of pH in the dissociation area of the carboxylic group. (a) Hyaluronic acid (HA); (b) chondroitin sulfate A (CS-A), chondroitin sulfate C (CS-C) and dermatan sulfate (CS-B) and (c) heparitin sulfate (HeS) and heparin (HeP).

RESULTS

As a function of pH, the mobility rates indicate that all significant variations occurred below the pH range of pH 4.5 (Table I and Fig. 1). Keratan sulfate is, however, an exception. The pH value does not seem to have any influence on the mobility of this particular glycosaminoglycan.

The rapid rise between pH 1.6 and 4.4 indicates the ionisation of the acidic carboxylic groups in the curve. The turning points of these pH jumps were calculated by means of the least square method as the points were pH = pK, and are listed in Table II.

In the dissociation area of the carboxylic group of HA especially, a special form of the HA spot appeared in which the easily identifiable shadow spot had very often migrated in the opposite direction from starting point. In Fig. 2a there is a good example of this effect. Fig. 2b shows the reference migrations in the dissociation area where the special form of HA is also seen. Examples of this kind of cleavage of HA also exist when histidine buffer, pH 5.70, and even barbituric acid buffer, pH 8.50, were used.

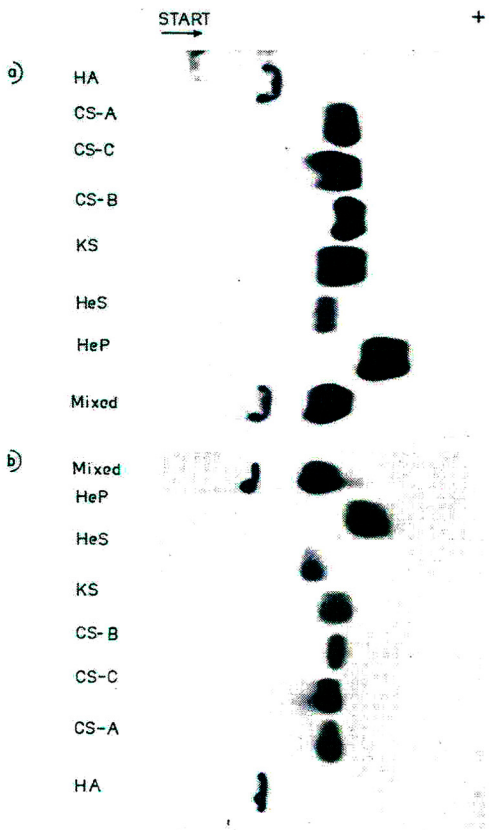


Fig. 2. (a) Electropherogram obtained using 0.02 *M* citric acid (pH 2.7), I = 0.164 (NaCl) as buffer solution. Note the cleavage of HA into the two spots having a typical form; (b) the reference run for GAGs on the acidic side. The buffer solution used in this experiment was 0.002 *N* HCl + 0.162 *M* NaCl, pH ≈ 2.50. The special form of HA is still present.

The influence of ionic strength on the mobility of the GAGs was investigated in both acidic and alkaline conditions. The effect of ionic strength seemed to be almost independent of that of pH. The ionic strength has, however, a great influence on the resolution ans sharpness of the spot. When a buffer solution with high ionic strength was used the migration was slow but the resolution was good (Fig. 3a). With a buffer with low ionic strength the migration improved, but on the other hand the sharpness of the spots was impaired as compared to the pherograms obtained from buffers with higher ionic strength (Fig. 3c).

DISCUSSION

The influence of pH variation on the separation of acidic GAGs has been investigated before [1] but never using such a wide pH range. The results of the present work show that the determination area of the pK values of the

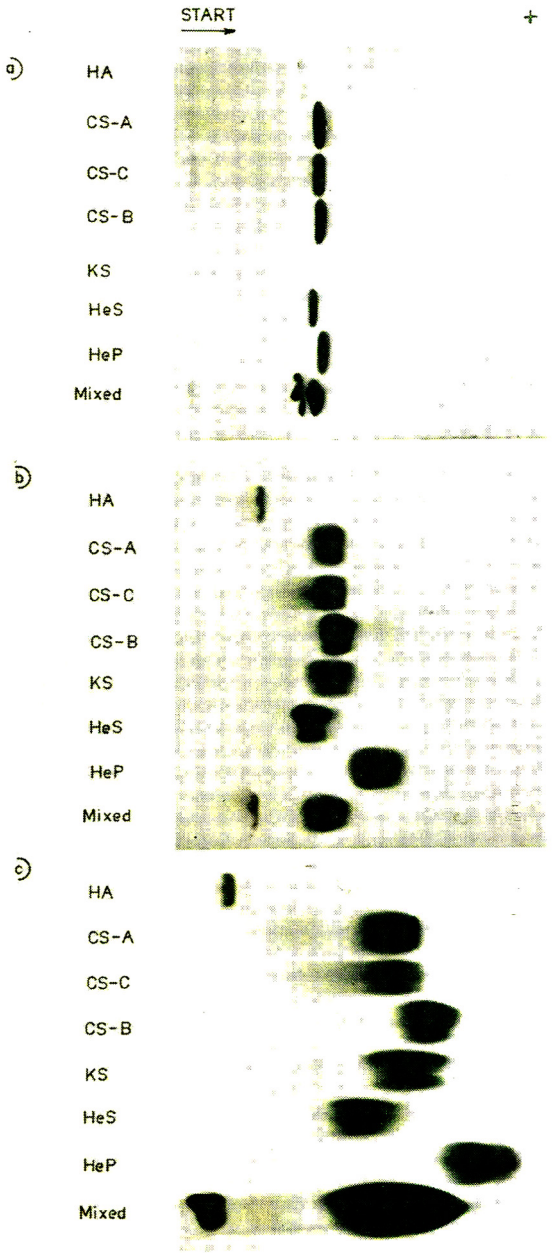


Fig. 3. The effect of ionic strength on the separation of GAGs on the acidic side (pH 2.1). (a) Buffer solution 0.01 *N* HCl + 0.5 *M* NaCl (high ionic strength); (b) 0.01 *N* HCl + 0.154 *M* NaCl (normally used ionic strength) and (c) 0.01 *N* HCl + 0.01 *M* NaCl (low ionic strength).

sulfate group of glycosaminoglycans, as seen in the case of keratan sulfate, is so acidic that it would be impossible to determine the pK values because of the drying of the cellulose acetate membrane during the run.

The effect caused by the variation of ionic strength is quite obvious. More ions in the electrolytical system require stronger current, and vice versa. The accuracy of the spot seems to be much better in the case of high ionic strength, indicating the pooling effect generally applied in column chromatography.

Although the pK values determined in this work are inaccurate, especially the pK value of HeP, it is seen that the acidity of GAGs decrease in the order HeP < CS-A < HA < HeS < CS-B < CS-C. Using the tritation method Laurent [17] obtained the value of pK = 2.95 for HA when I = 0.2 (NaCl). This is in good agreement with the value (pK = 3.0, I = 0.164 (NaCl), temperature, 10 ± 2°C) obtained by our electrophoretical method. The pK values of other GAGs have not, to our knowledge, been determined before.

The rapid rise of mobility as a function of pH seems to be connected with the GAGs having the carboxyl group in the molecule. The rise seems to be quite linear in the two co-adjacent pH units (i.e. 1 pH unit after the pK value). This experimental result also corresponds well with the findings obtained by Kiso and co-workers [15, 18].

From Fig. 2 and Table I it is easy to see that on the most acidic side many GAGs, HA, HeS and to some extent chondroitin sulfates, especially CS-C, have increasing mobility as a function of decreasing pH. According to Jokl's [19] observations this should be a result of (a) an increase in the charge of the GAG molecule or (b) a decrease in the molecular weight. The latter suggestion is out of the question, because the spot was still very accurate after the run.

The cleavage of the HA into two compounds having an opposite charge is difficult to explain. The splitting effect was found to be clearest in the dissociation range of the carboxylic group of HA, i.e. from pH 2 to 4, where the spot also has a special form caused by undecided migration direction (see Fig. 2a). Examples of cleavages having a wide distance with histidine buffer, pH 5.7, and even with barbituric acid buffer, pH 8.5, also existed, indicating that the cleavage is not only connected with the dissociation of HA. Some electrolytic solutions seem to have a stronger tendency to cause this cleavage of HA than others. A good example is citric acid near the pK range of HA. This pH area is also recommended as a useful one for the separation of acidic GAGs based on pH variation (Fig. 1 and e.g. Fig. 2b, pH 2.50).

The role of ionic strength is seen in Fig. 3. Using variable salt concentration it seems to be possible to regulate the separation procedure of GAGs as a function of mobility and sharpness of the spot. Because of the high ionic strength on the highly acidic side the KS-1 spot disappeared.

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CHROMBIO. 657

ISOTACHOPHORETIC DETERMINATION OF URINARY CITRATE IN NATIVE URINE

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SUMMARY

The technique of isotachophoresis has been used to develop a specific and sensitive method for the determination of citrate in unprocessed urine. The specificity of the isotachophoretic method was assessed using citrate lyase which caused disappearance of the isotachophoretic citrate signal. The isotachophoretic method compared favourably with the enzymatic method (citrate lyase) for urinary citrate. The normal range for urinary citrate in 25 healthy individuals, as found by isotachophoresis, was 0.33—2.89 mmol/24 h with a mean of 2.1 mmol/24 h.

INTRODUCTION

The determination of urinary citrate is of interest in urolithiasis research because of the known ability of citrate to form soluble complexes with calcium [1]. Consequently, the calculation of the activity products of the ions involved in stone formation [2] requires knowledge of the citrate concentration in urine. Currently, the determination of citrate in biological fluids can be performed using the pentabromoacetone method [3] or the enzymatic method [4] using citrate lyase (E.C. 4.1.3.6.) (CL).

Isotachophoresis has been successfully applied to the determination of mandelic acid, phenylglyoxylic acid, hippuric acid and purines or pyrimidines in urine [5, 6]. This promising method has not been systematically applied to the determination of other naturally occurring urinary constituents. The present investigation describes the conditions under which urinary citrate can be quantitated by isotachophoresis.

MATERIALS AND METHODS

Urine samples

Urine (24-h sample) was collected from 25 healthy individuals (laboratory staff), 10 male, 15 female, who were under their usual self selected diet and had no known renal or other diseases. Urine was collected without additives in plastic bottles and kept at 4°C during the collection period.

Measurement of urinary citrate by citrate lyase

The estimation of urinary citrate was performed by using the commercially available test kit of Boehringer (Mannheim, G.F.R.) using Welshman and McCambridge's method [7]. In the experiments designed for complete degradation of citrate in urines, the final concentration of the enzyme was 1 U/ml at a pH of 7.5 in 50 mmol imidazol—HCl buffer with an incubation time of 10 min.

Measurement of urinary citrate by isotachophoresis

The isotachophoretic analyses were performed in an LKB 2127 Tachophor (LKB, Bromma, Sweden) equipped with a 23 cm × 0.5 mm I.D. capillary. The starting voltage was 2 kV with a final voltage varying between 4 and 12 kV at 150 μA and 12°C. UV absorption at 254 nm was used for quantitation. The thermosignal could be used reliably for identification of the citrate. The time of analysis depended on the electrolyte content of the sample and varied between 20 and 60 min.

The chemicals used were commercially available and of analytical grade. The water used was distilled twice. In addition, the purification procedure for the terminating electrolyte solution was performed, as proposed by Everaerts et al. [8]. Leading electrolyte: $5 \cdot 10^{-3}$ *M* hydrochloric acid Suprapur (E. Merck, Darmstadt, G.F.R.); $1 \cdot 10^{-3}$ *M* sodium chloride (p.a. grade, E. Merck); 0.4% hydroxypropylmethylcellulose (Methocel 90 HG, 15,000 cps, Dow Chemicals, Midland, MI, U.S.A.). No adjustment of the pH (2.2) was carried out.

Terminating electrolyte: acetic acid, $5 \cdot 10^{-3}$ *M* (p.a. grade, E. Merck). Injection: the samples were injected through a septumless syringe injector as in Fredriksson's method [9] with an AA 701SN 10-μl Hamilton microsyringe equipped with a platin needle and tungsten plunger. The volume of injection varied in the range 2—10 μl. Citrate was quantitated by measuring the zone length with a graticula or a ruler. The chart speed was 0.5 or 1 mm/sec.

RESULTS

Measurement of citrate in aqueous solutions and urines

Aqueous solutions of citrate were tested over a range of 0.05—13 mmol/l. The injected volume was adjusted appropriately so that 5—10 nmol of citrate were injected. In aqueous solutions, the citrate signal, i.e. a non-UV-absorbing zone, could be easily identified by the specific stepheight of the thermal signal. It was preceded and followed by UV-positive zones (impurities?). Similarly, in urine, the citrate signal was sandwiched between even more pronounced UV-

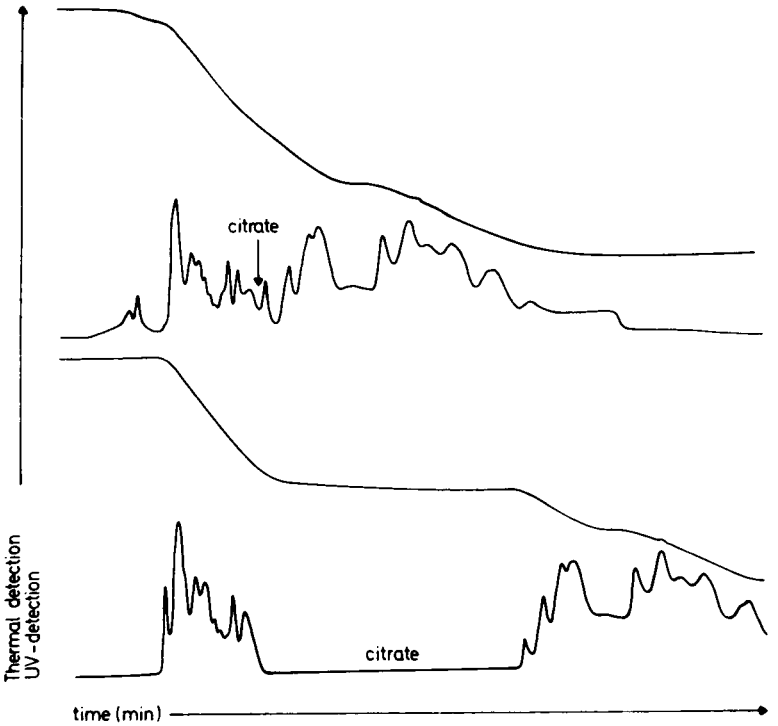

Fig. 1. Typical isotachopherograms of native urine before (lower two curves) and after (upper two curves) treatment with CL. Citrate can be reliably identified by both the non-UV-absorbing zone and by the specific step height of the thermal signal. After treatment with CL, a small, non-UV-absorbing incisure is detectable, which could correspond to non-converted citrate. Identification or quantitation of this zone is not possible. Injected volume: 10 μl native urine; incubation medium and procedure as given in text. The UV- (and thermal) traces of Figs. 1, 2 and 4 represent the middle parts of the complete isotachopherograms. Phosphate is the UV-negative zone preceding the (unidentified) UV-absorbing zones before the citrate.

absorbing zones (Fig. 1). This was obtained in all the urine samples examined. In some samples of freshly voided urine, significant changes with time were observed in the UV-absorbing zones which preceded the citrate zone. The zones preceding citrate diminished while at the same time the citrate zone length increased (Fig. 2). This process ceased after approximately 2.5 h. Thereafter no further changes of the length or shape of the UV-absorbing zones or the citrate zone were observed for up to 16 h. The following experiments therefore were all conducted with urine, which had been allowed to stand at least for 5 h at room temperature.

The calibration curve for standard aqueous solutions as well as the recovery curve of citrate standard added to pooled urine are given in Fig. 3.

The specificity of the isotachophoretic signal for citrate was examined by analysis of its reaction to treatment with CL. In none of the urines examined (n = 47) could a non-UV-absorbing zone, corresponding to citrate, be measured or detected after treatment with CL (Figs. 1, 4). The conditions of CL treat-

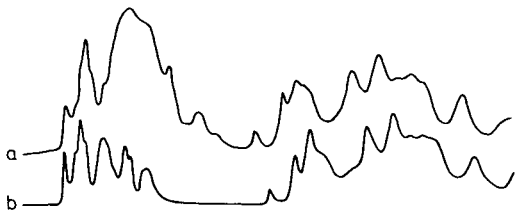

Fig. 2. Isotachopherograms of freshly voided urine. Injections: (a) immediately after voiding and (b) after 60 min standing at room temperature. The significant qualitative and quantitative changes in the zone length of citrate and the UV-absorbing zones preceding citrate suggest dissolution of citrate-containing complexes. Injected volume: 8 μl, procedure as in Fig. 1.

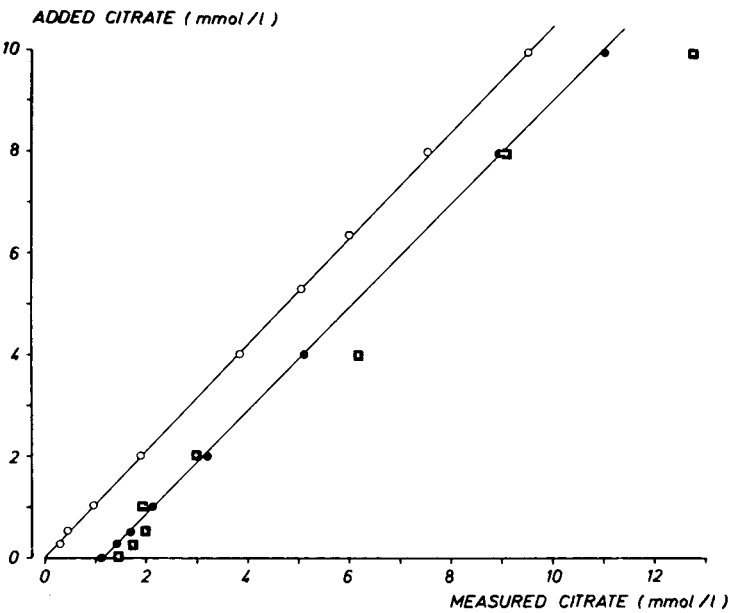

Fig. 3. Calibration curves: (○) citrate in aqueous solutions (isotachophoretic method); (●) internal standard (citrate added to pooled urine, isotachophoretic method) and (■) internal standard (enzymatic method). For details see text.

ment were: 1 U/ml; pH 7.5; 50 mmol imidazol—HCl buffer; this medium does not interfere with the isotachophoretic analyses.

The minimum amount of citrate which would be detected and measured under the above conditions is 0.2—0.4 nmol. In aqueous solutions, the conversion of citrate to oxaloacetate by the action of CL could be followed quantitatively, oxaloacetate being recorded as a zone with weak UV-absorption preceding the UV-negative citrate zone. In urine however, no clearcut detection of oxaloacetate was possible.

Recovery and reproducibility

The recovery of 1 mmol/l citrate added to 15 urine samples was 100 ± 2%

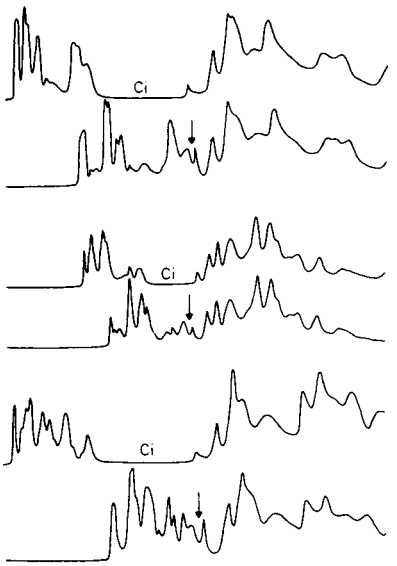

Fig. 4. Isotachopherograms (UV recording) of three urines before and after treatment with CL. Ci: non-UV-absorbing citrate zone. The arrows indicate a small non-UV-absorbing zone, which might correspond to non-converted citrate. Injected volume: 5 μl of native urine, procedure as in Fig. 1.

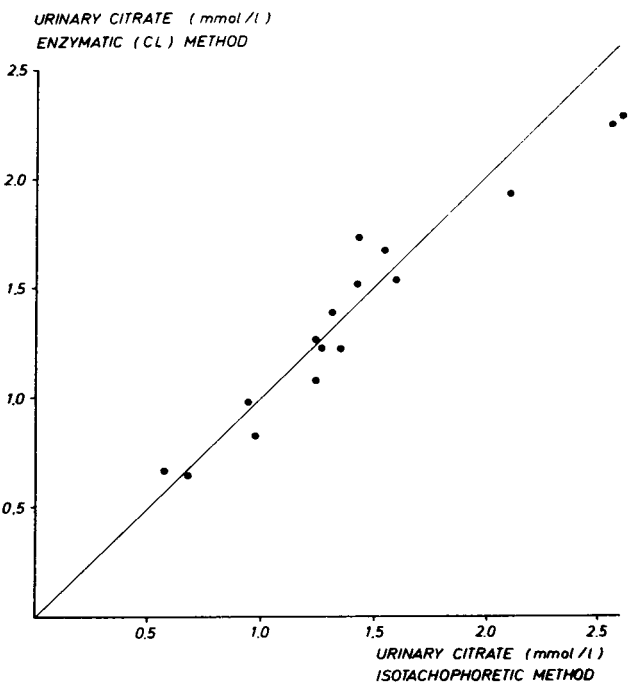

Fig. 5. Plot of 24-h urinary citrate determinations by the isotachophoretic method against the enzymatic method. The straight line indicates the line of identity. The results show a high degree of correlation (r = 0.997). For details see text.

(x ± S.D.). The reproducibility in aqueous solutions (10 sequential measurements) was 100 ± 0.7% and 102 ± 1.6% in urine samples.

Effect of storing and freezing

Storing at −25°C for up to one week and repeated freeze thawing did not markedly (± 5%) after the isotachophoretic measurements of citrate in urine.

Comparison of the enzymatic and the isotachophoretic methods for determination of urinary citrate

The citrate content of 24-h urine samples (n = 16) was analysed both by the isotachophoretic and by the enzymatic method. A close correlation (r = 0.997) between the results of the two methods was found (Fig. 5). In addition, the recovery of added citrate in pooled urine (n = 15) was also analysed by both methods (Fig. 3). While the recovery of added citrate analysed by isotachophoresis was virtually 100% and independent of the amount of added citrate, the values obtained with the CL method showed greater deviations from the expected values.

DISCUSSION

Isotachophoresis is based on the principle that the net mobility of each of the participating ions is constant under defined conditions. At equilibrium, therefore, the ion concentration in each unit length of the given zone is constant. Quantitation can then simply be achieved by measuring the length of each sample zone. The present work shows that quantitative determination of urinary citrate is possible by isotachophoresis with high accuracy and precision. The method is applicable to unprocessed urine samples either voided or stored for prolonged periods of time.

The results obtained by isotachophoresis are comparable with those obtained by the enzymatic CL method and show even less variance than the latter method. The values found for the 24-h urinary excretion of citrate with isotachophoresis are in accordance with values reported in the literature for the CL method [7, 10]. The values reported for the pentabromoacetone method are higher [11] — this is presumably due to its lack of specificity. The CL method was originally developed for the estimation of citrate in fruit juices etc. [4]. It has been applied to urine by Welshman and McCambridge [7].

While the isotachophoretic measurement of urinary citrate seems to be more precise than the CL method, isotachophoresis has one draw back, i.e. a relatively long run-time. However, the method offers one distinct advantage, i.e. the possibility of co-determination of other urinary anions (e.g. oxalate, sulfate, glycolic acid, uric acid, orotic acid [8, 12]).

The isotachophoretic method, using the technical details described above, provides a useful investigational tool for examining the role of urinary citrate in renal stone formation [2, 10] or other disease processes [13].

ACKNOWLEDGEMENT

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CHROMBIO. 640

SENSITIVE AND SELECTIVE METHOD FOR THE DETERMINATION OF CHLORMEZANONE IN PLASMA BY ELECTRON-CAPTURE GAS CHROMATOGRAPHY

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SUMMARY

A sensitive and selective determination method of chlormezanone in plasma has been divised. Chlormezanone in plasma was extracted with toluene at pH 4.5, and converted into *p*-chlorobenzaldehyde in 0.1 *N* NaOH. Using *p*-bromobenzaldehyde as an internal standard, the hydrolysis product and the internal standard were extracted with *n*-hexane, and the extract was concentrated in vacuo in the presence of isoamyl alcohol to prepare the sample solution. The sample solution was submitted to electron-capture gas chromatography. Chlormezanone was determined by use of the peak height ratio of *p*-chlorobenzaldehyde against the internal standard. The method was utilized successfully for pharmacokinetic studies of chlormezanone in plasma.

INTRODUCTION

The tranquilaxant chlormezanone (CM), 2-(4-chlorophenyl)-3-methyl-4-*m*-thiazanone-1,1-dioxide, has been widely used in the treatment of spastic paralysis, low back pain and neurosis, etc., whereas only one paper [1] has described briefly the pharmacokinetics of the drug in man. One report [2] has clarified the metabolism and pharmacokinetics of CM in mice and rats using ^{14}C-labelled CM, and indicated that CM exerts its medicinal effect in unchanged form. Therapeutic and toxic responses to CM appear to be related to its plasma concentration. Therefore it may be important to monitor the plasma levels of CM in patients treated with the drug.

Only a colorimetric method [1] has been presented for the determination of CM in plasma; however, the method was not sufficiently sensitive and

selective to measure therapeutic levels of CM. A more sensitive and selective method was required for reliable pharmacokinetic studies of CM in man.

Gas chromatography with electron-capture detection (ECD-GC) is a powerful tool for highly sensitive analyses of electrophilic compounds. In applications of ECD-GC to the determination of CM, it was found that CM hydrolyzed readily in alkaline solutions to form volatile *p*-chlorobenzaldehyde (*p*-CBA). By applying the hydrolysis product, *p*-CBA, to ECD-GC, a highly sensitive and selective method for the determination of CM in plasma was developed. This method was sufficiently suitable for the routine analysis of CM at plasma levels of 0.1—2 μg/ml.

This paper describes the method devised and the pharmacokinetics of CM in man.

EXPERIMENTAL

Chemicals and reagents

CM was of J.P.IX grade (Sterling-Winthrop, New York, NY, U.S.A.). All other chemicals were of reagent grade (Tokyo-kasei Kogyo, Tokyo, Japan, or Kishida Chemicals, Tokyo, Japan). A stock solution of CM was prepared by dissolving 5.0 mg of CM in 100 ml of acetate buffer solution (pH 4.5), and stored at 5°C protected from the light. Standard samples were prepared by spiking blank plasma with the stock solution at concentrations of 0.1—2 μg/ml, and stored at −20°C. An internal standard solution was prepared by dissolving *p*-bromobenzaldehyde (*p*-BBA) in *n*-hexane at a concentration of 0.5 μg/ml. An acetate buffer solution (pH 4.5) was prepared with 0.1 *M* acetic acid and 0.1 *M* sodium acetate.

Plasma samples

Plasma samples were obtained at six time intervals up to 48 h after single doses of 200 mg of CM in the form of a commercial tablet. Two tablets were administered to five healthy male subjects together with 200 ml of water. Each subject was fasted for 3 h both before and after drug administration. Blood was collected in heparinized syringes by venipuncture, and centrifuged in the usual manner to separate the plasma. The plasma samples were stored at −20°C until they were analyzed.

Analytical procedure

Sample preparation. To 0.1—1-ml plasma samples corresponding to 0.1—2 μg of CM in a 12-ml glass-stoppered centrifuge tube were added 1 ml of acetate buffer solution (pH 4.5) and 6 ml of toluene. The tube was shaken vigorously for 15 min and centrifuged at 2000 *g* for 5 min. A 5-ml volume of the toluene layer was pipetted into a 12-ml glass-stoppered centrifuge tube and evaporated in vacuo to dryness. The residue was dissolved in 1 ml of 0.1 *N* NaOH and allowed to stand for 15 min at room temperature. After addition of 2.0 ml of internal standard solution, the reaction mixture was extracted in a similar manner as the plasma. The aqueous layer was frozen in dry-ice—acetone then all of the *n*-hexane layer was transferred to a glass tube. After 1 ml of isoamyl alcohol had been added to the *n*-hexane extract,

the extract was concentrated in vacuo to a volume of about 1 ml.

Gas-chromatographic conditions. A Shimadzu gas chromatograph, Model 5A, equipped with a ^{63}Ni-ECD (Shimadzu Model-ECD-5), was used. The column was a 1 m × 3 mm I.D. glass tube packed with 5% EGS polyester on 80—100 mesh Gas-Chrom Q. The column was kept at 100°C isothermally, and the injection port and the ^{63}Ni-ECD were held at 150°C and 120°C, respectively. Highly purified nitrogen was used as carrier gas at a flow-rate of 40 ml/min. The sensitivity and the range were set at 10^2 MΩ and 16 mV, respectively. The sample size was 1—2 μl.

Calculations. The concentrations of CM in the plasma samples were determined from a calibration curve prepared by plotting peak height ratio of *p*-CBA against the internal standard (*p*-BBA). The calibration curve was obtained by using the standard samples in the same manner as described under *Plasma samples.*

Conversion rates of CM into p-CBA

The stock solution of CM was diluted five times with water of different pH values (0.5 *N* HCl—0.2 *N* NaOH) to prepare test solutions (10 μg/ml). The test solutions were allowed to stand at room temperature, and the ultraviolet (UV) absorption curves were then measured at a certain time interval with a Hitachi 323 recording spectrophotometer. The conversion rates were calculated from the absorbance at 260 nm due to *p*-CBA.

Identification of the hydrolysis product of CM

The hydrolysis product *p*-CBA was identified by means of gas chromatography—mass spectrometry using an Hitachi gas chromatograph—mass spectrometer, Model RMU-6MG. The mass spectrum was measured at 70 eV with an ion source temperature of 160°C.

Temperature dependence of p-CBA and p-BBA on ^{63}Ni-ECD response

An injection solution containing about 100 ng of both *p*-CBA and *p*-BBA in 1 ml of *n*-hexane was used. The gas chromatograms were obtained using 5.0 μl of the solution under similar GC conditions to those described under *Analytical procedure.* The ^{63}Ni-ECD temperature was adjusted from 80°C to 240°C at intervals of 20°C. The relative sensitivity was calculated from peak height.

Recovery test

The stock CM solution was diluted with water to prepare reference CM solutions at concentrations of 0.1—2 μg/ml. Both the standard samples described in *Chemicals and reagents* and the reference CM solutions were analyzed by means of the analytical procedure. The values of CM for the standard samples were compared with those for the reference CM solutions; the difference was calculated as the recovery of CM from plasma.

RESULTS AND DISCUSSION

Gas chromatography

Attempts to submit CM to GC without any chemical modifications failed because of the thermal decomposition of CM during chromatography. A volatile derivative derived from CM was required for GC analysis of the drug. In some examinations for converting CM into volatile compounds, it was found that CM hydrolyzed readily in alkaline solution to give *p*-CBA. This finding lead to the idea that *p*-CBA be used for the GC analysis of CM.

Fig. 1 shows the typical gas chromatograms resulting from blank plasma

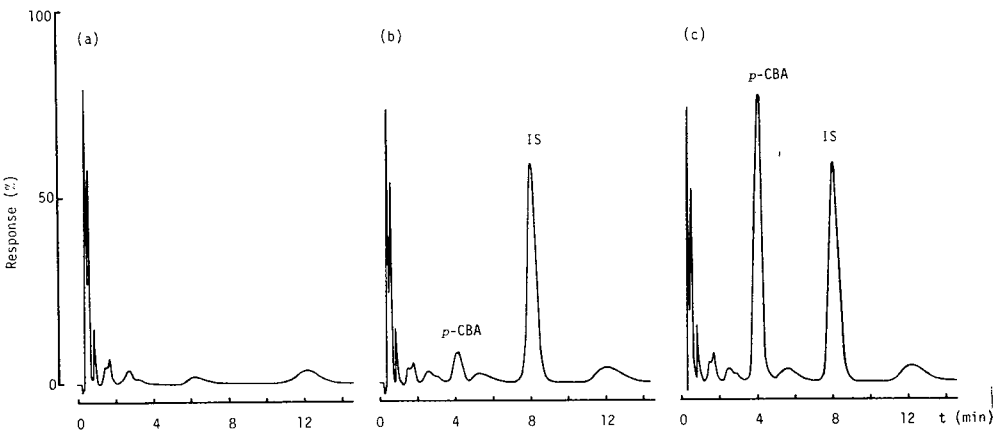

Fig. 1. Gas chromatograms of blank plasma (a), and standard samples prepared by spiking blank plasma with chlormezanone at concentrations of 0.2 μg/ml (b) and 2 μg/ml (c). *p*-CBA = *p*-chlorobenzaldehyde; IS = internal standard (*p*-bromobenzaldehyde).

and standard samples prepared by spiking blank plasma with CM. *p*-CBA and the internal standard were eluted at 4.2 min and 8.1 min, respectively, and were resolved satisfactorily from the peaks due to endogenous plasma components and impurities in the reagents.

Conversion of CM to p-CBA

The UV absorption curve of CM suggested that CM hydrolyzed readily in alkaline solutions at room temperature. The absorbance at 228 nm due to CM disappeared within 15 min in 0.1 *N* NaOH coincident with the appearance of absorbance at 260 nm due to its hydrolysis product. The product *p*-CBA was identified by comparing both the retention time and mass spectrum with those of authentic *p*-CBA, whose mass spectrum is characterized by peaks at *m/z* 140, 139 (base peak) and 111 corresponding to $[M]^{+\cdot}$, $[M-H]^{+}$ and $[M-CHO]^{+}$, respectively. Fig. 2 summarizes the conversion rates of CM into *p*-CBA under several conditions.

On the other hand, CM was stable in acidic solutions as seen in Fig. 2. No changes were observed in acetate buffer solution at pH 4.5 even after 24 h at room temperature, and little CM changed in 0.5 *N* HCl under similar aging conditions. These results suggested that weak acidic conditions were

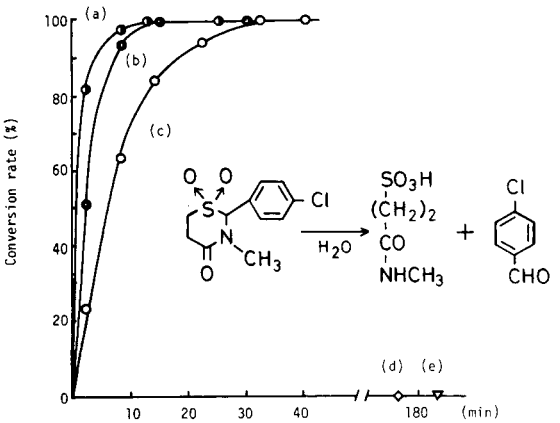

Fig. 2. Conversion rates of chlormezanone into *p*-chlorobenzaldehyde in 0.2 *N* NaOH (a), 0.1 *N* NaOH (b), 0.01 *N* NaOH (c), 0.5 *N* HCl (d) and acetate buffer (pH 4.5) (e), at room temperature.

desirable for the extraction and purification of CM in plasma samples to prevent the artifactual hydrolysis.

Detector temperature

It was presumed in the paper of Wentworth and Chen [3] that the ^{63}Ni-ECD response of *p*-CBA and *p*-BBA was dependent on the detector temperature; the response of the aromatic aldehydes would decrease with increasing detector temperature. Fig. 3 shows the temperature dependence of *p*-CBA and *p*-BBA with the ^{63}Ni-ECD used. The response of the aldehydes decreases with increasing detector temperature; furthermore, *p*-CBA is much more affected than *p*-BBA at about 140°C or above. This indicated that a lower detector temperature was desirable for sensitivity and precision. On

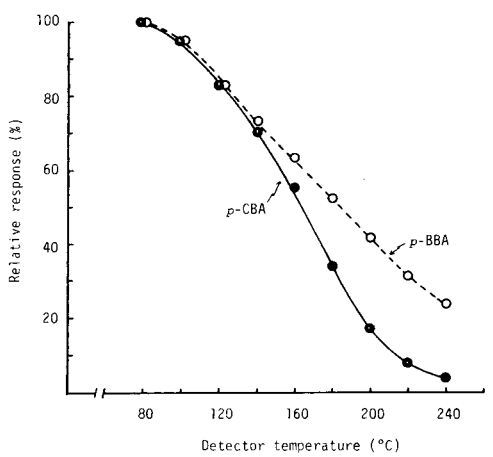

Fig. 3. Temperature dependence of *p*-chlorobenzaldehyde (*p*-CBA) and *p*-bromobenzaldehyde (*p*-BBA) with the ^{63}Ni-ECD used. The values are the mean of five measurements.

the other hand, a higher temperature would be desirable to avoid the accumulation of column bleed and/or plasma components in the detector. The detector temperature was set at 120°C by considering the conditions described above.

Selection of extraction solvents

Toluene was chosen for the extraction of CM from plasma in consideration of the extraction rate of CM and the removal of plasma components. CM in water was extracted completely by toluene, ethyl acetate and isoamyl alcohol; the toluene extract was less interfering than the others. *n*-Hexane was undesirable because of a low extraction rate for CM (about 30%) in spite of its being the least interfering of the solvents examined.

Benzene was as effective for both the extraction and clean-up as toluene; however, its use was avoided in view of it being a potential carcinogen.

However, *n*-hexane was chosen for the extraction of *p*-CBA from 0.1 *N* NaOH. *p*-CBA and internal standard (*p*-BBA) in 0.1 *N* NaOH were extracted completely by *n*-hexane, toluene, ethyl acetate and isoamyl alcohol. However, *n*-hexane was the most desirable of these solvents from the point of view of clean-up. The *n*-hexane extract contained fewer biological components than the others, and showed no interference at the retention times of *p*-CBA and *p*-BBA.

Calibration curve and recovery

The calibration curve showed good linearity (r = 0.9998) between peak height ratio and CM concentration in plasma in the range 0.1—2 μg/ml. Higher amounts of CM in the samples did not give quantitative results for the saturation of response. This may be due to a narrower dynamic range of the linear response, which is generally well-known as a characteristic of ECD [4]. The precision and recovery of the method are shown in Table I. CM in plasma could be determined within ± 7% coefficient of variation over the range 0.1—2 μg/ml. No significant differences were found between the values

TABLE I

PRECISION AND RECOVERY OF THE GAS-CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF CHLORMEZANONE (CM) IN PLASMA

Standard samples and reference CM solutions were prepared by spiking blank plasma and water with CM as described in Experimental. The values were calculated from a calibration curve obtained using authenic CM.

CM added (μg/ml)	CM found (μg/ml)						Percentage recovery (A/B × 100)
	Standard samples (A)			Reference CM solutions (B)			
	Mean ± σ	*n*	C.V. (%)	Mean ± σ	*n*	C.V. (%)	
0.103	0.98 ± 0.0063	9	6.4	0.103 ± 0.0058	9	5.6	95.1
0.412	0.408 ± 0.0167	7	4.1	0.411 ± 0.0173	7	4.2	99.3
1.03	1.03 ± 0.052	6	5.0	1.02 ± 0.050	6	4.9	101.0
2.06	2.01 ± 0.111	6	5.5	2.03 ± 0.083	6	4.1	99.0

of CM from standard samples and those from reference CM solutions.

The addition of isoamyl alcohol to the *n*-hexane extract was essential to prevent the sublimation of *p*-CBA and *p*-BBA in the concentration step. If this precaution were not taken, no quantitative results could be obtained.

In addition, it was suggested that CM metabolizes to form *p*-CBA [1]; however, this metabolite was not identified in mice and rats [2]. The method presented here can distinguish CM from *p*-CBA. *p*-CBA would be removed in the evaporation process of toluene extract even if it were present.

Application to the pharmacokinetics of CM in man

The presented method was utilized for the pharmacokinetics of CM in man. Table II summarizes the plasma levels of CM in healthy male subjects

TABLE II

PLASMA LEVELS OF CHLORMEZANONE (CM) AFTER ORAL ADMINISTRATION OF 200 mg/MAN IN THE FORM OF A COMMERCIAL TABLET (100 mg/TABLET)

Subjects	Plasma levels of CM (μg/ml)					
	1 h	2 h	4 h	8 h	24 h	48 h
S.F.	0.85	2.03	2.92	2.59	1.85	1.20
S.T.	0.53	2.48	2.66	2.40	1.53	0.90
K.S.	0.82	1.91	2.50	2.21	1.54	0.72
A.S.	0.08	2.82	2.51	2.39	1.39	0.76
H.T.	2.93	3.35	3.18	2.39	1.53	0.72
Mean	1.04	2.52	2.75	2.40	1.57	0.86
S.E.	0.49	0.27	0.13	0.06	0.08	0.09

after a single oral administration of 200 mg of CM. The CM was absorbed rapidly from gastrointestinal tract with an absorption rate constant of $7.97 \cdot 10^{-1}$ h^{-1} reaching a maximum plasma level of about 2.7 μg/ml 4 h after administration. Elimination occurred very slowly according to first-order kinetics with an elimination rate constant of $3.45 \cdot 10^{-2}$ h^{-1}, and CM remained in the plasma at a level of 0.86 μg/ml even after 48 h. The biological half-life of CM was calculated as 19.7 h. The elimination rate constant agreed well with that reported by McChesney et al. [1]. On the other hand, the absorption rate constant was a third of that reported by those authors [1]. This difference might be due to the CM formulation used, such as fine powder and tablet.

The method was also applicable to the determination of CM in whole blood. CM was extracted from hemolyzed whole blood as well as from plasma, and no effects of the red blood cells were found on either the extraction rate of CM or the gas chromatogram.

Several biomedical studies of CM are under investigation with successful results using the method. We believe that the method could be useful for the work of other laboratories.

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CHROMBIO. 648

RAPID QUANTITATIVE DETERMINATION OF UNDERIVATIZED CARBAMAZEPINE, PHENYTOIN, PHENOBARBITAL AND *p*-HYDROXYPHENOBARBITAL IN BIOLOGICAL FLUIDS BY PACKED COLUMN GAS CHROMATOGRAPHY

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SUMMARY

A method is described for measuring, without derivatization, the concentrations of phenobarbital, *p*-hydroxyphenobarbital, carbamazepine and phenytoin in biological fluids of epileptic patients undergoing long-term therapy. This method uses, at an isothermal temperature, a special column packing (GP-2% SP-2510-DA on 100—120 mesh Supelcoport).

The lower limit of detection for all substances analyzed is 1 μg/ml of biological material. The recovery of the compounds is about 95%, the reproducibility of the method is good (coefficient of variation, 4.3%). The mass spectra confirm the identity of substances eluting from the column. There is no interference from other commonly used antiepileptic drugs or endogenous substances.

The method has the advantages of high specificity, sensitivity and rapidity and it appears to be suitable for the routine monitoring of blood and urine concentrations in patients receiving multi-drug therapy.

INTRODUCTION

Numerous methods are available for the quantitative measurement of carbamazepine (CBZ), phenytoin (PHT), phenobarbital (PB) and *p*-hydroxyphenobarbital (*p*OHPB) in biological fluids by gas—liquid chromatography (GLC) and high-performance liquid chromatography (HPLC). A general survey of the various analytical techniques employed in the quantitative analysis of antiepileptic drugs and their metabolites has appeared in a recent book [1]. More recently other methods utilizing GLC [2—9] and HPLC [2, 10—12] have been published. However, some problems are still controversial, or may only be

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solved with methods that are too complicated to be adopted for routine use, especially for the determination of CBZ and *p*OHPB.

CBZ is rather unstable at high temperatures and at an acid pH, and it degrades into iminostilbene in high and non-constant proportions [1]. To avoid this degradation many methods use the formation of several derivatives [1, 2, 4—8].

Methods for the determination of *p*OHPB by GLC are carried out via the formation of a derivative (frequently a methyl derivative) that requires thorough purification of the biological samples. These procedures are both complicated and time-consuming [13—15]. For these reasons, we have tried to identify and measure these products, possibly together with PB and PHT, without derivatization by using a column packing developed by Supelco (Bellefonte, PA, U.S.A.) GP-2% SP-2510-DA [16].

Other methods have been described previously for the determination of some anticonvulsant drugs in plasma utilizing this phase at a programmed temperature [3, 9]. During the preparation of our manuscript a brief report has been published [17] utilizing a mixture of GP-2% SP-2510-DA on 100—120 mesh Supelcoport and 3% OV-17 on 100—120 mesh Gas-Chrom Q in equal parts by weight, at an isothermal temperature.

Our paper describes a gas chromatographic method for the quantitative determination of PB, CBZ, PHT and *p*OHPB without derivatization utilizing columns packed with SP-2510-DA at an isothermal temperature. Moreover, the substances eluting from the columns were analyzed in a mass spectrometer to confirm the identity of the drugs.

MATERIALS AND METHODS

Reagents and standards

All chemicals were of analytical grade. PB, CBZ, PHT, *p*OHPB, 5-(*p*-methylphenyl) hydantoin (MPH) and 5-(*p*-methylphenyl) 5-phenylhydantoin (MPPH) were obtained from Aldrich Europe (Beerse, Belgium).

Apparatus

A Carlo Erba Fractovap 2351 gas chromatograph equipped with a dual flame-ionization detector and a Hewlett-Packard 3380A recorder—integrator were used. The silanized borosilicate glass columns were packed with GP-2% SP-2510-DA on 100—120 mesh Supelcoport (Supelco).

For the analysis of PB and CBZ, the column length was 150 cm, and for the analysis of PHT and *p*OHPB it was 50 cm.

The following flow-rates were used: hydrogen, 15 ml/min; air, 250 ml/min; carrier gas (nitrogen), 50 ml/min (CBZ, PB) and 80 ml/min (PHT, *p*OHPB). The temperature of the columns was 245°C; the temperature of the injectors was 275°C.

The columns were conditioned by the following procedure: 15 min at room temperature with a carrier gas flow-rate (nitrogen) of 50 ml/min; 16 h at 265°C with a nitrogen flow-rate of 50 ml/min. After conditioning, and when the gas chromatograph was not in use, a nitrogen flow-rate of 10 ml/min was maintained in the column, and the oven temperature was kept at 100°C. This step seems very useful in holding the characteristics of the column constant.

Extraction procedures

CBZ, PB, PHT. To 1 ml of plasma or urine were added 15 μg of MPH (as an internal marker for PB and CBZ) and/or 15 μg of MPPH (as an internal marker for PHT), 0.2 ml of 1 *N* HCl and 8 ml of chloroform. To determine the calibration curves, various amounts of PB (10—80 μg), CBZ (2—20 μg) and PHT (5—40 μg) were added.

The test-tubes were shaken mechanically for 20 min, then centrifuged and the supernatant was discarded. Six millilitres of the chloroform layer were transferred to a second test-tube and evaporated to dryness at 65°C. The residue was redissolved in 100 μl of chloroform, and 1—2 μl of this solution were injected into the gas chromatograph.

Free pOHPB. To 1 ml of urine, either 1 *N* HCl or 1 *N* NaOH was added to bring the pH to about 5; 0.5 *M* acetate buffer (pH 5) was added to bring the final volume to 5 ml. To determine the calibration curve, various amounts of *p*OHPB (5—40 μg) were added.

To the buffered mixture 10 μg of MPPH (as internal marker) and 15 ml of ethylacetate were added. The test-tubes were shaken for 15 min, then centrifuged and 14—15 ml of the organic phase were transferred to a second test-tube. The organic phase was evaporated to dryness at 65°C with a stream of nitrogen. The residue was redissolved in 100 μl of acetone and 1—2 μl of this solution were injected into the gas chromatograph.

Total pOHPB. One millilitre of urine was incubated with an equal volume of 37% HCl at 100°C for 30 min. After cooling 6 *N* NaOH was added to it to bring the pH to about 5; 0.5 *M* acetate buffer (pH 5) was added to bring the final volume to 5 ml. The buffered mixture was extracted and analyzed by the procedure described above.

The conjugated *p*OHPB was estimated by the difference between total and free *p*OHPB.

Recovery

Analytical recoveries of substances were established as follows. For standards we added known quantities of the substances to pooled drug-free plasma (PB, CBZ and PHT) or to drug-free urine (*p*OHPB). Aliquots (1 ml) of the plasma or urine were taken through the extraction procedure without an internal marker being added. After drying, the extracts were reconstituted in 100 μl of chloroform containing 15 μg of MPH and/or MPPH; 1—2 μl of these solutions were injected into the gas chromatograph.

A second series of standards was prepared simultaneously by extracting 1 ml of drug-free plasma or drug-free urine and, after drying, adding the substances and the marker at the same concentrations as noted above.

We compared the peak area ratios of the extracted standard to the ratios obtained from the standard to which the compounds were added after extraction. The analytical recoveries so measured were corrected to absolute recoveries by the calculated ratio between the volume of chloroform added and the volume of chloroform taken out and evaporated during the extraction procedure.

For the urine extracts, the absolute recoveries were considered to be identical to the analytical recoveries because the volume of the organic phase taken out and evaporated was the same as the volume added.

Reproducibility

For the reproducibility studies, 24 plasma samples and urine samples (1 ml) containing three different concentrations of every drug (PB: 5, 30, 80 μg; *p*OHPB: 2.5, 15, 40 μg; PHT: 2.5, 15, 40 μg; CBZ: 2, 6, 15 μg; 8 samples for concentration) were prepared. Samples were stored at −20°C until analysis. The analyses were performed about once every ten days.

Biological material

Five adult epileptic patients were studied for their rate of elimination of *p*OHPB. The patients had been receiving a long-term antiepileptic drug regimen including PB, for at least six months.

A blood sample was obtained from the patients before the morning administration of the drugs (7 a.m.). Urine had been collected over the 24 h previous to blood collection (from 7 a.m. to 7 a.m.).

Plasma samples for the determination of PB, CBZ and PHT were obtained after separation of the heparinized blood of the patients. All specimens were stored at 4°C until analysis.

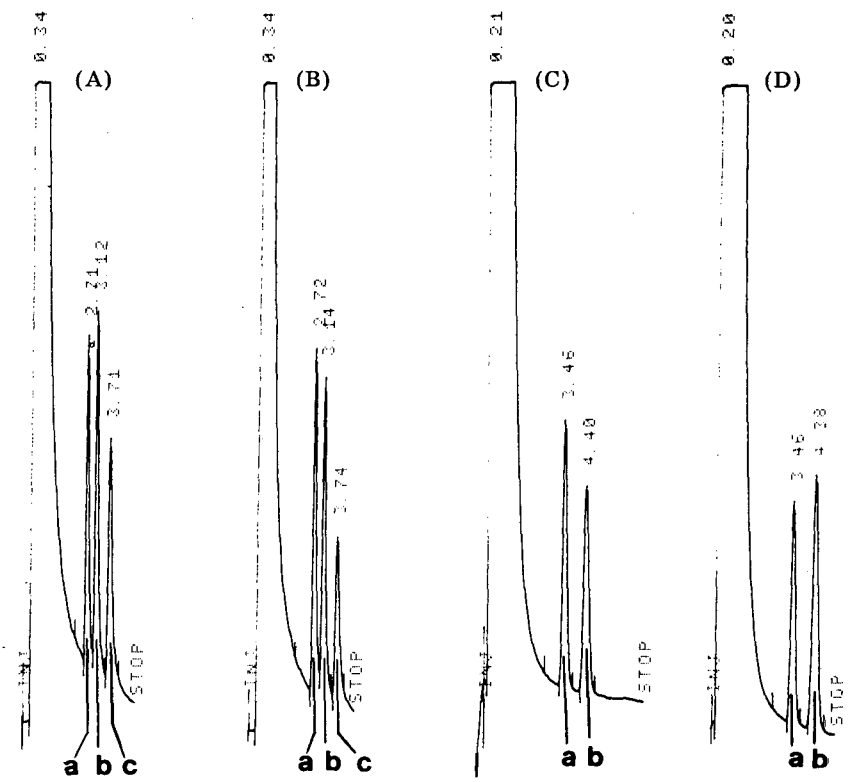

Fig. 1. Gas chromatographic responses obtained with: (A) 5(*p*-methylphenyl)hydantoin (a), phenobarbital (b) and carbamazepine (c), after extraction of a calibrated sample; (B) extracted plasma of a patient receiving phenobarbital and carbamazepine; (C) 5-(*p*-methylphenyl) 5-phenylhydantoin (b) and phenytoin (a) after extraction of a calibrated sample; and (D) extracted plasma of a patient receiving phenytoin.

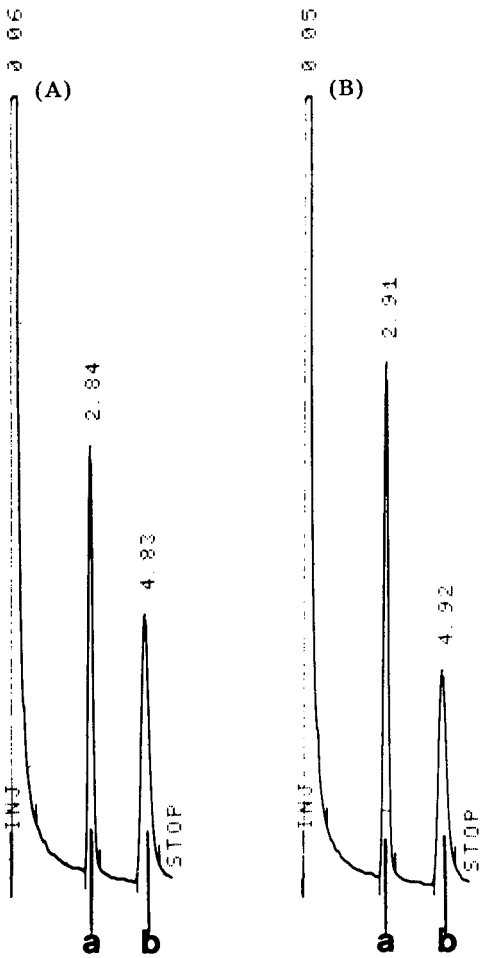

Fig. 2. Gas chromatographic responses obtained with: (A) 5(*p*-methylphenyl) 5-phenylhydantoin (a) and *p*-hydroxyphenobarbital (b), after extraction of a calibration sample; and (B) extracted urine after hydrolysis in a patient receiving phenobarbital.

RESULTS AND DISCUSSION

Typical chromatograms of PB, CBZ and PHT obtained from the injection of internal standards (calibrators) and from the injection of plasma extracts of patients are shown in Fig. 1. In Fig. 2 chromatograms of *p*OHPB obtained from the injection of internal standards and from the injection of urine extracts are shown. There is no interference from endogenous plasma or urine substances and metabolites; retention times are short.

The concomitant administration of ethosuximide, valproic acid, primidone, 5-methylphenobarbital, diazepam, clonazepam and clobazam does not interfere with the analysis of CBZ, PHT, PB and *p*OHPB.

Identification of the compounds eluting from the column was carried out by means of combined gas chromatography—mass spectrometry (LKB—9000). Fig. 3 shows the mass spectra of PHT, CBZ, PB and *p*OHPB. After comparison

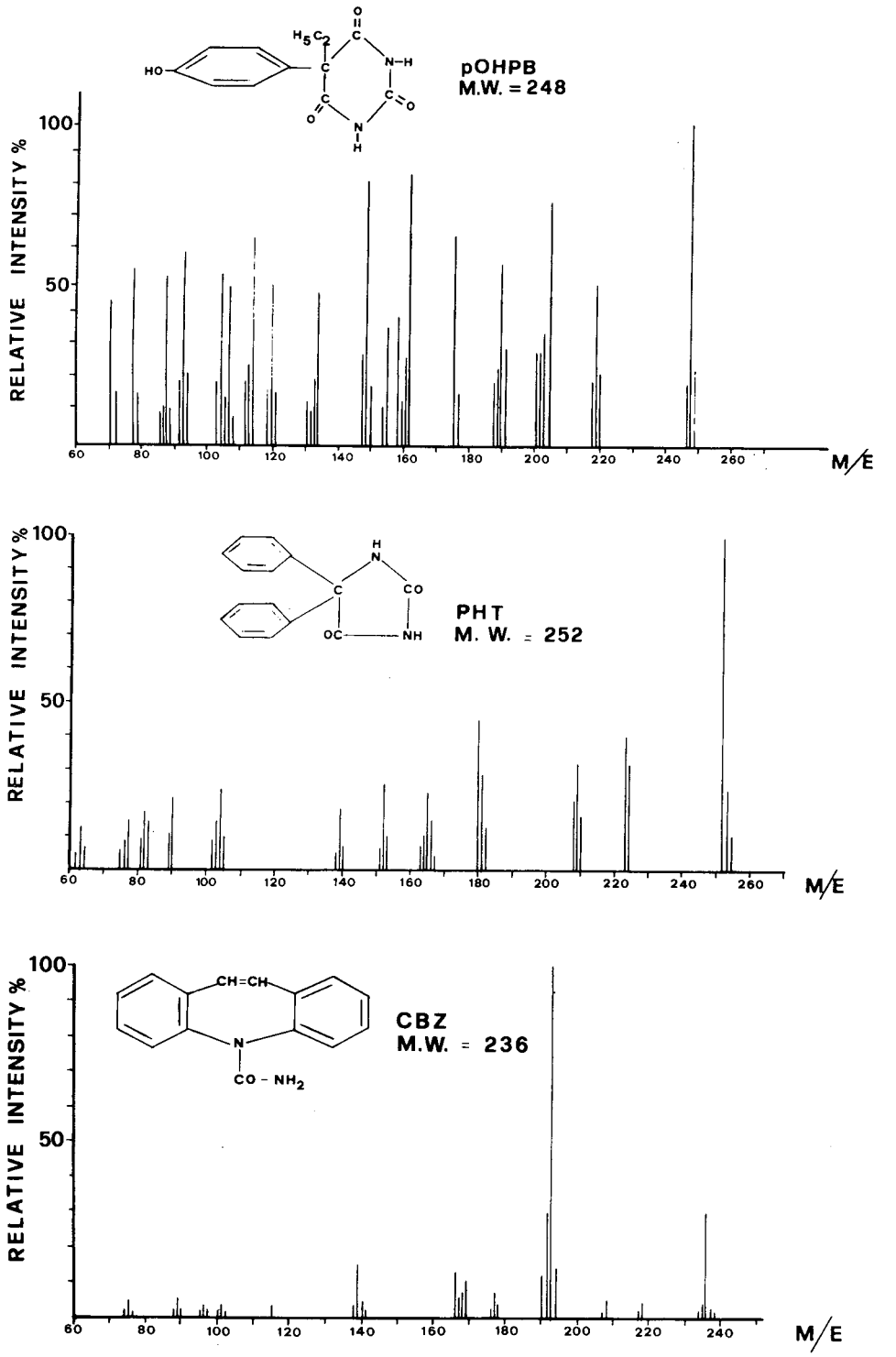
pOHPB
M.W. = 248
RELATIVE INTENSITY %
100
50
60
80
100
120
140
160
180
200
220
240
260
M/E
PHT
M. W. = 252
RELATIVE INTENSITY %
100
50
60
80
100
120
140
160
180
200
220
240
260
M/E
CBZ
M.W. = 236
CO - NH2
RELATIVE INTENSITY %
100
50
60
80
100
120
140
160
180
200
220
240
M/E

Fig. 3.

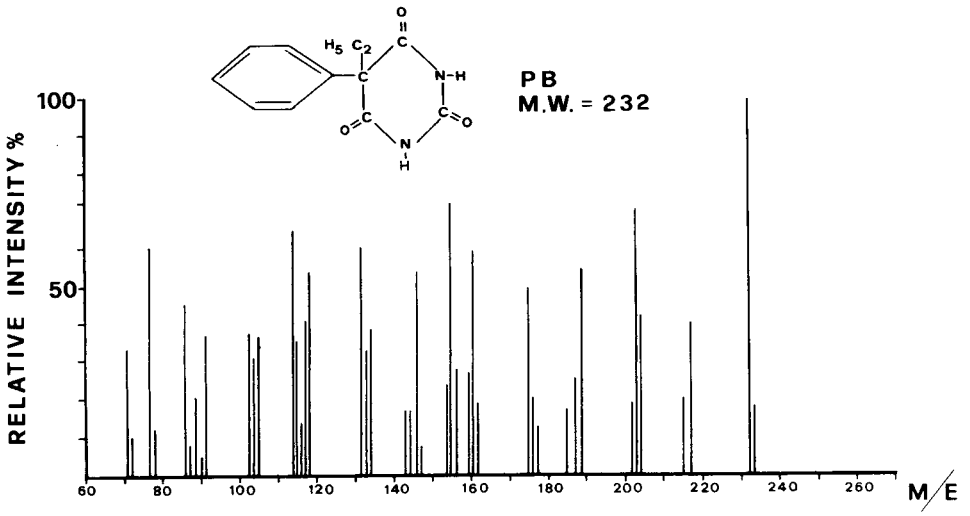

Fig. 3. Normalized electron-impact mass spectra of *p*OHPB, PHT, CBZ and PB obtained by applying a urine or plasma extract to the gas chromatograph—mass spectrometer.

of these mass spectra with the mass spectra of the pure reference substances obtained by direct inlet, it can be concluded that CBZ, PHT, PB and *p*OHPB leave the gas chromatographic column unchanged.

Therefore, in our procedure, these substances are being determined in intact form. The calibration curves of extracted substances are shown in Fig. 4. The linearity in the concentration ranges studied (2—20 μg/ml for CBZ; 5—40 μg/ml for PHT; 10—80 μg/ml for PB and 5—40 μg/ml for *p*OHPB) is very good for all the substances examined.

The minimal detectable amount using the described procedures is 1 μg/ml of plasma or urine for each substance. The recovery from human plasma (of PB,

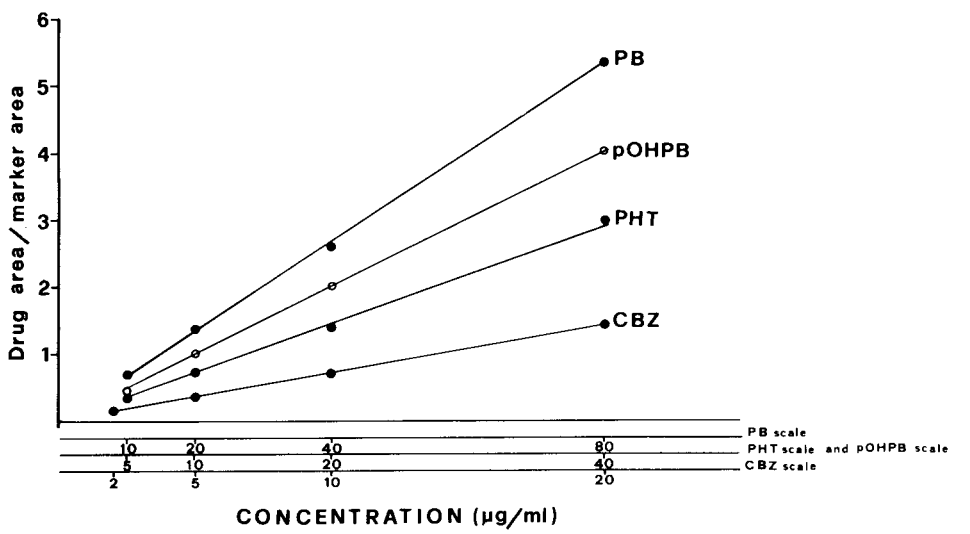

Fig. 4. Peak area ratio vs. concentration calibration curves for PB, *p*OHPB, PHT and CBZ. Marker for PB and CBZ is MPH; marker for PHT and *p*OHPB is MPPH.

PHT, CBZ) and from human urine (of *p*OHPB) is high and constant in the ranges examined, as reported in Table I. The PB standard curve derived from extracted urine is identical to that of the PB curve derived from extracted plasma. The day-to-day reproducibility of this procedure has given good results; data are shown in Table II.

On the column, CBZ undergoes a 5% degradation to iminostilbene at the various concentrations we analyzed over several months of working with different columns.

It is important for the stability of the columns to maintain a carrier gas flow-rate of about 10 ml/min at about 100°C when the columns are not in use. If this procedure is not followed, the peaks tail and the columns lose their sensitivity.

The excretion of PB and of free and conjugated *p*OHPB was studied in five epileptic patients; the results obtained are reported in Table III. Our results, similar to those obtained in other laboratories using different methods [13—15,

TABLE I

RECOVERY OF SUBSTANCES FROM HUMAN PLASMA (PB, CBZ AND PHT) AND FROM HUMAN URINE (*p*OHPB)

Amount added (μg/ml)		Amount found (μg/ml, mean ± S.D., $n = 4$)	Recovery (% ± S.D.)
PHT	5	4.8 ± 0.10	96.0 ± 2.00
	10	9.6 ± 0.19	96.0 ± 1.90
	20	19.4 ± 0.39	97.0 ± 1.95
	40	38.3 ± 0.95	95.7 ± 2.37
CBZ	2	1.9 ± 0.10	95.0 ± 5.00
	5	4.8 ± 0.10	96.0 ± 2.00
	10	9.6 ± 0.25	96.0 ± 2.50
	20	19.3 ± 0.47	96.5 ± 2.35
PB	10	9.7 ± 0.21	97.0 ± 2.10
	20	19.2 ± 0.30	96.0 ± 1.50
	40	38.4 ± 0.41	96.0 ± 1.02
	80	78.0 ± 0.91	97.5 ± 1.12
*p*OHPB	5	4.7 ± 0.22	94.0 ± 4.40
	10	8.9 ± 0.43	89.0 ± 4.30
	20	18.1 ± 0.41	90.5 ± 2.05
	40	35.3 ± 0.68	88.2 ± 1.70

TABLE II

REPRODUCIBILITY OF PHT, CBZ, PB ANALYSIS IN PLASMA SAMPLES AND OF *p*OHPB IN URINE SAMPLES

Drug	PHT			CBZ			PB			*p*OHPB		
Amount added (μg/ml)	2.5	15	40	2	6	15	5	30	80	2.5	15	40
Amount found (μg/ml)*	2.5	15.1	40.0	2.0	6.1	15.0	5.1	30.6	80.1	2.5	15.1	40.0
S.D.	0.14	0.45	0.13	0.13	0.23	0.55	0.23	1.12	1.60	0.29	0.61	1.17
C.V. (%)	5.57	2.99	2.62	6.50	3.70	3.67	4.52	3.66	2.00	11.44	4.08	2.94

*Mean of eight determinations.

TABLE III

PLASMA LEVELS AND RECOVERY OF PB DAILY DOSE AS PB AND *p*OHPB IN 24 HOURS

Urine collection was from five patients receiving long-term therapy. Percentage of daily PB dose is shown in parentheses.

Patients	Age (years)	Sex	Weight (kg)	PB daily oral dose (mg/kg)	Total daily drug regimen (mg/day)	PB plasma concentration (μg/ml)	PB urine amount (mg/24-h collection)	Free *p*OHPB urine amount (mg/24-h collection)	Conjugated *p*OHPB urine amount (mg/24-h collection)	Urine volume (ml)
C.F.	35	F	68	1.47	PHT = 350 PB = 100	24.6	14.2 (14.2)	3.2 (3.2)	7.5 (7.5)	1290
V.F.	20	F	60	2.50	PHT = 300 PB = 150	32.5	37.8 (25.2)	8.6 (5.7)	12.2 (8.1)	980
D.C.	35	F	55	1.82	CBZ = 1000 PB = 100	16.9	26.3 (26.3)	8.8 (8.8)	15.3 (15.3)	970
C.M.	45	F	56	2.23	PB = 125	18.3	24.7 (19.8)	5.1 (4.1)	11.3 (9.0)	980
V.P.	33	M	70	1.42	PB = 100	21.0	25.0 (25.0)	5.0 (5.0)	13.1 (13.1)	1016

18—22], confirm the relatively low elimination of PB either in intact form or as a *p*-hydroxy metabolite.

The possibility of measuring *p*OHPB by GLC without derivatization permits simplification of the extraction procedures. In fact, our method, in contrast to previously published papers, is based on simple solvent extraction from urine followed by sample concentration and direct injection onto the column. For these reasons our method is very simple and rapid; studies on PB metabolism can easily be performed in different situations (drug interactions, associated pathologies, etc.).

As described above, the gas chromatographic phase permits the determination of PB, PHT and CBZ without derivatization with good results for the linearity and reproducibility, which is an advantage especially for CBZ. Moreover, the gas chromatographic—mass spectometric analysis demonstrates that the substances are detected in intact form.

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SIMULTANEOUS DETERMINATION OF THEOPHYLLINE AND ITS MAJOR METABOLITES IN URINE BY REVERSED-PHASE ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A new, highly selective high-performance liquid-chromatographic (HPLC) assay for theophylline and its major metabolites in urine is described. The method utilizes an ion-pair extraction followed by separation and quantitation by reversed-phase ion-pair gradient-elution HPLC. Comparison with several other methods showed that interferences were present in too many blank urine samples to allow for the accurate quantitation of the metabolites of theophylline by direct injection—isocratic HPLC assays. Sample processing involving ion-pair complexing and extraction together with gradient-elution systems is recommended for accurate pharmacokinetic studies.

INTRODUCTION

Theophylline and its related salts comprise one of the more extensively used groups of bronchodilating agents in the treatment of chronic obstructive pulmonary diseases. The development of analytical techniques that permit the reliable determination of theophylline in plasma have contributed immensely to its therapeutic success as the clinical effects of the drug are highly correlated with the concentration in plasma. However, it is exceedingly difficult to predict a priori patient dosage requirements because of the large inter-individual variability in the clearance of the drug [1—4]. Moreover, it has been suggested that the clearance of the drug may be capacity limited since a disproportionate increase in steady-state blood concentration occurs when the dose is increased [5]. Also when concentrations above 20 mg/l are reached, the traditional log—linear decay curve is not observed; rather, the curve

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follows expected capacity-limited kinetics [6, 7]. Recently Monks et al. [8] have reported capacity-limited formation of 3-methyl xanthine in adults administered a 100-mg dose of theophylline labelled with carbon-14.

Diseased states, diet, smoking, genetic and environmental factors have been shown to markedly influence the clearance of theophylline [9—12]. Since the drug is eliminated primarily by hepatic metabolism [2], these factors presumably cause an increase or a decrease in the rate of one or more of these metabolic pathways. Studies on the metabolism of theophylline have shown that approximately 10% of the drug is eliminated by renal excretion, while the remainder is metabolized to 3-methyl xanthine (13—35%), 1-methyl uric acid (15—19%) and 1,3-dimethyl uric acid (35—40%) [13—16]. However, the inter-individual variability in these studies was very high and the urinary recovery of the drug highly variable. These and more recent studies used assays for theophylline and its metabolites which proved tedious, difficult to reproduce or gave inadequate resolution between the compounds of interest and endogenous interfering compounds in urine [13—18].

For these reasons, a new assay for urinary theophylline and its metabolites has been developed which is based on a combination of normal and ion-pair liquid—liquid extraction, with subsequent quantitation by reversed-phase ion-pair gradient elution high-performance liquid chromatography (HPLC).

MATERIALS AND METHODS

Instruments and instrumental conditions

The assay was performed on an HPLC system consisting of two Altex Model 100A pumps, an Altex Model 420 solvent gradient programmer and an Hitachi Model 100-30 variable wavelength ultraviolet (UV) detector set at 280 nm (Altex, Berkeley, CA, U.S.A.).

The column was a reversed-phase 5-μm Ultrasphere ODS, 25 cm × 4.6 mm I.D. (Altex); a slurry-packed precolumn (4.0 cm × 2.5 mm I.D.) of Lichrosorb RP-2 (10 μm) was attached to the system between the injector and analytical column.

Injections were made by means of a Waters Intelligent Sample Processor, Model 710A (Waters Assoc., Milford, MA, U.S.A.).

Data analysis was performed with a Spectra-Physics SP 4100 computing integrator (Spectra Physics, Santa Clara, CA, U.S.A.).

Reagents

Sources of the xanthine derivatives were: theophylline and β-hydroxyethyl theophylline from Sigma, St. Louis, MO, U.S.A.; caffeine from Eastman Kodak, Rochester, NY, U.S.A.; 3-methyl xanthine, 1-methyl xanthine, 1,7-dimethyl xanthine, 1-methyl uric acid, and 1,3-dimethyl uric acid from Adams Chemical, Round Lake, IL, U.S.A.

Sodium acetate (anhydrous), sodium bicarbonate and ammonium sulphate were of analytical grade. Tetrabutyl ammonium hydrogen sulphate (TBA) was obtained from Aldrich, Milwaukee, WI, U.S.A. Chloroform and ethyl acetate were of UV grade, the methanol was HPLC grade and they were all purchased from Burdick & Jackson Labs., Muskegon, MI, U.S.A. Isopropanol, reagent grade, was supplied by Mallinckrodt, St. Louis, MO, U.S.A.

Solvent A was a 0.01 *M* solution of sodium acetate and 0.005 *M* tetrabutyl ammonium hydrogen sulphate in distilled water, with the pH adjusted to 4.75 by 10 *M* NaOH. Solvent B contained the same amounts of salts, but included 50% (v/v) of methanol.

Solvent A was filtered through a Millipore filter type HA 0.45 μm and solvent B through a Millipore filter type BD 0.60 μm (Millipore, Bedford, MA, U.S.A.).

The internal standard solution was prepared by dissolving β-hydroxyethyltheophylline in methanol (50 mg/l). The extraction solution consisted of ethyl acetate—chloroform—isopropanol (45:45:10, v/v). A buffer solution (pH 11) used in the extraction procedure was prepared from 90 ml of 0.1 *M* anhydrous sodium carbonate and 10 ml of 0.1 *M* sodium bicarbonate.

Procedure

A 1.0-ml volume of the internal standard solution was evaporated to dryness in a 15-ml centrifuge tube at 35° C under a stream of nitrogen. An aliquot (0.5 ml) of the urine to be assayed was transferred to the tube and mixed with 0.5 ml of a 0.1 *M* TBA solution and 1.0 g of ammonium sulphate was added, followed by vortexing for 60 sec. A 250-μl volume of buffer (pH 11) was added to adjust the pH to approximately 6.0—6.5 using pH indicator paper.

The mixture was extracted with 10 ml of ethyl acetate—chloroform—isopropanol (45:45:10, v/v) by vortexing for at least 2 min. After centrifugation (5 min at 2000—3000 rpm), 5 ml of the organic layer were transferred and evaporated to dryness at 35° C under a stream of nitrogen.

The residue was vortexed for 60 sec to dissolve it in 0.5 ml of a 0.01 *M* solution of sodium acetate containing 10% (v/v) methanol adjusted to pH 4.75 by 10 *M* NaOH. Then a second 0.5-ml volume of a solution containing 0.01 *M* sodium acetate and 0.05 *M* TBA adjusted to pH 4.75 was added and the vortexing procedure was repeated for 60 sec. The two-step reconstitution procedure was adopted to ensure adequate dissolution of several of the poorly soluble methyl uric acid ion pairs. The concentration of TBA was necessary to avoid dissociation of the ion pairs which caused several split peaks in the resultant chromatograms.

The analysis was performed by solvent gradient elution controlled by the Altex solvent gradient programmer in which the concentration of methanol in the elution was varied from 4.5% to 23%.

The program was started with 9% solvent B and increased by a five-step* gradient program to a final concentration of 46% B. This was accomplished over a 31-min period. The solvent gradient was reduced back to 9% in 2 min and allowed to equilibrate for 15 min between automatic injections.

Quantitation of theophylline and metabolite peaks is achieved by the internal standard peak area ratio method. A standard curve for each compound was prepared by spiking blank urine obtained from a volunteer who had abstained for at least 48 h from caffeine-containing food and beverages (chocolate, tea, coffee, cola, etc.). The standard samples are prepared by evaporat-

*9—12% in 10 min, 12—30% in 15 min, 30—40% in 5 min, 40—46% in 1 min, 46—99% in 2 min.

ing, together with the internal standard solution, appropriate volumes of methanolic stock solutions containing theophylline and metabolites. The residue was reconstituted in 0.5 ml of blank urine (60 sec) followed by assay as described above.

RESULTS

The results of the analysis of a solution containing the test compounds,

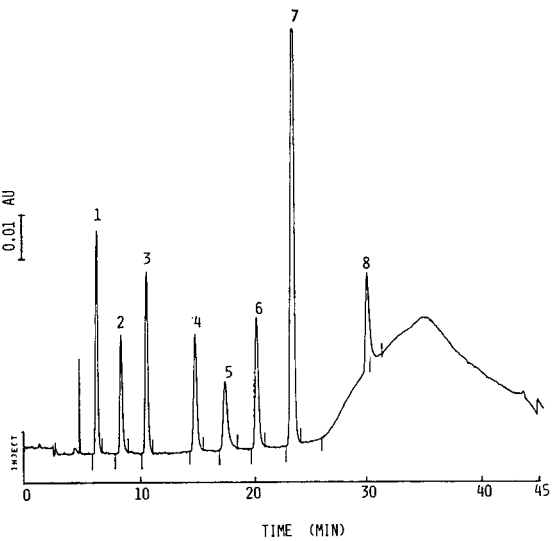

Fig. 1. Urine spiked with standard mixtures: (1) 3-MX, (2) 1-MX, (3) 1-MU, (4) 1,3-MU, (5) 1,7-MX, (6) 1,3-MX (theophylline), (8) caffeine, each at 20 μg/ml; and (7) internal standard (β-hydroxyethyltheophylline) at 50 μg/ml.

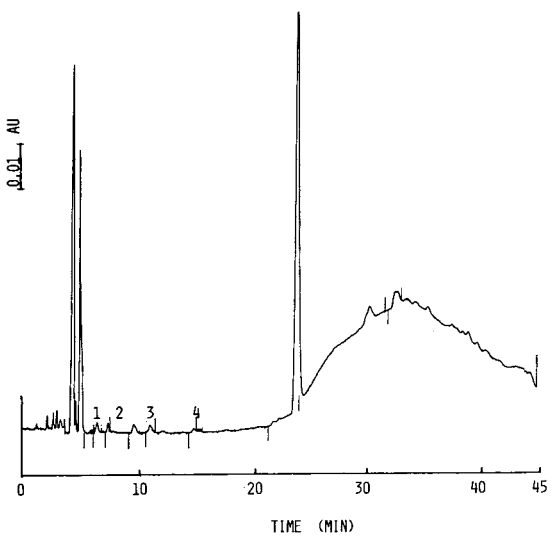

Fig. 2. Chromatogram of a patient's urine collected before theophylline administration, spiked with internal standard (50 μg/ml).

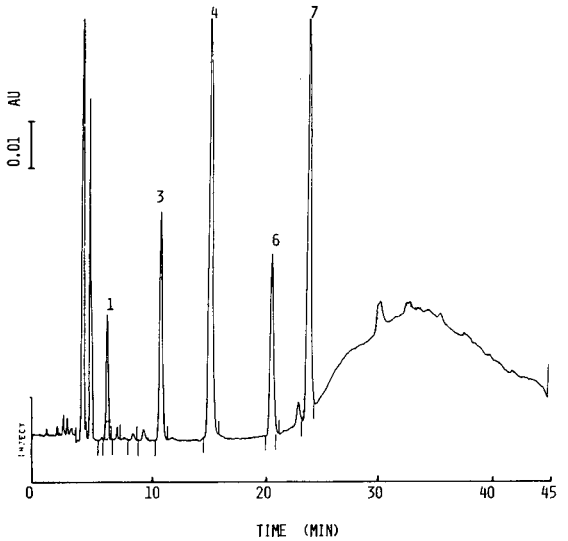

Fig. 3. Chromatogram of a patient's urine collected 3 h after theophylline administration. (1) 3-MX, (3) 1-MU, (4) 1,3-MU, (6) theophylline, and (7) internal standard.

of a blank urine sample and of a sample of urine taken from a volunteer 12 h after a 320 mg dose of theophylline, are shown in Figs. 1, 2 and 3, respectively. Recovery, assay precision and accuracy studies were performed six times for each compound at seven concentrations, varying from 2 to 150 μg/ml, and the results are shown in Table I.

DISCUSSION

Extraction procedure

In order to minimize sample workup, direct injection of samples was attempted under various circumstances, but was unsuccessful as interfering peaks did not allow the adequate resolution of the compounds of interest. Attempts to improve selectivity by pre-extraction at several pH values and with various extraction solvents were also unsuccessful.

Due to the large difference in physico-chemical properties of the methyl xanthines and the methylated uric acids, selection of a simple organic extraction solvent providing a high extraction coefficient for both groups was not possible. Thus, even at low pH, where both the methyl xanthines ($pK_a \approx 8.5$) and the methyl uric acids ($pK_a \approx 5.5$) are predominantly in the unionized form, extraction of the methyl uric acids from aqueous to organic (chloroform—isopropanol, 95:5, v/v) solvent was very inefficient (less than 15%) by virtue of their high polarity. Increasing the polarity of the extraction solvent (chloroform—isopropanol, 50:50, v/v) increased the extraction efficiency, but also resulted in an unacceptable degree of extraction of other, interfering, compounds.

For these reasons, extraction conditions were selected such that the methyl xanthines could be extracted in the unionized form by normal liquid—liquid

TABLE I

RECOVERY AND PRECISION STUDIES

For percentage recovery $n = 6$; C.V. = coefficient of variation.

Concentration (mg/l)	1,3-MX		3-MX		1-MU		1,3-MU		1-MX	
	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)
2	95.5	12.0	84.3	5.8	100.3	17.6	105.3	11.1	86.3	13.4
5	103.9	5.4	95.5	6.2	87.8	16.3	96.1	3.9	97.1	6.6
10	101.9	5.5	94.6	3.3	95.4	14.2	97.9	4.5	100.8	4.9
25	101.0	1.8	94.6	2.2	92.0	11.2	95.1	1.9	98.12	2.3
50	104.5	4.7	97.0	2.6	94.0	10.7	98.2	2.8	99.5	5.3
100	94.9	2.0	92.6	3.9	91.3	2.3	94.4	3.1	94.5	1.3
150	103.6	4.4	94.4	3.5	98.6	18.2	96.5	4.4	97.5	4.2
r^2	0.9970		0.9973		0.9592*		0.9975		0.9976	

*For individual calibration curves $r^2 > 0.998$ (see text).

extraction and the more polar methylated uric acids by means of an ion-pair liquid—liquid extraction. The latter type of extraction has been shown to be particularly efficient for polar and ionized compounds [19, 20]. A pH of 6.0—6.5 was determined to be optimal in accomplishing the extraction; at this pH range, the methyl xanthines are unionized and can be extracted with slightly polar organic solvents or mixtures (e.g., chloroform or ethyl acetate with a small percentage of isopropanol). On the other hand, the methyl uric acids are ionized at this pH, and form an ion pair with the tetrabutyl ammonium counter ion, which can be efficiently extracted in the solvents mentioned above. Extraction was enhanced by adding high concentrations of ammonium sulphate to the system to produce a salting-out effect. The optimum counterion concentration was found to be 0.1 *M*. Lower concentrations produced low and variable extraction efficiencies for 1-methyl uric acid (1-MU) and 1,3-dimethyl uric acid (1,3-MU), while higher concentrations permitted an unacceptable degree of co-extraction of interfering polar compounds. Similar observations were made on the effect of varying isopropanol and ammonium sulphate concentrations. Thus, with low concentrations of isopropanol or ammonium sulphate, poor extraction of both the methyl xanthines and the methyl uric acid ion pairs was noted, while high concentrations, again, produced an unacceptable degree of co-extraction of interfering polar compounds, resulting in much-reduced selectivity.

Chromatographic procedure

Similar considerations prompted the use of an ion-pairing chromatographic system. Thus, in the absence of the counter ion, the retention of the methyl-uric acids was inadequate for acceptable resolution. Addition of the tetrabutylammonium counterion to the solvent system markedly increased the retention of these compounds despite the fact that the pH of the analytical mobile phase was lower than their pK_a values. Small changes in pH (± 0.25 pH units) produced marked changes in the retention time of 1-MU and 1,3-MU. Thus, lowering the pH to 4.5 resulted in a marked reduction in the retention time and resolution of these compounds by virtue of reducing the fraction of these molecules in the ionic state, capable of ion pairing. Increasing the pH to 5.0 resulted in the opposite effect: the retention time of 1-MU and 1,3-MU increased by virtue of the higher equilibrium concentration of the paired, neutral species. The pH of 4.75 was optimal for resolution and was used in all analyses. The concentration of tetrabutyl ammonium ion was also varied at constant pH. As would be expected, reduced concentration of the ion caused a small reduction in the retention time of the methyl uric acids, and increased concentration produced an increased retention, although this effect was not as marked as that of pH. A tetrabutyl ammonium concentration of 0.005 *M* was determined to be optimum.

All chromatographic conditions were optimized by maximizing resolution and selectivity between small endogenous peaks and by optimized chromatography of the standard mixture shown in Fig. 2, which contained a mixture of 3-methyl xanthine (3-MX), 1-methyl xanthine (1-MX), 1,3-dimethyl xanthine (1,3-MX, theophylline), 1,7-dimethyl xanthine (1,7-MX, paraxanthine), 1,3,7-trimethyl xanthine (caffeine), 1-methyl uric acid (1-

MU), 1,3-dimethyl uric acid (1,3-MU) and β-hydroxyethyltheophylline (internal standard). This system allows the selective determination of theophylline and its metabolites including 1,7-MX; these compounds are not resolved in many other HPLC procedures [16—18].

The retention times observed were: 3-MX, 6 min; 1-MX, 8 min; 1-MU, 11 min; 1,3-MU, 15 min; 1,7-MX, 17 min; theophylline, 20 min; β-hydroxyethyltheophylline, 23 min; and caffeine, 30 min. As the retention times of endogenous uric acid and xanthine are substantially shorter than the first peak of interest (3-MX) these compounds do not interfere with the present procedure.

The use of an exhaustively silylated column together with a replaceable precolumn resulted in maintained column performance after the injection of several thousand samples over a period of approximately one year. The high-efficiency column (number of theoretical plates was approximately 1000 per cm), together with a fast time constant detector, allowed for the resolution of rapidly eluting peaks when the system was run isocratically with 10% methanol. However, in order to maximize quantitative estimation of the peaks and to elute endogenous peaks with long retention times, the gradient elution system was ultimately used for routine assays. The choice of 280 nm as the detection wavelength was to optimize the absorbance of both the methyl xanthines (λ_{max} = 270 nm) and methyl uric acids (λ_{max} = 290 nm). Although both of these groups of compounds have significant absorbance at 254 nm, more interference and spurious peaks were observed at this wavelength.

Quantitation, reproducibility and accuracy

Peak area ratios appeared to give more reproducible results than peak height ratios. The values for recovery show that theophylline, 3-MX and 1,3-MU can be extracted reproducibly at concentrations of up to 400 μg/ml. Above 150 μg/ml the standard curve for 1-MU became non-linear. The individual calibration curves for 1-MU (between 2 and 150 μg/ml) showed excellent linearity ($r^2 > 0.998$) but relatively large differences in slopes between assays were seen (see Table I). This was probably due to very minor variations in extraction conditions; e.g. pH, organic extractant composition between assays. For the other compounds the standard curve was linear over a wider range and showed good reproducibility. The limit of sensitivity (defined as three times the baseline noise) for the compounds measured as a pure substance in aqueous solutions was approximately 100 ng/ml. However, even though some subjects abstained from caffeine intake for longer than 48 h, their blank urine samples contained small residual peaks at retention times corresponding to 3-MX, 1-MU and 1,3-MU, which represents approximately 1—2 μg/ml of these compounds. Thus the limit of sensitivity of the assay is dependent upon these blank values, which possibly originate from unavoidable dietary intake of various xanthine derivaties.

These results indicate that the procedure has sufficient selectivity, sensitivity, precision and accuracy to be suitable for pharmacokinetic studies.

Comparison with other HPLC assays of urinary theophylline and metabolites

Desiraju et al. [17] proposed a direct assay of theophylline and metabolites

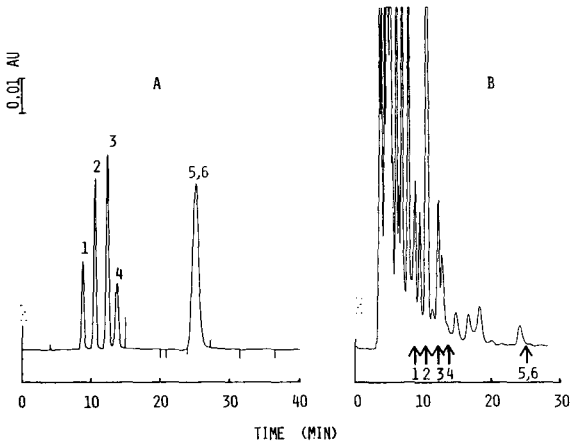

Fig. 4. Chromatograms of a standard mixture (A) and blank urine (B) run using the method of Desiraju et al. [17]. Arrows indicate elution times for compounds in standard test mixture. See Fig. 1 for identification code.

in which urine was directly injected into a 30-cm Waters Bondapak C_{18} column using 12% methanol in 0.05 *M* KH_2PO_4 as the eluent. With this system, the authors reported that the major endogenous urinary constituents eluted in the first 7 min and that only the peaks for hypoxanthine and xanthine were subject to interference since theophylline metabolite peaks eluted at longer retention times. We have been unable to validate these results. Fig. 4A is a reproduction of the chromatogram of a standard mixture (10 μl; 50 μg/ml) in water under the above conditions. Fig. 4B is a chromatogram of a typical blank urine sample (10 μl) under the above conditions. The arrows indicate the positions where theophylline and its metabolites should elute. It can be seen that interferences of the order of 10—100 μg/ml are present in this region. Similar results were obtained with other blank urine samples. Clearly, the degree of accuracy and precision required for pharmacokinetic studies can not be met in all circumstances.

Grygiel and co-workers [18] also reported a urinary assay which was a modification of the plasma assay of Orcutt et al. [21]. These workers had to extract the acidified urine with dichloromethane to assay theophylline; however, they report being able to separate the metabolites of theophylline by direct injection of 10 μl of urine into a 5-μm reversed-phase column (15 cm) using 10 m*M* acetate buffer (pH 5) as the eluent. The retention times were reported as 1-MU = 4.5, 3-MX = 10, 1-MX = 12.5, 1,3-MU = 15.5 min. Two separate assay procedures were therefore employed to measure theophylline and its metabolites in urine. Fig. 5A represents the chromatogram of a standard mixture of theophylline and metabolites under the above conditions using a 25 cm column, and Fig. 5B is a typical blank urine. The arrows once again indicate the positions where the metabolites should elute. Elimination of methanol from the mobile phase allows better resolution of some peaks (e.g., 2 and 4) but interferences were still present. In addition, carry-over peaks from previous injections, representing compounds with long retention times, were frequently seen. Other urines gave similar results. Examination

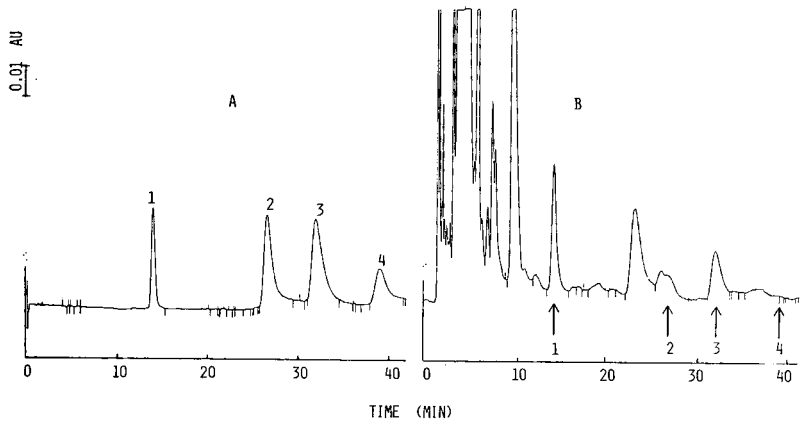

Fig. 5. Chromatograms of a standard mixture (A) and blank urine (B) run using the method of Grygiel et al. [18]. Arrows indicate elution times for compounds in standard test mixture. See Fig. 1 for identification code.

of Fig. 4A and B and Fig. 5A and B indicate the difficulties of utilizing these assays for the quantitative analysis of theophylline and its metabolites in most urine samples.

In addition to these assays, Aldridge et al. [22] investigated caffeine metabolism in the newborn using an HPLC assay to estimate the metabolites. The assay involves an extraction step from urine saturated with ammonium sulphate using chloroform—isopropanol (85:15, v/v) as the solvent. The residue of the organic layer was separated using a μBondapak C_{18} column and a solvent gradient comprising 1.5% (v/v) acetonitrile up to 7.5% in 0.5% (v/v) acetic acid over 15 min. The authors reported that the extraction step eliminated interfering peaks from endogenous compounds. With the important exception that 1,3-MX and paraxanthine were not resolved, the method appears to separate the metabolites of theophylline. However, the extraction efficiency of the methylated uric acids was low (in particular 1-MU, 36% ± 12%) by virtue of the polarity of these compounds.

The use of the ion-pair extraction procedure reported herein improves markedly the extraction efficiency of the uric acid derivatives, particularly of 1-MU with an average extraction efficiency of approximately 95% (see Table I).

ACKNOWLEDGEMENTS

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DETERMINATION OF THE QUINIDINE ANALOG, 7′-TRIFLUOROMETHYLDIHYDROCINCHONIDINE·2HCl IN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A rapid and specific high-performance liquid chromatographic (HPLC) assay was developed for the determination of the antiarrhythmic quinidine analog, 7′-trifluoromethyldihydrocinchonidine·2HCl ([I]·2HCl) in plasma and urine. The overall recovery of [I] from plasma was 86 ± 9% with a sensitivity limit of detection of 0.2 μg/ml.

The assay involves extraction of [I] into benzene—methylene chloride (9:1) from plasma or urine made alkaline with 0.1 *N* sodium hydroxide (pH 13) and saturated sodium chloride, the residue of which is dissolved in methylene chloride, an aliquot of which is analyzed by HPLC using adsorption chromatography on silica gel with UV detection at 254 nm. The mobile phase composed of methylene chloride—methanol—conc. ammonium hydroxide (95.5:4:0.5) yields baseline resolution of quinidine used as the internal (reference) standard, compound [I] and dihydroquinidine, a common contaminant in quinidine.

The assay was applied to the analysis of plasma and urine samples taken from a dog administered a single 20 mg/kg dose via intravenous and oral routes. The stability of [I] in human plasma for up to 37 days of storage at −17°C was also demonstrated.

INTRODUCTION

The compound 7′-trifluoromethyldihydrocinchonidine·dihydrochloride ([I]·2HCl) is a member of a series of synthetic quinidine analogs [1] with antiarrythmic activity [2]. It differs from quinidine mainly in the presence of a trifluoromethyl group on the C_7' quinoline moiety instead of a methoxy group on C_6' (Fig. 1). Quinine, quinidine and other quinoline analogs have long been the mainstay of therapeutic agents with antimalarial [3] and antiarrhythmic [4,5] activity.

Numerous publications have appeared on the analysis of quinidine in biolog-

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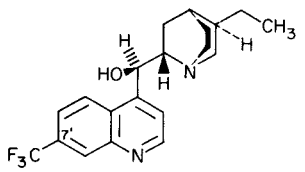
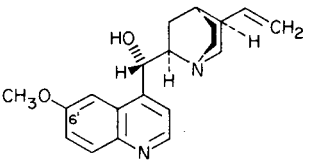
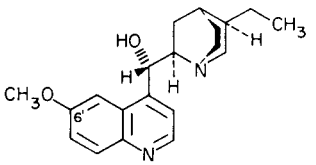


I] 7'-Trifluoromethyl-Dihydrocinchonidine



[II] Quinidine



[III] Dihydroquinidine

Fig. 1. Chemical structures of the compounds referred to in the text.

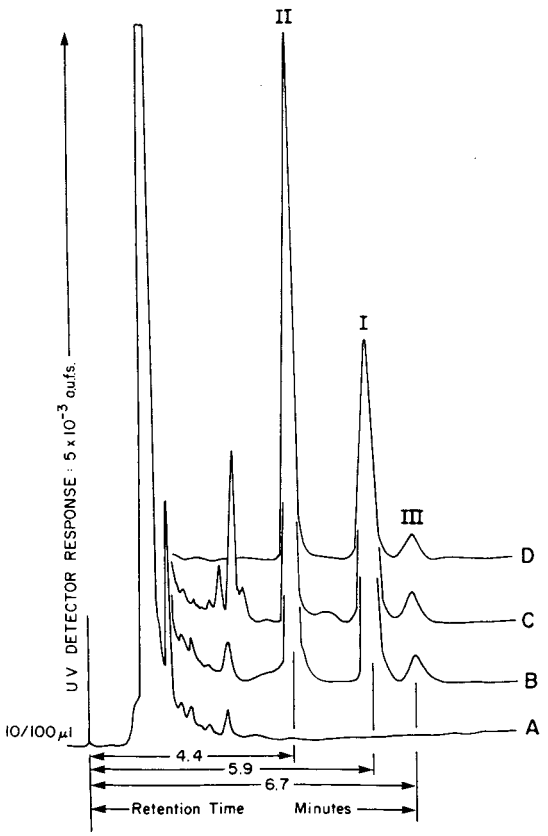


Fig. 2. Chromatograms of compound [I], compound [II], quinidine, used as the internal (reference) standard, and compound [III], dihydroquinidine, an impurity in quinidine. (A) Control dog plasma extract, (B) control dog plasma extract with recovered authentic standards, (C) in vivo dog plasma extract, (D) authentic standards.

ical samples using spectrofluorometric [6,7], thin-layer chromatographic [8—10], gas chromatographic (GC) [7,11,12], gas chromatographic—mass spectrometric (GC—MS) [7,13,14] and high-performance liquid chromatographic (HPLC) [15—24] methods.

The projected clinical dosage of [I]·2HCl is in the multi-milligram per kilogram range; therefore an HPLC assay would provide adequate sensitivity and specificity for quantitation. Quinidine sulfate [II] is a readily available compound, hence it is a logical choice for use as the internal (reference) standard in this assay. However, commercially available quinidine contains a significant percentage of dihydroquinidine [III] as a contaminant [25]. Consequently, the chromatographic resolution of these two components from each other and from [I] and its metabolites is necessary. Baseline resolution of all the components in plasma (Fig. 2) and in urine (Fig. 3) was obtained with the method developed, monitoring the effluent with a fixed-wavelength UV detector at 254 nm.

The assay was applied to the analysis of plasma and urine samples taken from a dog administered a single 20 mg/kg dose via intravenous and oral routes.

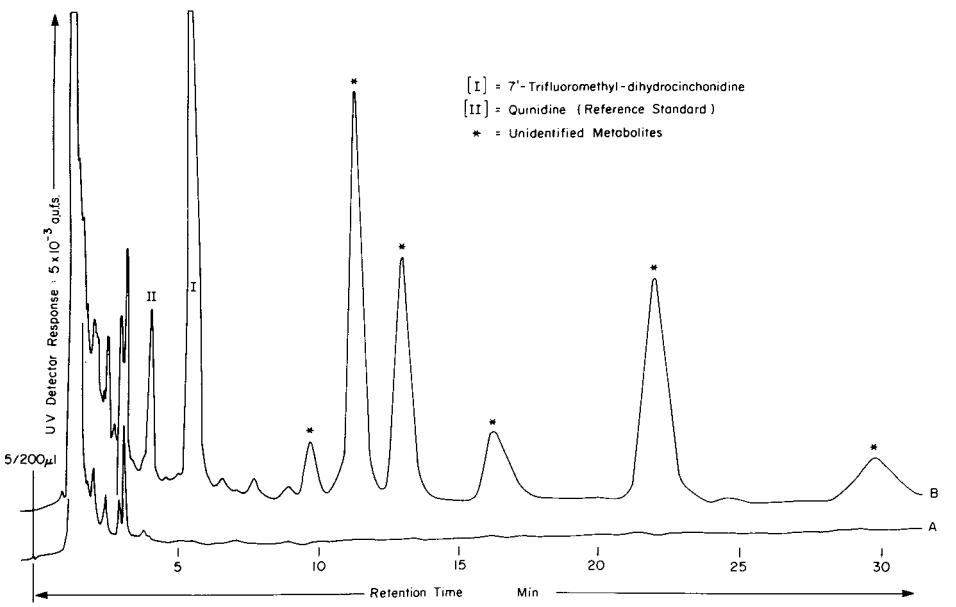


Fig. 3. Chromatograms of 2-ml extracts of the unconjugated fraction of urine (24—48 h post dose) from a dog administered a 20 mg/kg intravenous dose of (A) control urine extract, (B) in vivo dog urine extract.

EXPERIMENTAL

Column

A prepacked 25 cm × 4.6 mm I.D. stainless-steel column containing 10-µm Partisil silica gel, generating 39,100 plates per meter (Whatman, Clifton, NJ, U.S.A.), was used.

Instrument parameters

The HPLC system (Waters, Milford, MA, U.S.A.) consisted of a Model 6000 reciprocating piston pump, a Model U6K loop injector and a Model 440 fixed-wavelength absorbance detector with a UV filter at 254 nm. The isocratic mobile phase used was a mixture of methylene chloride—methanol—conc. ammonium hydroxide (95.5:4:0.5) at a constant flow-rate of 2 ml/min and a column head pressure of ca. 700 p.s.i. at ambient temperature. The detector sensitivity was 5×10^{-3} a.u.f.s. and the chart speed on the Hewlett-Packard recorder (Model 7132 A) was 0.5 in./min at 10 mV input. Under these conditions, 0.2 μg per 10 μl injected of [I], [II] and [III] gave nearly full-scale peak response. The retention times of [I], [II] and [III] were 5.9 min, 4.4 min and 6.7 min (Fig. 2), with corresponding capacity factors (k') of 3.35, 2.15 and 4.00, respectively.

Reagents

0.1 *N* NaOH (pH 13); saturated NaCl solution (1.5 g/ml); benzene, nano-grade (Mallinckrodt, St. Louis, MO, U.S.A.); methylene chloride and methanol (Burdick & Jackson Labs., Muskegon, MI, U.S.A.); conc. ammonium hydroxide (29.3% ammonia), Baker analyzed reagent grade (J.T. Baker, Phillipsburg, PA, U.S.A.).

Analytical standards

Compound [I]·2HCl (M.W. = 437.33, m.p. = >184°C decomp.) and quinidine·sulfate·hydrate ($[II]_2 \cdot H_2SO_4 \cdot H_2O$) (M.W. = 764.95, m.p. = 201—202.5°C) of pharmaceutical grade purity were obtained from the Chemical Research Division, Hoffmann-La Roche Inc. (Nutley, NJ, U.S.A.), while dihydroquinidine [III] was obtained from K + K Labs (Plainview, NY, U.S.A.).

Preparation of analytical standards

Weigh out separately 12.00 mg of [I]·2HCl and 11.79 mg of quinidine·sulfate·hydrate (equivalent to 10 mg of free base) and dissolve separately in 10 ml of methanol to yield stock solutions A and B containing 1 mg equivalent of [I] or [II] (free base) per ml, respectively.

Prepare working standard solutions a—f by mixing appropriate aliquots of A and B diluted to 10 ml with methylene chloride as follows:

Solution	Aliquots (μl) of Solution A + Solution B		Final conc./100 μl of solution	
	A	B	[I]	[II]
a	20	200	200 ng	2 μg
b	50	200	500 ng	2 μg
c	100	200	1 μg	2 μg
d	200	200	2 μg	2 μg
e	500	200	5 μg	2 μg
f	–	200	–	2 μg

Aliquots (100 μl) of solutions a,b,c,d or e are added to the residue of separate 1-ml specimens of control plasma as the external calibration standards

and mixed well on a Vortex mixer. Aliquots (10 μl) of these solutions (equivalent to 20, 50, 100, 200 and 500 ng of [I] and 200 ng of [II]) are injected to establish an external standard calibration curve, to verify the linearity and performance of the HPLC system. Addition of the drug to the residue of control plasma is necessary due to a chromatographic "enhancement effect" which compounds [I] and [II] exhibit; i.e., the peak height response is greater when the compounds are chromatographed in the residue of biological extracts than when chromatographed as pure standards, probably due to decreased adsorption losses caused by deactivation of the column by the lipids in the extract.

The internal standard [II] is added (100 μl of solution f) to all unknown plasma or urine samples.

Assay procedure

Into a 15-ml (PTFE No. 13) stoppered conical centrifuge tube, transfer a 100-μl aliquot of solution f (equivalent to 2 μg of [II], the internal standard), add 1 ml of unknown plasma or 0.5—2 ml of urine, 1 ml of saturated sodium chloride solution and 2 ml of 0.1 *N* sodium hydroxide (pH 13), mix for a few seconds very gently (at setting 2) on the Super-Mixer (Lab-Line Instruments, Melrose Park, IL, U.S.A.). Extract the samples with 6 ml of benzene—methylene chloride (9:1) by shaking for 15 min on a reciprocating shaker (Eberbach, Ann Arbor, MI, U.S.A.) at ca. 90 strokes/min. Centrifuge the samples in a refrigerated centrifuge (Model PR-J, Rotor No. 253, Damon/IEC, Needham, MA, U.S.A.) at 5°C for 10 min at ca. 2600 rpm (1500 *g*), and transfer the supernatant benzene—methylene chloride layer into a conical 15-ml glass stoppered centrifuge tube. Extract the sample a second time with an additional 6 ml of benzene—methylene chloride, centrifuge and combine the extracts. Evaporate to dryness at ca. 50°C in a N-EVAP evaporator (Organomation, Worchester, MA, U.S.A.) under a stream of dried nitrogen. Rinse the walls of the tube with 1—1.5 ml of benzene—methylene chloride (9:1) to concentrate the sample in the tapered end of the tube. (The reconstituted sample residues may be stored overnight at −20°C prior to subsequent analysis.) Evaporate the solution to dryness and dissolve the residue in 100—200 μl of methylene chloride and inject a 5—10-μl aliquot for HPLC analysis.

Along with the samples, process a 1-ml specimen of control plasma or 0.5—2 ml of urine and five 1-ml specimens of control plasma or 0.5—2 ml of urine to which 100 μl of standard solution a, b, c, d or e (equivalent to 200 ng, 500 ng, 1 μg, 2 μg or 5 μg of [I] and 2 μg of [II] each per ml of plasma or urine, respectively) are added; these samples are used to establish the calibration curve for the direct quantitation of the unknowns.

Calculations

The concentration of [I] in the unknowns is determined by interpolation from the calibration curve of the standards processed along with the unknowns using peak area ratios (peak area of [I] to peak area of internal (reference) standard [II] versus concentration). The calibration curve is linear from 200 ng to 5 μg of [I] per ml of plasma or urine.

RESULTS AND DISCUSSION

Percent recovery and sensitivity limits

The overall recovery of [I] and [II] from plasma was 86 ± 9% and 96 ± 6% (S.D.), respectively, over the concentration range of 0.20 to 5.0 μg of [I] per ml of plasma or urine. It was necessary to add the external standards to the residue of control plasma to obtain an apparent recovery of ⩽100% due to the aforementioned "enhancement" effect. The sensitivity limit of the assay was 0.2 μg of [I] per ml of plasma. The ammonia content in the mobile phase was critical to good chromatographic resolution, peak shape and response of these compounds.

The HPLC system is flushed initially with methanol to remove accumulated deposits from previous use. Fresh mobile phase is prepared before each analysis, and allowed to recycle through the system until equilibration with the column is attained, as indicated when a stable baseline is obtained. Several 10-μl aliquots of the mixed standard solution c are then injected until a reproducible response is obtained before the analysis of the biological samples is attempted.

It has been observed that the k' values of the compounds tended to increase throughout the day's run, probably due to a change of the ammonia content of the mobile phase. Prolonged use of the column will also tend to increase k' values.

Specificity of the assay

The extensive biotransformation of compounds such as [I]·2HCl [26] and quinidine [II] [15,20,24] necessitates the use of chromatographic procedures in order to ensure the specificity and accuracy of quantitation of the many compounds present in biological fluids. Although meaningful data have been reported on the bioavailability [27] and pharmacokinetics [28] of quinidine [II] and of dihydroquinidine [III] [29] using non-specific spectrofluorometric methods, more meaningful pharmacokinetic data on parent drug and metabolite profiles and therapeutic efficacy were obtained using specific chromatographic methods, especially HPLC with either UV absorption [17,18,20] or fluorometric detection [15,21—23,30]. Since therapeutic plasma concentrations of quinidine and its metabolites are high (μg/ml range) and its half-life relatively long (5—12 h), the tandem use of UV and fluorescence detection enables quantitation in plasma over a wide concentration range (e.g. 100 μg/ml to 5 ng/ml), establishing HPLC as the method of choice for the determination of this class of compounds.

Gas chromatographic behavior of [I]

Compound [I] has no intrinsic fluorescence, but its UV absorbance at 254 nm is sufficiently intense to attain a sensitivity limit of 200 ng/ml. The trifluoromethyl group at C_7' is an electron-withdrawing group which tends to delocalize the π electrons in the quinoline ring, reducing its UV absorption and any useful fluorescence. The converse is true with quinidine whose methoxy group at C_6' is electron-donating which enhances the aromaticity of the quinoline nucleus and contributes significantly to its excellent UV absorbance and fluorescence emission characteristics. Efforts at improving the sensitivity

limits of detection of [I] led to the investigation of electron-capture—gas—liquid chromatography (EC—GLC) and gas chromatographic—chemical ionization—mass spectrometric (GC—CI—MS) analysis as alternative methods.

Electron-capture—gas—liquid chromatography

The parent compound [I] and its trimethylsilyl (OTMS) ether (prepared by reaction with bis(trimethylsilyl)trifluoroacetamide + 10% trimethylchlorosilane heated at 60°C for 1 h, the residue of which was dissolved in *n*-hexane for EC—GLC analysis), were chromatographed on 4 ft. × 4 mm I.D. 3% OV-1 and 3% OV-17 columns using a 15 mCi ^{63}Ni EC detector (Tracor Instruments, Austin, TX, U.S.A.) (see Table I).

Compound [I] showed good intrinsic sensitivity to EC detection; the chromatograms however, showed a major tailing peak for [I] and smaller peaks as evidence of either thermal instability or due to small amounts of isomers. Chromatograms of compound [I]-OTMS derivative, however, showed a sharp symmetrical Gaussian-shaped peak of high sensitivity with two minor peaks probably due to trace amounts of the isomers of [I]. The silylation of [I] was confirmed by GC—electron impact (EI)—MS analysis of [I] which gave a molecular ion *m/z* 364 compared to the [I]-OTMS derivative which gave a molecular ion *m/z* 436 and a base peak at *m/z* 138 common to both.

The sensitivity of the response of [I]-OTMS to EC detection was sufficient to determine less than 50 ng/ml.

TABLE I

RETENTION TIMES (min) OF [I] AND ITS TRIMETHYLSILYL ETHER BY EC—GLC

Temperature for GC of [I] 220°C, and for GC of [I]-OTMS 200°C.

GC column	[I]	Smaller peaks		[I]-OTMS	Smaller peaks	
3% OV-1	4.2	3.0	3.5	6.5	10.0	13.5
3% OV-17	6.2	4.2	—	4.6	7.6	8.6

GC—MS analysis

Analysis of [I] and [II] as their respective OTMS derivatives by GC—CI—MS using both positive ion (PI) and negative ion (NI) modes of detection, was also investigated on a Finnigan Model 1015 modified for both PI [31,32] and NI [33] capability. The CI—mass spectra gave more intense ions for both compounds in the PI mode than in the NI mode. This was expected for [II]-OTMS which does not have an electronegative group in the aromatic ring required for good NI response. The major ions for [I]-OTMS in the NI mode were at *m/z* 378 $[M-58]^-$, *m/z* 360 $[M-76]^-$, *m/z* 346 $[M-90]^-$, but were not sufficiently intense for quantitative use.

The PI—CI—mass spectra for both compounds are shown in Fig. 4. The major ions for [I]-OTMS (Fig. 4A) were at *m/z* 477 $[M+41]^+$, *m/z* 465 $[M+29]^+$, *m/z* 437 $[M+H]^+$, *m/z* 421 $[M-15]^+$ and *m/z* 417 $[M-19]^+$, while those for [II]-OTMS (Fig. 4B) were at *m/z* 438 $[M+42]^+$, *m/z* 425 $[M+29]^+$, *m/z* 397 $[M+H]^+$, *m/z* 381 $[M-16]^+$. The additional ions at *m/z* 477 and *m/z* 465 for

[I]-OTMS and at *m/z* 438 and 425 for [II]-OTMS, respectively, are adducts due to methane used as the reagent gas. The intense $[M+H]^+$ ions at *m/z* 437 and *m/z* 397 are used for the quantitation of [I]-OTMS, using [II]-OTMS as the internal standard and carrier. The total ion chromatograms (see inset in Fig. 4) for [I]-OTMS gave two distinct peaks indicating the possible presence of an isomer as an impurity in the sample, while that for [II]-OTMS gave a single major peak, and a minor peak for [III]-OTMS, a known impurity.

Plasma samples spiked with known amounts of [I] and [II] were extracted as described, the residue silylated and analyzed by GC—CI—PI—MS. Linear calibration curves for both authentic pure standards and the compound recovered from plasma were obtained over a concentration range of 25—200 ng of [I]-OTMS injected, on a 4 ft. × 2 mm I.D. packed column containing 3% SE-30 on 120—140 mesh Gas-Chrom Q (Applied Science Labs., State College, PA, U.S.A.) at 230°C using methane as both the carrier and reagent gas. The $[M+H]^+$ ions at *m/z* 437 and *m/z* 397 were monitored at retention times of 1.75 and 3.42 min for [I]-OTMS and [II]-OTMS, respectively. Improvement in sensitivity may yet be obtained in either PI or NI modes using either isobutane or methane—ammonia as the reagent gas. These leads can be further developed to yield a more sensitive and specific assay for [I] using the deuterated analog of [I] as the internal standard, if needed for use in future clinical pharmacokinetic studies.

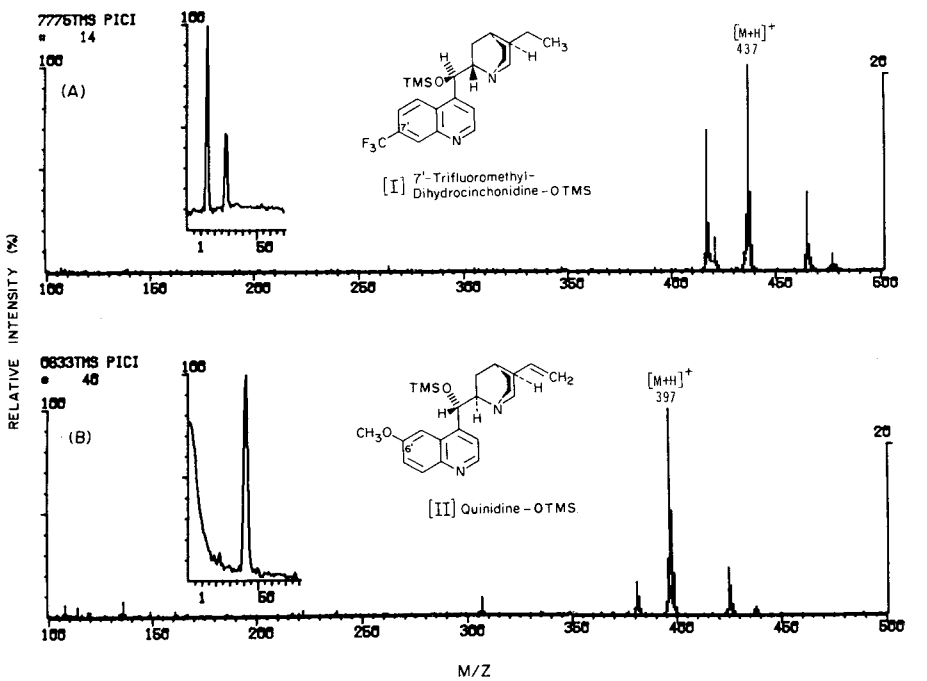


Fig. 4. GC—CI—PI—mass spectra of (A) compound [I] and (B) quinidine [II] as their respective trimethylsilyl derivatives. The inset in each panel is a total ion chromatogram for the compound.

Application of the HPLC method to pharmacokinetic studies in the dog

Plasma analysis. Plasma concentrations of [I] were determined in a dog (Table II) following the intravenous and oral administration of single doses of an aqueous solution of [I]·2HCl equivalent to 20 mg (free base) per kg. Following intravenous administration, a plasma concentration of 7.85 μg/ml at 5 min declined to a plateau of about 4.4 μg/ml from 30 min to 30 h, then declined to 1.41 μg/ml at 72 h. Following oral administration of the drug, a peak plasma concentration of 9.52 μg/ml was observed at 26 h, declining to 1.11 μg/ml at 100 h.

TABLE II

PLASMA CONCENTRATIONS OF [I] IN A DOG FOLLOWING THE ADMINISTRATION OF SINGLE DOSES OF AN AQUEOUS SOLUTION OF THE 2 HCl SALT EQUIVALENT TO 20 mg (FREE BASE)/kg BY INTRAVENOUS AND ORAL ROUTES

NST = No sample taken. NM = Non-measurable, < 0.2 μg/ml.

Time	Intravenous (μg/ml)	Oral (μg/ml)
5 min	7.85	NST
7.5 min	6.34	NST
10.0 min	5.84	NM
12.5 min	5.54	NST
15 min	5.64	NST
20 min	5.31	1.31
30 min	4.66	2.41
45 min	4.52	2.54
1 h	4.93	2.78
1.5 h	4.05	3.83
2 h	4.44	3.53
3 h	4.09	3.88
4 h	4.15	4.39
6 h	4.00	5.34
8 h	4.18	5.36
10 h	4.73	5.91
24 h	4.83	8.11
26 h	NST	9.52
28 h	NST	8.45
30 h	4.70	NST
32 h	NST	7.05
48 h	3.01	5.62
52 h	NST	5.09
56 h	NST	4.34
72 h	1.41	2.61
76 h	NST	2.42
80 h	NST	2.02
96 h	NST	1.20
100 h	NST	1.11

Urine analysis. The 0—24 h and 24—48 h urine samples from the dog administered a single intravenous [20 mg (free base)/kg] dose of the 2HCl salt equivalent to 224 mg of free base [I] were analyzed. The parent compound [I] in the unconjugated form represented 2.4% of the dose (Table III). Significant peaks representing at least 6 metabolites were seen in the unconjugated form in both the 0—24 h and 24—48 h urine samples (Fig. 3); the same peaks were also seen in lesser amounts in the glucuronide conjugate fraction. These metabolites were completely resolved from the parent drug, hence do not interfere with the specificity of the assay.

Studies on the in vitro metabolism of [I] have yielded five hydroxylated metabolites [26], which were characterized by NMR and mass spectrometry. The identity of the in vivo metabolites seen in urine must await the synthesis of authentic reference compounds.

TABLE III

URINARY EXCRETION OF UNCONJUGATED [I] (μg/ml) IN A DOG FOLLOWING THE INTRAVENOUS ADMINISTRATION OF AN AQUEOUS SOLUTION OF THE HCl SALT EQUIVALENT TO 224 mg (20 mg/kg) OF FREE BASE

Excretion period (h)	Volume voided (ml)	μg/ml	Total excreted (mg)	% of dose
0—24	140	25.90	3.63	1.6
24—48	410	4.43	1.82	0.8
		Total	5.45	2.4

Stability of [I] in human plasma on prolonged storage

The stability of [I] in plasma (determined by HPLC analysis) was evaluated at three concentrations following storage at −17°C and −70°C for a period of 7 and 37 days. The plasma samples were prepared as follows: Transfer a 0.5-ml aliquot of stock solution A (see Experimental section) into a 50-ml glass stoppered volumetric flask, dilute to 50 ml with human plasma, mix well in a sonic bath, to yield a plasma stock solution 1 whose concentration is 10 μg/ml (representing an upper limiting value on the calibration curve). Transfer 5 ml of solution 1 into a 25-ml volumetric flask, dilute to mark with plasma, and mix well, to yield plasma solution 2 whose concentration is 2 μg/ml (representing a mid-point value on the calibration curve). Transfer 0.5 ml of plasma stock solution 1 into a 25-ml volumetric flask, dilute to mark with plasma, to yield plasma solution 3 whose concentration is 0.2 μg/ml (representing the sensitivity limit of the assay).

Storage

Transfer four 3.5-ml aliquots of solutions 1, 2 and 3 (10, 2 and 0.2 μg/ml, respectively) into 7.5-ml glass vials with plastic caps for storage at −17°C (12 samples).

Additionally, transfer two 3.5-ml aliquots of solutions 1, 2 and 3 into 7.5-ml glass vials for storage at −70°C (6 samples).

The stored stability indicating plasma samples are analyzed as unknowns in triplicate on appropriate days along with the external standards and internal standards added to fresh control plasma as described previously.

The mixed standard solutions a—e prepared fresh on day 0 are stored at 5—10°C for evaluation of solution stability. A 10-μl aliquot of each standard is injected and compared to the response of a corresponding 10-μl injection from a set of freshly prepared mixed-standard solutions on each day of analysis.

The stability indicating plasma samples from day 0, days 7 and 37 stored at −17°C were analyzed and the data tabulated in Table IV. A correlation coefficient (determined by the method of least squares) of at least 0.95 was arbitrarily selected as the lower limit of acceptable stability of the compound under storage. The data indicated that [I] was stable throughout the storage interval at −17°C, with an overall coefficient of correlation between day 0 to 7 and day 0 to 37 of 0.9999. In the event that the samples stored at −17°C showed instability, then the analysis of the samples stored at −70°C would have been undertaken, using the same statistical criteria.

TABLE IV

STABILITY OF [I] IN PLASMA STORED AT −17° FOR 37 DAYS

Day 0 compared to day 7: coefficient of determination = 0.9998; coefficient of correlation = 0.9999. Day 0 compared to day 37: coefficient of determination = 0.9999; coefficient of correlation = 0.9999.

Sample conc. (μg/ml)	Day	n	Mean conc. found (μg/ml)	S.D.	S.D. (%)
0.2	0	3	0.26	± 0.01	4.5
2.0	0	3	1.98	± 0.05	2.4
10.0	0	2	9.52	± 0.01	0.1
0.2	7	3	0.29	± 0.02	8.1
2.0	7	3	2.01	± 0.10	4.9
10.0	7	3	10.14	± 0.27	2.7
0.2	37	3	0.20	± 0.02	12.3
2.0	37	3	2.03	± 0.09	4.4
10.0	37	3	10.55	± 0.17	1.6

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CHROMBIO. 647

SIMULTANEOUS DETERMINATION OF *d*- AND *l*-PROPRANOLOL IN HUMAN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method for the determination of *d*- and *l*-propranolol in human plasma is described. The method involves extraction of propranolol from plasma, and the formation of diastereomeric derivatives with the chiral reagent N-trifluoroacetyl-1-prolylchloride. Separation and quantitation of the diastereomeric propranolol derivatives are carried out by a reversed-phase high-performance liquid-chromatographic system with fluorimetric detection. The reproducibility in the determination of *d*- and *l*-propranolol in human plasma was 4.5% (relative standard deviation) at drug levels of 10 ng/ml.

In two subjects who received a single 40-mg tablet of racemic propranolol the plasma levels of the *d*-isomer were lower than of the *l*-propranolol. The half-lives of *d*- and *l*-propranolol were similar.

INTRODUCTION

The propranolol available for use as an adrenergic beta-receptor antagonist is a racemic mixture of two optical isomers, dextro (*d*) and laevo (*l*) propranolol. The *l*-isomer is more potent than the *d*-isomer and it is probably responsible for almost all the pharmacological effect [1, 2]. After administration of the racemate to animals the isomers have different disposition kinetics [3—5]. In man

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the half-life of *l*-propranolol was longer than that of *d*-propranolol after separate administration of the isomers [6]. However, data are lacking for the disposition of the isomers in man after administration of racemic propranolol. Such data are necessary especially in studies of concentration—effect relationships, since the concentration ratio between the two isomers may vary under different conditions and since only the *l*-isomer should contribute to the clinical effect.

This lack of human kinetic data on the *d*- and *l*-isomers of propranolol after administration of the racemate is due to the lack of suitable analytical methods. Previous methods used for separation of the isomers require sophisticated equipment and the use of isotopes [5, 7], immunological techniques of uncertain specificity [3], or gas—liquid chromatographic techniques which are not sensitive enough for studies of concentration—effect relationships in man [4].

This project was initiated to study the kinetics and effects of active *l*-propranolol in the presence of almost inactive *d*-propranolol after giving small doses of commercial racemic propranolol to man. To achieve this goal we developed a high-performance liquid chromatographic (HPLC) method which does not require complicated equipment nor isotopes but which is capable of quantitating the concentrations of *d*- and *l*-propranolol in plasma from subjects taking small doses of commercial propranolol. The method is based on the principles of forming diastereomeric derivatives with the chiral reagent N-trifluoroacetyl-1-prolyl chloride followed by separation of these propranolol derivatives on a reversed-phase column and fluorimetric detection.

MATERIALS AND METHODS

Chemicals

Racemic, dextro (*d*) and laevo (*l*) propranolol HCl were kindly supplied by Imperial Chemical Industries (Macclesfield, Great Britain). N-Trifluoroacetyl-1-prolyl chloride (N-TFA-1-prolyl chloride or TPC-reagent) 0.1 *M* in chloroform was obtained from Regis Chemical Co., Chicago, IL, U.S.A. Acetonitrile was of Grade S quality and purchased from Rathburn Chemicals, Walkerburn, Great Britain. LiChrosorb RP-8 and RP-18 were obtained from E. Merck, Darmstadt, G.F.R., and μBondapak phenyl from Waters Assoc. (Milford, MA, U.S.A.). All other reagents were of analytical or equivalent grade and used without further purification.

Apparatus

The pump was an Altex Model 100 solvent delivery system and the injector a Valco Model CV-6-UHPa (7000 p.s.i.). A Schoeffel FS 970 L.C. fluorimeter was used as the detector. It was operated with an excitation wavelength of 210 nm and a 340 nm cut-off emission filter. The mass spectrometer was an LKB 2091 equipped with a digital PDP 11/05 computer system and operated in the electron-impact mode at 70 eV.

Chromatographic technique

Chromatographic analyses were performed at room temperature. The mobile

phases were made with phosphate buffer (pH 2.2) with an ionic strength of 0.05. The mobile phases were degassed in an ultrasonic bath and brought to room temperature before use.

Column preparation

The column was made of 316 stainless-steel with a polished inner surface, equipped with modified Swagelok connections and Altex stainless frits (2 μm). The columns were 250 × 3.2 mm packed with either LiChrosorb RP-18, RP-8 with a mean particle diameter of 5 μm, or with μBondapak phenyl with a 10-μm particle size. The columns were packed by a modification of the ordinary balanced density slurry technique [8]. The supports were suspended in 15 ml of chloroform which was poured into the packing column. This was then filled with hexane and coupled to a Haskel pump. Acetone was used as driving liquid in the pump which was operated at 5000 p.s.i. After packing the columns were washed with 50 ml of acetonitrile and then with 100 ml of acetonitrile—water (50:50) before equilibration with the mobile phase consisting of 45% acetonitrile in phosphate buffer (pH 2.2) (μ = 0.05).

All columns were tested after packing in a test system with the mobile phase composition described above. *l*-Propranolol derivatized with the TPC reagent was used as solute for this test. Columns having a reduced plate height ($h = H/d_p$) of less than 10 at a flow-rate of 1 ml/min and an asymmetry factor of less than 1.5 were accepted for this study. The asymmetry factor was calculated by drawing a perpendicular to the base-line from the vertex formed by the two peak tangent lines. The back part of the peak base-line divided by the front part gives the asymmetry factor.

Extraction and derivatization of propranolol

One millilitre of 1 *M* carbonate buffer (pH 9.85) was added to 1.0-ml plasma samples containing propranolol. Propranolol was then extracted with 6.0 ml of water-saturated diethyl ether for 15 min. The tubes were centrifuged for 3 min at 4200 *g* and 5.0 ml of the ether layer were collected and evaporated at 40°C in a stream of dry nitrogen. The residue was dissolved in 0.5 ml of chloroform containing 25 μl of triethylamine (prepared just before use). One hundred microlitres of 0.1 *M* TPC reagent were added and reacted for 15 min at room temperature (see below). The reaction was stopped by the addition of 3.0 ml of water and 6.0 ml of water-saturated ether to two tubes at a time. After extraction for 2 min the tubes were centrifuged for 3 min at 4200 *g*. A 5-ml aliquot of the ether layer was evaporated to dryness at 40°C under nitrogen. The samples were dissolved in 170 μl of mobile phase and 150 μl were injected onto the column. The reaction time needed to obtain maximum yield of the propranolol derivatives was determined using six standard solutions containing each propranolol isomer at 25 ng/ml. The reaction was stopped in two tubes at a time at 2, 15 and 30 min, respectively.

Purity of propranolol enantiomers

The optical purity of *d*- and *l*-propranolol were determined by chromatography of the enantiomers in underivatized form in an ion-pair HPLC system using LiChrosorb Diol as support and a mobile phase consisting of dichloromethane, 1-pentanol with *d*-camphersulfonic acid as the counterion [9].

Standard curves

Two sets of six samples containing 1, 5, 10, 25, 40 and 50 ng/ml each of *d*- and *l*-propranolol hydrochloride in 1 *M* carbonate buffer (pH 9.85) were added to 1.0 ml of drug-free plasma. Extraction and derivatization were performed as described above. Peak heights were measured and plotted against the concentration of *d*- and *l*-propranolol.

Reproducibility studies

Reproducibility studies for analysis of *d*- and *l*-propranolol were performed at four different concentrations (1, 10, 25 and 50 ng/ml) of *d*- and *l*-propranolol hydrochloride dissolved in 1 *M* carbonate buffer (pH 9.85). Five 1.0-ml samples at each concentration were added to 1.0 ml of drug-free plasma and analyzed according to the procedure described above and the relative standard deviations were calculated.

Identification of the derivative

Eluted peaks were collected from the HPLC column to confirm the identity of the propranolol derivatives. The acetonitrile was evaporated and the derivative was extracted with ether. The ether was evaporated and the residue redissolved in chloroform; an aliquot was injected directly into the ion source of the mass spectrometer.

Subjects

After fasting for 8 h, two healthy male volunteers (37 years old) each took a single commercial Inderal® (ICI) tablet containing 40 mg of racemic propranolol. At timed intervals venous blood was drawn into heparinized Venoject tubes according to a protocol approved by our Ethical Committee. Plasma was separated immediately after collection and frozen until analyzed.

RESULTS AND DISCUSSION

Extraction procedure

The acid dissociation constant (pK_a) for the secondary amino function of propranolol is 9.5 [10]. Plasma samples and standard solutions were adjusted to pH 9.85 by 1 *M* carbonate buffer. This gave > 98.0% extraction of propranolol into diethyl ether with a phase volume ratio (V_{org}/V_{aq}) of 2.5.

Derivatization of propranolol

Derivatization of enantiomers with an optically active reagent yields diastereomers which often can be separated by HPLC [11] or gas–liquid chromatography [12]. In this assay procedure an HPLC system was used to separate and quantitate the diastereomeric derivatives formed by reaction of propranolol in human plasma with the chiral reagent N-trifluoroacetyl-l-prolyl chloride (TPC). The utility of this reagent as a resolving agent for enantiomers was demonstrated by Weygand et al. [13, 14]. Mass spectrometry was used to confirm the identity of the diastereomeric derivatives. The mass spectra of the diastereomers were essentially identical (Fig. 1). The fragmentation pattern coincided with that for the trimethylsilyl derivative of TPC-propranolol [12].

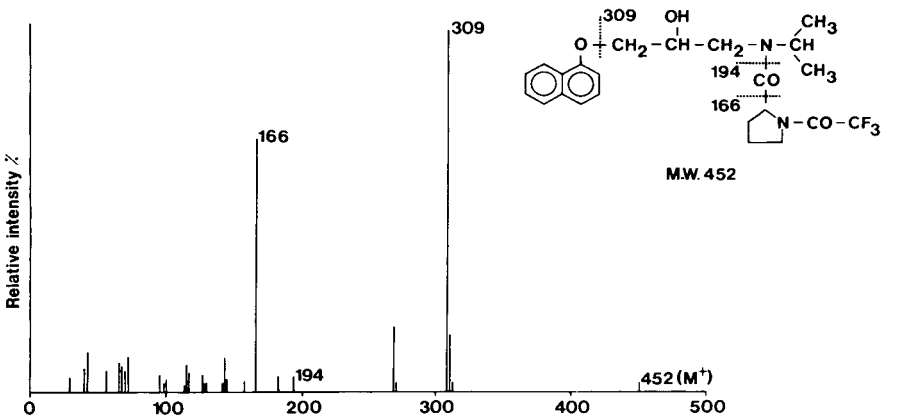

Fig. 1. Mass spectrum of N-trifluoroacetyl-l-prolyl chloride propranolol derivative.

The reaction time needed to obtain maximum yield was 15 min. No decrease in the yield was observed after 30 min of reaction time which indicates that no reaction takes place at the hydroxy function of propranolol during the time period studied. The reaction mixture was cleaned up by extraction with 3.0 ml of water and 6.0 ml of ether.

Chromatographic studies

A reversed-phase liquid chromatographic system was used to separate the diastereomers formed by reaction of propranolol with the chiral reagent. Several types of stationary phases (μBondapak phenyl, LiChrosorb RP-8 and RP-18) were tested to find which gave the highest selectivity for the diastereomers. LiChrosorb RP-18 gave the best selectivity with an α value of 1.2 with a mobile phase composition of 45% acetonitrile in phosphate buffer at pH 2.2 and a flow-rate of 1.0 ml/min. With this mobile phase the *l*-propranolol derivative had a capacity factor of 11.9 and the *d*-propranolol derivative a capacity factor of 14.4. The peaks of the *l*- and *d*-propranolol derivatives have asymmetry factors of 1.3 and 0.9, respectively.

A small increase in the asymmetry factor and the *H* value of the two diastereomeric derivatives have been observed over a long period of use, but after four months' use the *H* values of the *l*- and *d*-propranolol derivatives were 0.05 and 0.08, respectively. Fig. 2A shows a separation of the diastereomeric propranolol derivatives from human plasma using a four-month-old column. There were no disturbances of endogenous substances in plasma but four unexplained negative peaks can be seen in chromatograms of standard and plasma samples (Fig. 2B).

Quantitation of d-TFA-prolyl chloride in the reagent

Because commercial TPC-reagent contains small amounts of the *d*-isomer, four diastereomeric derivatives are formed upon reaction with racemic propranolol. Chromatographic experiments showed that *l*-propranolol derivatized with the TPC-reagent gave one main peak and a minor peak constituting 8.5 ± 0.2% (n = 5) of the main peak eluted with the same capacity factor as *d*-propranolol 1-TFA proline. *d*-Propranolol derivatized in the same way gave an equal

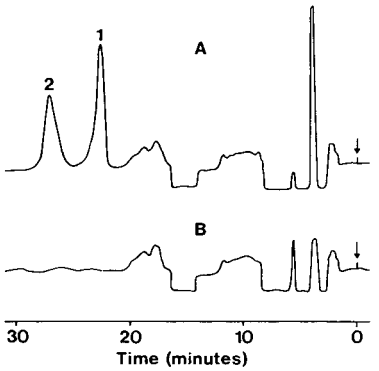

Fig. 2. Chromatograms of (A) a plasma sample containing *l*-propranolol (1) at 12.3 ng/ml and *d*-propranolol (2) at 8.9 ng/ml, and (B) of a blank plasma sample.

peak with the same capacity factor as *l*-propranolol 1-TFA proline. A likely cause of the minor peaks is that the *d*-TFA-proline fraction of the reagent forms *l*-propranolol *d*-TFA proline and *d*-propranolol *d*-TFA proline. This is supported by the fact that the manufacturer reports 8.2% of *d*-TFA prolyl chloride in the reagent which is in accordance with the above results. This may also indicate that no racemization takes place during the derivatization of propranolol. To confirm that the minor peaks were reaction products of the propranolol enantiomers and *d*-TFA-prolyl chloride, a racemic TPC reagent was prepared according to the description of Bonner [15]. Reaction of a solution of *d*- and *l*-propranolol (100 ng/ml) separately with the racemic reagent gave two peaks in each chromatogram. The peak heights were measured and the concentration in each peak was calculated from the standard curve and was found to be 50 ng/ml. A mixture of equal amounts of the two reaction mixtures gives only two peaks in the chromatogram with the same concentration and with the same capacity factors as obtained for racemic propranolol derivatized with the commercial TPC-reagent. The above experiment was performed with *d*- and *l*-propranolol each with an enantiomeric purity of > 99.5%. The optical purity of the propranolol enantiomers was checked as described in Materials and methods.

Correction of the results due to contamination of the TPC-reagent

Because the TPC-reagent contained *d*-TFA-prolyl chloride it was necessary to correct the concentrations obtained from peak height measurements according to the following equations

$$X = X_{\text{Chrom}} + AX - AY \quad (1)$$

$$Y = Y_{\text{Chrom}} + AY - AX \quad (2)$$

where X and Y represent the concentrations of *d*- and *l*-propranolol, respectively, that would be obtained if no *d*-TFA-prolyl chloride were present in the TPC-reagent. X_{Chrom} and Y_{Chrom} are the concentrations calculated from the peak heights in the chromatograms of *l*- and *d*-propranolol, respectively, when working with a contaminated reagent with respect to *d*-TFA-prolyl chloride, and A

is the percentage of *d*-TFA-prolyl chloride in the reagent. From eqns. 1 and 2 it can be seen that when TPC-reagent contaminated with *d*-TFA-prolyl chloride reacts with racemic propranolol, no correction of the results obtained has to be made because AX is in this case equal to AY. Eqns. 1 and 2 then reduce to $X = X_{Chrom}$ and $Y = Y_{Chrom}$, respectively. However, the situation is different when the concentration ratio of *d*-propranolol/*l*-propranolol is smaller or larger than unity, as is the case in patients' plasma. Combination of eqns. 1 and 2 gives

$$Y = \frac{Y_{Chrom}(1 - A)}{1 - 2A} - \frac{AX_{Chrom}}{1 - 2A} \quad (3)$$

where $\frac{1 - A}{1 - 2A} = K_1$ and $\frac{A}{1 - 2A} = K_2$. In this case the *d*-TFA-prolyl chloride content in the reagent is 8.5% ($A = 0.085$), giving $K_1 = 1.1$ and $K_2 = 0.1$ which means that

$$Y = 1.1\ Y_{Chrom} - 0.1\ X_{Chrom} \quad (4)$$

In analogy

$$X = 1.1\ X_{Chrom} - 0.1\ Y_{Chrom} \quad (5)$$

Optimization of the fluorimetric response

The method is sensitive enough to quantitate therapeutic concentrations of the two propranolol enantiomers in human plasma. The high sensitivity with this method is largely the result of using 210 nm as the excitation wavelength which we have found to give optimal fluorimetric response. Other investigators working with the quantitation of propranolol use an excitation wavelength of 295 nm, which gives only 6% of the response obtained with an excitation wavelength of 210 nm [16, 17] and a 340 nm cut-off emission filter. Further reasons for the high sensitivity obtained with our method are the small internal diameter of the column (3.2 mm) giving rise to a small area of cross-section and interstitial volume [18], and the optimal geometry and small volume (5 μl) of the flow-cell of the detector.

Standard curve and reproducibility studies

The reproducibility of the method was determined as described under Materials and Methods. The study was performed at four different concentrations (1, 10, 25 and 50 ng/ml) of *d*- and *l*-propranolol hydrochloride and the relative standard deviations found were 5.9, 4.5, 2.8 and 2.4%, respectively. Standard samples were extracted after the addition of 1.0 ml of drug-free plasma, which has the advantage of giving the same degree of propranolol extraction from standard and plasma samples thus making compensation due to different recoveries unnecessary.

A standard curve is constructed by plotting the peak height against the concentration of each enentiomer. Linear regression equations of the standard curve for *l*- and *d*-propranolol were calculated and are given below.

$Y_l = 5.634\ x + 0.3396$
$Y_d = 4.428\ x + 0.8428$

The linearity of the standard curves in the range 1.0—45 ng of base per ml for each enantiomer was determined by calculating the correlation coefficients which were found to be 0.9994 and 0.9991 for the *l*- and *d*-propranolol derivatives, respectively.

Pharmacokinetics

The plasma concentrations of *d*- and *l*-propranolol measured in two subjects who received a single, oral 40-mg dose of racemic propranolol are shown in Fig. 3. After half an hour the concentrations of *d*- and *l*-propranolol were similar in one subject (Fig. 3A). Later the concentration of *d*-propranolol was always lower than that of the *l*-isomer. The plasma half-lives were similar for the enantiomers, 3.4 h. In the other subject (Fig. 3B), the *d*-propranolol concentration was lower at all time points. Thus the area under the plasma concentration—time curve (AUC) was lower for *d*- than for *l*-propranolol after a 40-mg oral dose (racemate). Also in this subject the half-lives were the same, 3.2 h. These data are in agreement with the report that the plasma concentration of *d*-propranolol is lower than that of *l*-propranolol for up to 1.5 h after giving the separate isomers orally [6] and also with in vitro human liver data where we have shown that *d*-propranolol is more rapidly oxidized than *l*-propranolol [19]. These data disagree with findings in dogs, where the AUC in plasma was lower for the *l*-isomer [5]. We have no definite explanation of this discrepancy, and we have only studied two subjects. However, the data may indicate a species difference with regard to the metabolism of propranolol between dog and man.

In this study the plasma half-lives of *d*- and *l*-propanolol were similar after oral administration of racemic propranolol (Fig. 3). This is consistent with theories on hepatic elimination of "high-clearance" drugs [20] since both *d*- and *l*-propranolol are highly extracted by the liver. George et al. [6] found that the half-life of *d*-propranolol was shorter than that of *l*-propanolol when the isomers were given separately. This may be the result of reduced hepatic blood flow caused by *l*- but not *d*-propranolol [21]. Decreased hepatic blood flow should prolong the half-life of a "high-clearance" drug like propranolol. Our

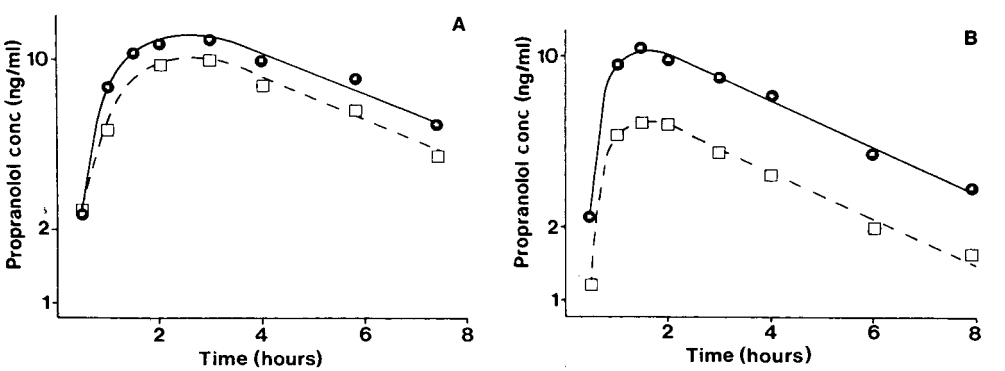

Fig. 3. Plasma concentrations of *d*-propranolol (□ --- □) and *l*-propranolol (● — ●) in two subjects (A and B) receiving 40-mg commercial tablets containing racemic propranolol. Values are corrected according to eqns. 4 and 5.

data and those of George et al. [6] are not inconsistent since after administration of the racemate the clearance of both *d*- and *l*-propranolol would be affected by the decreased liver blood flow caused by *l*-propranolol. However, if only *d*-propranolol is given, the liver blood flow should not be decreased and thus the plasma half-life would be shorter.

In conclusion, a high-performance liquid chromatographic method was developed by which both *d*- and *l*-propranolol can be separated and quantitated separately in clinical plasma samples after administration of small doses of racemic propranolol. The method requires no complicated techniques or the use of isotopes. We use this method in human pharmacodynamic studies.

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CHROMBIO. 660

SENSITIVE METHOD FOR THE DETERMINATION OF CHLOROQUINE AND ITS METABOLITE DESETHYL-CHLOROQUINE IN HUMAN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method has been developed for the rapid quantitative analysis of chloroquine and its metabolite desethyl-chloroquine in plasma, blood and urine using high-performance liquid chromatography. An ethylene dichloride extract of the alkalinized biological samples was extracted with dilute acid and chromatographed on a reversed-phase column. Phosphate buffer in acetonitrile was used as the mobile phase with perchlorate as the counter-ion. Ultraviolet absorption at 254 or 340 nm or fluorescence detection was used. The fluorescence spectra and the fluorescence quantum yield of the substances were determined.

Chloroquine and desethyl-chloroquine concentrations in the range of 10 nmol/l (UV-detection) and of 0.5 nmol/l (fluorescence detection) could be accurately measured with a relative standard deviation of 12%. The method should be adequate for therapeutic and pharmacokinetic studies.

INTRODUCTION

Chloroquine (CQ) is a potent antirheumatic drug [1, 2] and is used frequently as an antimalarial drug. Although the drug has been used for more than 40 years the pharmacokinetic behaviour of this drug has not yet been clarified. This is most probably due to the lack of highly sensitive and selective analytical methods allowing measurements of chloroquine in biological samples after administration in therapeutic doses. The methods used so far have been liquid extraction with fluorescence detection [3, 4]. These methods are not sensitive enough and are unselective since the major metabolite desethyl-chloroquine (CQM) is determined simultaneously [4].

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The aim of the present work was to develop a selective and sensitive method for the determination of CQ, an amine, in human plasma and urine. The method includes a separation by ion-pair high-performance liquid chromatography (HPLC), of the protonated forms of CQ and CQM using the perchlorate ion as a counter-ion. Ion-pair partition chromatography has been used for the separation of various synthetic amines [5] and biogenic amines [6]. Recently a method for the separation and determination of divalent amines as perchlorate ion-pairs has been described [7]. The choice of the conditions for the extraction and chromatographic procedure is discussed in more detail elsewhere [8]. The method provides complete separation of CQ and the main metabolite CQM. Furthermore, it is sensitive enough to determine the low concentrations anticipated for pharmacokinetic studies and is comparatively rapid, which permits its use in the routine monitoring of therapeutic plasma concentrations [9]. These have been reported in the range of 0.60—2.5 μmol/l serum during chronic treatment of rheumatoid patients (0.25 g/day) using liquid extraction with fluorescence detection [9].

EXPERIMENTAL

Instruments

A Laboratory Data Control pump, Model 712-74, and a Rheodyne Model 7120 high-pressure injection valve provided with a 150-μl loop were used. The detectors were a Schoeffel Model FS 970 fluorescence detector or a Waters Model 440 UV detector. The excitation wavelength of the fluorescence detector was set at 335 nm with a 370 nm emission cut-off filter. The UV detector had a measuring wavelength of 254 or 340 nm. The detector output was connected to a Vitatron 2001 recorder. The pH was measured with a Radiometer PHM 64.

Chemicals and reagents

The molecular structures of CQ, CQM and the internal standard 6,8-dichloro-4-(1-methyl-4-diethylamino-butylamino)-quinoline (D) are presented in Fig. 1. They were kindly supplied by Sterling-Winthrop (Skärholmen, Sweden). Quinine bisulphate, pharmacopoeial grade (Apoteksbolaget, Solna, Sweden) was recrystallized from ehtanol—water mixtures. Acetonitrile (chromatographic purity, Merck, Darmstadt, G.F.R.) and ethylene dichloride (Merck) were used. All aqueous solutions were prepared using high-purity water obtained from a Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.). All other chemicals were of analytical quality. Glassware and centrifuge tubes were cleaned by standing overnight in 5 *M* nitric acid, and were then ultrasonically cleaned and rinsed with Milli-Q water.

Extraction and chromatography conditions

It has been found that divalent 4-amino-quinoline derivatives give quantitative extractions > 99% from pure aqueous solutions at pH 10.5—11.0 in ethylene dichloride, methylene chloride and chloroform using equal phase volumes. When extracting biological samples from the solvents mentioned ethylene dichloride gave the lowest blank disturbance of the chromatogram

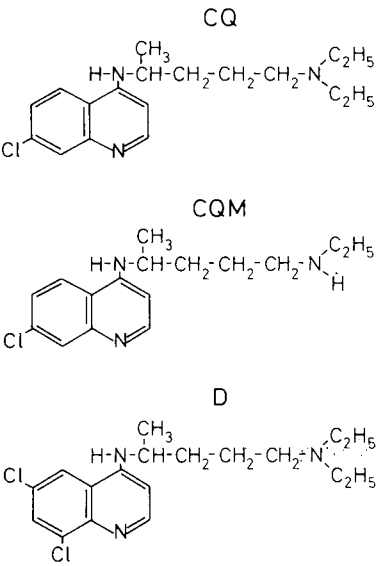

Fig. 1. Molecular structures of chloroquine (CQ) desethyl-chloroquine (CQM) and internal standard (D).

when using fluorescence detection. Acetonitrile (40%, v/v) has been found a suitable organic modifier for the separation of the 4-amino quinolines when perchlorate (75 mmol/l) is used as a counter-ion [8]. A mobile phase without perchlorate gave no separation of CQ and CQM [8]. An optimal chromatographic efficiency was obtained at a low pH (pH < 4.5) of the mobile phase. Above this pH (> 4.5) a decrease in chromatographic efficiency was seen.

In the present study the analytical column (200 × 4.0 mm I.D.) was packed by the upward-slurry packing method with pure methylene chloride as described by Bristow [10] using modified Nucleosil C_{18} 5 or 10 μm (Macherey, Nagel & Co., Düren, G.F.R.). When method II (direct injection) was used the analytical column was protected by a guard column (30 × 1.4 mm I.D.) packed with LiChrosorb RP-8, 10 μm (Brownlee Labs, Santa Clara, CA, U.S.A.). The flow-rate of the mobile phase [acetonitrile—phosphate buffer (40:60), ionic strength 0.1, pH 3, and perchlorate 75 mmol/l] was 0.8—1.0 ml/min. The mobile phase was degassed ultrasonically immediately before use and the experiments were performed at room temperature.

When using fluorescence detection a T-union was used to combine the column eluate with 0.2 *M* borate buffer, pH 11.5. The fluorescence signal was maximal at a flow-rate of approximately 0.30 ml/min, which gave a pH in the detector of 9.3—9.6. To eliminate noise in the detector a short stainless-steel coil (150 × 0.2 mm I.D.) was inserted after the T-union.

Analytical procedure

Method I

Injection after extraction (plasma, whole blood and urine). A 0.5--2.0-ml sample was made alkaline with 2.0 ml of 1 *M* sodium hydroxide and extracted for 15 min with 7.0 ml of ethylene dichloride containing the internal standard (D) at 100—200 nmol/l. The aqueous phase was discarded after centri-

fugation (step 1). Then 4.0 ml 0.05 *M* borate buffer, pH 11.0, was added. After shaking for 10 min and centrifuging the aqueous phase was removed by aspiration (Step 2). Next, 300 μl 0.1 *M* hydrochloric acid was added. After shaking for 10 min and centrifuging, 150 μl of the aqueous phase were injected. Whole blood was hemolyzed by diluting with distilled water and freezing at −70° C. Step 2 in the extraction was repeated.

Method II

Injection without extraction (plasma, urine). If high concentrations were anticipated:

Plasma. A 500-μl sample of plasma was deproteinized by adding it dropwise to a solution (2.0 ml) of trichloroacetic acid 5% (w/v) in methanol containing the internal standard (D) in concentrations of 100—200 nmol/l, during vigorous vortex mixing for 30 sec. The sample was left standing at room temperature for 10 min and after centrifugating for 20 min, 150 μl of the supernatant were used for chromatographic determination. Microprecipitates were easily removed by a 0.45-μm filter inserted before the injection loop.

Urine. A 200-μl sample of urine was diluted to 5.0 ml with the mobile phase containing the internal standard (D) at 1 μmol/l. The mixture was vortex mixed for 30 sec, then 150 μl were injected.

Calibration curves based on peak ratios (CQ/D, CQM/D) were prepared daily by means of spiked drug-free plasma and urine samples which were carried through the whole analytical procedure. The choice of the internal standard D was justified by the distribution, extraction and fluorescence properties or UV characteristics similar to those of CQ and CQM [8]. The CQ and CQM peaks were identified on the basis of their retention times in relation to the internal standard.

D was eluted after CQ and CQM in the chromatography system and its peak was well separated from the other peaks. By selecting an internal standard which is eluted after CQ, interference with other polar metabolites can be avoided.

Fluorescence quantum yield measurements. These were performed using an Aminco-Bowman spectrofluorimeter equipped with Hanovia 150 W xenon lamp and IP 21 photomultiplier tube using a bandwidth of 12 nm. They were determined for CQ, CQM and D by the comparative method of Chen [11] using quinine bisulphate as a reference standard [12]. Spectra were corrected in quantum yields measurements.

RESULTS AND DISCUSSION

The fluorescence detection of CQ and CQM was found to be 20 times more sensitive than UV detection at 254 or 340 nm. The fluorescence of CQ and CQM was also found to be pH dependent with a maximum at pH 9.5. Since the chromatographic column is unstable at pH > 8, a post-column addition of borate buffer (pH 11.5) was made in order to obtain the maximal fluorescence in the detector. In Table I, the quantum yields of CQ and CQM are given and found to be 0.13 and 0.14, respectively, when the excitation wavelength was 330 nm. The internal standard (D) had a lower quantum yield.

Fig. 2 demonstrates calibration curves in plasma determined by method I and Fig. 3 shows a calibration curve in urine obtained with method II. Using method I it was possible to determine 0.5 nmol/l of CQ and CQM with a relative standard deviation of 12%. Method II had a considerably lower sensitivity, > 200 nmol/l, and was therefore used only when concentrations > 200 nmol/l were anticipated. The results from a comparative study of methods I and II when applied to plasma and urine samples are given in Table II. Method II is thus suitable for the rapid quantitation of CQ and CQM.

TABLE I

FLUORESCENCE QUANTUM YIELDS AND SPECTRAL PROPERTIES

Chloroquine, desethyl-chloroquine and the internal standard in borate buffer pH 9.5; quinine bisulphate 0.05 mmol/l in 0.1 mol/l H_2SO_4.

Substance	Excitation maximum (nm)	Emission maximum (nm)	Quantum yield = ϕ
Chloroquine	334	392	0.13
Desethyl-chloroquine	334	392	0.14
D (internal standard)	343	400	0.08
Quinine bisulphate	345	450	0.55*

*Ref. 12.

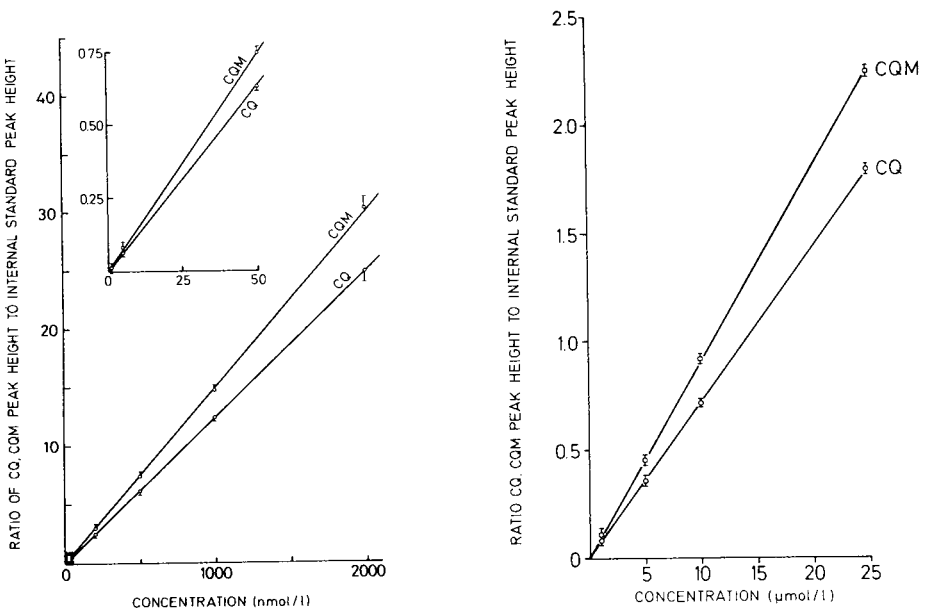

Fig. 2. Calibration standard curves of CQ and CQM determined by method I in plasma with fluorescence detection. The shaded area in the large figure is magnified in the upper part of the figure. Each point represents the mean value and S.D. of four determinations carried out on different days. The length of the bar corresponds to the estimated standard deviation.

Fig. 3. Calibration standard curves of CQ and CQM determined by method II in urine with UV detection. Each point represents the mean value and S.D. of four determinations carried out on different days. The length of the bar corresponds to the estimated standard deviation.

TABLE II

COMPARISON OF METHODS I AND II

Method I (x)	Method II (y)	Number of samples	Correlation coefficient	Regression equation
Plasma				
CQ	CQ	9	0.974	$y = 0.913x + 0.012$
CQM	CQM	9	0.994	$y = 0.987x - 0.014$
Urine				
CQ	CQ	15	0.996	$y = 0.964x - 1.29$
CQM	CQM	15	0.998	$y = 0.965x + 0.42$

TABLE III

PRECISION OF THE ANALYTICAL METHODS

R.S.D. = relative standard deviation.

	Concn. (nmol/l)	S.D.	R.S.D. (%)	n
Method I with fluorescence detection				
Within-day: plasma				
CQ	0.6	0.07	11.7	9
	4.9	0.15	3.1	9
	51.4	2.0	3.9	5
	821	32	3.9	5
CQM	0.5	0.05	10.0	9
	5.1	0.27	5.3	9
	49.7	2.1	4.2	5
	782	27	3.5	5
Day-to-day: plasma				
CQ	50.2	6.7	13.4	5
	775	40	5.2	5
CQM	51.0	4.6	9.1	5
	790	35	4.4	5
Method II with fluorescence detection				
Within-day: plasma				
CQ	213	15	7.0	7
	725	38	5.2	7
CQM	264	20	7.5	7
	770	27	3.5	7
Within-day: urine*	(μmol/l)			
CQ	5.0	0.19	3.8	5
	25.5	0.40	1.6	5
	49.4	0.36	0.7	5
CQM	5.0	0.20	4.0	5
	25.3	0.66	2.6	5
	49.7	0.32	0.6	5

*UV-detection.

Table III gives some results of tests of the within-day and the day-to-day reproducibilities for both methods.

The absolute recoveries of CQ and CQM from drug-free plasma, whole blood and urine, were also determined; the samples were analyzed by methods I and II. The absolute recovery was obtained by comparison of the peak heights from these samples with a calibration curve obtained by directly injecting known amounts of CQ and CQM into the chromatograph. The results are presented in Table IV.

None of the following drugs interfered with the determination of CQ and CQM in plasma: ibuprofen; phenylbutazon; naproxen; prednisolon; indometacin; salicylic acid; salicylazosulphapyridine. The drugs are commonly used in combination therapy for rheumatoid diseases.

Fig. 4 shows the plasma concentration—time data of CQ and CQM ob-

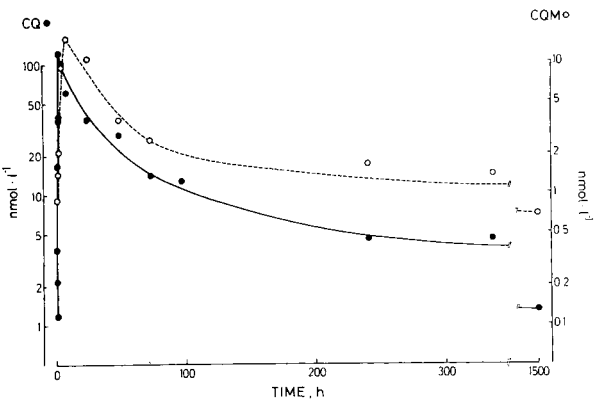

Fig. 4. Plasma concentration—time data of CQ and CQM obtained from a patient after a single oral dose of 0.25 g of chloroquine phosphate. Method I with fluorescence detection.

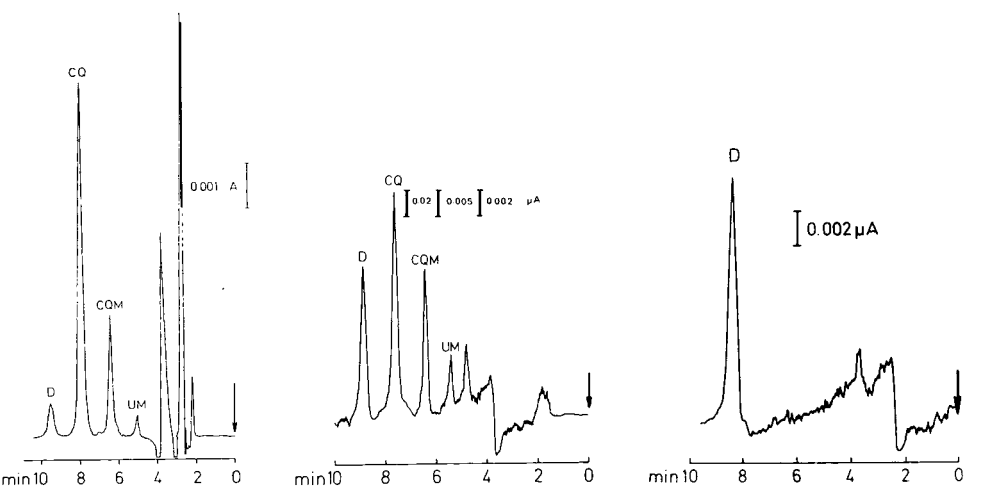

Fig. 5. Chromatograms of plasma and urine samples obtained from the same patient as in Fig. 4. UM is an unidentified metabolite. 1, Plasma, CQ, 82 nmol/l; CQM, 16 nmol/l; D, internal standard; sample was processed by method I with fluorescence detection. 2, Urine, CQ, 8.5 μmol/l; CQM, 3.1 μmol/l; D, internal standard; sample was processed by method II with UV detection at 340 nm. 3, Drug-free plasma with internal standard D was processed by method I with fluorescence detection.

TABLE IV

RECOVERY OF THE ANALYTICAL METHODS

$n = 4$.

	Recovery (% ± S.D.)									
	Concentration (nmol/l)									
	0.5	5	10	25	50	200	500	800	1000	1500
Method I: plasma										
CQ	100 ± 11.4	97 ± 5.3			97 ± 3.2	86 ± 2.0	87 ± 1.5		88 ± 2.0	
CQM	104 ± 9.4	92 ± 6.9			94 ± 3.0	90 ± 4.0	85 ± 1.4		90 ± 1.2	
Method I: whole blood										
CQ			87 ± 2.5			72 ± 3.4		75 ± 3.5		
CQM			89 ± 1.1			75 ± 5.0		70 ± 2.1		
Method II: plasma										
CQ						102 ± 5.4		98 ± 6.1		103 ± 3.4
CQM						94 ± 3.0		100 ± 2.8		100 ± 3.7
Method II: urine	Concentration (μmol/l)									
CQ	100 ± 1.5		99 ± 0.7	98 ± 1.7	100 ± 1.0					
CQM	97 ± 1.4		97 ± 1.1	97 ± 3.2	99 ± 1.3					

tained after the first single dose of 0.25 g chloroquine phosphate to a rheumatoid patient. Venous samples were taken and analyzed by method I (fluorescence detection) at regular intervals. The plasma concentration of CQ is approximately 10 times higher than that of CQM during a dosage interval. It is obvious that the disposition patterns of CQ and CQM are similar and seem to follow a multi-exponential curve. The chromatogram of this patient's plasma and urine (see Fig. 5) indicates that another yet unidentified metabolite is present.

CONCLUSIONS

The analytical method described exhibits good reproducibility and permits the simultaneous determination of CQ and its metabolite CQM at concentrations of 0.5 nmol/l in plasma and urine. If higher concentrations (> 200 nmol/l) are anticipated, the samples (plasma, urine) can be injected directly into the liquid chromatograph after dilution. The chromatographic system has a stable column life for up to 10 months and allows analysis of 20—30 samples daily. The method could be a valuable tool for the further elucidation of CQ disposition and pharmacokinetics in man and an aid in the monitoring of chronic CQ therapy which has to be followed closely to avoid the undesirable side effects [9].

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CHROMBIO. 664

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF NALIDIXIC ACID AND HYDROXYNALIDIXIC ACID IN PLASMA WITH A DYNAMIC ANION-EXCHANGE SYSTEM

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SUMMARY

A rapid high-performance liquid chromatographic method has been developed for monitoring plasma levels of patients treated intravenously with nalidixic acid. The major metabolite (in vitro also active) can be determined as well; 50-μl plasma samples are sufficient. Use is made of a dynamic anion-exchange system. Different parameters such as adsorption of the surfactant cetrimide onto the column; pH and ionic strength of the eluent, and the critical micelle concentration of the surfactant in the eluent have been studied.

INTRODUCTION

Nalidixic acid (1-ethyl-1,4-dihydro-7-methyl-4-oxo-1,8-naphthyridine-3-carboxylic acid; NA) is occasionally used intravenously for the treatment of systemic infections. Renal failure is common amongst these patients and it is therefore necessary to monitor the plasma concentrations in order to prevent toxic or, on the other hand subtherapeutic levels. The major metabolite, hydroxynalidixic acid (1-ethyl-1,4-dihydro-7-hydroxymethyl-4-oxo-1,8-naphthyridine-3-carboxylic acid; HNA) is in vitro also active against Gram-negative micro-organisms and that is a reason for determining the plasma levels of this product too.

Several methods have been described for the assay of NA and HNA in biologic media. The original fluorescence method [1] and a modification thereof [2] are not suitable for monitoring plasma levels. Two high-performance liquid chromatographic (HPLC) methods have been reported; the first makes use of an ion-exchange column [3] and in the second method [4]

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NA is methylated and chromatographed on a reversed-phase system. A gas—liquid chromatographic (GLC) method [5] has also been described.

The aim of this study was to develop a procedure for the determination of NA and HNA in plasma, in which the advantages of the methods reported previously are combined, such as speed, selectivity, sensitivity and the use of small plasma samples. A dynamic ion-exchange system is used, which was introduced by Knox and co-workers [6, 7] under the name "soap chromatography". In this system a reversed-phase column is used and a surfactant such as cetrimide is added to the eluent. The cetrimide can be retained by the stationary phase, resulting in a "dynamically coated" column. The quaternary nitrogen groups of the bonded cetrimide molecules give the column ion-exchange properties, although ion-pair formation remains another possibility of the retention mechanism. The influence of several parameters on the chromatographic behaviour of NA, HNA and the internal standard used in the assay (pentothiobarbital) have been investigated. The critical micelle concentration (cmc) of the surfactant cetrimide in the mobile phase is a special point of interest in this study.

EXPERIMENTAL

Chemicals

Nalidixic acid and hydroxynalidixic acid were kindly supplied by Sterling Winthrop (Haarlem, The Netherlands). Pentothiobarbital was obtained by extraction with chloroform from an acidified aqueous solution of Pentothal® (Abbott, Campoverde, Italy). Methanol and chloroform were of analytical grade and were obtained from Merck (Darmstadt, G.F.R.). Cetrimonium bromide (cetrimide) was of European Pharmacopoean quality (OPG, Utrecht, The Netherlands).

Apparatus

The HPLC system consisted of a solvent delivery system 6000A, a universal injector U6K, a UV absorbance detector operated at 313 nm, a Model R401 differential refractometer and a μBondapak C_{18} column (particle size 10 μm), 30 cm × 3.9 mm I.D., all from Waters Assoc. (Milford, MA, U.S.A.). Peak areas were measured by means of a Spectra-Physics SP4000 data system (Santa Clara, CA, U.S.A.).

Conductance was measured with a Metrohm conductometer E518 in combination with a type EA645 titration cell (Metrohm, Herisau, Switzerland). Chromatographic and conductance measurements were carried out at 25°C.

Gas chromatography—mass spectrometry (GC—MS) analysis was performed on a Hewlett-Packard 5810 gas chromatograph; colum 2 m × 2.5 mm I.D., 3% OV-1 on Gas-Chrom Q (Chrompack, Middelburg, The Netherlands), 80—100 mesh; oven temperature 145°C; injection port temperature 300°C; carrier gas helium: flow-rate 25 ml min^{-1}; detection MS/total ion current. The mass spectrometer was a JEOL D300, with JMA-2000 data system; chamber voltage 70 eV; ion source temperature, 200°C; trap current 300 μA; accelerating voltage, 3 kV.

Adsorption of cetrimide onto the packing material (μBondapak C_{18})

The amount adsorbed was measured in the following way. A water—methanol (1:1, w/w) mixture containing an aqueous phosphate buffer pH 7.4 (final concentration of phosphate in the eluent, 0.016 mol kg^{-1}), was passed through the column. After equilibrium was reached, 0.12% (w/w) cetrimide was added to the eluent. The breakthrough volume of cetrimide was detected with a refractometric detector. Fractions were collected at the end of the chromatographic system and analyzed by GC—MS after evaporation of the solvent and reconstitution in methanol. The amount of cetrimide would then be $(V_R - V_0)C$, where V_R = breakthrough volume, V_0 = void volume and C = concentration of cetrimide in the eluent.

Determination of the critical micelle concentration

The cmc of cetrimide in the eluent was measured by titrating the eluent (without cetrimide), containing different percentages (0—50%, w/w) methanol, with a 50% (w/v) solution of cetrimide. Portions of 5 μl (at 0% methanol) up to 50 μl (at 50%, w/w, methanol) titrant were added stepwise to the eluent. After each addition the conductance was measured and plotted against the cetrimide concentration. The cmc is the concentration of cetrimide at the breakpoint of the titration curve.

Determination of NA and HNA in plasma

A 50-μl plasma sample was acidified with 50 μl 0.1 *M* hydrochloric acid and subsequently extracted with 1.50 ml chloroform containing the internal standard pentothiobarbital, 1.5 μg ml^{-1}. After mixing with a Vortex mixer for 30 sec and centrifugation at 2500 *g* for 2 min, the chloroform layer was transferred to another centrifuge tube and evaporated to dryness. The residue was dissolved in 50 μl eluent; 20 μl of this solution was injected into the chromatograph. The eluent consisted of a water—methanol (1:1, w/w) mixture, containing a phosphate buffer pH 7.4 (measured in water). The final concentrations of phosphate and cetrimide in the eluent are 0.016 mol kg^{-1} and 0.12% (w/w), respectively. The flow-rate was 1.5 ml min^{-1}; detection, UV absorbance at 313 nm.

RESULTS AND DISCUSSION

Adsorption of cetrimide onto the column

The method of measuring breakthrough volumes for the determination of the amount of cetrimide adsorbed onto the packing material has been described in the literature [6, 8]. Knox and Laird [6] collected 0.2-ml fractions of the eluate, which were added to a two-phase mixture of water, methylene chloride and propanol, containing the dye sunset yellow. Cetrimide, if present in the eluate, was extracted with the dye as ion-pair into the organic layer. However, the use of a differential refractometer, as described by Terweij-Groen et al. [8] is much more convenient.

In this study the latter method was used, but fractions of the eluate were still collected for GC—MS analysis.

Fig. 1 shows the refractometer response when 0.12% (w/w) cetrimide was added to the eluent.

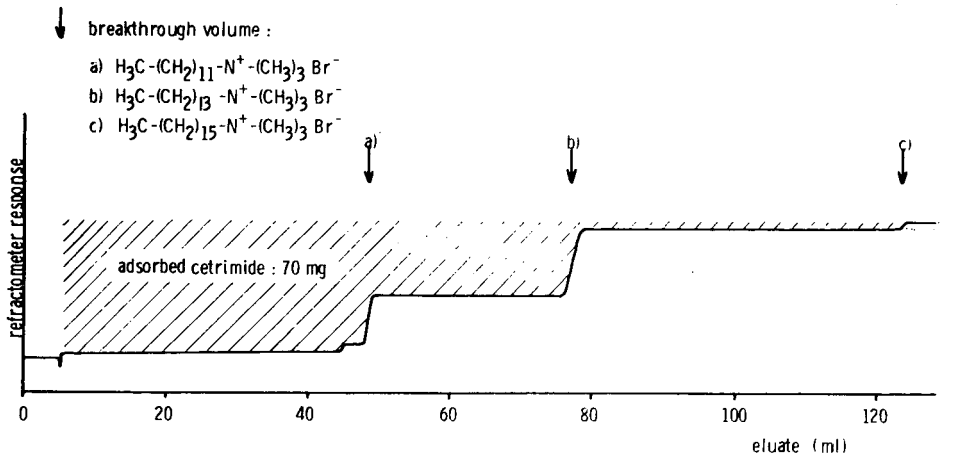
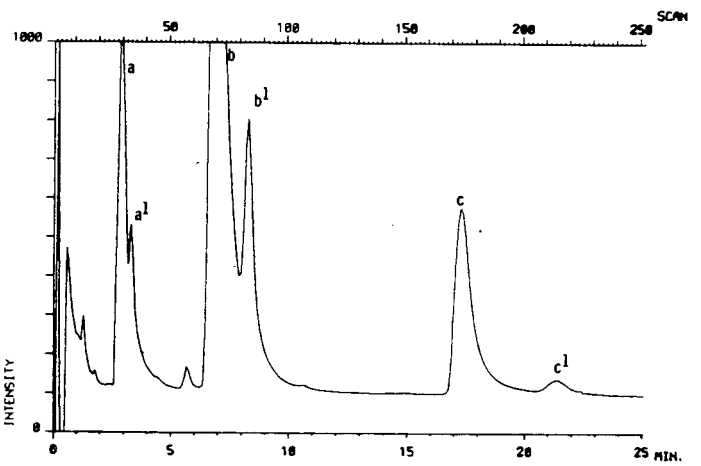


Fig. 1. Adsorption of cetrimide onto a μBondapak C_{18} column. Eluent: cetrimide, 0.12% (w/w); phosphate (pH = 7.4), 0.016 mol kg^{-1}; methanol—water (1:1, w/w).



peak	formula	m/e
a	$H_3C-(CH_2)_{11}-N(CH_3)_2$	213
a^1	$H_3C-(CH_2)_{11}Br$	248 (Br^{79}), 250 (Br^{81})
b	$H_3C-(CH_2)_{13}-N(CH_3)_2$	241
b^1	$H_3C-(CH_2)_{13}Br$	276 (Br^{79}), 278 (Br^{81})
c	$H_3C-(CH_2)_{15}-N(CH_3)_2$	269
c^1	$H_3C-(CH_2)_{15}Br$	304 (Br^{79}), 306 (Br^{81})

Fig. 2. GC—MS pyrogram of a cetrimide solution in methanol. For conditions, see Experimental.

Fig. 2 shows a pyrogram of cetrimide. The identity of the different compounds, as established by MS, is also displayed. When cetrimide is pyrolysed in the injection port of the gas chromatograph, two reactions predominate under the present conditions.

$$CH_3(CH_2)_nN^{\oplus}(CH_3)_3Br^{\ominus} \xrightarrow{\Delta T} CH_3(CH_2)_nN(CH_3)_2 + CH_3Br \quad (1)$$

$$CH_3(CH_2)_nN^{\oplus}(CH_3)_3Br^{\ominus} \xrightarrow{\Delta T} CH_3(CH_2)_nBr + N(CH_3)_3 \quad (2)$$

A comparable degradation pattern was observed for cetrimide in the presence of phosphate (same concentration as in the eluent). Choi et al. [10] described a method for the determination of cetrimide in pharmaceutical preparations, also based on GLC pyrolysis. They observed pyrolytic degradation according to pattern (1) only; however, their experimental conditions were different (a Carbowax KOH column was used and a much higher injection port temperature, 450° C, was applied).

Pyrolysis of different fractions of the eluate showed, that the three steps observed with the refractometer (Fig. 1) correspond with the breakthrough volumes of the three components of cetrimide, which differ in the length of the alkyl group.

Influence of the chromatographic conditions on the elution behaviour of NA, HNA and pentothiobarbital

In Fig. 3 the capacity ratio (k') is plotted versus the pH of the phosphate buffer, added to the eluent; the concentration of cetrimide and of phosphate were kept constant. On increasing the pH, the k' value of pentothiobarbital also increases. A phosphate buffer of pH 7.4 was chosen for the determination of NA and HNA; a good separation was then obtained.

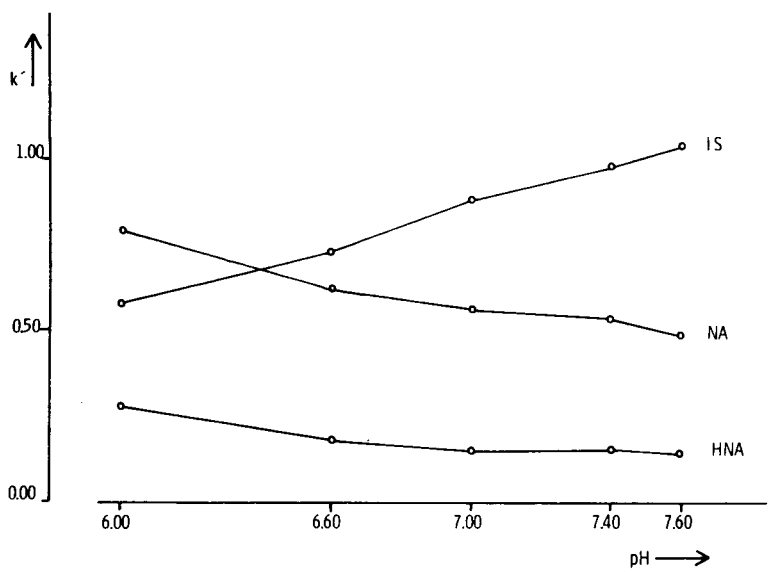


Fig. 3. Influence of the aqueous pH of the phosphate buffer on k'. Eluent: cetrimide, 0.12% (w/w); phosphate, 0.016 mol kg^{-1}; methanol—water (1:1, w/w).

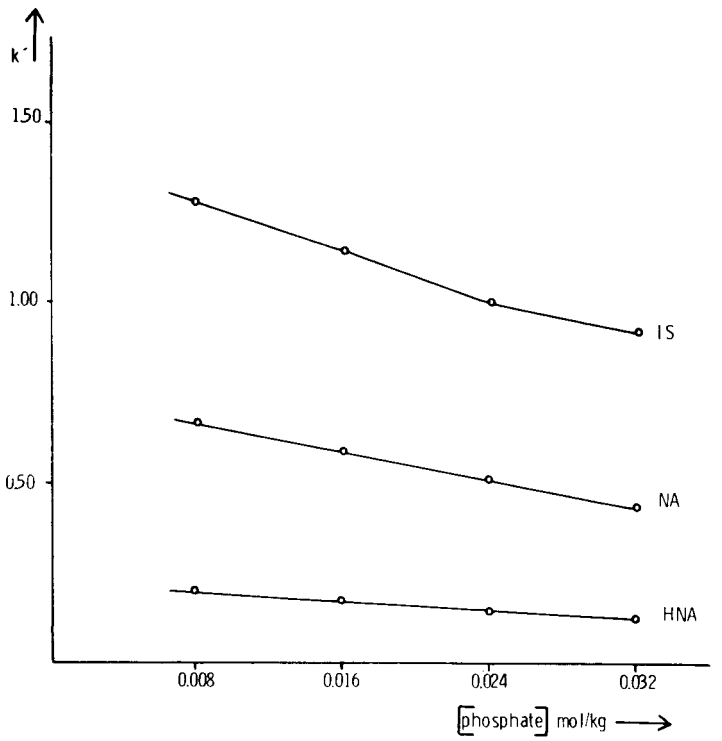


Fig. 4. Influence of the phosphate concentration at aqueous pH 7.4. Eluent: cetrimide, 0.12% (w/w); methanol—water (1:1, w/w).

Fig. 4 shows the relation between the phosphate concentration and k'. On increasing the phosphate concentration the retention times decrease. This is consistent with an ion-exchange process, which is thought to be the most important mechanism in these solvent-generated ion-exchange systems [7, 8]. With increasing phosphate concentration less solute anions will be retained, due to the competition with the phosphate ions, resulting in shorter retention times. Arbitrarily a concentration of 0.016 mol kg^{-1} phosphate was chosen for the determination of NA and HNA.

In Fig. 5 the cmc value, as calculated from the conductimetric titrations, is plotted versus the methanol concentration. For the determination of NA and HNA a concentration of 0.12% (w/w) cetrimide and 50% (w/w) methanol was chosen. In this eluent cetrimide micelles cannot be present, because the cmc in this eluent is about 1.5% (w/w) (see Fig. 5).

Fig. 6 shows the relationship between the cetrimide concentration and k' for NA, HNA and the internal standard. In the lowest cetrimide concentration region a small increase of the cetrimide concentration results in a relatively large increase of the retention times; indicating that partitioning of the undissociated acids between the eluent and the stationary phase is of secondary importance. With increasing cetrimide concentration in the mobile phase the amount of cetrimide adsorbed onto the stationary phase increases. Knox and Laird [6] showed that in their experiments the adsorption of cetrimide could

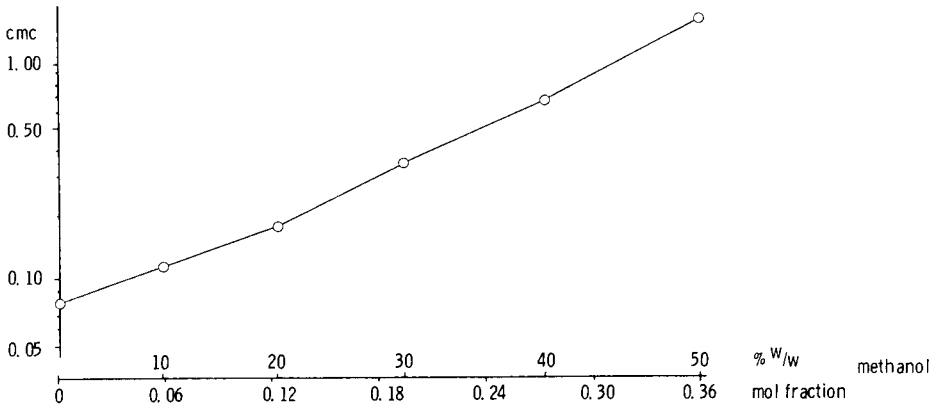


Fig. 5. Influence of the methanol concentration on the cmc of cetrimide in the eluent. Each solution contained a phosphate buffer, aqueous pH 7.4, with a final phosphate concentration of 0.016 mol kg^{-1}.

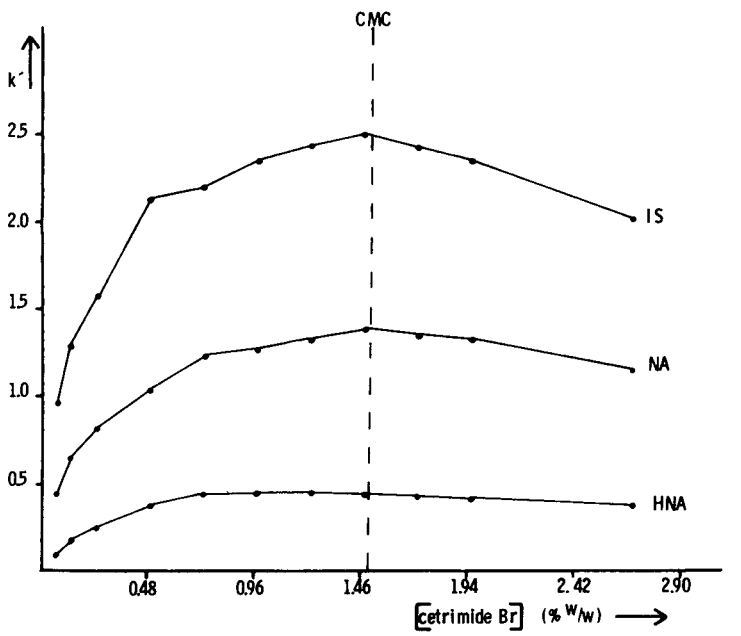


Fig. 6. Influence of the cetrimide concentration on k'. Eluent: phosphate (aqueous pH 7.4), 0.016 mol kg^{-1}; methanol—water (1:1, w/w).

be described by the Freundlich isotherm. The total amount of cetrimide adsorbed onto the stationary phase will hardly increase anymore at higher cetrimide concentrations in the mobile phase, resulting in an almost constant contribution to the retention mechanism by the ion-exchange process. The cetrimide concentration at which k' values are at a maximum, practically coincides with the cmc of cetrimide in the eluent. The decrease of k' values at cetrimide concentrations above 1.5% (w/w) can be explained by partitioning of

the solute anions between the mobile phase and the cetrimide micelles. A plot similar to Fig. 6 was obtained by Ghaemi and Wall [9]. Under the experimental conditions used in their investigations, the cmc of cetrimide* was also found to coincide with the cetrimide concentration at which maximal values for k' were obtained. It, therefore, appears that the role of cmc of cetrimide, and possibly of related compounds, in the mobile phase cannot be ignored as was suggested by Terweij-Groen et al. [8].

Determination of NA and HNA in plasma

Pentothiobarbital was chosen as the internal standard (IS), because it is extracted from acidified solutions, it can be detected at 313 nm and it has the required chromatographic properties. Following the procedure as described before, no decomposition of the internal standard was observed, even when after evaporation of the organic layer the residue was heated for 2 h at 60°C.

A calibration curve was constructed by analyzing 14 plasma samples (as described under Experimental) spiked with NA and HNA with concentrations ranging from 1 μg ml^{-1} up to 100 μg ml^{-1}. The peak area ratios (y), NA/IS and HNA/IS, were plotted against the concentration (x) of NA and HNA, respectively. The equations for the straight lines obtained for both species were calculated by the method of least squares and were found to be: $y = 0.0058 (\pm 0.0001)x + 0.0017 (\pm 0.0031)$ ($r^2 = 0.9995$) for NA, and $y = 0.0044 (\pm 0.0001)x + 0.0137 (\pm 0.0040)$ ($r^2 = 0.9987$) for HNA.

The reproducibility of the method was examined by analyzing two series of six samples each, to each of which was added NA and HNA, at concentrations for both compounds of 2 μg ml^{-1} and 20 μg ml^{-1}, respectively. The coefficients of variation were for NA and HNA 10% and 8%, respectively at 2 μg ml^{-1} and 2.7% and 3.5% at 20 μg ml^{-1}. The absolute recoveries of NA and HNA from plasma samples were determined following the procedure and calculated to be 90% for both species.

Using 50-μl plasma samples the detection limit for NA and HNA is about 1 μg ml^{-1}. Therapeutic levels for NA are in the range of 20—50 μg ml^{-1} plasma, so the method is sensitive enough.

Fig. 7 shows chromatograms obtained from blank plasma and from a plasma sample of a patient suffering from a complicated infection and renal failure, who was treated intravenously with 4 g NA per day. Apparently HNA levels in these patients are sufficiently high to be quantified by our method.

Recently Cuisinaud et al. [11] published a method comparable to the one described in this paper for the determination of NA, HNA and another metabolite, 1-ethyl-1,4-dihydro-4-oxo-1,8-naphtyridine-3,7-dicarboxylic acid (which has not been found in plasma). The influence of the various parameters on the chromatographic behaviour of NA and its metabolites was not reported. In this method [11] a double extraction procedure of 1-ml plasma samples was applied. This double extraction is necessary because a detection wavelength of 254 nm was chosen. In our experiments we observed that a number of plasma peaks interfere in the chromatogram at this wavelength after single

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*This brand of cetrimide has as its major component $H_3C-(CH_2)_{15}-N^{\oplus}(CH_3)_3Br^{\ominus}$.

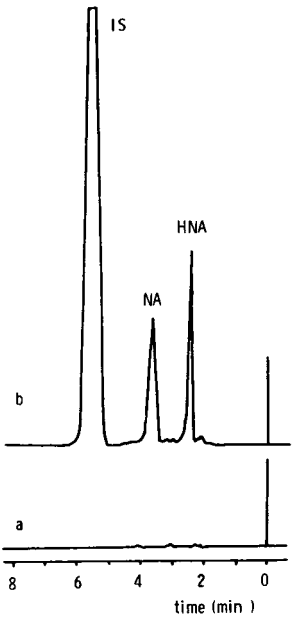

Fig. 7. Chromatograms obtained by analysis of 50 μl plasma. For conditions see Experimental. (a) Plasma blank; (b) plasma of a patient treated with nalidixic acid; NA, 17 μg ml^{-1}; HNA, 21 μg ml^{-1}.

extraction; these peaks are not detected at 313 nm. However, measuring at 254 nm has the advantage of lower detection limit.

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CHROMBIO. 665

EXTRACTION AND QUANTIFICATION OF DAUNOMYCIN AND DOXORUBICIN IN TISSUES

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SUMMARY

The measurement of intracellular concentrations of the anti-cancer drug doxorubicin was performed by the application of a simple cell extraction technique combined with a rapid high-performance liquid chromatographic separation. Quantitation was done by fluorescence detection. The extraction procedure was non-degradative and the mean recovery of drug was 95%. A high drug extraction efficiency was confirmed with radiolabeled [^{3}H]doxorubicin. The method is applicable to normal and neoplastic tissue.

INTRODUCTION

Quantitation of the tissue content of two important anti-cancer agents, doxorubicin and daunomycin, has been difficult. Assays have been carried out using either demanding thin-layer chromatography techniques [1] or fluorometric measurements that are unable to resolve metabolites of the drugs [2].

Measurements of cell levels of these drugs are of potential therapeutic importance, since resistance to these agents has been shown to be related to alterations in drug transport [3—5] in several experimental tumors. Whether or not changes in drug transport are important in drug resistance to these agents in human tumors is yet to be demonstrated.

Recently, high-performance liquid chromatographic (HPLC) techniques

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have been described for analyzing daunomycin and doxorubicin in urine and plasma [6—8]. The present study describes a rapid tissue extraction method modified from Israel et al. [7] coupled with a simple HPLC separation that can be used to determine cellular drug levels of daunomycin or doxorubicin. The extraction efficiency is high and is confirmed by measurement with radiolabeled doxorubicin.

EXPERIMENTAL

Reagents

Doxorubicin was obtained from commercial sources (Adria Laboratories, Dublin, OH, U.S.A.). Radiolabeled doxorubicin ([^{3}H] doxorubicin) was a gift of New England Nuclear (Boston, MA, U.S.A.), specific activity 0.5 Ci/mmol. The purification of the labeled material is described below. Doxorubicinol and doxorubicinone were prepared from doxorubicin [9]. Daunomycin was obtained from the National Cancer Institute (Bethesda, MD, U.S.A.). Methanol (distilled in glass) was obtained from Burdick & Jackson Labs., Muskegon, MI, U.S.A. R.P.M.I. 1640 was obtained from Associated Biomedic Systems, Buffalo, NY, U.S.A.

Tissues

The cells used in experiments were either peripheral blood (PB) mononuclear cells obtained from heparin anticoagulated venous blood drawn from normal volunteers and then separated on Ficoll-Hypaque gradient (PB lymphocytes) or HL-60 cells grown from a stock kindly provided by R. Gallo (National Cancer Institute). HL-60 is a recently described cell line derived from a patient with acute progranulocytic leukemia [10]. HL-60 cells were grown in R.P.M.I. 1640 medium supplemented with 20% fetal calf serum, 1 μM glutamine, and 100 units penicillin per ml and 50 μg streptomycin per ml in Corning T-30 flasks in 95% air, 5% carbon dioxide.

Cell incubations

Cells were suspended at 2×10^6/ml in sterile Dulbecco's phosphate buffered saline (PBS), pH 7.4, at 37°C with drug concentrations and incubation times as specified. Incubations were in screw-capped glass tubes in air. Aliquots of HPLC purified, ^{3}H-labeled doxorubicin (1.85×10^4 disintegrations per second/10^6 cells) were added to incubation mixtures. Tubes were protected from light during the incubations.

Drug extractions

Samples of cells were extracted following incubations. Cells were centrifuged at 225 *g* for 5 min. The supernatant incubation medium was removed and cells were washed twice in 0.5 ml PBS. The incubation medium and all the washes for each sample were combined and subsequently handled for extraction in the same manner as the cell pellet. The washed cell pellet was resuspended in 1 ml PBS. One extract was prepared from the resuspended cell pellet and another from the pooled washes and incubation medium for each sample. These extracts were obtained by adjusting the pH with 200 μl Tris

buffer, pH 8.4, then extracting twice with four volumes of chloroform—methanol (9:1). The organic phase was removed, evaporated on a Buchi Rotavapor (Brinkmann Instruments, Westbury, NY, U.S.A.) over a 40°C water bath and the residue redissolved in the HPLC mobile phase for injection as described below. All steps in the extraction procedure were conducted in reduced room light. Samples redissolved in mobile phase were stable when stored at −20°C for at least 1 week.

High-performance liquid chromatography

The chromatographic separations were accomplished on a DuPont Model 850 liquid chromatograph (DuPont, Wilmington, DE, U.S.A.) equipped with a 30 cm × 3.9 mm I.D. μBondapak C_{18} column of 10 μm particle size (Waters Assoc. Waltham, MA, U.S.A.). An isocratic solvent system (mobile phase) was used consisting of 0.05 *M* NaH_2PO_4—methanol (35:65, v/v). Solvent was degassed by continuous helium sparging. The flow-rate was 1.5 ml/min and the column oven temperature was 36°C.

Ultraviolet absorption was monitored with a DuPont Model 850 fixed wavelength absorbance detector set at 254 nm. Fluorescence was measured with a Model SF-970 liquid chromatography fluorometer (Schoeffel Instrument, Westwood, NJ, U.S.A.). The excitation wavelength was 482 nm (tungsten lamp) and the emission wavelength was 580 nm (Schoeffel filter No. 2-73, low cut-off at 550 nm).

Triangulation of the peak area (UV or fluorescent detector) was used for quantitation. The standard curve was linear to at least 100 ng (185 pmol) with a correlation coefficient of 0.9998. The lower limit of sensitivity for accurate quantification was 5 ng per injection.

Radioactivity was measured using 10 ml/vial of Beckman Ready Solv HP scintillator in a Beckman LS150 liquid scintillation spectrometer. The ^3H counting efficiency was 30—40%. Counts were corrected by reference to a quench curve derived from external standard ratio measurements.

The [^3H]doxorubicin supplied by New England Nuclear was purified by collection of fractions corresponding to the fluorescent peak on HPLC injection. On re-injection the material was determined to be 94% pure. The [^3H]doxorubicin required periodic (approximately every 2 weeks) purification before experimental use.

RESULTS

HPLC separation

Injection of a mixture of daunomycin, doxorubicin, and two compounds reported to be metabolites of doxorubicin, namely doxorubicinol and doxorubicinone, showed excellent resolution of native drug from metabolites (Fig. 1). The compounds were recovered in the sequence doxorubicinol, doxorubicin, doxorubicinone, and daunomycin with retention times of 3.4, 3.9, 4.7, and 5.6 min, respectively.

Tissue extraction studies

In studies employing cell incubation with drug, the cell pellets were extracted and the washes were pooled with the incubation medium for

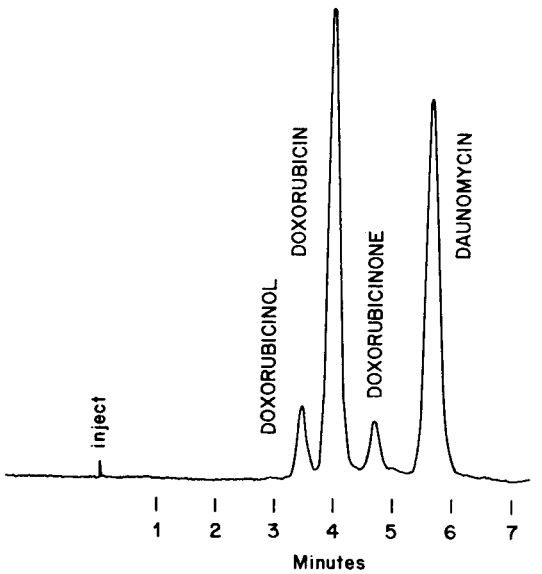

Fig. 1. HPLC separation of doxorubicin and daunomycin from related compounds using fluorescence detector. This represented the injection of an aliquot of a 1:6:1:6 mixture of doxorubicin, two metabolites of doxorubicin (doxorubicinol and doxorubicinone) and daunomycin with the solvent conditions as described under Experimental.

extraction separate from the cells. Aliquots of these extracts were injected into the liquid chromatograph. The drug content of the samples was determined both by triangulation of area of the fluorescence peak compared to the standard curve and by scintillation counting of the HPLC effluent fraction corresponding to the observed fluorescent peak. The lower limit of recognition was approximately at the level of 2 ng per sample; the limit for precise quantification was 5 ng per injection.

As shown in Table I, the cellular content of drug increased as the incubation time increased from 1 to 2 h with normal peripheral blood mononuclear cells. In addition, the cellular content of drug increased in a linear manner (Fig. 2)

TABLE I

DOXORUBICIN RECOVERY FOLLOWING INCUBATION WITH 10^6 PB LYMPOCYTES IN 0.5 ml PBS WITH 142 ng [^{3}H]DOXORUBICIN

Mean of 4 representative experiments.

	Drug in cells (ng)		Drug in media and washes (ng)		Total drug			
	fluorescence	^{3}H	fluorescence	^{3}H	fluorescence		^{3}H	
					ng	%	ng	%
1 h	33.75	37.6	124.75	132.1	158.5	112	169.7	119
2 h	48.32	49.1	87.0	85.5	135.32	96	134.6	94.7

as the drug concentration was increased during incubation in HL-60 cells (Table II).

The close agreement in both sets of experiments of drug assay by HPLC fluorescence measurement and by ^{3}H-label scintillation counting supported the observation that the extraction procedure did not result in loss of ^{3}H label. The mean total drug recovery was 95% in these experiments. The efficiency of total drug recovery in these experiments indicates that but little of the cellular content of drug might have been unextractable. Hence, the use of an internal standard, such as [^{3}H]doxorubicin added prior to the extraction, can be justified.

Careful analysis of the HPLC patterns in repeated runs and the recovery data revealed no significant evidence of drug metabolite formation with these cell types under these incubation conditions.

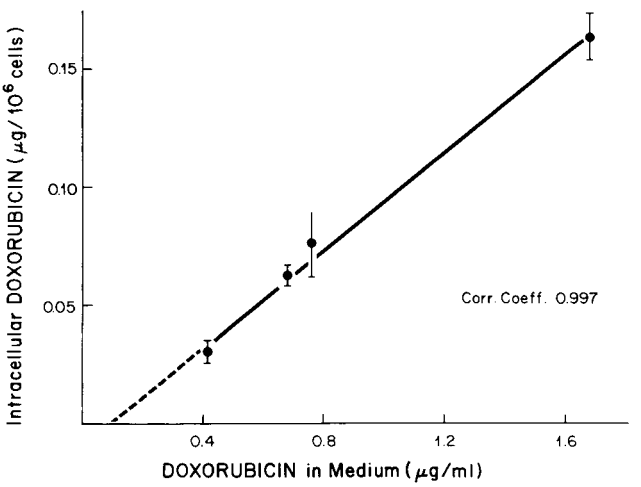

Fig. 2. Intracellular doxorubicin concentration in HL-60 cells. The recovery of intracellular doxorubicin following 1-h incubation of HL-60 cells with increasing concentrations of the drug in the incubation medium.

TABLE II

DOXORUBICIN RECOVERY FOLLOWING INCUBATION WITH HL-60 IN 0.5 ml PBS WITH INCREASING QUANTITIES OF [^{3}H]DOXORUBICIN FOR 1 h AT 37°C

Mean of 4 representative experiments.

Drug added (ng)	Cellular drug content (ng)		Drug in medium and washes (ng)		Total drug recovered			
	Measured by fluorescence	Measured by radiolabeling	fluorescence	^{3}H	Measured by fluorescence		Measured by radiolabeling	
					ng	%	ng	%
206	30	28	142.0	164.2	172.0	83	192.2	93
340	67.3	57.1	256.7	259.6	323.0	95	316.7	93
382	76	75.1	298.3	285.5	374.3	97.9	360.6	94.3
843	160.2	165.3	593.3	605.2	753.3	89.3	770.5	91.3

DISCUSSION

In the therapy of cancer in man the most effective drugs currently available are doxorubicin and daunomycin. Techniques providing accurate measurement of intracellular doxorubicin content and capable of detecting metabolites are therefore potentially important in exploring mechanisms of drug resistance in human tumors and for understanding the effect of agents that may modify the toxicity of doxorubicin.

The analytic procedures were modified from those of Israel et al. [7], who studied drug content in biological fluids. Their systems were not applicable to tissues, because they resulted in drug degradation and poor recovery. These problems led to the present extraction and analytic steps.

The present study shows that using highly purified ^{3}H-labeled doxorubicin we can confirm the efficient, non-degradative extraction of doxorubicin from cells and its reliable measurement in a HPLC system.

The results with HL-60 cells and peripheral blood lymphocytes show that, under these incubation conditions, cells rapidly concentrate doxorubicin. Brief incubation of HL-60 cells in doxorubicin in the range of drug concentrations used does result in inhibition of growth. Therefore, this technique provides the opportunity to determine cellular drug concentrations for correlative analysis with target cell cytotoxicity.

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CHROMBIO. 655

Note

Gas chromatographic and gas chromatographic—mass spectrometric studies on α-keto-γ-methylthiobutyric acid in urine following ingestion of optical isomers of methionine

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In current gas chromatographic (GC) analysis, the flame photometric detector (FPD) has come into frequent use in the specific detection of sulfur compounds. In 1977, Favier and Caillat [1] reported an analytical method of determining urinary α-keto-γ-methylthiobutyric acid by the use of a GC—FPD system for patients with hypermethioninemia. In our earlier studies using this

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analytical system, a remarkable difference in dimethyl sulfide exhalation was observed after the administration of optical isomers of methionine [2, 3].

In the present investigation, a quinoxalinol derivative of α-keto-γ-methylthiobutyric acid was analyzed using GC and gas chromatography—mass spectrometry (GC—MS) with the view to study first the metabolic difference between the optical isomers of methionine and, secondly, the urinary excretion of α-keto-γ-methylthiobutyric acid after the ingestion of L- or D-methionine.

MATERIALS AND METHODS

Extraction and derivatization

α-Keto-γ-methylthiobutyric acid (ACMTB) was obtained from Sigma (St. Louis, MO, U.S.A.). A 5-ml standard solution of ACMTB or urine samples of 5 to 25 ml were acidified by hydrochloric acid to pH 1.0 and extracted five times with diethyl ether. The pooled ether phase was concentrated to 5 ml under vacuum and was further extracted three times with a 5% aqueous solution of $NaHCO_3$. The water phase was re-acidified by hydrochloric acid to pH 1.0, and ACMTB was converted to the quinoxalinol derivative with *o*-phenylenediamine hydrochloride using the method of Favier and Caillat [1].

Apparatus and analytical conditions

A gas chromatograph (Model GC-6APFp, Shimazu, Kyoto, Japan) equipped with FPD was used for the present investigation. The glass column (3 m × 3 mm I.D.) was packed with 2% SP-2250 on Chromosorb W AW DMCS 100—120 mesh. The column temperature was initially isothermal at 180°C for 5 min, then increased to 240°C at the rate of 2°C/min with a hold at 240°C. The FPD with a 394-μm filter was operated at 750 V; the detector temperature was 300°C. Nitrogen was the carrier gas at a flow-rate of 50 ml/min.

The quinoxalinol derivative of ACMTB was identified with a gas chromatograph—mass spectrometer (Hitachi RMU-6M, Hitachi, Tokyo, Japan) equipped with a 002B Datalyzer. Electron energy was 20 eV and 60 μA; accelerating potential, 3200 V; ionization source temperature, 220°C and pressure, $1.2 \cdot 10^{-7}$ torr. Since the background mass spectra of SP-2250 had *m/e* 220, SE-30 was used as the liquid phase for the analytical column in GC—MS analyses.

Methionine loading test

Both L- and D-methionine were obtained from Wako (Osaka, Japan). After overnight fasting, three healthy subjects (one male and two females) ingested 3 g of L-methionine or 3 (or 1) g of D-methionine and urine samples were collected every hour for 4 h. After the determination of creatinine concentration (Technicon AA-II, Technicon, Tarrytown, NY, U.S.A.), urine samples, spiked with thymol, were stored at −20°C. The interval between each test per person was three weeks or more.

RESULTS AND DISCUSSION

The retention time of the quinoxalinol derivative of ACMTB by GC analysis

was 22.75 min. This derivative was quantitated by absolute calibration on logarithmic paper and its detection limit was 5 ng. As stated above, this derivative was further analyzed with GC—MS using SE-30 as the liquid phase for the analytical column (Fig. 1).

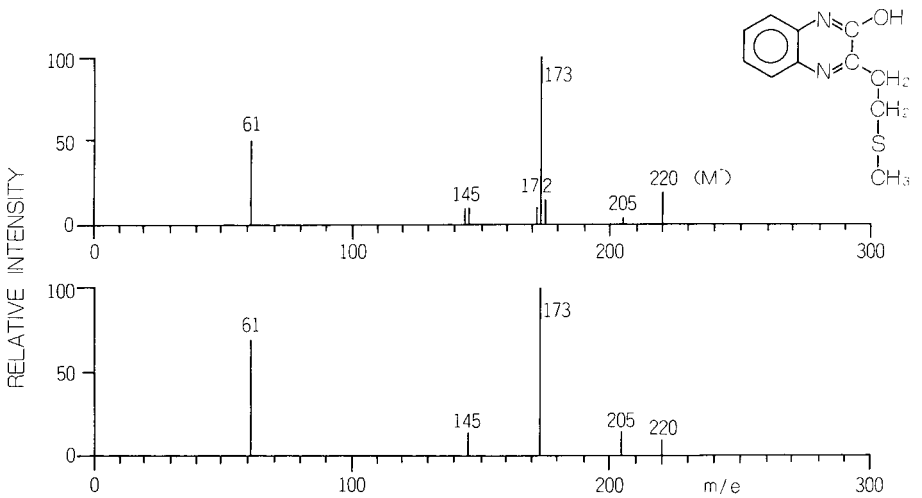

Fig. 1. Mass spectra of the quinoxalinol derivative of α-keto-γ-methyl-thiobutyric acid (ACMTB). Top, standard ACMTB solution; bottom, urine sample of 36-year-old female after ingestion of 3 g of D-methionine.

TABLE I

URINARY EXCRETION OF α-KETO-γ-METHYLTHIOBUTYRIC ACID AFTER THE INGESTION OF D- OR L-METHIONINE

Experimental subjects			Methionine ingested (g)	Urinary α-keto-γ-methylthiobutyric acid (μg/mg Cr)		
Subject	Sex and	age (years)		Before ingestion	Pooled urine A (1st and 2nd hour)	Pooled urine B (3rd and 4th hour)
A	female	36	D-methionine 3	0.11	578.1	766.6
B	male	42	D-methionine 3	0.05	155.8	81.5
B	male	42	D-methionine 1	0.29	272.0	63.5
B	male	42	L-methionine 3	0.21	0.2	0.4
C	female	36	L-methionine 3	0.09	29.7	58.5

The urinary ACMTB concentration per ml was converted into μg per mg of creatinine (μg/mg Cr) (Table I). The concentration of ACMTB before ingestion of the methionines was 0.15 ± 0.10 (mean ± S.D.) μg/mg Cr (n = 5) in all the exprimental subjects. In the case of 3 g of D-methionine ingestion, the concentration increased noticeably in pooled urine A (1st and 2nd hour) and pooled urine B (3rd and 4th hour) and this tendency was also observed even in case of 1 g of D-methionine ingestion. In the case of 3 g of L-methionine ingestion,

however, the increase in concentration was apparently smaller than in the case of D-methionine ingestion.

In our earlier studies in healthy subjects, it was demonstrated that the concentration of dimethyl sulfide in expired alveolar gas (alv-DMS) increased markedly from the fasting level of 2.0 ± 1.9 to the maximum concentration of 66.0 ± 42.1 ng/dl (mean ± S.D.) (n = 6) following the ingestion of 1 g of D-methionine. In contrast, alv-DMS increased only slightly after the administration of 2 g of L-methionine; the fasting level was 2.7 ± 1.3 and the maximum concentration was 4.6 ± 2.6 ng/dl (n = 4). In spite of the marked changes in alv-DMS concentration, the amount of methyl mercaptan in expired alveolar gas (alv-MM) did not always increase and it did not behave parallel to alv-DMS [2, 3].

It is believed that deamination, followed by reamination to the L-isomer, is the method by which the body uses the so-called unnatural isomer of many amino acids [4]. However, an alternate pathway for methionine metabolism has been recently demonstrated in vitro in rat and monkey liver homogenate, independent of S-adenosyimethionine formation. This pathway is thought to involve conversion of methionine to its keto analogue which then is oxidatively decarboxylated to 3-methylthiopropionic acid [5]. If 3-methylthiopropionic acid is further methylated to dimethyl β-propiothetin, dimethyl sulfide may be produced via this pathway. Dimethyl sulfide will be also produced by the methylation of methyl mercaptan, which has been stated to arise from methionine itself or from ACMTB [6, 7], or it may be formed from α-keto-γ-methylthiobutyric acid-δ-dimethyl sulfonium produced beforehand by the methylation of ACMTB.

In view of our results with urinary ACMTB and alv-DMS, it seems likely that a significant amount of D-methionine may be metabolized via the proposed alternative pathway and L-methionine may be metabolized via the normal trans-sulfuration pathway which involves formation of homocysteine. Further studies are in progress to investigate the metabolism of methionine isomers and their intermediates.

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CHROMBIO. 646

Note

Glass capillary gas chromatographic determination of N^{τ}-methylhistidine in urine

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Since the potential utility of measuring urinary N^{τ}-methylhistidine [His-(τMe)] for determining the catabolic rate of skeletal muscle protein was first suggested [1], confirmation has been provided by several workers [2—6]. Catabolic rates of muscle proteins under various nutritional conditions have been estimated by this method [7—10] and recently the use of His(τMe) excretion as an index of myofibrillar protein breakdown was reviewed [11].

Accurate determination of the concentration of His(τMe) in muscle protein is essential because its concentration is very low, but no specific assay for His (τMe) has been reported. Most workers have determined His(τMe) by ion-exchange chromatography and a number of methods have been published specifically for the analysis of methylamino acids [3,12—16].

This paper describes a method for the isolation from biological specimens and quantitative determination of His(τMe) by glass capillary gas chromatography (GC).

EXPERIMENTAL

Isolation of His(τMe) by ion-exchange column chromatography (Method A)

Ion-exchange chromatography was performed using 200—400 mesh AG 50W-X8 (Bio-Rad Labs., Richmond, CA, U.S.A.) as described by Ward [17]. The resin was packed by gravity into a column (5 × 1 cm I.D.) to give a bed height of 3.5 cm. The column had previously been equilibrated with 0.2 *M* pyridine (20 ml). Since some His(τMe) is excreted in the N-acylated form [2],

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urine was hydrolyzed before analysis by heating in an equal volume of 12 *N* HCl at 110°C overnight in sealed vials. The hydrolyzates were evaporated to dryness under vacuum and the residues were dissolved in 0.2 *M* pyridine and applied to the column in a volume of 2 ml (corresponding to 0.25 ml of urine). The acid and neutral amino acids were eluted with 20 ml of 0.2 *M* pyridine. His(τMe) was then eluted with 10 ml of 1 *M* pyridine. The eluent flow-rate was 10 ml/h. The His(τMe) fraction (also containing 1-methylhistidine and histidine) thus obtained was evaporated to dryness under vacuum. The dried samples were then derivatized to make them suitable for GC analysis.

Isolation of His(τMe) by charcoal column chromatography (Method B)

Column adsorption chromatography was performed using charcoal—Celite (1:1, w/w) (BDH, Poole, Great Britain) suspended and washed with 1 *N* HCl. Columns (1.5 × 1 cm I.D.) were silanized with 10% Dri Film (Pierce, Rockford, IL, U.S.A.) in carbon tetrachloride, then washed with 20 ml of water and buffered at pH 5 with 0.33 *M* acetate buffer (5 ml). The hydrolyzates were evaporated to dryness under vacuum and the residues were dissolved in 0.33 *M* acetate buffer (pH 5) and an amount corresponding to 0.25 ml of urine was applied to the charcoal—Celite column. After washing with 20 ml of water followed by 5 ml of 80% acetone, His(τMe) was eluted with 30 ml of dichloromethane—methanol—33% ammonium hydroxide (70:25:5). The eluates were evaporated to dryness under vacuum.

This second analytical procedure was routinely used for His(τMe) determination in human and rat urine, because of its speed, ease and simplicity.

Derivatization of His(τMe) for gas chromatography

The preparation of the His(τMe) derivative is a two-step process, initially requiring that the carboxyl group be esterified. From among the esterification agents the mixture of dry acetyl chloride 5% in propanol was chosen; 5 ml of the esterification mixture were added to the residue. Each tube was capped, mixed and left to react overnight at room temperature. Samples were evaporated to dryness under vacuum, then N-acylated with 150 μl of trifluoroacetic anhydride and 200 μl of methylene dichloride for 30 min at room temperature. The His(τMe) derivative was evaporated to dryness under vacuum and redissolved in a methylene dichloride solution of the reference standard (*n*-triacontane 50 μg/ml) before GC analysis. His(τMe) was completely derivatized as the propyl ester, N-trifluoroacetate, checked by thin-layer chromatography.

GC analytical conditions

The His(τMe) derivative gives sharp and symmetrical peaks on common stationary phases such as pretested SE-30, OV-1, OV-17, OV-101 (Applied Science Labs., State College, PA, U.S.A.) in packed columns, but when biological samples are analyzed, resolution is not quite as good as that of the standard pool because of interfering peaks. For this reason it was preferred to work with glass capillary columns.

The gas chromatograph was a high-resolution dedicated gas chromatograph 3900-B (Dani, Monza, Italy) equipped with a flame-ionization detector. The glass capillary column (20 m × 0.85 mm O.D., 0.30 mm I.D.; Duran 50) was

prepared according to the barium carbonate procedure described by Grob et al. [18] and given a 0.15-μm thick Pluronic F-68 coat using the static procedure. The split injection mode was used. Temperatures were as follows: oven 200°C, detector 280°C, injector 300°C. Carrier gas was hydrogen (O_2 free) with a flow-rate of 0.7 ml/min. Splitter flow was 15 ml/min. The mass-spectrometric assays were done on an LKB-9000 (Bromma, Sweden) interfaced with a 3% OV-1 packed column.

The practicable sensitivity limit is 12 nmol/ml. The use of an electron-capture detector (ECD) increased the sensitivity about 100-fold, but this detector was not needed because human and rat urine contain large amounts of His-(τMe). The ECD should be very useful for measuring low concentrations of His(τMe), in muscle protein, for example.

Quantitation

This important step was performed using the method of internal standardization with *n*-triacontane. The calibration curves for His(τMe) derivative concentrations ranging from 2.5 to 100 ng/μl indicated a linearity in the response within this range of concentrations.

Urinary creatinine determinations were performed on samples of the urine using 3,5-dinitrobenzoic acid according to the instructions supplied with Eurochima Kit (Elvi-Milano, Milan, Italy).

RESULTS

A typical gas chromatogram of rat urine samples is illustrated in Fig. 1.

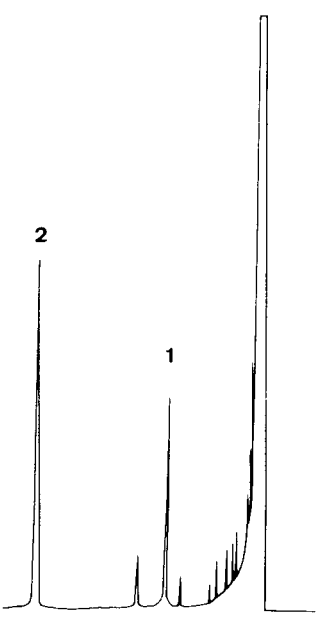

Fig. 1. Gas chromatogram of human urine sample. 1 = His(τMe) derivative; 2 = reference standard (*n*-triacontane 50 μg/ml).

TABLE I

EXCRETION OF TOTAL HIS(τMe) BY YOUNG HEALTHY ADULT MEN AND BY ADULT MALE RATS

	Body weight (kg)	Creatinine excretion (mg/ml)	His(τMe) excretion (μg/ml)	Ratio His(τMe)/creatinine (μg/ml)
Humans				
M.C.	53	1.84	30.35	16.49
E.Z.	63	1.42	23.84	16.78
M.S.	79	2.93	49.10	16.75
P.D.P.	66	2.64	41.44	15.69
Mean		2.20±0.3	36.18±6.0	16.40±0.2
Rats*				
1	—	0.34	14.10	41.22
2	—	0.57	21.45	41.73
3	—	0.66	29.35	44.00
4	—	0.58	24.05	41.11
Mean		0.54±0.08	22.26±3.81	42.01±0.72

*Male CD-COBS rats (Charles River, Italy) of body wt. 250 g were used.

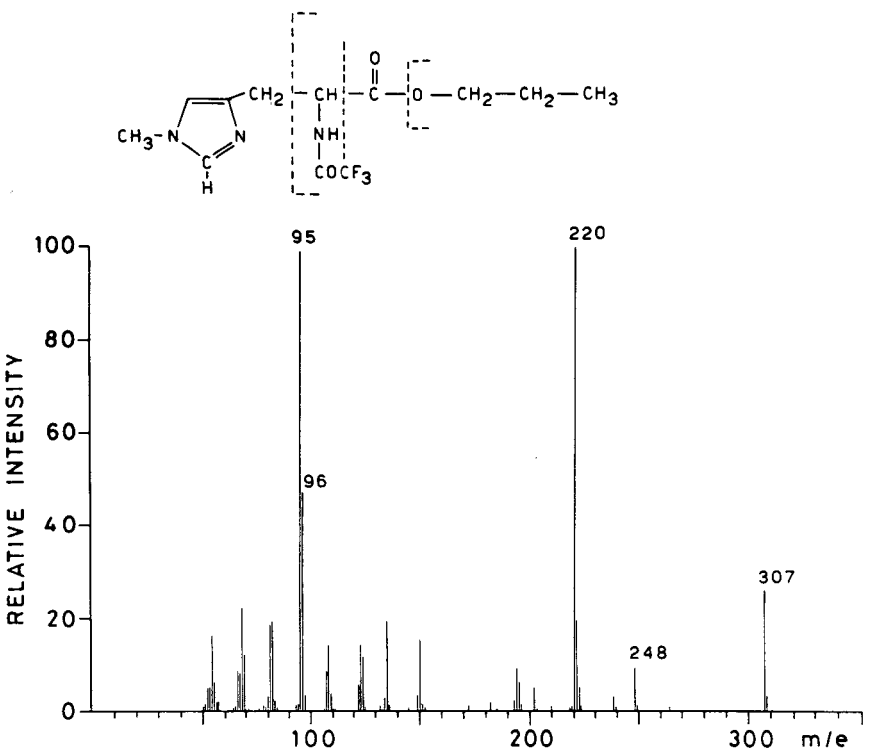

Fig. 2. Mass spectrum of His(τMe) derivative.

Human samples give a similar GC trace. Recoveries for His(τMe) from human and rat urine were, respectively, 99±1% and 98±1%, the mean ± S.E.M. of four determinations. No significant differences were found between recoveries for different His(τMe) concentrations (25, 50, and 100 μg). The basal levels of His(τMe) in human and rat urine are reported in Table I.

Mass spectrometric analysis confirmed that the His(τMe) derivative is a propyl ester, N-trifluoroacetate, as shown in Fig. 2.

DISCUSSION

During the extraction step, 1-methylhistidine and histidine are eluted together with His(τMe). Subsequently they are derivatized with His(τMe), but, whereas 1-methylhistidine is easily derivatized, the histidine derivative is very difficult to prepare because of the presence of both the monoacyl and diacyl derivatives. This difficulty has been reported by several workers [19—21]. For this reason our interest was restricted to His(τMe) analysis because otherwise the analytical procedure for biological samples becomes very complicated. Chromatographic peaks of biological samples were identified with precision by comparison of the mass spectra of the biological compound and of authentic reference standards obtained by electron impact. Critical factors during derivative formation are that the reagents must be free of any trace of moisture, and that the preceding reagent must be completely eliminated by evaporation before the next derivatization step is undertaken.

The present method, with a sensitivity of 5 ng/μl, appears to be reliable for determining His(τMe) in urine of animals and man in normal and pathological conditions. The use of an ECD increases the sensitivity about 100-fold, thus making it suitable for measuring very low His(τMe) levels, in studies of the catabolic rates of muscle proteins, for instance.

Further studies are in progress to measure muscle His(τMe) turnover in animals and man.

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Note

Gas chromatographic determination of monoamine oxidase activity using mixed substrates

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Monoamine oxidase (MAO) catalyzes the oxidative deamination of monoamines. A mitochondrial preparation has been reported to contain two forms of MAO with different specificities: form-A and form-B, these forms may be distinguished by their differences in sensitivity to inhibitors and substrate specificity [1].

Mixed substrate experiments have been carried out with the mitochondrial MAO from rat liver [2], human brain [3] and rat brain [4] in order to study substrate specificity and amine competition for MAO. However, we are not aware of any report dealing with the simultaneous determination of resulting products in mixed substrate experiments for the determination of MAO activity. The purpose of using a mixed substrates method in the present paper is to establish a useful method for estimating effects on MAO caused by various diseases as a change of MAO activity which is difficult to distinguish by the use of a single substrate. Various methods have been used for the assay of MAO activity: oxygen electrode [2, 5], spectrophotometry [6—8], fluorometry [9—14], radioisotopic assay [4, 15—20] and gas chromatography (GC) [21]. Reports dealing with GC for measuring enzymatic activity are not numerous, but a GC method generally makes it possible to study enzyme reactions with

mixed substrates, offering an advantageous technique for the simultaneous determination of resulting products. A comparison of the enzyme reaction patterns obtained from mixed substrates may be applicable to clinical diagnosis, without an absolute determination of individual products. In the present study, mixed substrate experiments have been carried out with rat liver mitochondrial MAO. MAO activity has been determined by GC with benzylamine and β-phenylethylamine as the substrates. The products, benzaldehyde and phenylacetaldehyde were converted to their pentafluorophenylhydrazones and detected with an electron-capture detector.

EXPERIMENTAL

Materials

Reagents. Pentafluorophenylhydrazine (PFPH) was purchased from Aldrich (Milwaukee, WI, U.S.A.); benzylamine hydrochloride and β-phenylethylamine hydrochloride from Tokyo Kasei (Tokyo, Japan); benzaldehyde from Yoneyama Yakuhin (Osaka, Japan); phenylacetaldehyde from Wako Junyaku (Osaka, Japan), respectively. The aldehydes were distilled before use and kept under nitrogen.

Substrate solutions. Benzylamine (20 μmoles/ml) and β-phenylethylamine (10 μmoles/ml) solutions were prepared in the buffer solution.

Buffer solution. A 50 m*M* potassium phosphate solution, pH 7.4 was used.

Internal standard (IS) solution. An aldrin solution (0.25 μg/ml) was used in *n*-hexane.

Enzyme. Male Wister strain rats weighing 150—200 g were decapitated and their livers removed and homogenized in 6 volumes of 50 m*M* potassium phosphate buffer (pH 7.4). The mitochondrial fraction was prepared by differential centrifugation using the method of Hogeboom et al. [22]. The mitochondria were washed once by resuspending them in 50 m*M* potassium phosphate buffer and used as the enzyme preparation. This preparation was divided into small volumes and kept frozen until use. The enzyme protein content was estimated by the method of Lowly et al. [23] and was in the range of 5—10 mg/ml.

Apparatus and conditions

A Shimadzu GC-4APE gas chromatograph equipped with a 10-mCi ^{63}Ni electron-capture detector (ECD) was used. The GC conditions were as follows: a 2 m × 3 mm I.D. glass column packed with 3% XE-60 on 80—100 mesh Celite 545 (AW DMCS): column temperature 190°C; detector temperature 200°C and chart speed 0.25 cm/min.

Procedure for the assay of MAO activity

The incubation medium contained 1 μmole of benzylamine hydrochloride and 0.5 μmole of β-phenylethylamine hydrochloride in a total volume of 0.15 ml of 50 m*M* phosphate buffer, pH 7.4. The mixture was pre-incubated at 37°C for 5 min in a 10-ml centrifuge tube, after which the reaction was started by adding 0.1 ml of the mitochondrial preparation. After shaking for 30 min in an incubator at 37°C, the reaction was stopped by adding 0.01 ml of 60%

perchloric acid. *n*-Hexane (4 ml) containing 1 μg of aldrin as IS, was added to the reaction solution, followed by saturating with sodium chloride and the resulting aldehydes were extracted by mixing the solution on a homomixer. To 1 ml of the *n*-hexane extract in another tube, 0.1 ml of PFPH solution (3 mg/ml, 10% acetic acid in ethanol) was added and the mixture was allowed to stand for 1 h at room temperature. The reaction solution was washed with 1 ml of 6 *N* hydrochloric acid to remove any excess of PFPH, diluted five-fold with *n*-hexane and an aliquot of the solution was applied onto the GC column. A series of blanks was prepared by incubating the substrates in the absence of the mitochondrial preparation. The enzyme source was added immediately, followed by perchloric acid treatment, prior to extraction.

RESULTS AND DISCUSSION

The only report dealing with GC of MAO activity is that by Farris et al. [21], who reported a microassay for the determination of MAO activity using ECD, where *m*-iodobenzylamine was employed as a substrate and the oxidation product, *m*-iodobenzaldehyde was extracted into cyclohexane and measured by electron-capture GC. This technique was successfully applied to a sensitive and specific assay for determining MAO activity in serum and platelets. The selection of *m*-iodobenzylamine as a substrate for their study was based on three important considerations. First, the aldehyde formed from the oxidative deamination of the amine was quite stable to further oxidation; secondly, *m*-iodobenzylamine was a very good substrate for form-B MAO and finally, *m*-iodobenzaldehyde was highly sensitive to detection by ECD. However, their method suffers from the disadvantage of being limited to the use of a halogen-containing substrate, so a procedure of derivatizing the aldehydes formed to halogen-containing compounds which are very sensitive to ECD is general with regard to the possibility of using various substrates. On the other hand, it appears that most of the substrates which are attacked by MAO are of the β-phenylethylamine type. In this study, benzylamine and β-phenylethylamine were chosen as one of the simplest combinations of two substrates.

3% XE-60 was chosen as a suitable column packing for the GC separation, because it gave sharp symmetrical peaks and good separations. Aldrin was used as the internal standard. Fig. 1A shows a gas chromatogram of a standard solution containing IS.

A condensation reaction of PFPH and aldehydes in aqueous solution is known to be an effective derivatization process [24], which gives volatile, ECD-sensitive derivatives, extractable with organic solvents. The condensation reaction proceeded more readily in neutral media, but in the enzyme reaction solution, strong acidity derived by adding 0.01 ml of 60% perchloric acid and co-existing enzyme protein seemed to remarkably disturb the condensation reaction. A series of preliminary investigations was carried out in order to find suitable conditons for the extraction of aldehydes and the reaction with PFPH. *n*-Hexane was chosen as the solvent for extraction. Salting-out improved the extraction yield. The reagent concentration was about 60 times greater than that of the aldehydes and the reaction period was fixed at 1 h at room temperature in order to obtain constant reaction yields. PFPH was used as a

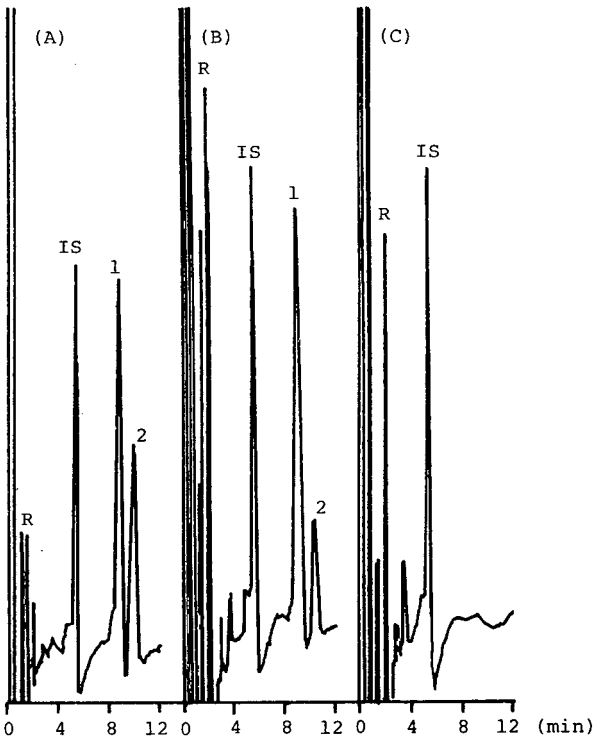

Fig. 1. Chromatograms obtained from 1-μl injections of (A) 47 pg/μl benzaldehyde and 150 pg/μl phenylacetaldehyde standard, (B) the product of a 30-min incubation of a rat liver mitochondrial preparation, and (C) the product of a 30-min incubation of substrate only. Peaks: 1 = phenylacetaldehyde; 2 = benzaldehyde; R = reagent, PFPH and IS. Internal standard, aldrin.

solution in ethanol. A small amount of acetic acid was added to the reaction solution in order to accelerate the condensation reaction. Prior to injection into the gas chromatograph, any excess of PFPH was easily eliminated by washing with 1 ml of 6 *N* hydrochloric acid, to minimise damage to the ECD.

A series of standards, ranging in concentration from 2 to 20 nmoles of benzaldehyde and from 5 to 50 nmoles of phenylacetaldehyde per 4 ml of *n*-hexane containing IS was converted to the corresponding pentafluorophenylhydrazone and was separated by GC. Standard calibration curves were prepared from the data obtained by plotting peak height ratios of hydrazone—aldrin against the concentration of each aldehyde, and showed a linearity passing through the origin. The concentration of aldehyde generated in an enzyme reaction on a mitochondrial preparation was determined by using these curves.

To determine the optimal substrate concentration for the incubation mixture, assays were performed using different concentrations of benzylamine and β-phenylethylamine. Linear Lineweaver—Burk plots were obtained with observed K_m values of 0.38 m*M* for benzylamine and 0.20 m*M* for β-phenylethylamine. In the mixed substrate experiments, the concentration of substrate was fixed at 4 m*M* for benzylamine and at 2 m*M* for β-phenylethylamine, and MAO activities of mitochondrial preparations from rat liver were measured

according to the procedure described in Experimental. Fig. 1 shows a set of three chromatograms obtained using the standard assay procedure.

To investigate the effect of different amounts of enzyme on the rate of aldehyde production, we performed a series of incubations containing various quantities of the mitochondrial preparation and linear calibration curves were obtained up to 1.1 mg protein/0.1 ml of the enzyme concentration for benzylamine and β-phenylethylamine.

The precision of our method was determined by five repeated assays on an identical mitochondrial preparation according to the standard procedure. A standard deviation of 1.6% for benzylamine, 1.5% for β-phenylethylamine and mean aldehyde production rates (nmoles/mg protein/min) of 0.16 for benzylamine and 0.97 for β-phenylethylamine were obtained, respectively.

Using a mixture of two substrates, we examined the inhibitory effect on MAO activity. The reaction rates for both substrates were simultaneously examined by the standard procedure at five different benzylamine concentrations and constant β-phenylethylamine concentration (Fig. 2A). The ratios of the amounts of both aldehydes formed are illustrated in Fig. 2B. Plotting of the ratios against five different benzylamine concentrations shows a linear curve passing through the origin.

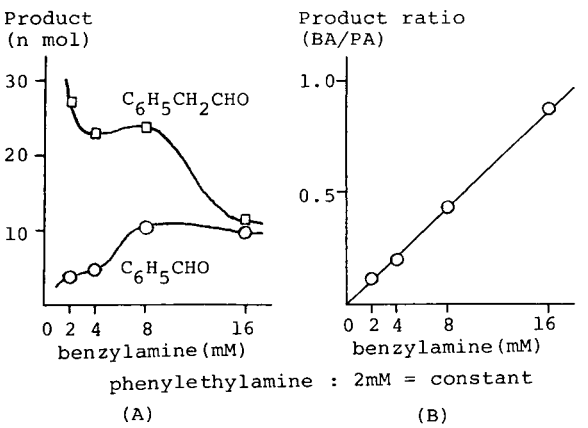

Fig. 2. Effect of substrate concentration ratio on the enzymatic reaction.

It seems likely that this technique could be applied to the analysis of MAO activity in serum and platelet samples. The method with mixed substrates is expected to offer an interesting means for studies on substrate specificity, differentiation of iso-enzymes and the simultaneous determination of some co-existing enzymes.

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Note

Determination of nicotinamide in human plasma and urine by ion-pair reversed-phase high-performance liquid chromatography

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Nicotinamide is used in the prevention or treatment of dietary vitamin deficiencies as well as for other therapeutic purposes [1]. In connection with clinical pharmacological research with nicotinic acid and nicotinamide it was desirable to determine plasma and urine levels of nicotinamide in the presence of large amounts of nicotinic acid. Microbiological [2], colorimetric [3], fluorimetric [4] and ion-exchange [5] methods have been described for the analysis of nicotinamide in plasma and (or) urine, but lack specificity or are too lengthy for serial determinations.

A method combining sample pre-cleaning with a short column and the separation of nicotinamide with an ion-pair reversed-phase high-performance liquid chromatography (HPLC) system was developed using isonicotinamide as internal standard.

MATERIALS AND METHODS

Apparatus

An HPLC system consisting of a Model 6000A pump, a U6K injector, a Model 440 UV detector (all from Waters, Königstein, G.F.R.), a strip chart recorder Model BD 8 (Kipp Analytica, Solingen, G.F.R.) and an Autolab system I computing integrator (Spectra-Physics, Darmstadt, G.F.R.) were used for the chromatographic separation and quantitation. Centrifugations were effected at 2000 *g*.

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Reagents

All test substances were analytical grade. Methanol (for spectroscopy; Merck, Darmstadt, G.F.R.); distilled water (B. Braun, Melsungen, G.F.R.) and sodium dioctylsulfosuccinate (USP XII; Serva, Heidelberg, G.F.R.) for the mobile phase, were used without further purification. Sep-Pak C_{18} cartridges were from Waters.

Standard solutions

Nicotinamide was dissolved in water in concentrations of 1000, 200 and 50 mg/l for the plasma calibration, and 5000, 2000 and 500 mg/l for the urine calibration; isonicotinamide was used at concentrations of 400 mg/l and 5000 mg/l as an internal standard for the plasma and urine analyses, respectively. Solutions were kept cold.

Procedure

Blood and urine samples were obtained from healthy volunteers at regular intervals after the administration of nicotinamide or nicotinic acid; blood was heparinized, immmediately centrifuged and stored at −23°C; urine was stored at the same temperature; before analysis they were again centrifuged.

Plasma purification. Plasma (1 ml) was mixed with 10 µl internal standard solution, 10 µl water, vortexed for 30 sec and then slowly pumped through a Sep Pak C_{18} cartridge (previously washed with 2 ml methanol and 5 ml water); it was washed with 0.5 ml water (which was discarded) and eluted with 1.5 ml of the chromatographic mobile phase; this fraction was collected, centrifuged for 2 min and injected into the column (150 µl for concentrations below 2 mg/l and 100—25 µl for higher concentrations). For nicotinamide concentrations above 10 mg/l, the sample was diluted with water previous to the purification procedure. The calibration curve was obtained in the same way, but 10 µl of the standard nicotinamide solutions were mixed in instead of 10 µl of water.

Urine purification. The procedure was the same as for plasma, except that the concentrations of nicotinamide and internal standard were those indicated under *Standard solutions.*

Chromatographic conditions

The mobile phase was prepared by dissolving 4.446 g sodium dioctylsulfosuccinate in 1450 ml water, adjusting the pH to 2.5 with formic acid and mixing with 1050 ml methanol; the solution was passed through a 0.45-µm filter and degassed before use; the flow-rate was 2.0 ml/min and the pressure was 2000—3000 p.s.i.; the UV detector was set at 254 nm with a sensitivity of 0.01 a.u.f.s.; quantitation was effected through peak area integration; the calibration regression line was obtained using the relationship area nicotinamide/area isonicotinamide vs. added nicotinamide concentration (mg/l). The guard column (25 × 4 mm I.D.; filled with Bondapak C_{18} Corasil (Waters) was renewed every day. Plasma was analyzed with a µBondapak C_{18} (30 cm × 4 mm I.D.; Waters) or a LiChrosorb RP-18 10-µm (25 cm × 4 mm I.D.; Merck) column; for urine, two µBondapak C_{18} columns were used in series. All chromatograms were run at room temperature.

RESULTS

Fig. 1a shows the separation of a mixture of isonicotinic acid, nicotinic acid, nicotinuric acid, nicotinamide and the internal standard, isonicotinamide (retention times: 2.18, 2.63, 3.57, 4.60 and 6.00 min, respectively). Plasma extracts from blank samples show a very small peak from endogenous nicotinamide (20—50 μg/l) [4]. Fig. 1b displays a chromatogram from a volunteer's plasma, 40 min after the oral ingestion of 200 mg nicotinamide. Analysis time

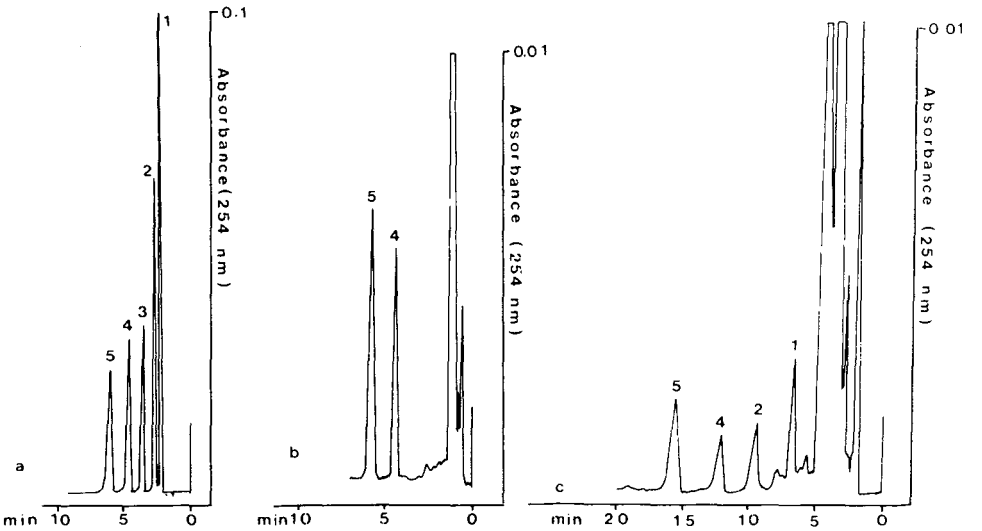

Fig. 1. Chromatograms of (a) mixture of isonicotinic acid (1), nicotinic acid (2), nicotinuric acid (3), nicotinamide (4) and the internal standard isonicotinamide (5) (each 1 μg); (b) plasma extract after nicotinamide ingestion; nicotinamide (4) (concentration, 2.10 mg/l), isonicotinamide (5) (concentration, 4 mg/l); 100-μl injection; (c) urine extract spiked with nicotinic acid (1), nicotinuric acid (2), nicotinamide (4) (each 20 mg/l) and isonicotinamide (5) (50 mg/l); 10-μl injection.

was 10—15 min per sample. The relationship, area nicotinamide/area isonicotinamide is linear in the range 0.1—10 mg/l and the statistical regression line can be represented as $y = 0.2924x + 0.0093$; $r = 0.999$. The recoveries of nicotinamide and isonicotinamide in plasma at a concentration of 10 mg/l were 91.8 ± 4.1% and 92.4 ± 4.1% ($n = 4$) respectively, and the same data were obtained from aqueous solutions. Urine analyses recoveries were in the range 1—50 mg/l with a linear regression line $y = 0.0227x + 0.0275$; $r = 0.999$; the analysis time was 20—30 min per sample. Fig. 1c shows the chromatogram of urine spiked with nicotinic acid, nicotinuric acid, nicotinamide and isonicotinamide (retention times were 6.02, 8.67, 10.91 and 14.29 min, respectively).

The sensitivity of the method is 0.1 mg/l for plasma and 1 mg/l for urine; precision and accuracy are shown in Table I.

The following substances showed no interference in the analysis: nicotinic acid; isonicotinic acid; nicotinuric acid; picolinic acid, 6-hydroxy nicotinic acid; nicotinic acid-N^1-oxide; nicotinamide-N^1-oxide; N^1-methyl nicotinamide

TABLE I

REPRODUCIBILITY OF PLASMA AND URINE ANALYSES FOR NICOTINAMIDE

	Plasma (mg/l)			Urine (mg/l)		
Concentration given	0.5	2	10	5	20	50
Concentration found	0.53	1.98	10.0	5.1	19.8	50.0
Coefficient of variation (%, n = 10)	3.4	4.5	4.8	6.8	5.1	5.6

chloride; N′-methyl nicotinamide; N′-N′-diethyl nicotinamide; picolinamide; salicylic acid; acetylsalicylic acid; salicyluric acid; salicylamide; benzoic acid; hippuric acid; thiamine hydrochloride; biotin; ribofavine-5′-phosphate; D-panthenol; and pyridoxine hydrochloride.

DISCUSSION

Several stationary phases, eluting solvents, modifiers and extraction procedures were tried [3, 4, 6—8]; although in many cases the separation of the nicotinamide and isonicotinamide peaks was satisfactory, interfering peaks from plasma and urine could not be eliminated; the use of dioctylsulfosuccinate [9] as an ion-pair in combination with reversed-phase chromatography with a C_{18} modified stationary phase, preceded by purification through a small column circumvented these difficulties.

The concentration of nicotinamide could be determined even in the presence of a large excess of nicotinic and nicotinuric acid; other metabolites did not interfere, and plasma blanks showed no interfering peaks. The simultaneous determination of nicotinamide together with nicotinic and nicotinuric acid was not attempted as the latter showed analytical recoveries of 34% and 84%, respectively. Incubation and recovery experiments suggest that total plasma

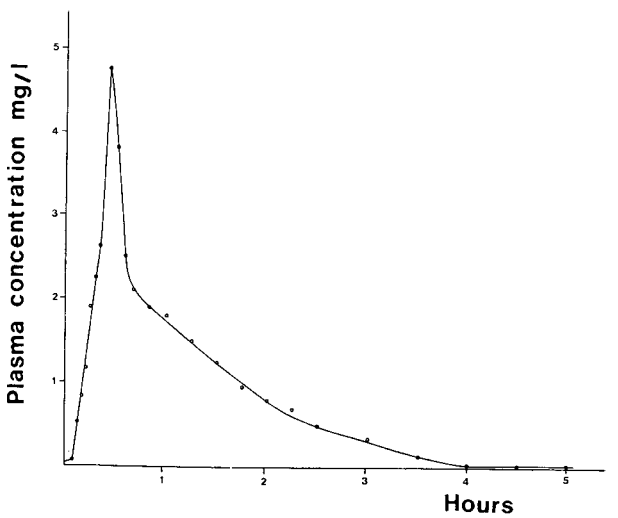

Fig. 2. Plasma concentration—time curve of nicotinamide after a single oral dose of a 200-mg tablet.

nicotinamide is determined. During a working day, 30—40 plasma or 10—15 urine analyses could be carried out, including the purification step. The method has been found to be rapid, simple, reproducible, specific and adequate for pharmacokinetic work, and has been used continuously over a period of a year. Fig. 2 shows a plasma concentration—time curve after nicotinamide ingestion. The results of these researches will be published elsewhere.

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Note

Simultaneous determination of theophylline and caffeine after extractive alkylation in small volumes of plasma by gas chromatography—mass spectrometry

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Theophylline is widely used as a bronchodilator in the treatment of reversible airway obstruction [1]. In the last few years it has also been used in the treatment of apnéa in premature infants [2]. Several rather specific and sensitive gas chromatographic (GC) and liquid chromatographic methods for its determination have been described [3—7]. These may also be used, after slight modification, for the determination of caffeine. However, in these methods, quantification of both theophylline and caffeine may be difficult to achieve in small sample volumes and may also involve tedious extraction procedures. A GC—mass spectrometric (MS) method with high sensitivity and specificity for the determination of caffeine only has been reported [8]. Sensitive immunotechniques such as radioimmunoassay (RIA) and enzyme multiplied immunotechnique (EMIT®) are also available for the determination of theophylline.

The aim of the present work was to design a method for determining theophylline and caffeine in the plasma of premature infants treated with theophylline for neonatal apnéa. In contrast to adults, such infants have been shown to metabolize theophylline to caffeine to a significant degree [9], producing plasma concentrations of caffeine that probably have pharmacological effects. The method described is capable of handling small sample volumes (50 μl) and involves only one extraction step, which is combined with the derivatization stage. The effect of variations in the extraction yield on the

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precision of the method can be reduced by the use of deuterated analogues of theophylline and caffeine as internal standards.

MATERIALS AND METHODS

Instrumental

A Finnigan 4000 gas chromatograph—mass spectrometer was used for the measurements. It was equipped with a multiple ion monitoring unit (Finnigan, Sunnyvale, CA, U.S.A.). The injector, of the Grob capillary type, was operated at 225°C in a splitless mode, and was equipped with valves which were programmed to vent the injector 60 sec after injection. The glass capillary column was 25 m OV-225. The column temperature was programmed from 170—210°C at a rate of 10°C/min. A pressure of 20—25 kPa of helium was applied to the column which was directly coupled to the ion source. The electron energy was set at 70 eV. For injection, solid sample syringes (SGE, North Melbourne, Australia) were used. They were cleaned in a Hamilton syringe cleaner between injections.

Internal standards

Deuterated theophylline was synthesised by monomethylation of 1-methylxanthine [10] with trideuteromethyl iodide. Deuterated caffeine was synthesised by dimethylation of 1-methylxanthine with the same alkylating agent.

Method

Plasma (50 μl) in a 10-ml screw-capped tube was added to 100 μl of a solution containing trideuterotheophylline (4.2 μg/ml) and hexadeuterocaffeine (5.2 μg/ml), 1 ml of 0.5 *M* pH 10 carbonate buffer and 50 μl 0.1 *M* tetrabutylammonium ion solution (prepared by dissolving tetrabutylammonium hydrogen sulphate in molar amounts of sodium hydroxide solution and adding water to make up the volume). The mixture was shaken with 5 ml of dichloromethane containing 2.5% of ethyl iodide in a water bath at 50°C for 20 min. After centrifugation the organic phase was removed and evaporated (Büchler Vortex evaporator). The residue was dissolved in 20 μl acetone and 1—2 μl were then transferred to a solid sample syringe and injected into the chromatograph.

RESULTS AND DISCUSSION

Theophylline was extracted in an ionized form as an ion pair with tetrabutylammonium into the organic phase where it was ethylated. Since theophylline was consumed by ethylation in the organic phase the extraction was > 95%. Caffeine was extracted to the organic phase in a neutral form (ca. 95%).

Fig. 1 shows chromatograms of plasma samples obtained by monitoring the molecular ions obtained from ethylated theophylline and caffeine respectively.

The ethyl derivatives of 1,7-dimethylxanthine and 3,7-dimethylxanthine (theobromine) were not separated on the column used in the method

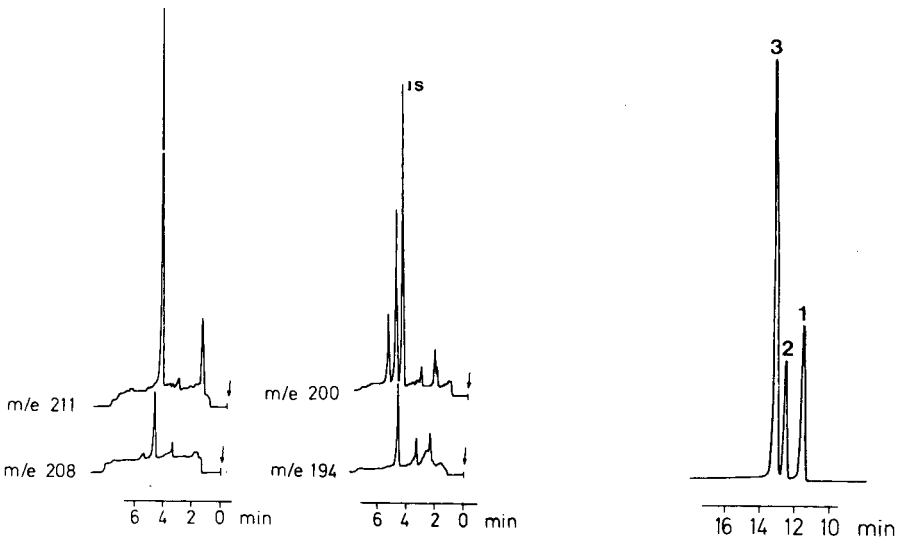

Fig. 1. Chromatograms from a plasma sample treated according to the method described. The sample contained 1.7 μg/ml of theophylline and 2 μg/ml of caffeine. An arrow indicates the injection. *m/z* 194, caffeine; *m/z* 200, hexadeuterocaffeine (internal standard, IS); *m/z* 208, ethyltheophylline and *m/z* 211, trideuteroethyltheophylline.

Fig. 2. Chromatogram of pentafluorobenzyl derivatives of dimethylxanthines on a 25-m OV-17 glass capillary column programmed from 190°C to 230°C at 10°/min. The molecular ions at *m/z* = 362 were monitored on the mass spectrometer. Peaks: 1 = theophylline; 2 = 1,7-dimethylxanthine and 3 = theobromine.

described. Since the molecular ions of these substances have the same *m/z* value as the ethyl derivative of theophylline, they will interfere with the determination of theophylline. This has no importance when determining theophylline and caffeine concentrations in premature infants because they have been reported to lack the ability to demethylate caffeine [11]. To accomplish the separation of these substances a more selective column has to be used. On a 25-m OV-17 capillary column it was possible to separate the ethyl derivative of theophylline from those of 1,7- and 3,7-dimethylxanthine, but the temperature had to be increased rather slowly.

Exchange of the alkylating agent ethyliodide for pentafluorobenzyl bromide in the described method will give the pentafluorobenzyl derivative of the dimethylxanthines. Fig. 2 shows the chromatogram obtained with this derivatization, making it possible to quantify caffeine and all three dimethylxanthines simultaneously.

The calibration graphs were constructed by analysing plasma samples spiked with theophylline (0—15 μg/ml) and caffeine (0—15 μg/ml). The peak height ratios of the ions *m/z* 208/211 and 194/200 were plotted against the concentration of theophylline and caffeine respectively.

The precision of the method was 3.9% for theophylline (0.6 μg/ml plasma, n = 10) and 3.2% for caffeine (0.6 μg/ml, n = 10). The detection limits were 20 and 40 ng/ml plasma for theophylline and caffeine, respectively. The higher detection limit for caffeine was due to the fact that it was eluted on the slope

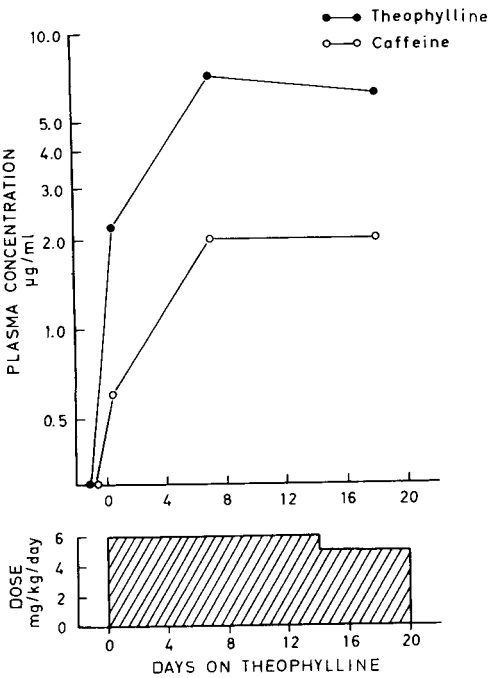

Fig. 3. Plasma concentrations of theophylline and caffeine in a premature newborn infant treated with oral theophylline for neonatal apnéa.

of a small background peak. By using a somewhat slower temperature programme the caffeine peak can be resolved and the detection limit is about the same as that for theophylline.

Fig. 3 shows the plasma concentrations of theophylline and caffeine in a premature newborn infant treated with oral theophylline. The plasma sample obtained before treatment did not contain detectable amounts of the substances. At steady state the caffeine plasma levels were approximately 1/3 of the theophylline levels, and this is in agreement with previous findings [12].

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Note

Simultaneous determination of dyphylline and theophylline in human plasma by high-performance liquid chromatography

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Dyphylline [7-(2,3-dihydroxypropyl)theophylline] and theophylline are used clinically as bronchodilators in the treatment of asthma. At present, their therapeutic effect appears to be dependent upon their concentration in the blood, with the effective range 10—20 μg/ml. In order to compare and correlate efficacy with plasma levels of dyphylline with those of theophylline in clinical studies, a fast and accurate analytical method for both drugs is needed in which other dietary xanthines do not interfere.

A number of procedures using high-performance liquid chromatography (HPLC) have been published for theophylline [1—12] and dyphylline [13—17]. But only the procedure of Maijub and Stafford [13] can determine both drugs simultaneously. However, this procedure requires silica column deactivation and extraction from plasma with 65% recovery for dyphylline. We have developed a rapid, precise and accurate method for the simultaneous determination of dyphylline and theophylline in the presence of caffeine and theobromine which is suitable for automated HPLC analysis of human plasma samples. The results of our experiments are described in this report.

EXPERIMENTAL

Materials

All reagents were analytical reagent grade. Aqueous solutions were prepared using deionized water (Mill-Q-Water System, Millipore, Bedford, MA, U.S.A.).

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Glass-distilled acetonitrile (Burdick & Jackson Labs., Muskegon, MI, U.S.A.) was used for HPLC. Theophylline was obtained from Pfaltz and Bauer (Stanford, CT, U.S.A.) and dyphylline (Lot No. CRM 1062) from Mallinckrodt Pharmaceutical Division (Mallinckrodt, St. Louis, MO, U.S.A.). Theobromine, caffeine and β-hydroxyethyltheophylline (β-HET) were obtained from Sigma (St. Louis, MO, U.S.A.).

High-performance liquid chromatography

The chromatograph was a Hewlett-Packard Model 1084B, equipped with a variable-wavelength spectrophotometric detector, automatic sampler and LC terminal. A 10-μm μBondapak C_{18} column (Waters Assoc., Milford, MA, U.S.A.) was used; the mobile phase was acetate buffer—acetonitrile (94:6). The buffer was prepared by adjusting the pH of a 0.01 *M* solution of sodium acetate in deionized water to 4.0 with reagent grade glacial acetic acid. The column oven temperature and solvent temperature were 40°C. A flow-rate of 2.0 ml/min was used yielding an operating pressure of 85 bar (approximately 1300 p.s.i.). The spectrophotometric detector had a 12-μl cell volume and was operated at a wavelength of 274 nm.

Standards

Standard solutions of dyphylline and theophylline (5 mg/ml) were prepared in buffer and stored at 4°C. These solutions were then diluted as necessary to prepare the appropriate plasma standards for each drug and each assay run. The internal standard of β-hydroxyethyltheophylline was also prepared in buffer (0.2 mg/ml) and stored at 4°C. Peak area ratios of dyphylline and theophylline to β-hydroxyethyltheophylline were determined for plasma standards.

Sample preparation procedure

To 1 ml of plasma (or standard) were added 100 μl of an internal standard solution and 100 μl of a 40% aqueous trichloroacetic acid solution. The mixture was vigorously stirred for 30 sec on a Vortex Genie Mixer (Scientific Products, Evanston, IL, U.S.A.), allowed to stand for 5 min, and then centrifuged for 15 min at 2000 *g*. The supernatant was transferred to a 2-ml vial, sealed and placed in the automatic sampler. A blank plasma sample (1.0 ml) was treated in an identical manner. The sampler injected a 25-μl volume on to the column of the high-performance liquid chromatograph.

Recovery and reproducibility

Drug recovery from plasma after protein precipitation was determined at concentrations of 2.5, 5.0, 10.0, 25.0 and 50.0 μg/ml in plasma by comparing the peak areas with those obtained for aqueous solutions containing known concentrations of dyphylline and theophylline. Reproducibility was determined for the same concentration range by quadruplicate analysis of samples at each concentration.

RESULTS AND DISCUSSION

A linear relationship between the peak area ratio and plasma concentration

of theophylline and dyphylline exists in the range of 2.5—50 μg/ml. The correlation coefficient is 1.0000.

The precision (reproducibility) of this method was determined by quadruplicate analyses of standard samples at each concentration. The results (Table I)

TABLE I

PRECISION (C.V.) AND ACCURACY (M.E.) OF THE SIMULTANEOUS DETERMINATION OF DYPHYLLINE AND THEOPHYLLINE IN HUMAN PLASMA IN THE RANGE 50.0—2.5 μg/ml

Theoretical (μg/ml)	Dyphylline			Theophylline		
	Calculated*	C.V. (%)	M.E. (%)	Calculated*	C.V. (%)	M.E. (%)
50	49.67 ± 1.14	2.3	0.7	47.94 ± 2.53	5.3	4.3
25	24.96 ± 0.70	2.8	0.1	24.09 ± 1.50	6.2	3.8
10	9.90 ± 0.18	1.8	1.0	9.94 ± 0.56	5.7	0.6
5	4.91 ± 0.14	2.9	1.8	5.33 ± 0.33	6.2	6.1
2.5	2.37 ± 0.11	4.8	5.7	2.89 ± 0.16	5.6	13.5

*As mean concentration ± S.D. (μg/ml).

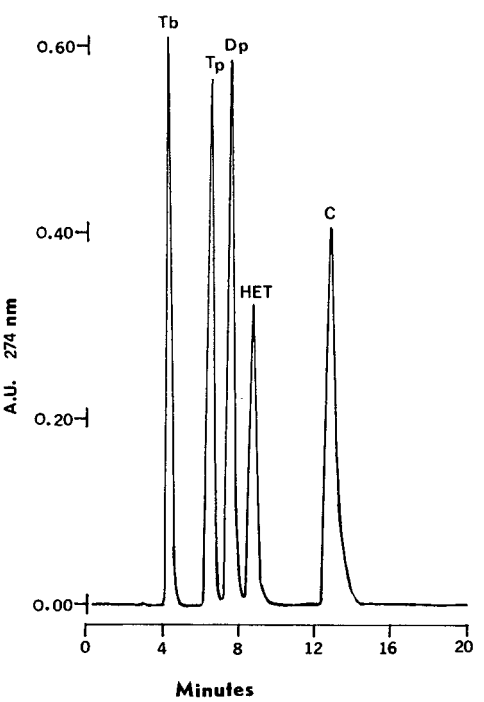

Fig. 1. Separation of theobromine (Tb), theophylline (Tp), dyphylline (Dp) β-hydroxyethyltheophylline (HET) and caffeine (C). Column, μBondapak C_{18}; mobile phase, 0.01 *M* sodium acetate (pH 4.0)—acetonitrile (94:6).

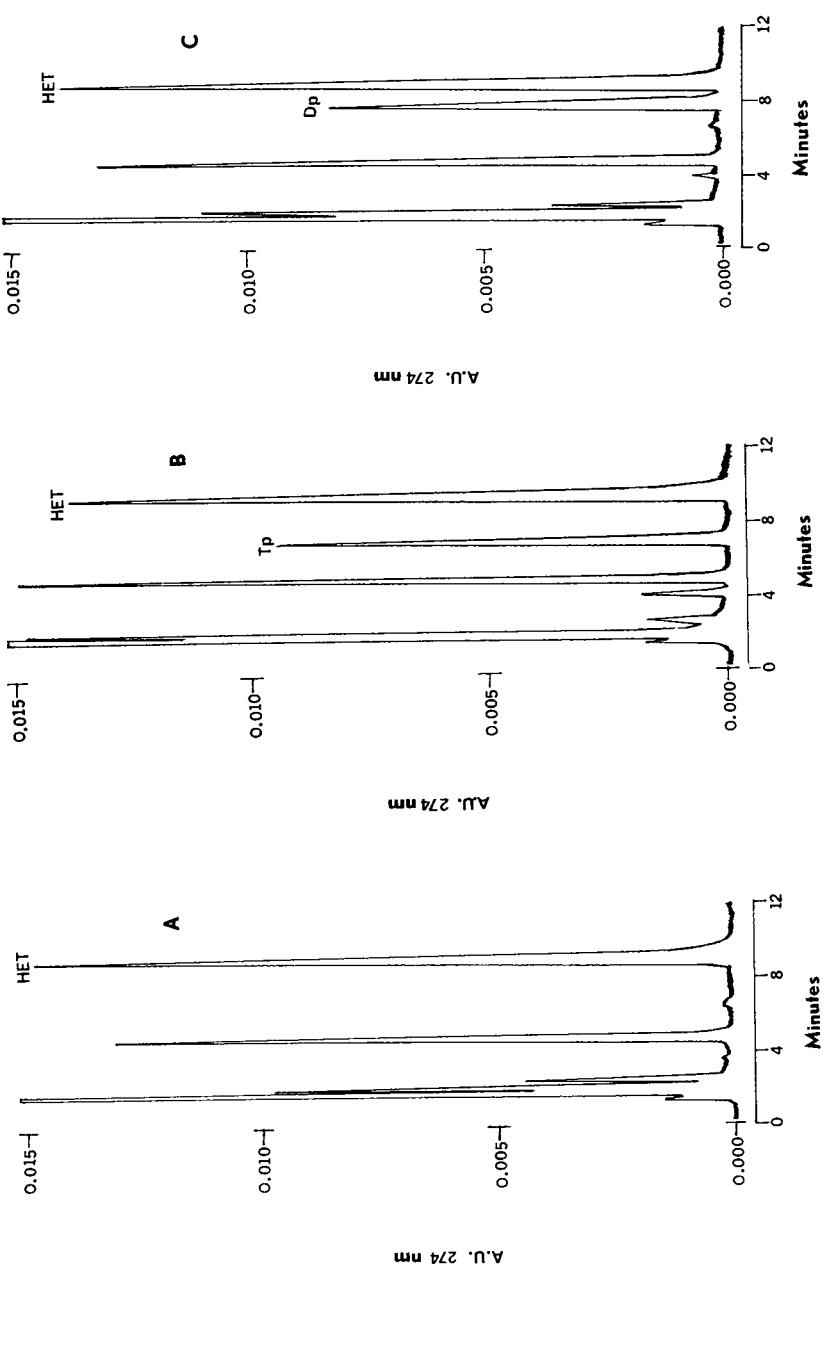

Fig. 2. Chromatogram of plasma from a patient before (A) and after a single oral dose of 6 mg/kg theophylline (B) or 10 mg/kg dyphylline (C). Column, μBondapak C_{18}; mobile phase, 0.01 *M* sodium acetate (pH 4.0)—acetonitrile (94:6).

show that the precision, expressed as the coefficient of variation (CV), was 4.8% or better for dyphylline and 6.2% or better for theophylline.

The accuracy, calculated as relative mean error (M.E.*) was 5.7 and 13.5% or better for dyphylline and theophylline, respectively. The accuracy is more commonly expressed as recovery, which for our method was 94.6—99.9% for dyphylline and 95.9—115.6% for theophylline.

In order to obtain a realistic estimate of the sensitivity of the assay, the limit of detection [18] was calculated based on the peak area ratio value for zero concentration as estimated from linear regression and the standard deviation for the lowest plasma concentration used. The limit of detection was found to be 0.4 μg/ml for dyphylline and 1.0 μg/ml for theophylline.

Complete (baseline) resolution of both drugs from each other and from caffeine and theobromine was considered a prerequisite for a good assay and was achieved by the use of the described mobile phase composition (see Fig. 1). An example of an analysis of plasma obtained 1 h after dosing from the same patient taking 6 mg/kg theophylline (Theolair tablet) or 10 mg/kg dyphylline (Lufyllin tablet) is shown in Fig. 2. The concentrations determined were 8.25 and 10.54 μg/ml, respectively.

The time needed for analysis was 30 min for sample preparation and 12 min for chromatographic analysis. In the automated mode many samples can be prepared for analysis within an hour, and with the automatic sampler capacity of 60 samples, all samples can be analyzed in a 12-h overnight run.

CONCLUSION

An automated HPLC assay has been developed that is sufficiently sensitive, accurate and precise for the routine clinical monitoring of dyphylline and theophylline. Caffeine and theobromine do not interfere.

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*Relative mean error = $\frac{\text{absolute value of theoretical value} - \text{determined value}}{\text{determined value}} \times 100.$

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Note

Simultaneous measurement of chloramphenicol and chloramphenicol succinate by high-performance liquid chromatography

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Chloramphenicol (CAP) is a broad spectrum antibiotic which is currently experiencing a resurgence of use in pediatrics for the treatment of ampicillin resistant *Haemophilus influenzae*.

Several recent papers [1,2] have recommended that CAP serum concentrations be routinely monitored during treatment to assure therapeutic levels of the drug and to avoid concentration-dependent toxicity. Analysis of CAP has been reported utilizing microbiological [3], colorimetric [4], gas chromatographic [5], enzymatic [6] and high-performance liquid chromatographic (HPLC) [7,8] methods. The HPLC methods are reliable, easy to perform and, due to the small serum volume required, ideally suited for CAP quantitation in pediatric patients. However, these methods do not describe the simultaneous analysis of chloramphenicol succinate (CAPS) and CAP.

CAP is administered intravenously as its succinate ester which is biologically inactive and must be hydrolyzed in the body to free CAP. We, as well as others [9], have found no correlation between the dose of CAPS administered and blood levels of free CAP. The purpose of this paper is to describe an improved HPLC method for simultaneous quantitation of CAP and CAPS in serum, urine and cerebrospinal fluid (CSF).

MATERIALS AND METHODS

Chromatography

All assays were performed on a Perkin-Elmer model 601 high-performance liquid chromatograph equipped with a LC55 UV/VIS variable-wavelength detector and interfaced with a Sigma 10 data system (Perkin-Elmer Corp., Norwalk,

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CT, U.S.A.). The data system provided a print-out of the digitally integrated area under the peaks and the retention times for CAPS, CAP and the internal standard. A Perkin-Elmer C-18, ODS-HC-SIL-X-1 reversed-phase column was used. The oven temperature was maintained at 50°C, the flow-rate was 1.5 ml/min, and the detection wavelength was 272 nm.

Reagents

CAP and CAPS were gifts from Parke Davis & Company (Ann Arbor, MI, U.S.A.). 5-Ethyl-*p*-tolylbarbituric acid (ETBA), used as the internal standard, was obtained from Aldrich (Milwaukee, WI, U.S.A.). Sodium acetate, low absorbance grade, was obtained from Matheson, Coleman and Bell (Norwood, OH, U.S.A.). Acetonitrile, ethyl acetate and methanol were HPLC grade and distilled in glass (Burdick & Jackson Labs., Muskegon, MI, U.S.A.). Glacial acetic acid was reagent grade (J.T. Baker, Phillipsburg, NJ, U.S.A.).

The mobile phase was acetonitrile—0.1 *N* sodium acetate (15:85). The pH of this solution was adjusted to 6.4 by the addition of a few drops of glacial acetic acid. 1.0 *N* sodium acetate solution (pH 4.6) was prepared in a similar manner.

Stock standards of CAP, CAPS and ETBA were individually prepared in methanol to yield a final concentration of 1 g/l of each. Working standards were prepared in drug-free serum with appropriate dilutions from the stock solution. The working internal standard (ETBA) was added to the extraction solvent (ethyl acetate) to yield a final concentration of 20 mg/l.

Procedure

Fifty μl control or patient serum, CSF, or urine diluted 1:10 with distilled water, was placed in a 1.5-ml Eppendorf centrifuge tube. An amount of 100 μl 1.0 *N* sodium acetate was added and mixed. 1.0 ml of extraction solvent (ethyl acetate) containing ETBA was then added. The tubes were vortexed vigorously for at least 1 min and then centrifuged in a Brinkman table-top micro centrifuge. The upper organic phase was transferred to a clean glass tube (10 × 13 mm) and evaporated to dryness at 40°C under nitrogen. The samples were sequentially reconstituted with 50 μl methanol, vortexed, and 10 μl were injected onto the column.

RESULTS

Fig. 1A shows a typical chromatogram for blank serum containing the internal standard. The first two peaks appearing in the chromatogram are unidentified artifacts which do not interfere with the determination of CAPS, CAP or ETBA. Fig. 1B illustrates a chromatogram obtained from drug-free serum to which 20 mg/l of CAP and CAPS were added. Fig. 1C is a chromatogram obtained from a patient sample in which the determined concentrations of CAPS and CAP were 12.7 mg/l and 9.4 mg/l, respectively. There were two peaks for CAPS, with retention times of 2.2 (CAPS I) and 2.5 (CAPS II) min. Free CAP and the internal standard had retention times of 3.5 and 5.0 min, respectively. Similar chromatograms were obtained with urine and CSF.

CAP and CAPS were added to drug-free serum to yield concentrations of 5 to 100 mg/l. Concentrations and peak areas were linearly related over this

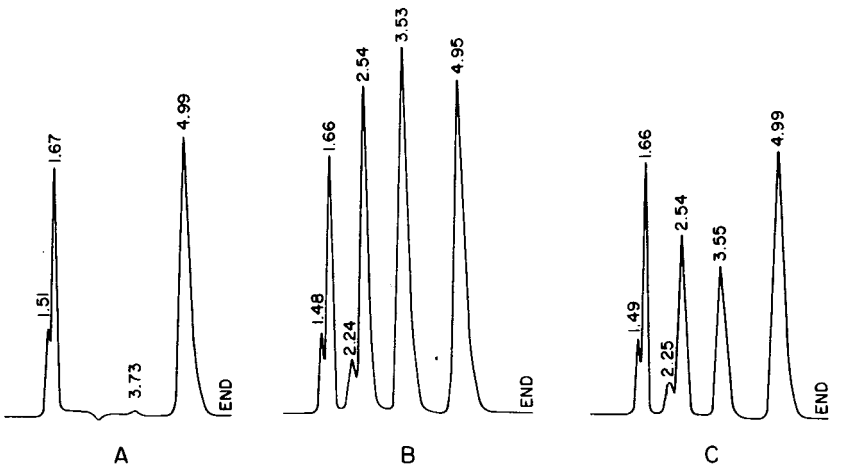

Fig. 1. Typical chromatograms of (A) blank serum containing internal standard; (B) drug-free serum reconstituted with 20 mg/l of CAP and CAPS each; and (C) patient's serum determined as 9.4 mg/l CAP and 12.7 mg/l CAPS. Retention times: CAPS I, 2.2 min; CAPS II, 2.5 min; CAP, 3.5 min; internal standard (ETBA), 5.0 min.

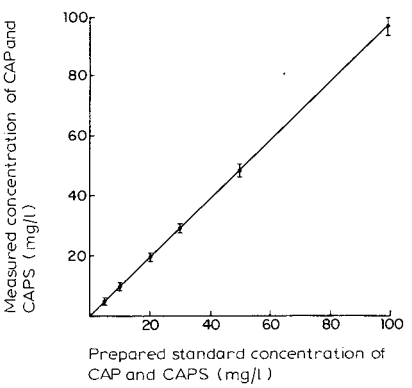

Fig. 2. CAP and CAPS linearity. Mean (⊙) and range at each concentration for both compounds (n = 5). The standard curves for both compounds are superimposed.

range (Fig. 2). Urine standards of CAP and CAPS prepared in an analogous manner also exhibited linearity over this range.

Within-run precision was evaluated by processing aliquots of a prepared standard serum pool containing 20 mg/l of both CAP and CAPS (Table I). Aliquots of a pool containing CAP and CAPS were individually frozen, and stability studies were conducted by analyzing samples taken from this pool (Table II) for a period of four weeks. The stability studies showed no difference in values obtained from these samples compared to those which were freshly prepared (Table I). Table III indicates the recovery data for different concentrations of both CAP and CAPS. At each concentration, the ratio of the two peak areas of CAPS was constant (20 CAPS I : 80 CAPS II).

TABLE I

PRECISION OF THE ANALYSIS OF FRESHLY PREPARED STANDARDS OF CAP AND CAPS

	Amount added (mg/l)	Mean ± S.D. (mg/l)	*n*
CAP	20	19.9 ± 1.4	22
CAPS	20	20.0 ± 1.2	22

TABLE II

STABILITY OF THE ANALYSIS OF A FROZEN POOL OF CAP AND CAPS OVER A 4-WEEK PERIOD

	Amount added (mg/l)	Mean ± S.D. (mg/l)	*n*
CAP	20	20.0 ± 1.4	14
CAPS	20	19.8 ± 1.9	14

TABLE III

ANALYTICAL RECOVERY OF KNOWN AMOUNTS OF CAP AND CAPS ADDED TO HUMAN PLASMA

Five samples were analyzed at each concentration.

Added (mg/l)		Found (mg/l)		Recovery (%)	
CAP	CAPS	CAP	CAPS	CAP	CAPS
100	100	95	93	95	93
50	50	47	49	96	98
30	30	30	29	100	97
20	20	21	20	105	100
10	10	10.2	10.3	102	103
5	5	5.8	5.4	116	108

DISCUSSION

CAPS standard solutions, prepared in methanol and analyzed according to our procedure, produced a single chromatographic peak. In biological specimens or distilled water, two peaks were always observed (Fig. 1). The sum of the area counts from these two peaks equaled the total area counts obtained from the pure methanolic standards. Therefore, we chose to combine the areas of the two CAPS peaks to calculate the total CAPS concentration in biological fluids.

It has previously been reported [10] that in neutral or acidic solutions CAPS spontaneously forms the cyclic hemi-ortho ester, which exists in equilibrium with the non-cyclic 3-monosuccinate ester of chloramphenicol. We believe that CAPS I represents the cyclic form and CAPS II represents the non-cyclic form of the compound. The ratio of CAPS I to CAPS II (20:80) observed in our

patient samples is consistent with the ratio that has been previously observed in aqueous solutions [10]. As CAPS is eliminated from the serum during a dosing interval, CAPS I always disappears before CAPS II. This suggests that as the major form (3-monosuccinate ester) is hydrolyzed in the body, the equilibrium is shifted and the cyclic form reverts to the 3-ester which is subsequently hydrolyzed. Alternatively, the cyclic form itself may undergo hydrolysis to the active, free chloramphenicol. In either case, the cyclic form exists in lesser amounts and disappears first.

The generally accepted therapeutic range for CAP is 10—20 mg/l. The risk of reversible concentration-related toxicity increases progressively at levels above 30 mg/l. The linearity of this assay up to concentrations of at least 100 mg/l is such that elevated serum CAP concentrations can be accurately determined without the necessity of sample dilution.

CAP and CAPS concentrations in serum determined following refrigeration at 4°C for 24 h were not significantly different from those determined within 6 h of sample collection. However, samples left at room temperature for 24 h or stored refrigerated for longer time intervals showed a variable decrease in the CAPS concentration which did not always correspond to an increase in CAP concentration. The reason for the spontaneous disappearance of CAPS is unknown. CAP and CAPS are stable for at least one month when samples are frozen within 3 h after collection. Because of the apparent instability of CAPS, we recommend that patient samples be analyzed within 24 h from the time of collection or frozen as soon as possible so that erroneous results are avoided.

Since the sample volume used is only 50 μl for both CAP and CAPS, the assay is ideally suited to pediatric patients. This method has been in routine use at this institution for the past year. Our pharmacokinetic studies of CAPS in patients, submitted for publication elsewhere [11], indicate that CAPS is hydrolyzed in a highly variable and unpredictable manner in the body. This hydrolysis is an individual characteristic and may contribute to the lack of attainment of therapeutic levels of chloramphenicol in some patients. Additionally, we have also shown [11] that substantial and variable amounts of an administered dose of CAPS are excreted unhydrolyzed in the urine. We feel that it is important to quantitate CAPS in order to determine if a patient is capable of hydrolyzing the compound during the dosing interval.

In conclusion, a rapid and simple micro-assay for CAP and CAPS has been described. The sensitivity, precision, and accuracy of this method is such that routine clinical laboratories can easily implement it for monitoring levels of CAP and CAPS. Due to the lack of correlation between the administered dose of CAPS and blood levels of free CAP, we recommend that both compounds be measured in all patients receiving CAPS to help in selecting the appropriate dosage schedule and determine if the patient is adequately hydrolyzing the ester.

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Note

Determination of gentamicin in serum using liquid column chromatography

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Gentamicin (GTM) is an aminoglycoside antibiotic used in the treatment of serious infections. Maximum therapeutic efficacy occurs for peak levels in the range 5—10 mg/l [1]. Overdosing may result in renal impairment [2—4], whereas sub-therapeutic doses are not only ineffective but may lead to the development of antibiotic resistance [5]. GTM has been reported to have a serum half-life in patients with normal renal function in the magnitude of 2 h [2]. As a consequence serum concentrations determined 8 h after the medication have to be very low, and values above 1.5—2.0 mg/l indicate tissue accumulation of GTM. It is well established that monitoring serum GTM levels is the most effective ensurance for adequate therapy [5]. Until very recently these analyses were performed only by using microbiological (MB) methods. These methods are often time consuming due to the bacterial growth rate. However, the growth rate may be increased by using special test-strains [6] and higher incubation temperatures [7] allowing the plates to be read after 4—6 h. Furthermore the method is cheap and simple involving only the normal equipment of a bacteriological laboratory. However, faster methods would be an improvement. Radioimmuno assays have been used in the routine monitoring of serum GTM levels, but require the use of radiochemicals. High-performance liquid chromatography [8—13] has been reported to be an adequate technique. GTM is treated with reagents making the derivate detectable by means of fluorometry. Recently, a homogeneous enzyme immuno assay (EMIT®) has been described as a fast and specific analysis adequate for the routine monitoring of serum GTM level.

This paper reports on a method using high-performance liquid chromatography (HPLC) for the separation procedure with quantitation by means of ultra-violet (UV) absorption.

EXPERIMENTAL

Reagents

Methylene chloride, acetonitrile and concentrated borate buffer (pH 10) were all of analytical reagent grade from E. Merck (Darmstadt, G.F.R.). Benzene sulphonyl chloride (BSC) was of synthetic grade (E. Merck) and distilled once before use (1 mmHg, 108°C). Other commercial chemicals and solvents were of analytical grade.

Stock solutions (1 g/l) of GTM and of the internal standard, netilmicin, were prepared in distilled water. When kept in a refrigerator at 4°C they were stable for at least six months.

Extraction procedure

To a 500-μl serum sample were added 60 μl of sodium hydroxide (1 *N*), 500 μl of phosphate buffer (1/15 *M*, pH 7.4) and 12.5 μg netilmicin as internal standard. Acetonitrile (3 ml) was added and the tube was shaken vigorously for 10 sec resulting in a precipitation of the serum proteins. The specimen was then centrifuged for 3 min at 1000 *g* after which the supernatant was transferred to another tube containing 1500 μl of phosphate buffer (1/15 *M*, pH 7.4) and methylene chloride (3 ml). The contents were mixed for 5 min at 20 rpm on a rotary mixer, and centrifuged. The buffer phase (upper layer) was transferred to another tube using a pasteur pipette moistened with distilled water. A volume of 50 μl sodium hydroxide (1 *N*) and 600 μl BSC solution in acetonitrile (1%, v/v) was added. The mixture was reacted at 75°C for 10 min in an open system. After reaction, the tube was filled with nitrogen, stoppered and placed in the freezer for 10 min to cool the reaction mixture to 0°C. Borate buffer (3 ml, pH 10) and methylene chloride (3 ml) were then added. The mixture was extracted for 5 min at 20 rpm, centrifuged and the methylene chloride (lower layer) was transferred to a tapered tube and evaporated to dryness (40°C) under a stream of nitrogen. The residue was dissolved in 100 μl of the mobile phase.

Liquid chromatography

A liquid chromatograph (Pye Unicam, Cambridge, Great Britain) type LC3 equipped with an UV detector LC3 was used. The column (25 cm × 4.6 mm I.D.) was filled with LiChrosorb RP-18, particle size 10 μm. The mobile phase was acetonitrile—methylene chloride—water—methanol (80 : 10 : 8 : 4) with a flow-rate of 4.0 ml/min. The detection was carried out at 230 nm.

Calculations

The serum concentrations were read from standard curves constructed from chromatograms of serum samples containing varying, but known amounts of GTM, giving concentrations of up to 10 mg/l. The peak height ratios between GTM and netilmicin were plotted against the concentrations.

Enzyme immuno assay

The analyses were carried out using the instruments and the reagent kits as recommended by the Syva Corporation (Palo Alto, CA, U.S.A.).

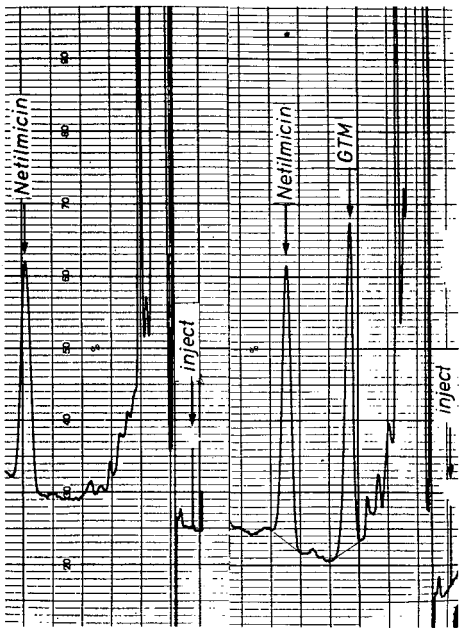

Fig. 1. Chromatograms of serum samples containing 0 (left) and 7.5 mg gentamicin per l (right). Gentamicin and netilmicin appear 3.5 and 5.5 min respectively, after the injection.

RESULTS

Fig. 1 illustrates chromatograms of serum samples containing 0 (left) and 7.5 mg GTM per l (right), respectively. Under the conditions described GTM and netilmicin appeared 3.5 and 5.5 min respectively, after the injection. A calibration graph constructed on the basis of serum samples containing various concentrations of GTM in the range from 0 to 10 mg/l, demonstrated linearity between the GTM concentrations and the ratio between the peak heights of GTM and netilmicin (Table I).

TABLE I

CORRESPONDING VALUES BETWEEN THE GTM SERUM CONCENTRATION AND THE PEAK HEIGHT RATIO (R) OF GTM TO NETILMICIN

Concentration GTM added (mg/l)	R
0.0	0.00
2.5	0.40
5.0	0.79
7.5	1.20
10.0	1.58

TABLE II

ACCURACY OF GTM DETERMINATIONS

Number of samples, 10.

Concentration added (mg/l)	Calculated concentration* (mg/l)	Coefficient of variation (%)
2.5	2.6 ± 0.13	5.0
5.0	5.1 ± 0.14	2.7
7.5	7.6 ± 0.39	5.1
10.0	9.9 ± 0.20	2.0

*Mean ± S.D.

TABLE III

RANDOMLY SELECTED GTM SAMPLES DETERMINED BY HPLC, EMIT®, AND MB

HPLC	EMIT®	MB
0.3	0.1	<1
0.8	0.6	<1
1.3	1.2	1.6
0.8	0.6	<1
4.9	4.6	4.5
1.3	1.1	<1
1.2	1.0	<1
3.3	3.3	2.9
1.3	1.4	<1
3.6	3.1	3.8
4.3	4.2	5.6
2.0	2.2	4.5
0.8	1.2	1.0
2.0	1.7	2.2
7.0	6.3	11.0
2.4	2.3	
4.8	4.4	
7.0	6.3	
9.1	7.5	
9.1	8.1	
10.7	9.4	
5.7	5.5	
13.8	11.7	
7.4	6.9	

The sensitivity was defined as the lowest concentration giving a peak at least ten times higher than the noise on the base line. In this way the lowest concentration giving safe quantitation was 0.2 mg/l.

The accuracy and reproducibility for clinical use was found to be within acceptable limits (Table II).

Tests of specificity, i.e. adding different drugs to the samples, were not carried out but we never detected any interfering peaks.

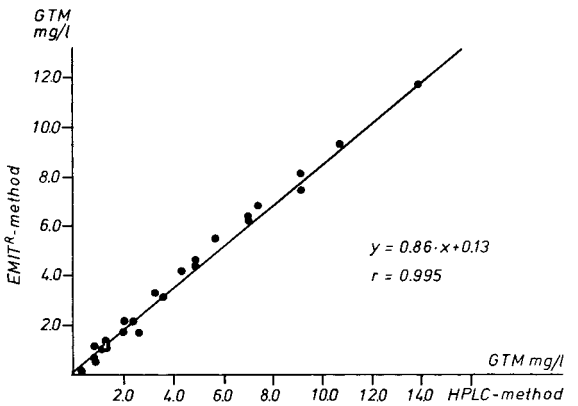

Fig. 2. Relationship between the EMIT® and the HPLC procedures. For both methods 24 serum samples were measured.

The composition of the mobile phase makes it possible to inject a new sample every 6 min, giving a (theoretical) capacity equal to 10 samples per h.

Inter-methods correlations were carried out by analysing randomly selected serum samples from patients treated with ordinary dosages of GTM. The samples were analyzed using the HPLC, MB and the EMIT® methods. The results obtained are given in Table III. As seen the methods gave almost identical results for concentrations up to 13 mg/l. Fig. 2 demonstrates corresponding values from the HPLC and the EMIT® methods. A correlation coefficient of 0.995 was obtained. The equation for the straight line, $y = 0.86x + 0.13$, further confirms the uniformity between the methods.

DISCUSSION

Direct UV detection of GTM is impossible because of the lack of an UV chromophore. Chemical derivatization with e.g. BSC is necessary to enhance UV absorbance. The isolation of GTM from other serum constituents is difficult due to the excellent solubility of GTM in water or phosphate buffer. Direct derivatization of GTM in human serum samples is obstructed by albumin, therefore precipitation of this compound is necessary before the reaction. Acetonitrile is found to be an excellent reagent for precipitation of proteins, due to the formation of a solid mass that adheres to the wall of the tube [8]. Addition of methylene chloride removes most of the acetonitrile into the lower organic phase, resulting in an increased concentration of GTM in the upper layer.

Many procedures for the determination of GTM using liquid chromatography have been reported [8—13], and the method described in this paper can not be claimed to be superior to these assays, as the demanded sample volume, the detection limit, the specificity and the accuracy of all these methods are almost similar. However, in one point our method differs from the other liquid chromatographic methods, as no separation of the three constituents of GTM (C_1, C_{1a}, C_2) is obtained (Fig. 1). No attempts have been made to achieve specific estimations of each of the three compounds, as the main purpose with this assay was to design a routine method.

The derivatization with BSC is a critical point in the procedure, as too long a reaction time and too high a temperature apparently destroy the formed compound. The reaction time and temperature have to be maintained within narrow limits. The addition of sodium hydroxide immediately before the reaction with BSC is important to ensure a pH between 7 and 9 after the derivatization. Experiments have demonstrated the importance of a certain ratio between the amounts of sodium hydroxide and the BSC.

The results given in Fig. 2 show that the EMIT® and the HPLC methods give almost identical results when applied to the same serum samples drawn from patients in ordinary therapeutic treatment. This indicates an acceptable specificity achieved by both methods, when identical results are obtained using totally different analytical techniques.

ACKNOWLEDGEMENTS

We are greatful to Jørgen Bang, M.D. for helpful suggestions concerning the manuscript, and to Schering, U.S.A. for supplying pure substances of netilmicin. We are indebted to Mr. Gert Rode, Syva Denmark for the loan of the EMIT LAB SYSTEM 5000 which made it possible to correlate the HPLC method with the immuno assay method.

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Note

Quantification of prazosin in plasma by high-performance liquid chromatography

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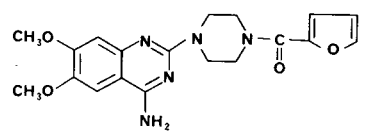
The antihypertensive drug, prazosin (I) is often administered in low doses to humans and requires sensitive methods for its detection in plasma. Of the assay methods reported the fluorometric—wet chemistry method [1] lacks sensitivity and the high-performance liquid chromatographic (HPLC) assay [2] has a high coefficient of variation and interfering plasma peaks eluting with the prazosin. The latter method also requires a lengthy extraction procedure.



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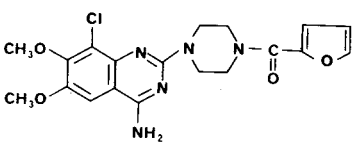

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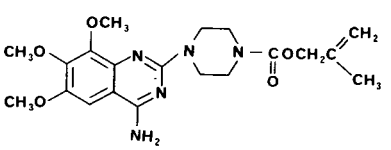

III

The assay procedure presently described employs a new more suitable internal standard, a single extraction step and evaporation followed by HPLC with fluorescence detection.

EXPERIMENTAL

Reagents

All reagents were analytical grade and aqueous solutions were prepared using glass distilled water. Chloroform was Nanograde from Mallinckrodt, St. Louis, MO, U.S.A. Specially purified acetonitrile (210 nm cut-off, Unichrom from Ajax Chemicals, Melbourne, Australia) was used for the high-pressure liquid chromatography. Prazosin hydrochloride and the internal standard (III) were provided by Pfizer, West Ryde, Australia and the internal standard, 8-chloroprazosin hydrochloride (II) was synthesized from vanillin. Information regarding 8-chloroprazosin can be obtained from the author.

Standards

Stock solutions of I and II were prepared in methanol (500 nmol/l each) and stored in the dark at 4°C (stable for at least 1 week). The prazosin solution was used to prepare the appropriate plasma standards for each assay run. Peak height ratios of I to the internal standard II were determined for plasma standards and unknowns and quantification performed by reading unknown values from a plotted standard curve.

Extraction procedure

Plasma (2 ml) was pipetted into a 15-ml glass-stoppered tube. The internal standard solution (200 μl) was added, followed by 0.5 ml of 1.0 *N* potassium hydroxide and 5 ml of chloroform; the mixture was then vortexed for 30 sec. The phases were separated by centrifugation (1100 *g* for 10 min) and the aqueous layer removed by vacuum aspiration and discarded. The organic layer was poured into autosampler tubes (diSPotubes from Scientific Products, State College, PA, U.S.A.) (75 mm × 12 mm) and evaporated under a stream of pure dry nitrogen at 50°C. The residue was reconstituted in 0.5 ml of mobile phase [1.5 m*M* phosphoric acid—acetonitrile (77:23)]. One hundred microlitres of this solution were injected into the chromatograph.

High-performance liquid chromatography

A chromatograph (Spectra-Physics Model SP 8000) equipped with a ternary solvent system, helium degass and automatic data reduction facilities was used. Files for the instrument operation and integration were stored on disc (Spectra-Physics Model SP 4010 disc module). The reversed-phase column used measured 300 mm × 4 mm I.D. and was packed with alkyl phenyl bonded to 10-μm silica (μBondapak/Phenyl from Waters Assoc., Milford, MA, U.S.A.). Column oven temperature was 50°C. The mobile phase was automatically mixed by the instrument and consisted of 1.5 m*M* aqueous phosphoric acid—acetonitrile (77:23) at a flow-rate of 2 ml/min. The instrument was operated in the constant flow mode and all solvent lines from the column to the detector were carefully thermally insulated. The column effluent was monitored using a fluorescence detector (Schoeffel Model 970) at an excitation wavelength of 246 nm with an emission cut-off filter allowing 90% transmission at 389 nm.

The fluorimeter sensitivity was 3.5, range 0.05 μA full-scale and time constant 7.0 sec. Samples were injected automatically using a 100-μl sample loop and an autosampler (Spectra-Physics Model 8010).

Recovery and reproducibility

Recovery of the HPLC assay was determined at concentrations of 1.25, 2.5, 5.0, 25 and 50 nmol/l in plasma by comparison of the peak height of the prazosin peak with that obtained for a solution in mobile phase containing a known concentration of I (500 nmol/l) injected directly into the chromatograph.

Intra-assay reproducibility of the assay was determined at concentrations of 1.25, 2.5 and 50 nmole/l by assaying four plasma samples at each concentration. Inter-assay reproducibility was determined by assaying a single plasma sample containing added I (50 nmol/l) in each assay run.

Interference by other drugs

Samples of the drugs and metabolites listed in Table I were dissolved in mobile phase (500 nmol/l of each) and injected into the high-performance liquid chromatograph. The retention times were obtained if a peak was observed.

TABLE I

RETENTION TIMES OF DRUGS AND METABOLITES IN THE HPLC ASSAY FOR PRAZOSIN

Drug	Retention time (sec)
Prazosin (I)	350
8-Chloroprazosin (II)	625
Internal standard (III)	1270
Propranolol	490
4-Hydroxypropranolol*	235
N-Desisopropylpropranolol*	275
Atenolol	150
Practolol	—
Metoprolol	235
Labetolol	360
Pindolol	—
Timolol	234
3-Methyl-*s*-triazolo[3,4-*a*]phthalazine**	265
3-Hydroxymethyl-*s*-triazolo[3,4-*a*]phthalazine**	150
s-Triazolo[3,4-*a*]phthalazine**	200
Quinidine	230
Dihydroquinidine	265
3-Hydroxyquinidine***	160
Imipramine	1220
Desipramine	1070

*Propranolol metabolites.
**Hydralazine metabolites.
***Quinidine metabolite.

RESULTS AND DISCUSSION

To separate prazosin from eluted plasma peaks in the HPLC assay published [2], a lower percentage of acetonitrile in the mobile phase was required which resulted in an unacceptably long retention time for internal standard III. Internal standard II (8-chloroprazosin) had a considerably shorter retention time (Table I) and was used in preference. Chromatograms obtained for blank plasma, a plasma standard, plasma from a volunteer following a single oral dose of prazosin (2 mg) and from a patient at steady-state are shown in Fig. 1. Using the extraction procedure described here only minor plasma peaks were observed and recovery was 99.6 ± 6.8% and linear over the concentration range 1.25—50 nmol/l (0.48—19.2 ng/ml). Coefficients of variation for the assay are shown in Table II. The detection limit of the asssay was 40 pmol/l determined at a peak height of twice the noise level by injecting the entire extract from 2 ml of plasma.

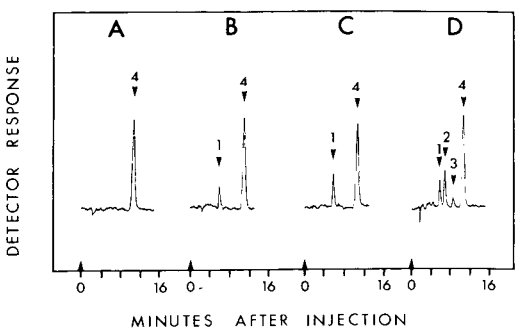

Fig. 1. Chromatograms obtained for the high-performance liquid chromatographic assay of prazosin. (A) Blank plasma, (B) plasma standard containing 1.25 nmol/l of prazosin, (C) plasma drawn from a volunteer 1.0 h after single oral dose of prazosin and containing 2.1 nmol/l of prazosin, (D) plasma sample from a patient at steady-state on prazosin and propranolol and containing 1.5 nmol/l of prazosin. Peaks: 1 = prazosin, 2 = prazosin metabolite, 3 = propranolol, 4 = 8-chloroprazosin (internal standard).

TABLE II

COEFFICIENTS OF VARIATION FOR THE PRAZOSIN ASSAY

	Concentration (nmol/l)	C.V. (%)
Intra-assay	50.0	2
	2.5	3
	1.25	3
Inter-assay	50.0	3

No interference was observed from propranolol, 4-hydroxypropranolol, N-desisopropylpropranolol and other drugs and metabolites shown in Table I. In plasma samples from patients at steady-state on prazosin an additional peak with retention time 416 sec was observed which was probably a metabolite of prazosin (peak 2, Fig. 1).

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Note

Determination of the vasodilator UK33274 by high-performance liquid chromatography using fluorescence detection

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UK33274 is a vasodilator which is structurally based on prazosin (Fig. 1). It has potential clinical advantages over prazosin in that its longer duration of action appears to make it suitable for once daily administration and its slower onset of action appears to lessen the tendency to first-dose hypotension which is a characteristic of prazosin action. In common with prazosin, UK33274 is effective at very low concentrations and in order to define clearly the dispositional characteristics of the drug it is necessary to employ an analytical method capable of detecting concentrations as low as 1 ng/ml. We describe here a method using high-performance liquid chromatography (HPLC) with fluorescence detection, which is based on an assay previously described by one of us for prazosin [1].

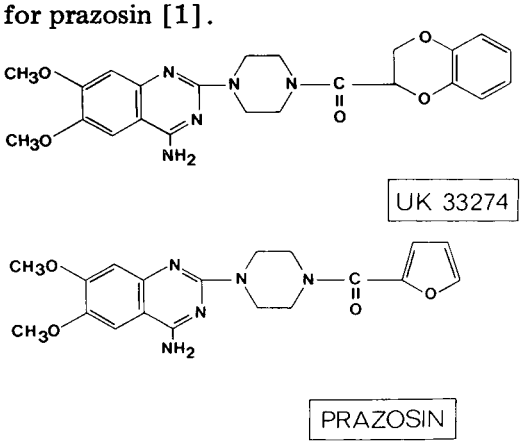

Fig. 1. Structures of UK33274 and prazosin.

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EXPERIMENTAL

Reagents and materials

Unless otherwise stated the reagents and methods of sample preparation and assay calibrations are as previously described [1].

UK33274 [1-(4-amino-6,7-dimethoxy-2-quinazolinyl)-4-(1,4-benzodioxan-2-ylcarbonyl)piperazine methanesulphonate] and prazosin [1-(4-amino-6,7-dimethoxy-2-quinazolinyl)-4-(2-furanylcarbonyl) piperazine hydrochloride] as the internal standard were supplied by Pfizer (Sandwich, Great Britain).

Chromatography

A Hewlett-Packard 1084B high-performance liquid chromatograph fitted with a Spherisorb 5 μm ODS C_{18} bonded reversed-phase column (250 × 4 mm I.D.) was used for the analysis. One pump contained a 0.01 *M* solution of pentane sodium sulphate and a 0.02 *M* solution of tetramethylammonium chloride in water adjusted to pH 3.4 with glacial acetic acid (solvent A). The other pump contained the same concentration of pentane sodium sulphate and acetic acid as solvent A in methanol (solvent B). Both solvents were filtered before use. An isocratic solution of 60% solvent B and 40% solvent A was used with daily minor adjustments in solvent composition (1—2%) to maintain optimum baseline separation of UK33274 and the internal standard. The flow-rate of the mixture was 210 ml/h with a column input pressure of 200 bar (2900 p.s.i.). A Pye Unicam LC-FL detector was used with deuterium lamp and Pye Unicam filters, 254 nm interference for excitation and 370 nm cut-off for emission. A Hewlett-Packard 7985OB LC terminal was employed for recording.

RESULTS AND DISCUSSION

Under the chromatographic conditions described above the retention times of UK33274 and internal standard were 5.3 and 3.7 min, respectively. Fig. 2A shows the chromatogram of an analysis of 1 ml of whole blood containing 10 ng of UK33274 and 20 ng of internal standard. Fig. 2B shows a chromatogram

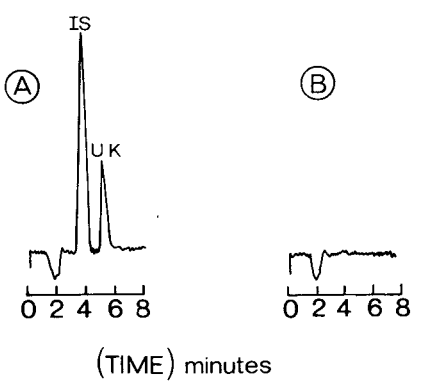

Fig. 2. (A) Chromatogram of extracted whole blood containing 10 ng of UK33274 and 20 ng of prazosin (IS). (B) Chromatogram of 1 ml of control blood taken through the analysis.

of 1 ml of control whole blood taken through the analysis. No peaks corresponding to the peaks shown in Fig. 2A have been found in assaying control blood samples from six subjects. The sample recovery through the procedure averaged 50%.

A typical calibration curve for UK33274 in whole blood is linear in the range 2—50 ng/ml with a regression coefficient of 0.99. The average coefficient of variation for the normalised peak height ratio over this range is 5.1%. The limit of detection, arbitrarily defined as three times baseline noise, is 1 ng/ml. Reproducibility studies using eight samples each at various concentrations gave the following coefficients of variation: 2 ng, 7.4%; 5 ng, 5.5%; 10 ng, 2.7%; and 20 ng, 2.8%.

After storage at −20°C for six weeks, six samples at each of four different initial concentrations gave the following results: 2 ng/ml, 1.9 ± 0.1 ng/ml (standard deviation); 5 ng/ml, 4.8 ± 0.3 ng/ml; 10 ng/ml, 9.6 ± 0.4 ng/ml; and 20 ng/ml, 18.9 ± 1.2 ng/ml.

The chromatographic conditions used in this assay were similar to those previously employed in the analysis of prazosin [1]. It can therefore be anticipated that the same interference from other cardiovascular drugs will be found and for further information the reader is referred to the earlier paper.

In conclusion, we have described a rapid and sensitive analysis in whole blood of a potentially useful new vasodilator.

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Book Review

Electrophoresis '78 (Proc. Int. Conf. Electrophoresis, Cambridge, MA, April 19—21, 1978), edited by N. Catsimpoolas, Elsevier/North-Holland, Amsterdam, 1978, XI + 443 pp., price Dfl. 117.00, US$ 52.00, ISBN 0-444-00294-4.

A number of scientific branches connected with the study of living matter are demanding ever more of separation methods, both analytical and preparative. The high resolving power and the non-destructive procedures of electromigration (electrophoretic) methods, naturally stimulates the interest in further development of the theory and practice of electromigration separations. This interest is most felt in the study of biopolymers and cell particles.

Nowadays regularly organized international conferences of top scientists in the field of electrophoresis afford collections of valuable contributions of a theoretical, instrumental and applicable nature. Electrophoresis '78, edited by N. Catsimpoolas, in the series Developments in Biochemistry, Vol. 2, originated from such an international conference organized in 1978 in Massachusetts. The Editor, although faced with the difficulties owing to the complexity of some of the papers, has organized a publication of ca. 440 pages, divided into eight thematically different sections.

The first part, containing 56 pages, discusses fundamental problems. The effort to present a new, unifying view of the electromigration processes should be especially appreciated where the accent is put on charge fractionation methods, i.e. isotachophoresis and isoelectric focusing, and on the treatment of the highly topical question of the separation of membrane proteins in the presence of detergents, the properties of which are also surveyed.

The second, shorter section (19 pages), is devoted to new instrumentation developments in the analytical separation of cellular materials using carrier-free methods. The orientation of the instrumentation towards automation will obviously contribute, in the near future, to the expansion of the spectrum of clinical investigations concerning the interpretation of charge properties of cells in the field of cancer research.

The section Computer Analysis (29 pages), is aimed directly at automation. Its content should serve as a higher evaluation of analytical data of electro-

pherograms through numerical suppression of the effect of noise in one- and two-dimensional electromigration separations of protein mixtures.

The next section of the same length is devoted to capillary isotachophoresis. In addition to instrumentation, aimed towards the increase in sensitivity of universal detection, a new possibility of microanalysis of proteins in body liquids appears, using the formation of soluble immunocomplexes.

A full quarter of the book is devoted to one-dimensional methods. With one exception, it deals with gel separations. The instrumental section may also be applied to two-dimensional techniques. The method of quantification of proteins and DNA's at the 1-ng level is also of a more general nature. The same is true of the interpretation of the mobilities for the determination of molecular mass, with the accent on SDS-complexes of proteins.

The section on two-dimensional methods is obviously devoted to the application of flat-bed polyacrylamide and/or agarose gels. The combination of thin-layer isoelectric focusing and SDS—gel electrophoresis with crossed immunoelectrophoresis dominates owing to its resolving power. Protein mapping is enriched by the characterisation of p*I* and the molecular masses of individual components at a very high resolving power.

Special questions on the analytical zone electrophoresis of DNA's are dealt with in 19 pages. The technically simple urea—agarose gel method yields excellent resolution of smaller DNA species of up to 1000 base pairs. The increasing interest in electrophoretic separation and characterisation of living cells, for example lymphocytes, thymocytes and in general sub-populations of cells of immunologic interest, is documented by seven reports in the cell electrophoresis section. Laser-Doppler spectroscopy fulfils the requirement of rapid evaluation of the distribution of mobilities or surface charge of the cells. Some of the other articles, however, tend to follow preparative goals as well, and they also touch on the problems of separation of cells in weightless state, general problems of electrokinetic potential of the separation chambers. The flow-through methods of zone electrophoresis are, of course, mainly applied here.

As usual, preparative methods are relatively less represented in this book. The bulk of contributions mainly concerns, and is most informative on, the field of complex analysis of proteins, cell populations and DNA's.

Electrophoresis '78, with its spectrum of analytical methods and applications, offers those interested in electromigration analysis in the field of biochemistry, molecular biology and clinical analysis new stimuli and therefore this publication can be recommended to them.

Prague (Czechoslovakia) Z. PRUSÍK

PUBLICATION SCHEDULE FOR 1980

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