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DETERMINATION OF HOMOVANILLIC, ISOHOMOVANILLIC AND VANILLYLMANDELIC ACIDS IN HUMAN URINE BY MEANS OF GLASS CAPILLARY GAS—LIQUID CHROMATOGRAPHY WITH TEMPERATURE-PROGRAMMED ELECTRON-CAPTURE DETECTION

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(First received February 28th, 1980; revised manuscript received May 16th, 1980)

SUMMARY

The determination of urinary homovanillic, isohomovanillic and vanillylmandelic acids as their trifluoroacetylhexafluoroisopropyl ester derivatives by glass capillary gas—liquid chromatography has been studied. It was shown that even with high column efficiencies a single peak—single compound relationship could not be assumed and for reliable quantitation it was necessary to check determinations with a second gas—liquid chromatography column.

INTRODUCTION

Many methylated phenolic compounds derived from aromatic amino acids and catecholamines have been discovered in urine. Synthetic and degradative routes have been published [1]. Homovanillic (HVA), isohomovanillic (IHVA) and vanillylmandelic (VMA) acids have been widely studied because of their clinical importance in cases of neuroblastoma, Parkinsonism and neural chest tumours and other conditions [1,2]. Various techniques have been used for their determination such as colorimetry [3], fluorimetry [4, 5], spectrophotometry [6], thin-layer chromatography [7—9], paper chromatography [10], high-performance liquid chromatography [11—15], gas chromatography [16—36] and mass fragmentography [2,37—39]. Mass fragmentography is probably the best method for specifically determining small quantities of these metabolites in biological fluids, because it can eliminate interference due to other compounds, but the method is only available to a limited number of workers.

Gas—liquid chromatography (GLC) with the electron-capture detector (ECD) offers a method of high sensitivity, but problems arise because of the multiplic-

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ity of peaks obtained with urine samples. Thus it is necessary to analyze samples on different liquid phases in order to confirm the purity and identity of compounds represented by individual peaks. With GLC—ECD the nature of the metabolites shown on the chromatogram can also be inferred.

Urine samples show large differences between the retention times of a range of compounds and temperature programming is necessary in order to obtain satisfactory peaks for both quickly and slowly migrating compounds. The ECD is normally used in conjunction with isothermal conditions with packed columns, but more recently capillary columns have been introduced [40—46].

We report here on the determination of HVA, IHVA and VMA in human urine using glass capillary gas chromatography with temperature programming and ECD. This work was developed for the particular study of urine in high-pressure neurosyndrome.

EXPERIMENTAL

Materials

Vanillylmandelic acid (VMA), homovanillic (HVA) and bis(4-hydroxy-3-methoxyphenylglycol) piperazine salt (HMPG) were obtained from Sigma (St. Louis, MO, U.S.A.), homovanillyl alcohol (HMPE) from Aldrich (Milwaukee, WI, U.S.A.), 3-(*p*-hydroxyphenyl)propionic acid (*p*-OHPPA) and *p*-hydroxyphenylacetic acid (*p*-OHPAA) from R.N. Emanuel (Wembley, Great Britain), hexafluoroisopropanol (HFIP), trifluoroacetic anhydride (TFAA), *p*-hydroxydiphenyl (*p*-OH-diphenyl), ethyl acetate, sodium chloride and hydrochloric acid were obtained from BDH (Poole, Great Britain). Dexsil 300GC was from Analabs (North Haven, CT, U.S.A.), OV-101 and SP-2250 were from Chromatography Services (Wirral, Great Britain), OV-225 was from Phase Separations (Queensferry, Great Britain) and polypropylene sebacate (PPSeb) from Griffin and George (Alperton, Great Britain). Glass 1-ml ampoules were obtained from Baird and Tatlock (Chadwell Heath, Great Britain). Isohomovanillic acid (IVHA) was a gift from Roche Products (Welwyn Garden City, Great Britain).

Gas chromatography

Gas chromatography was carried out with a Hewlett-Packard Model 7620A gas chromatograph fitted with a 2 mCi ^{63}Ni electron-capture detector. Peak areas were determined with a Hewlett-Packard integrator Model 3370A. Capillary columns were drawn with a Hupe-Busch machine from 1.5 m of Pyrex glass tubing (9 mm O.D. and 3 mm I.D.) to give 75 m of coiled length. This was coated with 5% Chromosorb R and 5% liquid phase by a single step method and 12.5-m lengths at each end were removed and discarded. Sample volumes were usually restricted to 0.3 μl by Hamilton syringe injected directly into the lumen of glass-lined tubing. The columns gave approximately 1400—2500 theoretical plates/m with the HVA peak.

Preparation of samples

It was necessary to standardise the method for urine samples. The creatinine content of urine was determined and a volume of urine [47] was then taken (usually 0.7—1.5 ml) which contained 1.15 mg of creatinine. This was made up to 2.0 ml with distilled water. Excess sodium chloride to saturate (500 mg) was added, followed by 50 μl of 6 *M* hydrochloric acid (pH < 1) and a known quantity of internal standard, HMPE or *p*-OHPPA. The solution thus obtained was extracted three times with 1-ml volumes of ethyl acetate. A similar method was shown with [^{14}C]HVA to give 99.9 ± 1.9% extraction [21]. From the combined ethyl acetate extracts 0.25-ml aliquots were transferred to 1-ml ampoules. These were taken to dryness in vacuo over phosphorus pentoxide. The dried residue from urine samples was derivatized with 100 μl of a mixture of HFIP—TFAA (1:1, v/v) in sealed ampoules at 100°C for 1 h. The ampoule was cooled and the excess of reagents removed under a stream of nitrogen. The dried residue was dissolved in 150 μl of ethyl acetate and 0.3 μl was injected onto the GLC column. It was found that pure HVA, IHVA and VMA (500 pmol to 15 nmol) were completely derivatized with HFIP—TFAA (3:1, v/v) at 100°C in 15 min, but no change was seen with incubation for 2 h.

RESULTS AND DISCUSSION

It is advisable to use as low an injection port temperature as possible consistent with good peak efficiency particularly for heat-sensitive compounds. An inlet temperature of 270°C was used. Although there was substantial loss of hippuric acid at this temperature, no decomposition products were observed on the chromatogram. Below 250°C reduced responses were obtained due to inadequate volatilization of the sample.

Fig. 1 shows the response curves for different compounds (pmol) against the peak area response. The linear dynamic range was approximately 100 for each compound under the conditions quoted. It is possible to increase the linear range by increasing the pulse interval. But increasing the pulse interval led to increased size of dips in the baseline with corresponding peak integration difficulties. It was found that the size of the dips varied with the liquid phase and we do not attribute the dips to a dirty detector as suggested previously [48].

The derivatization procedure was complicated by the presence of carboxylic, phenolic and alcoholic groups on the compounds being studied. Recoveries were dependent on the amount derivatized as well as the ratio of reagents used.

Variable results due to leakage were obtained when using glass-stoppered test-tubes and Reactivials [27]. Sealed glass ampoules were used.

The effect of changing the HFIP:TFAA ratio six-fold from 1:2 to 3:1 showed no effect on the derivatization of HVA and IHVA but VMA in urine showed low recoveries with TFAA in lower ratio than 1:1 (v/v).

The time course of the reaction for VMA, HVA and IHVA in urine using a reaction mixture of HFIP—TFAA (1:1, v/v) showed that the reaction at 100°C was completed in 15 min, but no losses were incurred by incubation for a period of 90 min. These results agree with other workers who used HFIP—TFAA (1:2, v/v) at 100°C for 1 h for HVA and IHVA [27]. HVA and IHVA were derivatized with HFIP—TFAA (1:3, v/v) at 65°C for 15 min [28] but in a later paper [39] HFIP—TFAA (1:2, v/v) incubation at 75°C for 60 min was used. We were unable to obtain good yields for VMA at 75°C. Similarly we were unable to use the conditions (75°C, 90 min) or reagent ratio alcohol:anhydride (1:10, v/v) described for the pentafluoropropionyl-pentafluoropropyl ester derivative of HVA [34]. Hippuric acid always gave a big detector response

TABLE I

RELATIVE RETENTION DATA FOR METABOLITES RELATIVE TO HMPE ON DIFFERENT LIQUID PHASES

GLC conditions as in Fig. 1.

Liquid phase	Initial temperature (°C)	°C/min	HMPE (min)	*p*-OHPAA	VMA	HVA	IHVA	HA	HMPG
Dexsil 300GC	110	4	16.38	0.53	0.67	0.76	0.79	0.84	0.97
OV-101	100	4	12.95	0.57	0.79	0.83	0.86	0.88	1.04
OV-225	115	4	12.75	0.49	0.67	0.70	0.73	0.88	1.09
SP-2250	100	4	12.52	0.46	0.58	0.73	0.77	0.82	0.91
QF-1	115	4	12.05	0.56	0.84	0.80	0.82	0.87	1.20
GE SE-54	105	4	12.05	0.55	0.74	0.82	0.85	0.87	1.03
Dexsil 300GC + OV-17 (2:1, w/w)	100	4	17.79	0.52	0.62	0.71	0.75	0.78	0.87
OV-17 + OV-101 (1:1, w/w)	100	4	13.39	0.52	0.68	0.78	0.81	0.85	0.96
PPSeb	125	*	26.14	0.17	0.21	0.47	0.54	**	***

*Isothermal.

**Not observed.

***Not run.

TABLE II

RELATIVE MOLAR RESPONSE VALUES OF METABOLITES RELATIVE TO HMPE TAKEN AS 1.0 ON DIFFERENT LIQUID PHASES

GLC conditions as in Fig. 1.

Compound	Dexsil 300GC (n = 5)	OV-101 (n = 4)	SP-2250 (n = 5)	QF-1 (n = 5)	GE SE 54 (n = 4)	Dexsil 300GC + OV-17 (2:1, w/w) (n = 4)	OV-17 + OV-101 (1:1, w/w) (n = 4)
p-OHPAA	2.51 ± 0.12	2.62 ± 0.21	2.58 ± 0.11	2.41 ± 0.07	2.69 ± 0.13	2.51 ± 0.08	2.62 ± 0.11
VMA	4.56 ± 0.27	4.42 ± 0.29	4.87 ± 0.21	4.34 ± 0.15	4.42 ± 0.16	4.62 ± 0.21	4.34 ± 0.18
HVA	3.51 ± 0.21	3.45 ± 0.12	3.58 ± 0.11	3.35 ± 0.12	3.55 ± 0.12	3.57 ± 0.08	3.47 ± 0.08
IHVA	2.79 ± 0.18	2.65 ± 0.03	2.83 ± 0.13	2.66 ± 0.07	2.79 ± 0.08	2.79 ± 0.11	2.67 ± 0.09

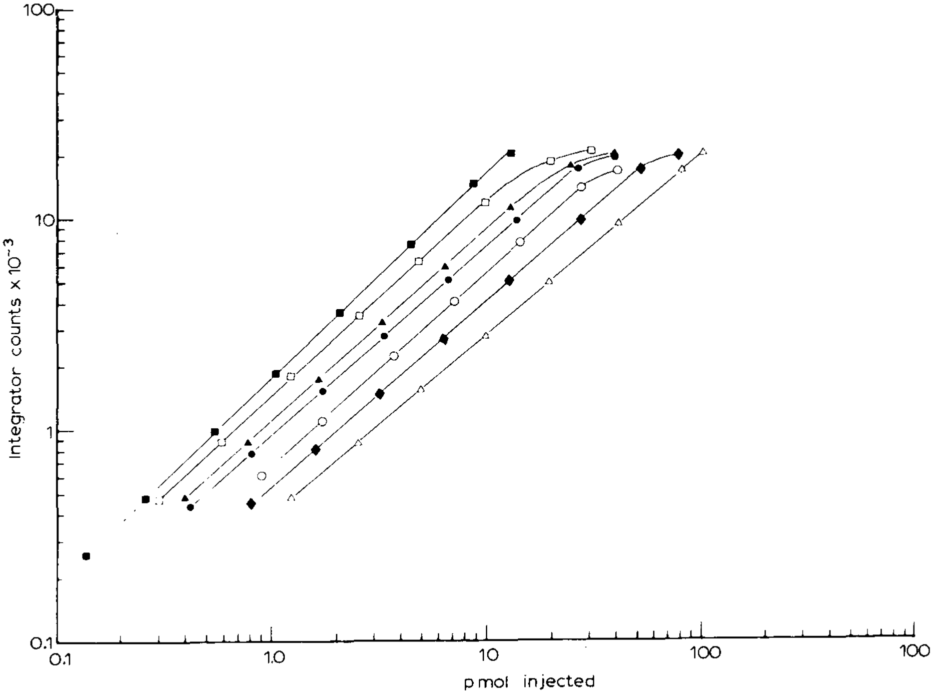

Fig. 1. Response—concentration curve for trifluoroacetyl-hexafluoroisopropyl ester derivatives. Preparation of sample: see Experimental. GLC conditions: 50 m × 0.27 mm I.D. coated with 5% Chromosorb R and 5% SP-2250 SCOT column; carrier gas, hydrogen at a flow-rate of 3 ml/min; make-up gas, argon—methane (90:10) flow-rate, 50 ml/min; temperatures, detector, 290°C; injector, 275°C; column, 100°C programmed at 4°C/min; pulse interval, 15 μsec; attenuation, 2×10^2; sample size, 0.3 μl. ■ = HMPG, □ = VMA, ▲ = HVA, ● = IHVA, ○ = p-OHPAA, ◆ = hippuric acid, △ = HMPE.

and in order to minimize this the derivatization reaction was standardized at 100°C for 60 min with HFIP—TFAA (1:1, v/v). This loss of hippuric acid was not investigated. Additional peaks corresponding to benzoic acid or glycine were not observed on the chromatogram.

The recoveries of metabolites added to urine samples were as follows: VMA, 95 ± 4.5%; HVA, 96 ± 3.8%; IHVA, 98 ± 3.5% (n = 4). Using the same derivatives with GLC determination recoveries from urine were reported as follows: VMA, HVA and IHVA 95—105% [39].

Choice of stationary phase

Relative retention times of six metabolites on nine different liquid phases are given in Table I. All columns with the exception of PPSeb were temperature-programmed and baseline separation was obtained for all the compounds listed. Exceptionally VMA had a longer retention time than HVA and IHVA with QF-1 liquid phase, although this did not occur with other polar stationary phases OV-225 and PPSeb.

The relative molar response (RMR) values of the metabolites were determined with different liquid phases (Table II). This work was done because it had been reported that recoveries varied with the liquid phase. Using the same derivatives with ECD, recoveries were reported of 79 ± 6% for HVA on a 3.8% SE-30 column, 65 ± 4% with a 2% SE-52 column and 65 ± 5% with a 2% QF-1 column [27]. VMA showed the highest standard deviation. Comparing liquid phases the RMR values showed some variation for all the metabolites but these may be attributed to the inherent problems with ECD where many critical parameters such as gas flow-rate and temperature have been specified [48—53].

Fig. 2a shows the separation of seven metabolite derivatives on a SP-2250 SCOT column (1420 theoretical plates/m) in about 12 min. Approximately a 15-fold reduction in quantity injected gave the same peak height as with a packed column in shorter time and with improved resolution. Fig. 2b shows the

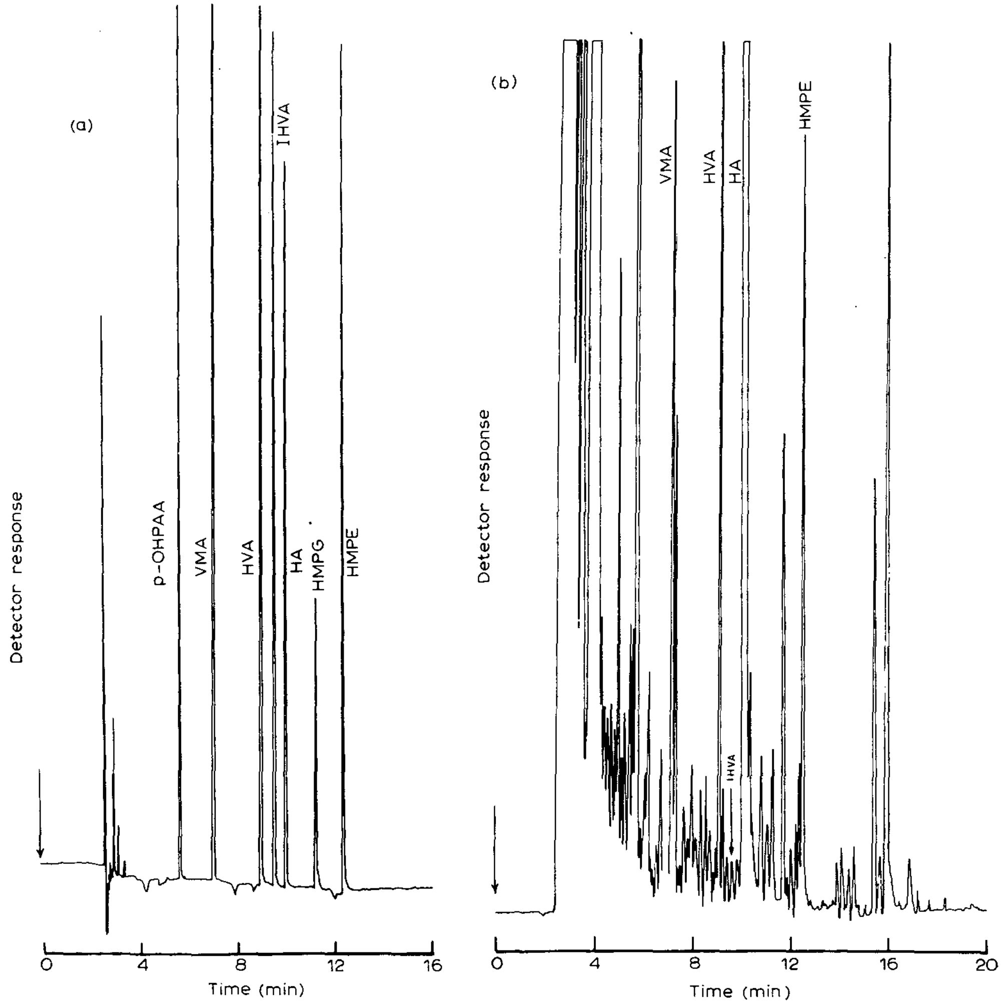

Fig. 2. Separation of trifluoroacetyl-hexafluoroisopropyl ester derivatives on SP-2250 SCOT column. GLC conditions as in Fig. 1. (a) Standard compounds injected: *p*-OHPAA, 4.6 pmol; VMA, 3.4 pmol; HVA, 4.3 pmol; IHVA, 4.5 pmol; hippuric acid (HA), 8.8 pmol; HMPG, 1.4 pmol; HMPE, 13.3 pmol. (b) Urine sample, with internal standard HMPE.

TABLE III

URINARY CONCENTRATIONS (nmol/ml) OF VANILLYLMANDELIC, HOMOVANILLIC AND ISOHOMOVANILLIC ACIDS DETERMINED ON DIFFERENT LIQUID PHASES

GLC conditions as in Fig. 1. $n = 3$.

Liquid phase	VMA	HVA	IHVA
OV-101	4.4	21.3	3.2
OV-225	18.5	22.9	*
QF-1	*	20.3	*
Dexsil 300GC	4.7	21.7	0.6
SP-2250	9.4	42.4	0.5
OV-101 + OV-17	4.8	19.6	*
Dexsil 300GC + OV-17	4.9	19.8	5.7

*Not determined owing to interfering peaks.

chromatogram obtained for the metabolites in a urine sample. The VMA peak was only partially resolved from some unknown peak. Changing the temperature programme did not improve the resolution.

Because of the multiplicity of peaks with urine samples and the possibility of overlap, quantitative recoveries were determined with seven different liquid phases (Table III). VMA gave spuriously high values with OV-225 and SP-2250 because there were obscured peaks with compounds having the same retention times. HVA gave an average value of 20.9 ± 1.26 nmol/ml urine on six liquid phases, but SP-2250 (as shown in Fig. 2b) gave the very high value of 42.4 nmol/ml urine because of an unseen interfering peak. The peak for IHVA was always very small and adjacent peaks made accurate integration difficult.

Fig. 3a shows the separation of standard metabolites on an OV-101 SCOT column and Fig. 3b shows the peaks obtained with a urine sample. Reliable values for VMA and HVA were obtained on this column but interference with the IHVA peak on this column resulted in a spuriously high value (see also Table III). Thus OV-101 could only be used to determine HVA and VMA but not IHVA in urine samples. HMPE was used as internal standard with this stationary phase [28,39]. It was reported that free HMPE occurred naturally in rat urine [54], but none was detected in human urine by these authors or in this work.

The liquid phases OV-225, GE SE-54 and QF-1 gave a satisfactory separation of standard metabolite derivatives, but they were not satisfactory for urine samples. The separation of the standard metabolites on Dexsil 300GC is shown in Fig. 4a and a urine sample in Fig. 4b. HMPE could not be used as an internal standard on this column because of interference by an unknown compound with a retention time of 16.5 min in Fig. 4b. Instead, *p*-OHPPA and *p*-OH-diphenyl were used as internal standards. A constant peak area ratio for these two standard compounds was a good indication that no urine metabolite was present having the same retention times. The values for VMA, HVA and IHVA (Table III) indicated that on Dexsil 300GC there were no interfering peaks leading to spuriously high values. But for IHVA this was not always true and it was necessary to analyze each urine sample on two columns as a check on accuracy of quantitation.

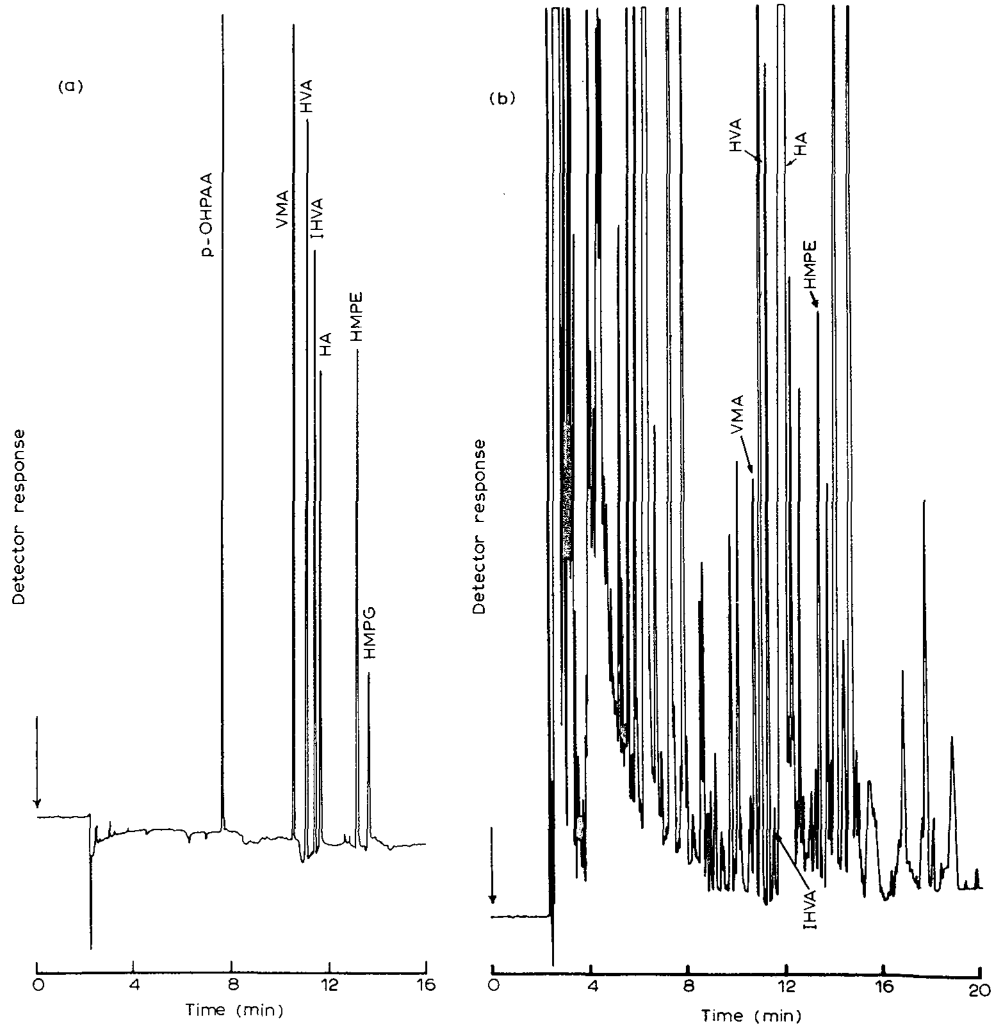

Fig. 3. Separation of trifluoroacetyl-hexafluoroisopropyl ester derivatives on OV-101 SCOT column. GLC conditions as in Fig. 1. (a) Standard compounds; (b) urine sample with internal standard HMPE.

Table IV shows the analysis of VMA, HVA and IHVA in five urine samples of male healthy volunteers obtained with two different GLC columns. The results are given as nmol per ml urine, μg per mg creatinine and mg/day in order to enable full comparison to be made with published results. There is general agreement for the VMA and HVA results on the two columns. IHVA determined on Dexsil 300GC shows values of 0.44—1.01 nmol/ml in urine, whereas on OV-101 the values are falsely high 0.99—3.85 nmol/ml (as expected, see Table III). Our results agree with other published values for HVA (4.05 mg/day) and IHVA (0.1 mg/day) obtained by mass fragmentography [39], HVA (3.30 ± 1.08 S.D. μg per mg creatinine) by GLC—ECD [21], HVA (4.45 ± 0.62 S.E.M. mg/24 h) by fluorimetry [55] and HVA (5.4 mg/24 h) and IHVA (0.05

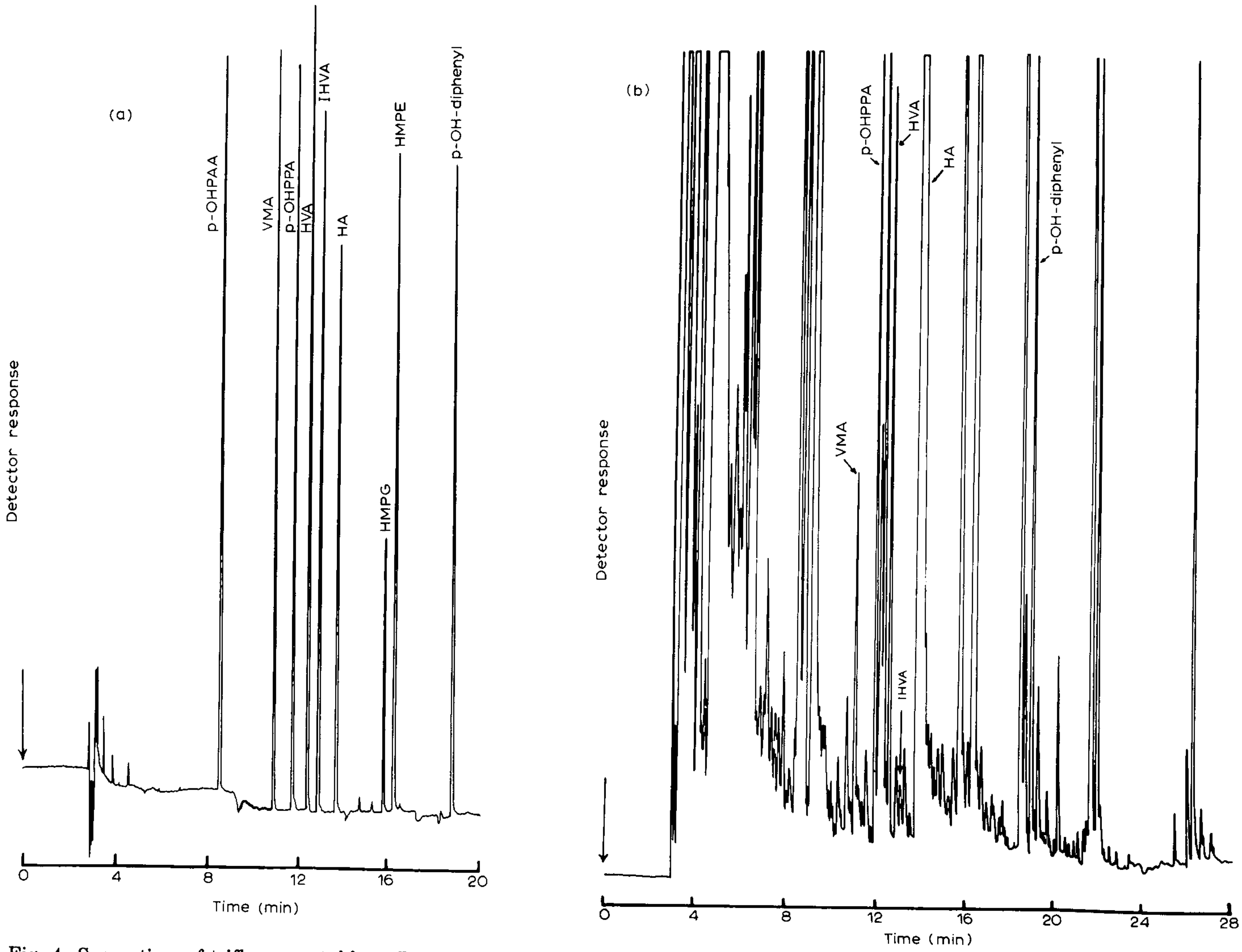

Fig. 4. Separation of trifluoroacetyl-hexafluoroisopropyl ester derivatives on Dexsil 300GC SCOT column. GLC conditions as in Fig. 1. (a) Standard compounds; (b) urine sample, with p-OHPPA and p-OH diphenyl

TABLE IV

VANILLYLMANDELIC, HOMOVANILLIC AND ISOHOMOVANILLIC ACIDS IN URINE DETERMINED WITH TWO GLC LIQUID PHASES

Compound	No.	OV-101			Dexsil 300GC		
		nmol/ml	µg/mg creatinine	mg/day	nmol/ml	µg/mg creatinine	mg/day
VMA	1	15.19	1.73	2.03	13.57	1.54	1.81
	2	13.99	1.86	3.04	15.12	2.01	3.28
	3	5.98	1.43	2.43	5.28	1.27	2.15
	4	14.46	1.89	3.43	15.30	2.00	3.63
	5	10.74	2.02	2.83	9.77	1.85	2.57
HVA	1	33.59	3.51	4.13	33.81	3.54	4.16
	2	39.36	4.81	7.85	40.86	5.00	8.15
	3	16.02	3.53	5.99	15.54	3.42	5.81
	4	44.69	5.37	9.75	44.28	5.33	9.66
	5	23.25	4.03	5.62	25.76	4.65	6.23
IHVA	1	2.38	0.24	0.29	0.59	0.05	0.06
	2	2.36	0.28	0.47	0.85	0.10	0.17
	3	0.99	0.21	0.37	0.44	0.09	0.16
	4	3.85	0.46	0.84	1.01	0.12	0.22
	5	1.83	0.31	0.44	0.65	0.11	0.15

mg/24 h) by paper chromatography and colorimetry [56].

Grob [57] has pointed to the problem of single peaks which may be due to several compounds. This work confirms the real need to check on single peaks obtained from urine samples even with high-efficiency capillary columns. We have tried to meet criticism that methods which are acceptable with pure metabolites should also be applicable to urine samples [58,59].

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DETERMINATION OF LEUCINE FLUX IN VIVO BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY UTILIZING STABLE ISOTOPES FOR TRACE AND INTERNAL STANDARD

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SUMMARY

A simple and reliable method is described for the determination of leucine flux in vivo using two stable isotopes of leucine and gas chromatography—mass spectrometry (GC—MS). [6,6,6-$^2H_3$]Leucine is administered as a primed-dose constant infusion in vivo and DL-[$^2H_7$]-leucine is added to plasma as an internal standard. Plasma leucine concentration and moles per cent enrichment of [$^2H_3$]leucine can be determined simultaneously by GC—MS and selected ion monitoring. Leucine flux calculated from the [6,6,6-$^2H_3$]leucine data was nearly identical to that obtained with L-[U-^{14}C]leucine in dogs. This method is readily applicable to the study of leucine metabolism in humans of all ages and laboratory animals.

INTRODUCTION

The branched-chain amino acids (BCAA) leucine, isoleucine and valine, cannot be synthesized de novo in mammals. Therefore the only source of these essential amino acids for protein synthesis is dietary intake or proteolysis of endogenous protein. In addition to their essential role as components of protein, BCAA may play a direct role in the regulation of protein metabolism [1—3] and serve as a major source of nitrogen for non-essential amino acids [4—6]. Currently there is no simple and reliable method for the determination of BCAA flux, and little is known about the factors which regulate their metabolism in vivo. In the present report a simple and rapid method is described for the simultaneous quantitation of plasma leucine content and the moles per cent enrichment of stable isotope tracer, using gas chromatography—mass spectrometry (GC—MS) and simultaneous ion monitoring of the tracer (L-[$^2H_3$]leucine) and an internal standard (DL-[$^2H_7$]leucine).

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METHODS AND REAGENT PREPARATION

DL-[4,5,5,5,6,6,6-$^2H_7$]Leucine ([$^2H_7$]leucine) and L-[6,6,6-$^2H_3$]leucine ([$^2H_3$]leucine) were obtained from Merck, Sharp and Dohme Isotope Division (Point Claire/Duval, Quebec, Canada). The DL-[$^2H_7$]leucine and L-[$^2H_3$]leucine were 91 and 97 moles per cent enriched, respectively. The [$^2H_3$]- and [$^2H_7$]-leucine were determined to be >99% pure by amino acid analysis and gas chromatography. L-[U-^{14}C]Leucine (300 mCi/mmole) was obtained from New England Nuclear (Boston, MA, U.S.A.). Natural L-leucine was obtained from Sigma (St. Louis, MO, U.S.A.).

The [$^2H_3$]leucine and [U-^{14}C]leucine were dissolved in 0.9% sterile saline, passed through a 0.2-μm filter (Gelman Instrument Co., Ann Arbor, MI, U.S.A.) into sterile vials and autoclaved prior to use. The [$^2H_3$]leucine was determined to be pyrogen free by an FDA approved laboratory. An approximately 30 μM solution of [$^2H_7$]leucine was made by dissolving the stable isotope in deionized water and its content was determined using an amino acid analyzer (Beckman Model 119CL, Palo Alto, CA, U.S.A.).

Leucine concentrations in plasma were determined by ion-exchange chromatography (Beckman Model 119CL). For determination of [^{14}C]leucine radioactivity, plasma was processed in a manner identical to that for quantitation of the plasma leucine concentration, except that water was substituted for ninhydrin and the analyzer effluent was collected in 2.0-ml aliquots. These aliquots were quantitatively transferred to 20-ml scintillation vials (Curtin-Matheson Industries, Houston, TX, U.S.A.) and suspended in 15 ml of scintillation medium (Aquasol®, New England Nuclear) for counting in a liquid scintillation spectrometer (Searle Corporation, Des Plaines, IL, U.S.A.). Quench correction was accomplished by the use of a ^{133}Ba external standard. The dpm/ml plasma were divided by the concentration (μmoles/ml) to obtain [^{14}C]leucine specific activity.

AG 50W-X8 and AG 1-X8 ion-exchange resins (Bio-Rad Labs., Richmond, CA, U.S.A.) were rinsed ten times with an equal volume of deionized water and stored at 4°C as a 1:1 (v/v) suspension in deionized water. Separate disposable 1 × 8 cm plastic columns (Quik-Sep columns with plastic filter disc; Isolab, Akron, OH, U.S.A.) were filled with 2.0 ml of the respective resin suspensions and rinsed with 2—4 ml of deionized water immediately before use. Screw-cap vials (4 ml) were obtained from Kimble (Toledo, OH, U.S.A.) and their caps fitted with Teflon liners (Arthur H. Thomas Co., Philadelphia, PA, U.S.A.). Bis(trimethylsilyl)trifluoroacetamide (BSTFA, Regisil®) was obtained from Regis (Morton Grove, IL, U.S.A.) and acetonitrile (silylation grade) was obtained from Pierce (Rockford, IL, U.S.A.). Ammonium hydroxide was obtained from Mallinckrodt (Davis, KY, U.S.A.). OV-11 was obtained from Supelco (Bellefonte, PA, U.S.A.).

PROCEDURE

One to two milliliters of blood are collected in an iced tube containing sodium heparin (Kimble-Terumo, Elkton, MD, U.S.A.) during infusion of [$^2H_3$]-leucine (or [^{14}C]leucine). Following centrifugation at 4°C for 10 min, the plasma is separated and stored at −80°C until the time of assay.

At the time of analysis, 200 μl are accurately transferred to a 10 mm × 75 mm test-tube and 40 μl of DL-[$^2H_7$]leucine are added making an approximately 6.0 μ*M* solution. The sample is deproteinized with 200 μl of 3 *M* perchloric acid; after mixing, the sample is centrifuged for 10 min, and 300 μl of supernatant are transferred to a second 10 mm × 75 mm test-tube. This latter solution is neutralized to a pH of 7 with 10% (w/v) potassium hydroxide solution, and centrifuged for 10 min. The resultant supernatant is applied to the AG1-X8 anion-exchange resin and the effluent is directly applied to the AG 50-X8 cation-exchange resin column to remove organic acids and to isolate plasma amino acids. The AG 50W-X8 column is rinsed with 4 ml of deionized water and the column effluent discarded. The amino acids are eluted from the AG 50W-X8 column with 2 ml of freshly prepared 4 *N* ammonium hydroxide solution. This latter eluate is collected in 4-ml reaction vials, frozen and taken to dryness by lyophilization. Trimethylsilyl derivatives are made by the addition of 50 μl each of BSTFA and acetonitrile; the reaction vial is tightly capped and allowed to stand at room temperature for 14—24 h.

One to two microliters of the derivatized sample are injected into a 6 m × 6 mm column packed with 3% OV-11 interfaced by a jet separator to a 5985B Hewlett-Packard gas chromatograph—mass spectrometer. The injection temperature is 250°C. Following 1 min at 90°C the column heat-bath temperature is increased at 4°C/min to 200°C. Utilizing positive electron-impact ionization (70 eV) and simultaneous ion monitoring the peak heights of 158, 161, and 165 *m/e* ions are determined using relative dwell times of 50, 250, and 100 msec, respectively.

The peak height ratios of (*m/e* 161/*m/e* 158) × 100 and (*m/e* 165/*m/e* 158) × 100 are calculated; the molar ratios are determined from the standard curve, and moles per cent enrichment calculated: moles per cent enrichment = 100/[1 + (1/molar ratio)]. Natural leucine concentration in plasma is calculated from the moles per cent enrichment of [$^2H_7$]leucine [MPE($^2H_7$)] by the formula

$$\mu\text{moles leucine/liter plasma} = \frac{6.0\ \mu\text{moles } [^2\text{H}_7]\text{leucine per liter plasma}}{[\text{MPE}(^2\text{H}_7)] \times \frac{1}{100}}$$

At steady-state, leucine flux was calculated by the formula [6]

$$R_a = R_d = \left(\frac{100}{\text{MPE}_e} - 1 \right) r^s$$

where R_a and R_d are the rates of appearance and disappearance of leucine, respectively, MPE_e is the moles per cent enrichment of [$^2H_3$]leucine at steady-state and r^s is the rate of infusion of [$^2H_3$]leucine (moles per cent enrichment × μmoles/min). r^s is subtracted from the apparent flux since it is infused at 1—2% of the estimated endogenous production rate.

Leucine flux utilizing [^{14}C]leucine was calculated by the formula

$$R_a = R_d = \frac{r^*}{\text{SA}_e} - r^s$$

where r^* is the rate of [^{14}C]leucine infused (dpm/min) and SA_e is the specific activity of leucine at steady-state.

STANDARD CURVES

To determine the effects of [$^2H_7$]leucine on the $^2H_3:{}^2H_0$ leucine standard curve, two separate sets of standards were made, one with various molar ratios (0—0.06) of $^2H_3:{}^2H_0$ leucine, and a second set of standards identical to the first set except that [$^2H_7$]leucine was added to create varying $^2H_7:{}^2H_0$ leucine molar ratios. These $^2H_7:{}^2H_0$ leucine ratios (0.015—0.12) would accommodate the physiologic range of plasma leucine concentrations (for example, 400 to 50 μM, respectively [7]).

To maximize any artifacts introduced by ion fragments of the [$^2H_7$]leucine at *m/e* 158 and 161, the standard with the highest molar ratio of $^2H_7:{}^2H_0$ leucine contained no [$^2H_3$]leucine. The contribution of the [$^2H_7$]leucine to *m/e* fragments 158 and 161 was 0.6% and 0.2% of the base peak (*m/e* 165). The contribution of [$^2H_7$]leucine to the *m/e* 158 ion, assuming the molar ratio of $^2H_7:{}^2H_0$ leucine was within our standard curve, would be $< 0.1\%$, below the level of sensitivity of the assay method. The two $^2H_0:{}^2H_3$ leucine standard curves derived from $^2H_0:{}^2H_3:{}^2H_7$ leucine and $^2H_0:{}^2H_3$ leucine mixtures were essentially identical (Fig. 1).

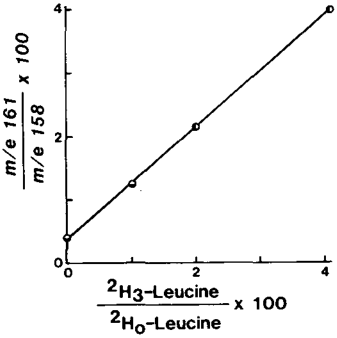
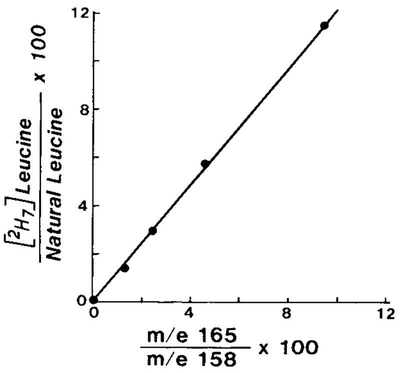


Fig. 1. The peak height ratio of *m/e* 161/*m/e* 158 and the molar ratio of L-[6,6,6-$^2H_3$]-leucine/natural leucine ($^2H_3:{}^2H_0$) in the presence (○) and absence (●) of DL-[4,5,5,5,6,6,6-$^2H_7$]leucine. Triplicate points are included within the area of each symbol.

The simultaneously derived $^2H_7:{}^2H_0$ leucine standard curve is depicted in Fig. 2. The linear relationship between peak height ratio and molar ratio suggests no significant interference from other potential contaminating ions at *m/e* 158 since the lowest enrichment of [$^2H_7$]leucine was in the standard with the highest enrichment of [$^2H_3$]leucine.

Fifteen plasma samples were analyzed for their leucine content both by amino acid analyzer and by GC—MS using [$^2H_7$]leucine as an internal standard (Fig. 3). The correlation coefficient between the two methods was $r = 0.99$. The coefficient of variation of ten replicate injections of the same sample was 0.95%. The coefficient of variation of leucine content determined on ten separate preparations of the same sample was 1.4%.



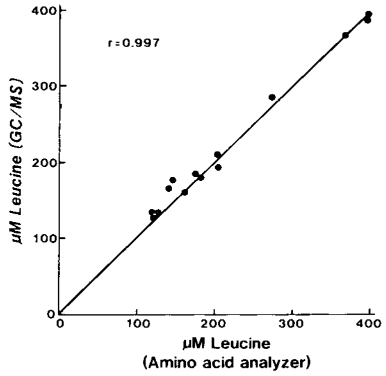


Fig. 2. The molar ratio of DL-[4,5,5,5,6,6,6-$^2H_7$]leucine to natural leucine and peak height ratio of *m/e* 165/*m/e* 158. Triplicate points are included within the area of each symbol.

Fig. 3. Plasma leucine content determined by an amino acid analyzer and by the addition of DL-[4,5,5,5,6,6,6-$^2H_7$]leucine to plasma as an internal standard (ca. 6 μmoles [$^2H_7$]leucine per l) and selected ion monitoring by GC—MS.

IN VIVO VALIDATION

Leucine production rates were estimated simultaneously with [^{14}C]leucine and [$^2H_3$]leucine in three dogs. The animals were fasted for 24 h and placed in a standing sling. Two plastic venous catheters were placed, one in a forelimb vein (isotope infusion) and a second in the saphenous vein (blood sampling). The animals were administered primed, continuous infusions of [^{14}C]leucine (0.225 μCi/kg, 0.015 μCi/kg per min for 4 h) and [$^2H_3$]leucine (60 μg/kg, 8 μg/kg per min for 4 h). Two hours were allowed for isotopic equilibrium and, subsequently, plasma samples were obtained at half-hourly intervals over the last 2 h of study for determination of plasma leucine content, moles per cent enrichment of [$^2H_3$]leucine and specific activity of [^{14}C]leucine.

Over the last 2 h of study, isotope and substrate equilibrium was observed. Leucine flux was essentially the same with both isotopes (4.00 ± 0.20 vs. 4.10 ± 0.27 μmoles/kg per min from [^{14}C]leucine and [$^2H_3$]leucine, respectively, Table I).

TABLE I

STEADY-STATE FLUX RATES OF LEUCINE SIMULTANEOUSLY DETERMINED BY [U-^{14}C]LEUCINE AND [$^2H_3$]LEUCINE

Dog	[$^2H_3$]Leucine (μmoles/kg per min)	[U-^{14}C]Leucine (μmoles/kg per min)
1	3.64	3.62
2	4.32	4.57
3	4.03	4.11
Mean ± S.E.	4.00 ± 0.20	4.10 ± 0.27

Following an overnight fast, five normal male volunteers received a primed-dose (ca. 60 μg/kg) continuous infusion (ca. 4 μg/kg per min) for 4 h to enrich the circulating leucine to 1—2 moles per cent. Following 2 h to allow for isotopic equilibration, venous blood was obtained at 30-min intervals over the last 2 h of study. The plasma leucine concentrations and moles per cent $[^2H_3]$-leucine enrichment reflect substrate and isotopic equilibration (Fig. 4). The leucine flux (production = utilization at steady-state) in these subjects was 2.2 ± 0.2 μmoles/kg per min.

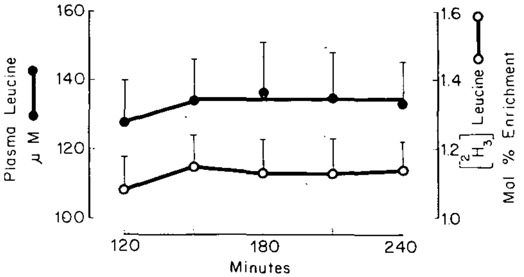


Fig. 4. Plasma leucine concentration and moles per cent enrichment of L-[6,6,6-$^2H_3$]leucine in five normal male volunteers during the last 2 h of a 4-h infusion of L-[6,6,6-$^2H_3$]leucine. Data are displayed as mean ± S.E.

DISCUSSION

Little information is currently available on the regulation of branched-chain amino acid production and utilization in vivo [8—11]. A previously described isotopic method for determination of leucine flux in vivo requires the use of $[^{14}C]$leucine and is tedious and time-consuming [9]. With the increased reliability and availability of GC—MS systems and the custom synthesis of stable isotopes, it is now possible to examine the metabolic regulation of a number of compounds in man with isotope dilution methodology without the use of ionizing radiation.

The method described herein utilizes two deuterium-labeled leucine molecules; one for in vivo infusion (L-$[^2H_3]$leucine) and one as an internal standard (DL-$[^2H_7]$leucine). Selection of the [6,6,6-$^2H_3$]leucine places the ^2H at a sufficient distance from the amino nitrogen group of leucine that transamination of the isotopically labeled leucine does not result in loss of enrichment. Subsequent metabolism of the leucine carbon chain by branched-chain α-keto acid dehydrogenase is irreversible [12], thus preventing re-entry of the deuterium label into the leucine pool. As a result, use of $[^2H_3]$leucine as tracer should accurately estimate the flux of the leucine carbon. This was confirmed in the present studies since simultaneous estimation of leucine flux with $[^2H_3]$- and [U-^{14}C]leucine gave identical flux rates in dogs. Moreover, the rates of leucine flux observed in normal adult humans utilizing $[^2H_3]$leucine in the present studies were not appreciably different from those found by Golden and Waterlow [9] in adults using [U-^{14}C]leucine as trace.

Stable isotopes have been widely used as internal standards for the quantitation of a number of compounds in plasma, including amino acids [13]. In the present method, careful selection of the internal standard is required to avoid artifactual contamination of the fragments of interest from the unlabeled leucine (*m/e* 158) and [$^2H_3$]leucine (*m/e* 161). Therefore, the internal standard must be of sufficient purity and enrichment to permit accurate quantitation of the unlabeled leucine and to avoid interference with the determination of the mole per cent enrichment of the infused trace (L-[$^2H_3$]leucine, *m/e* 161; 1—1.5 moles per cent enrichment). The DL-[$^2H_7$]leucine used in the present method as the internal standard has less than 1% the base peak at mass fragments of *m/e* 158 and 163. These fragments could potentially interfere with the quantitation of leucine and the determination of the [$^2H_3$]leucine enrichment. Addition of [$^2H_7$]leucine to the plasma sample, creating a plasma [$^2H_7$]leucine concentration equal to the plasma concentration of unlabeled leucine, increased the molar ratio of [$^2H_3$]leucine to natural leucine by 0.4%. At the concentration (6 μM) selected in the described methods, no significant error was introduced (Fig. 1).

The assessment of substrate flux requires the determination of both substrate concentration and isotopic enrichment (moles per cent enrichment or specific activity). The method described above provides a rapid and accurate technique for the simultaneous quantitation and determination of the moles per cent enrichment of the infused trace in large numbers of samples. It eliminates time-consuming procedures involving ion-exchange resin chromatography for the determination of leucine specific activity and the exposure of subjects and laboratory personnel to radioactive compounds. Moreover, since it requires small volumes of blood, it can be readily used for the determination of leucine flux in children and laboratory animals where the volume of blood available for sampling may be severely limited.

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CHROMBIO. 633

DETERMINATION OF AN ANTISECRETORY TRIMETHYL PROSTAGLANDIN E_2 ANALOG IN HUMAN PLASMA BY COMBINED CAPILLARY COLUMN GAS CHROMATOGRAPHY—NEGATIVE CHEMICAL IONIZATION MASS SPECTROMETRY

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SUMMARY

A method is described for measuring a trimethyl prostaglandin E_2 analog, TM-PGE$_2$, in human plasma. Trideuterated and monofluorinated analogs of TM-PGE$_2$ are added to plasma as internal standard and carrier, respectively. The plasma is adjusted to pH 3.0 and is extracted with a mixture of benzene—dichloromethane (9:1). The residue, following removal of the extracting solvent, is reacted consecutively with pentafluorobenzyl bromide and bistrimethylsilyltrifluoroacetamide. The excess derivatizing reagents are removed by evaporation, and an aliquot of the reconstituted residue is analyzed by capillary column gas chroma-

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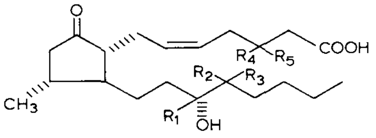
tography using methane as the carrier gas. A quadrupole mass spectrometer is set to monitor in the gas chromatographic effluent the $(M - C_7H_2F_5)^-$ fragment ion of TM-PGE$_2$ (*m/e* 449) and trideuterated TM-PGE$_2$ (*m/e* 452) generated by methane negative chemical ionization. Quantitation of unknowns is based on a comparison of the *m/e* 449 to *m/e* 452 ion ratio in each unknown to that obtained from the analysis of control plasma spiked with known amounts of TM-PGE$_2$ and fixed amounts of internal standard and carrier. The sensitivity limit of the assay is approximately 100 pg ml^{-1}, which is equivalent to 1 pg injected. The assay was used to measure the concentration of TM-PGE$_2$ in the plasma of two subjects following a single 10 μg kg^{-1} oral dose of the drug.

INTRODUCTION

The trimethyl prostaglandin E$_2$ analog, TM-PGE$_2$ (I), is an orally active inhibitor of gastric acid secretion in dog and man [1,2]. An assay for the compound is required to establish its pharmacokinetic profile and to verify its systemic absorption in toxicology studies. The assay must be extraordinarily sensitive because the drug is effective at a dose of only 10 μg kg^{-1} and highly specific because TM-PGE$_2$ is structurally similar to many naturally occurring prostaglandins. The procedure should be relatively simple for it to be useful in analyzing the large number of samples generated by a typical pharmacokinetic or toxicology study.



Compound	R_1	R_2	R_3	R_4	R_5
I	H	CH_3	CH_3	H	H
II	^2H	CH_3	CH_3	^2H	^2H
III	H	F	H	H	H

This paper reports a sensitive, specific and relatively simple gas chromatographic—mass spectrometric (GC—MS) procedure for TM-PGE$_2$. The method is based on the conversion of TM-PGE$_2$ to an electron-capturing (EC) pentafluorobenzyl ester and the GC analysis of the latter as its trimethylsilyl ether. A similar derivative has been used in an EC—GC assay for prostaglandin F$_{2\alpha}$ [3]. Sensitivity and specificity are provided by mass spectral analysis using both negative chemical ionization (NCI) and selected ion monitoring. Specificity is aided by capillary GC using a SCOT column. The assay features the use of a trideuterated analog of TM-PGE$_2$, compound II, as internal standard. Compound III is used as a carrier substance to ensure high recoveries at low drug concentrations. Because of the assay's relatively simple workup procedure, it can be used to analyze a great number of plasma samples in a relatively short period of time. Assay sensitivity is approximately 100 pg ml^{-1}.

EXPERIMENTAL

Equipment and operating conditions

Gas chromatograph. A Finnigan Model 9500 gas chromatograph was equipped with a 19 m × 0.5 mm I.D. glass SCOT column (SGE, Austin, TX, U.S.A.) and an SGE splitless SCI-AK injector. Prior to use, the column was conditioned overnight at 250°C with methane (69 kPa) as carrier gas. The injection port, column oven, interface oven and transfer line were operated at 280°C, 250°C, 230°C, and 225°C, respectively. A methane column pressure of 14 kPa was used for the assay. Under these conditions, the retention time of TM-PGE_2 was approximately 3 min.

EC—GC measurements were made using a Micro-Tek MT-220 (Tracor, Austin, TX, U.S.A.) gas chromatograph equipped with a ^{63}Ni detector. The detector was operated in the d.c. pulsed mode. The EC—GC parameters were adjusted to give maximum sensitivity.

Mass spectrometer. A Finnigan Model 3200 mass spectrometer was modified to permit negative ion chemical ionization as previously described [4]. The ion source voltages were set to give the maximum signal response consistent with satisfactory ion peak shape and unit mass resolution. Methane was used as GC carrier gas and was also added directly after the GC column, before the interface, as makeup gas and NCI gas. The ion source pressure was approximately 130 Pa. The continuous dynode electron multiplier was operated at —2100 V and the conversion dynode was operated at +2500 V.

Peak monitor. Selected ion recordings were obtained using a Finnigan PROMIM peak monitor. The responses were recorded on a multichannel paper-chart recorder (Rikadenki KA-41). The mass spectrometer was set to monitor *m/e* 449 and *m/e* 452. Both channels were operated at a gain of 10^8 V A^{-1}, 100 msec dwell time and a filter setting of 0.5 Hz. The recorder was operated at a chart speed of 2 cm min^{-1}.

Glassware. Culture tubes (16 ml, Pyrex No. 9825) equipped with Teflon®-lined screw-caps were used for plasma extraction. Conical centrifuge tubes (5 ml, Pyrex No. 8061) were used for evaporation of the solvent extracts. Both types of tubes were purchased from SGA, Bloomfield, NJ, U.S.A. All glassware, including the GC column, was cleaned with detergent, rinsed thoroughly with distilled water, treated with Prosil-28 (PCR Research Chemical, Gainesville, FL, U.S.A.) and, finally, was rinsed in an ultrasonic bath with methylene chloride and then methanol prior to drying in an oven at 105°C. Blood samples were collected in Vacutainer® No. 6527 from Becton-Dickinson.

Solvent evaporation. Solvents were removed at 50°C using a nitrogen evaporator (N-Evap, Organomation Assoc.).

Shaker. Extractions were performed by shaking (80—100 strokes min^{-1}) on a variable-speed reciprocating shaker (Eberbach Inc.).

Centrifuge. Centrifugation was done using a Damon/IEC Model CRU-5000 refrigerated centrifuge operated at 10°C and 1000 *g*.

Chemicals

TM-PGE_2 and compounds II and III were obtained from Dr. G. Holland, Chemical Research Department, Hoffmann-La Roche, Nutley, NJ, U.S.A. Nanograde methylene chloride, acetonitrile and benzene and reagent grade monobasic potassium phosphate and 85% phosphoric acid were purchased from Mallinckrodt (St. Louis, MO, U.S.A.). Pentafluorobenzyl bromide (PFBB) and diisopropylethylamine were obtained from Aldrich (Milwaukee, WI, U.S.A.). Bis-trimethylsilyltrifluoroacetamide (BSTFA) was purchased from Pierce (Rockford, IL, U.S.A.).

Solutions

1 M phosphate buffer (pH 3.0). Monobasic potassium phosphate (136 g) was dissolved in 1 l of distilled water and the pH of the solution was adjusted to 3.0 with 85% phosphoric acid.

TM-PGE$_2$ [I]. TM-PGE$_2$ (1.0 mg) was dissolved in 1 ml of methanol. A 0.010-ml aliquot of this solution was diluted to 100 ml with distilled water (solution A, 100 ng ml^{-1}). Ten milliliters of this solution were diluted to 100 ml with distilled water to give solution B (10 ng ml^{-1}).

Compound II. Compound II (1.0 mg) was dissolved in 1.0 ml of methanol. A 0.010-ml aliquot of this solution was diluted to 100 ml with distilled water (solution C, 100 ng ml^{-1}).

Compound III. Compound III (2.0 mg) was dissolved in 100 ml of methanol (solution D, 20 μg ml^{-1}).

35% PFBB in acetonitrile. PFBB (3.5 ml) was diluted to 10 ml with acetonitrile.

Procedure

Extraction. Depending on the expected concentration, either 0.5 or 1.0 ml of plasma and either 1.0 ng or 2.5 ng of compound II (10 or 25 μl of solution C) were added to a 16-ml culture tube containing 200 ng of compound III (10 μl of solution D). To this solution were added 3 ml of 1 *M* phosphate buffer (pH 3.0) and 6 ml of benzene—methylene chloride (9:1). The mixture was vortexed vigorously for 30 sec on a Vortex Genie mixer and was shaken for 30 min on a mechanical shaker. The sample was centrifuged and the organic phase was transferred to a 5-ml centrifuge tube and evaporated to dryness under a stream of nitrogen. If necessary, the residue can be stored overnight at this point in a vacuum desiccator.

Derivatization. The residue was dissolved in 30 μl of acetonitrile. Ten microliters of the 35% PFBB solution and 10 μl of a 10% diisopropylethylamine in acetonitrile solution were added. The reaction mixture was allowed to stand for 15 min at 40°C in the N-Evap water-bath. The solution was evaporated at 50°C under nitrogen and the residue was dried in a vacuum desiccator for 15 min. The vacuum-dried residue was dissolved in 50 μl of acetonitrile and 10 μl of BSTFA. This mixture was heated at 60°C for 15 min and the solvent was evaporated under nitrogen. The residue was stored in a vacuum desiccator until analyzed.

GC—MS analysis. Depending on the expected concentration, the residue was dissolved in between 25 and 100 μl of hexane and a 1-μl aliquot of this solution was injected into the GC—MS system with the divert valve open and the electron filament off. Fifty-five seconds after injection, the GC divert valve was closed. The filament power supply was turned on 5 sec later.

Calculation

The peak heights of the *m/e* 449 and *m/e* 452 ions were measured with a ruler and the *m/e* 449 to *m/e* 452 ion ratio was converted to a concentration using a standard curve. The standard curve was generated from the analyses of control plasma (0.5 ml) to which was added either 0 μl (0 ng), 10 μl (0.1 ng), 25 μl (0.25 ng), 50 μl (0.5 ng), or 75 μl (0.75 ng) of solution B, or 10 μl (1 ng), 50 μl (5 ng), 75 μl (75 ng) or 100 μl (10 ng) of solution A, along with the 2.5 ng of compound II (internal standard) and 200 ng of compound III (carrier). The resulting data of ion ratio vs. amount added from the spiked plasma were subjected to linear regression analysis using an appropriate program on a digital computer. The concentration in an unknown was determined using the slope and intercept values from the regression analysis.

Clinical samples

Two healthy male volunteers were fasted for 7.5 h prior to receiving a 10 μg kg^{-1} oral dose of TM-PGE_2. Aliquots (10 ml) of whole blood were drawn at —5, 20, 40, 60, 75, 90, 120 and 150 min post dosing. The blood was centrifuged for 30 min and the plasma was isolated and stored at —10°C.

RESULTS AND DISCUSSION

The methane NCI mass spectra of derivatized TM-PGE_2 and compound II show no molecular ions (Fig. 1). Intense fragment ions corresponding to the loss of the pentafluorobenzyl radical are found at *m/e* 449 in the mass spectrum of TM-PGE_2 and at *m/e* 452 in the mass spectrum of compound II, and these ions are used for quantitation. Two smaller fragment ions corresponding to the loss of $(CH_3)_3$ SiOH and $CH_2 = Si(CH_3)_2$ from the base peak are found at *m/e* 359 and *m/e* 377 and at *m/e* 362 and *m/e* 380 in the mass spectra of TM-PGE_2 and compound II, respectively.

Typical ion chromatograms from the analysis of 1 ml of control plasma spiked with either 0 (A) or 0.5 ng (B) of TM-PGE_2, in addition to 2.5 ng of compound II and 200 ng of compound III, can be seen in Fig. 2. The small TM-PGE_2 response in chromatogram A from undeuterated TM-PGE_2 in compound II and "ghosting" from previous injections currently limits the assay sensitivity. This response typically represents 0.025 ng ml^{-1} of TM-PGE_2. For any given set of samples, the assay sensitivity limit is considered to be three times the TM-PGE_2 response at *m/e* 449 in the ion chromatogram from control plasma. The TM-PGE_2 response in ion chromatogram B represents, at most, 5 pg of derivatized TM-PGE_2 injected on-column. It is estimated from the analysis of pure derivatized standards that the NCIMS response of TM-PGE_2 at *m/e* 449 is approximately five times greater than the EC response of TM-PGE_2 and is approximately 25 times greater than the response of the $[MH - (CH_3)_3\text{-}SiOH]^+$ base peak ion in the methane positive CI mass spectrum of the same derivative.

Assay precision and the recovery of TM-PGE_2 were determined by spiking six separate 1-ml plasma samples with 100 pg of the authentic compound and analyzing the samples using the procedure described. The mean (± S.D.) concentration found, 96 ± 5 pg ml^{-1}, indicates an inaccuracy of less than 10% and a precision (coefficient of variation) of 5% at this low concentration. This experiment was repeated with six 1-ml plasma samples each spiked with either 0.25 ng or 2.5 ng of TM-PGE_2. The mean concentrations (± S.D.) found were 0.26 ± 0.03 and 2.2 ± 0.03 ng ml^{-1} for the 0.25 and 2.5 ng ml^{-1} samples, respectively. The mean recovery (± S.D.) of the compound for these samples, based on a comparison of the response of derivatized TM-PGE_2 with the response

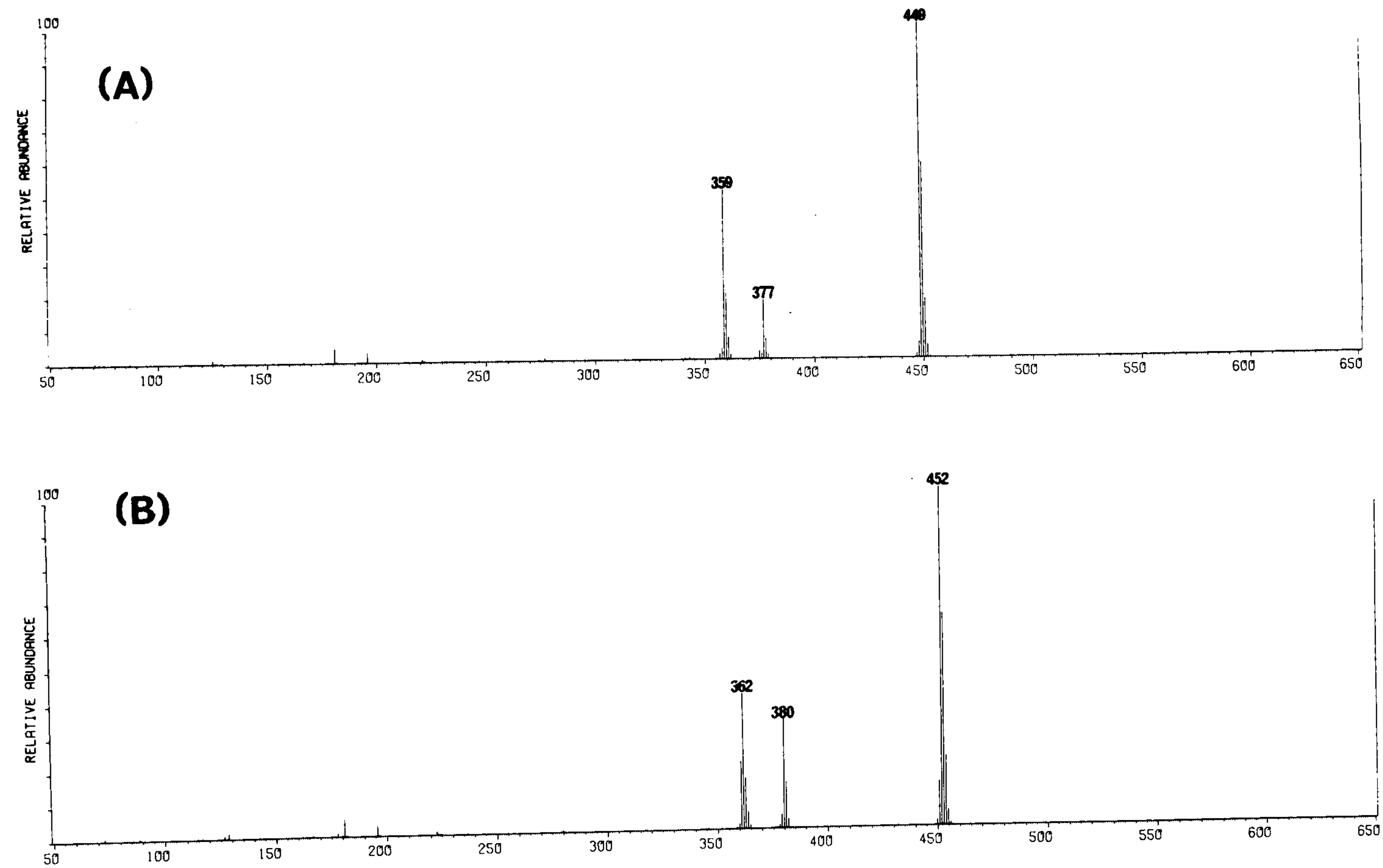


Fig. 1. Methane NCI mass spectra of TM-PGE$_2$ (A) and trideuterated TM-PGE$_2$ (B). Spectra were obtained under the GC—MS conditions described in the text.

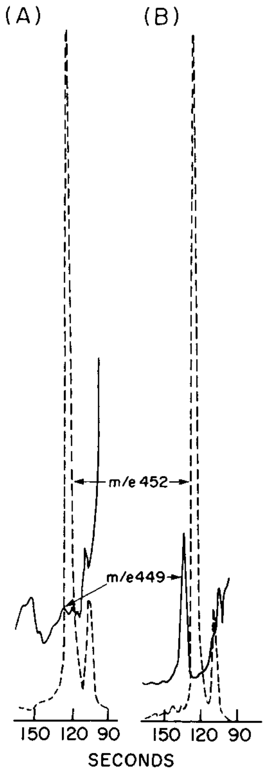


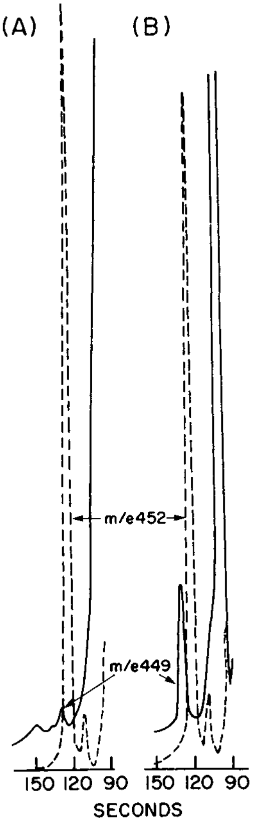
Fig. 2. Ion chromatograms from the analysis of 1 ml of control plasma spiked with either 0 ng (A) or 0.5 ng (B) of TM-PGE$_2$ (*m/e* 449), 2.5 ng of compound II (*m/e* 452) and 200 ng of compound III. For these ion chromatograms approximately 1 µl out of the available 100 µl was injected.

from the injection of known amounts of derivatized TM-PGE$_2$, was 56 ± 9%.

The effect of the blood collection container on assay accuracy was evaluated. A 25-ml sample of blood, freshly drawn into a glass syringe, was spiked with 62.5 ng of TM-PGE$_2$ (i.e. 2.5 ng ml^{-1}). The stoppers of six Vacutainers were removed. Aliquots (2 ml) of the spiked blood were transferred into the Vacutainers which were restoppered. Additional 2-ml aliquots of blood were transferred to six of the same 16-ml culture tubes which were used for plasma extractions. The culture tubes were capped and all twelve tubes were placed on a horizontal shaker and gently shaken at room temperature for 30 min. Three of the Vacutainer blood samples and three of the culture tube blood samples were centrifuged and the plasma collected. One milliliter each of the six plasma samples and 1 ml each of the six remaining whole blood samples were assayed for TM-PGE$_2$. The mean TM-PGE$_2$ concentration (± S.D.) measured was 2.30 ± 0.04 ng ml^{-1} for the blood samples exposed to the culture tubes and 2.18 ± 0.08 ng ml^{-1} for the blood samples exposed to the Vacutainers. The mean TM-PGE$_2$ concentration (± S.D.) measured was 3.26 ± 0.21 ng ml^{-1} for the plasma samples exposed to culture tubes and 3.51 ± 0.08 ng ml^{-1} for the plasma samples exposed to the Vacutainers. Thus, no adverse effect of the Vacutainer compared to the culture tube on assay accuracy could be established.

The stability of TM-PGE$_2$ in plasma and blood on prolonged storage was determined. To 50 ml of both control plasma and control blood were added 50 ng of TM-PGE$_2$. Duplicate 1-ml aliquots of the spiked blood were analyzed for TM-PGE$_2$ and the remaining blood and plasma were stored at −10°C. Duplicate 1-ml aliquots of the blood and plasma were analyzed on days 15, 21, and 133. The measured concentration of TM-PGE$_2$ for all these samples were within 6% of the initial blood concentration measured on day 1. Thus, given an assay precision of 5%, TM-PGE$_2$ seems to be stable in either frozen blood or plasma for at least four months.

Following these experiments, TM-PGE$_2$ was administered to two male volunteers. Ion chromatograms from the analysis of plasma from one of the volunteers obtained either before dosing (A), or 90 min post dosing (B), are shown in Fig. 3. The plasma concentration—time curves for the subjects can be seen in Fig. 4. Assuming that absorption is rapid and that distribution is complete after 40 min, the elimination half-lives were 26 and 20 min for subjects 1 and 2, respectively.



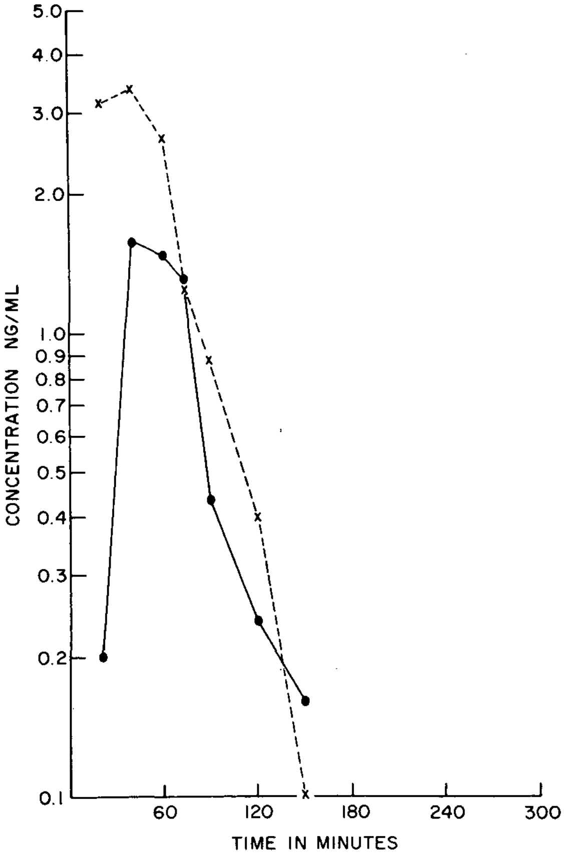


Fig. 3. Ion chromatograms from the analysis of 1 ml of plasma from a subject who had received a 10 μg kg^{-1} oral dose of TM-PGE$_2$. (A) Plasma taken just prior to dosing; (B) plasma taken 90 min post dosing. The TM-PGE$_2$ concentration at 90 min was 0.4 ng ml^{-1}.

Fig. 4. Plasma concentration—time curve for two male volunteers who had received a 10 μg/kg^{-1} oral dose of TM-PGE$_2$. (●) Subject 1, (X) subject 2.

CONCLUSIONS

(1) The pentafluorobenzyl ester—trimethylsilyl ether derivative most commonly used in the determination of prostaglandins by EC—GC can also be used for the analysis of these compounds by GC—NCIMS.

(2) Analysis by GC—NCIMS offers a significant increase in sensitivity over analysis by either positive ion GC—CIMS or EC—GC. The sensitivity limit of the GC—NCIMS assay for TM-PGE_2 is 100 pg ml^{-1}.

(3) The method was used successfully to determine TM-PGE_2 in plasma following a single 10 μg kg^{-1} oral dose of the drug in man.

(4) This analytical procedure should be useful in the measurement of other naturally occurring and xenobiotic prostaglandins.

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CHROMBIO. 631

QUANTITATIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF NUCLEOSIDES AND BASES IN HUMAN PLASMA

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SUMMARY

A reversed-phase high-performance liquid chromatographic (HPLC) method has been developed for the estimation of purines, pyrimidines and their congeners in biological fluids. An initial HPLC separation allowed the collection of a number of effluent fractions, each of which contained a single component of interest. The re-application of these fractions to a second HPLC separation permitted the resolution and quantification of nanogram amounts of these components. Isocratic elution with volatile buffers renders the samples amenable to automatic sampling procedures or lyophilisation. Data are presented on the application of the method to the analysis of nucleosides and bases in human plasma.

INTRODUCTION

Purine and pyrimidine antimetabolites such as 6-mercaptopurine, allopurinol and 5-fluorouracil have been in clinical use for many years. More recently, interest has grown in the use of naturally occurring nucleosides and bases to modulate the effects of anticancer agents [1—13]. We have been pursuing the reversal of methotrexate toxicity with nucleic acid precursors, both in animals and man [1—4].

For these studies a method was required which would allow us to monitor a number of purines and pyrimidines (hypoxanthine, Hx; thymine, T; oxypurinol, Op; allopurinol, Ap; and thymidine, TdR) in the plasma of patients. Methods available during the early stages of our studies were prohibitive in terms of sensitivity and the time involved in quantitation of single components [14—16]. Several methods have been published for the estimation of nucleosides or bases

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using high-performance liquid chromatography (HPLC) [17—29]. Unfortunately none of these was suitable for our purposes. The majority allowed only the estimation of one or two components, or combinations present in relatively high concentration in the samples used. The method described here was developed to allow us to monitor the particular nucleosides and bases of interest in single, relatively small plasma samples. With minor modifications the method has proved suitable for the estimation of a wide range of naturally occurring purines and pyrimidines and their congeners.

Selective absorption has proved valuable in certain cases where a class of related compounds has been separated from unwanted components. For example Gehrke and co-workers [22,24,27] used a boronate column to separate ribonucleosides from urine; Brown and Bye [19] used a Cu^{2+} Chelex-100 column to separate purine bases from plasma and urine. These methods could not be adapted to our purposes, since we were interested in deoxyribonucleosides and bases which are not retained on a boronate column. Further we wished to estimate both purines and pyrimidines. Group separations of nucleosides and bases on polyacrylamide gel columns as described by Khym [30] and used by Jackson et al. [6] were lengthy and complex and thus unsuitable for our purposes.

An initial separation system was evolved which allowed the collection of a number of effluent fractions each of which contained a single component of interest together with a limited number of contaminants. An isocratic reversed-phase HPLC system was used for the initial separation as this gave optimal resolution of IR and TdR from the large compound peak eluting between 17 and 20 min (Fig. 1b and c) under these conditions. It was found that different reversed-phase packings exhibited different retention characteristics for complex standard mixtures. Columns which were totally unsuitable for the initial separation were found to be extremely useful in the separation of components which co-eluted with the peaks of interest in the initial plasma run. Application of the fractions collected from the initial separation to an appropriate second chromatographic separation permitted the resolution and quantification of the peaks of interest. Isocratic elution conditions were again used for the second chromatographic separation as this facilitated the use of automatic sampling procedures without the problems of washing/re-equilibration or loss of sensitivity due to baseline fluctuations associated with gradient elution.

EXPERIMENTAL

Apparatus

Chromatographic studies were conducted with Waters Assoc. (Milford, MA, U.S.A.) equipment comprising two Model 6000A solvent delivery systems, controlled by a Model 660 solvent programmer; a two-channel (254 nm and 280 nm) Model 440 absorbance detector or Model 450 variable-wavelength detector. A Model U6K universal injector was used for initial plasma runs and a Waters intelligent sample processor (WISP) Model 710 was used for the application of fractions eluted from these runs. Absorbance changes were monitored using a Rikadenki Model B381L three-pen recorder. Columns were thermostatically maintained at 23.5°C using a Haake model FE constant temperature circulating bath connected to a column temperature control block (Waters Assoc.).

Columns used for this study were 10-μm μBondapak C-18 (Waters Assoc.), 10-μm Lichrosorb RP-18 (Merck, Darmstadt, G.F.R.) and 5-μm Zorbax C-8 (Dupont, London, Great Britain). LiChrosorb RP-18 and μBondapak C-18 columns were packed by a method similar to that of Broquaire [31]. A Magnus P6000 slurry packing unit connected to a Micromeritics 705 slurry reservoir was used. A stirred slurry of packing material in methanol—7.4 m*M* sodium acetate (80:20) was forced upwards into the column by pumping methanol—7.4 m*M* sodium acetate (50:50) into the reservoir.

Chemicals

Nucleosides and bases used in these investigations were obtained from Sigma (London, Great Britain) with the exception of allopurinol (Burroughs Wellcome, London, Great Britain) and 4,6-dihydroxypyrazole (3,4-D)-pyrimidine (oxypurinol) (Aldrich Milwaukee, WI, U.S.A.). Other chemicals of analytical grade were purchased from the following sources: methanol (James Burroughs, London, Great Britain); perchloric acid and potassium hydrogen carbonate (Hopkin & Williams, Chadwell Heath, Great Britain); acetic acid (Koch-Light, Colnbrook, Great Britain) and ammonia (Fisons, Loughborough, Great Britain).

Buffers

All buffers were prepared fresh daily using water from a Milli-QZD 20 230 00 reagent-grade water system (Millipore, Bedford, MA, U.S.A.). Water and buffers were filtered through a HA 0.45-μm filter (Millipore) and methanol through a 0.5-μm Fluoropore filter before use.

Sample preparation

Fresh heparinised blood samples were centrifuged at 600 *g* for 10 min. Plasma was collected on ice and 0.5 vol of 1 *N* perchloric acid added. The samples were first centrifuged at 2000 *g* for 10 min at 4°C. The supernatant was then subjected to a 10,000 *g* spin for a further 10 min at 4°C. This second centrifugation removed a fine haze which was apparent in many of the patients' plasma samples. The supernatants were then neutralised with solid potassium bicarbonate, and after standing on ice for 15 min, the potassium perchlorate precipitate was removed by filtration through a Millex 0.22-μm filter (Millipore).

Chromatography of deproteinised plasma samples

Initial separations were performed using a 300 mm × 4 mm I.D. μBondapak C-18 reversed-phase column (Waters Assoc.) running isocratically in 0.025 *M* ammonium acetate pH 5.0 at a flow-rate of 2 ml/min (Fig. 1). Injection volumes of 500 μl of deproteinised plasma were used, and appropriate fractions collected into sample bottles for the WISP and stored in batches for further analysis. Sample collection was determined by the elution profiles of appropriate standards injected onto the column immediately prior to the plasma run (Fig. 1a). Following the collection of the final fraction a 5-min gradient to 100% methanol was initiated. This served to wash lipophilic plasma

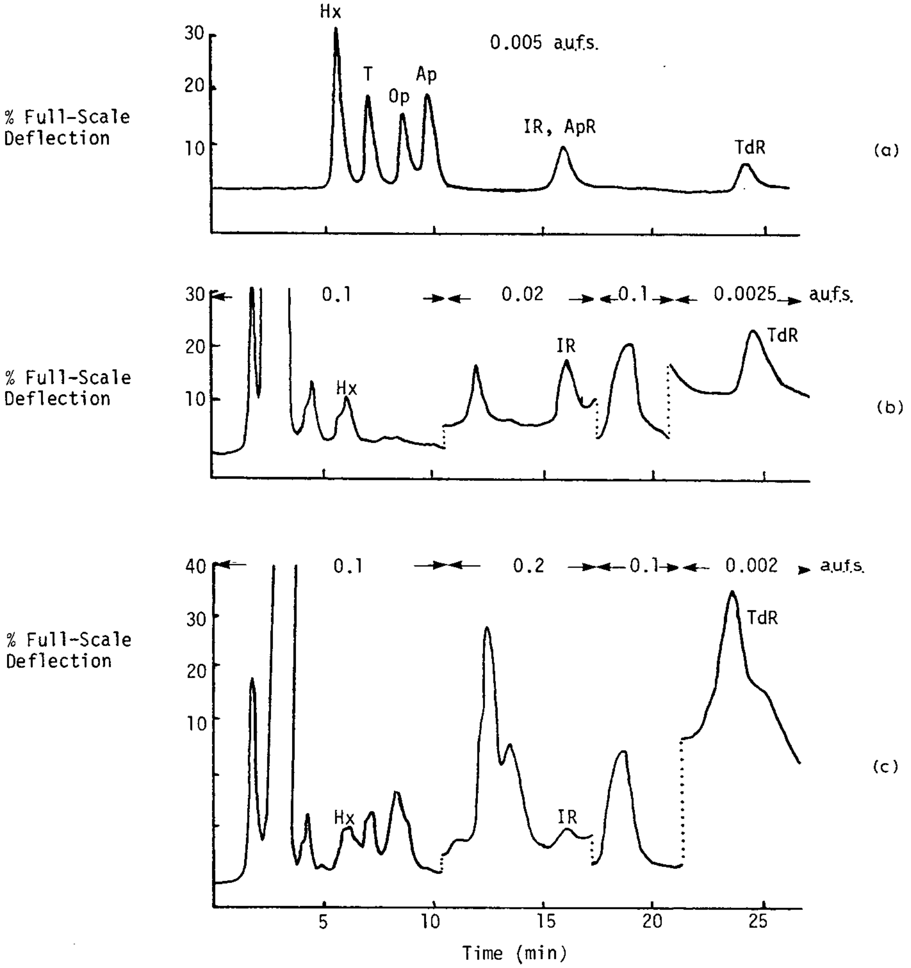

Fig. 1. Chromatograms obtained from the injection of 500-μl samples of (a) standards, (b) healthy human plasma extract, (c) patients' plasma extract onto a 300 mm × 4 mm I.D. μBondapak C-18 reversed-phase column eluted isocratically with 0.05 *M* ammonium acetate pH 5.0. Flow-rate 2 ml/min; temperature 23.5°C; detection 254 nm; a.u.f.s. as indicated between arrows. Peaks: HX, hypoxanthine; T, thymine; Op, oxypurinol; Ap, allopurinol; IR, inosine; ApR, allopurinol riboside; TdR, thymidine.

components from the column. Following the elution of these components a reverse gradient to buffer was selected and the column allowed to equilibrate before the application of further samples.

Analytical procedure

Fractions collected from the initial plasma runs were thawed and shaken to ensure homogeneity and placed in the carousel of the WISP together with a standard bottle in position 1. The WISP was then programmed to inject appropriate volumes of each sample together with reference injections of the standard after every five samples. The recorder was routinely used to monitor absorption at 254 nm at sensitivity settings of 0.02, 0.005 and 0.001 absorption units full scale (a.u.f.s.) allowing quantitation of a wide range of nucleoside or base concentrations in a single automated run.

Hypoxanthine analysis

For the quantitation of hypoxanthine a 300 mm × 4 mm I.D. LiChrosorb 10-μm RP-18 column was used in an isocratic system of 0.025 *M* ammonium acetate pH 5.0 with a flow-rate of 2 ml/min (Fig. 2). Sample volumes of 100 μl were used with a run time of 7 min which led to a total automated run time of about 8 h for the analysis of 40 samples.

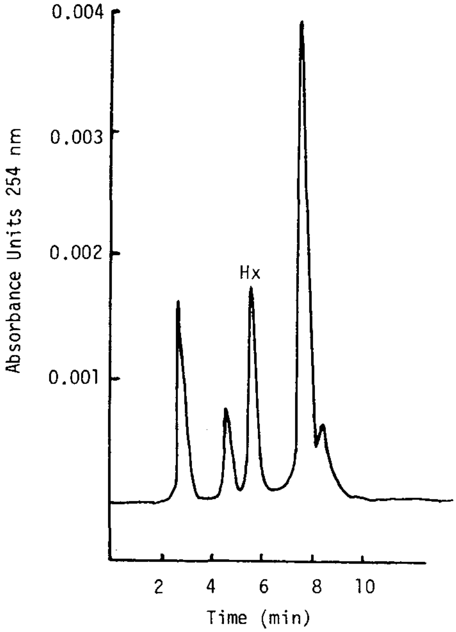

Fig. 2. Separation of hypoxanthine (Hx) from components which co-eluted with hypoxanthine during the initial plasma run. Column, 300 mm × 4 mm I.D. LiChrosorb 10 RP-18; injection volume 100 μl. Running conditions: isocratic elution with 0.025 *M* ammonium acetate pH 5.0 at a flow-rate of 2 ml/min; temperature 23.5°C.

Thymine, oxypurinol and allopurinol analyses

The fractions corresponding to thymine, oxypurinol or allopurinol were subjected to a second chromatographic separation using the same column and isocratic running conditions as for the initial plasma sample (Figs. 3—5). Using an injection volume of 100 μl with run times of 8, 9 and 11 min for thymine, oxypurinol and allopurinol respectively, 40 samples could be assayed within 10 h using the WISP.

Thymidine analysis

Thymidine was assayed using a Zorbax C-8 reversed-phase column running isocratically in 0.05 *M* acetic acid (Fig. 6); a 500-μl injection volume was used. With a run time of 18 min an automated run on the WISP allowed the assay of 40 samples within 16 h.

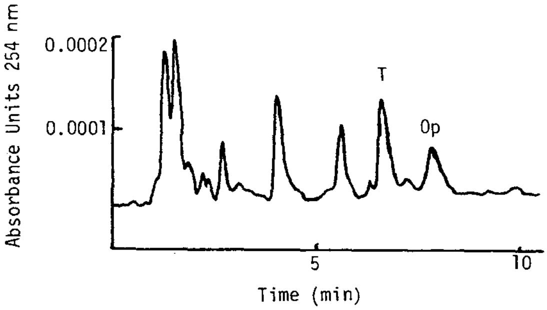

Fig. 3. Separation of thymine from components which co-eluted with thymine during the initial plasma run. Running conditions as for Fig. 1. Peaks: T, thymine and Op, oxypurinol.

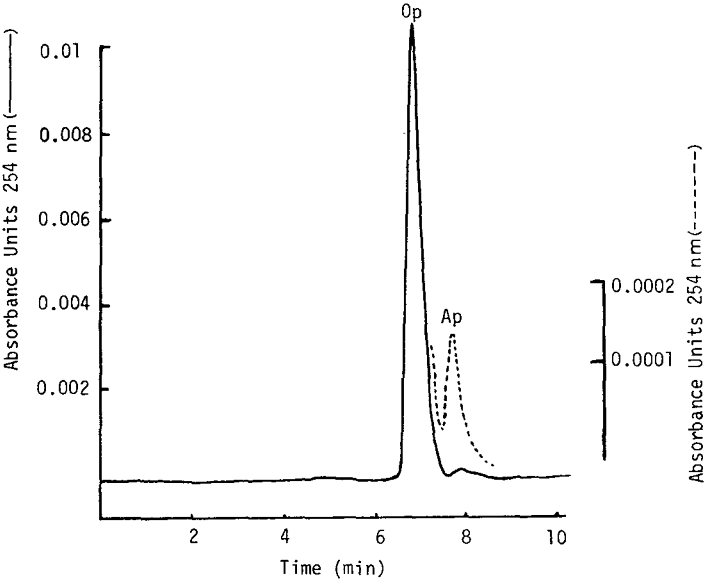

Fig. 4. Separation of oxypurinol from components which co-eluted with oxypurinol during the initial plasma run. Running conditions as for Fig. 1. Peaks: Op, oxypurinol and Ap, allopurinol.

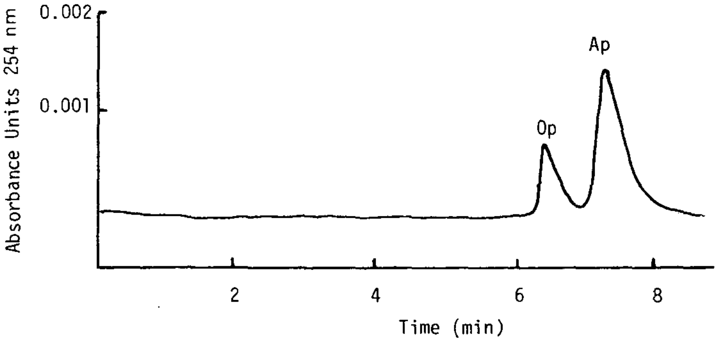

Fig. 5. Separation of allopurinol from components which co-eluted with allopurinol during the initial plasma run. Running conditions as for Fig. 1. Peaks: Op, oxypurinol and Ap, allopurinol.

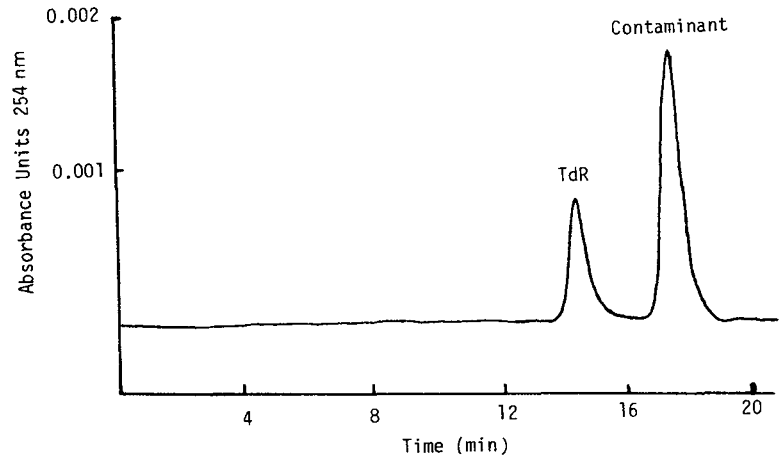

Fig. 6. Separation of thymidine (TdR) from an unidentified component which co-elutes with TdR during the initial plasma run. Column 300 mm × 4 mm I.D. Zorbax C-8; injection volume 500 μl. Running conditions: isocratic elution with 0.05 *M* acetic acid at a flow-rate of 2 ml/min; temperature 23.5°C.

Peak identification and quantification

During the evaluation of the method, fractions were collected from initial runs of plasma samples from mice, patients and healthy human volunteers. The conditions of the second chromatographic separation were varied until we were satisfied that each peak of interest was separated from any contaminant. This involved the comparison of retention times and 254/280 nm absorption ratios with standards under a range of column conditions. When large peaks were obtained, for example in samples from patients with high (ca. 50 *μM*) circulating hypoxanthine levels it was possible to compare absorption spectra using a Model 450 variable-wavelength detector (Waters Assoc.).

Quantification was achieved by the injection of 0.1—5000-μg samples of standard nucleosides and bases onto appropriate columns running under conditions identical to those used for sample separation.

Equivalent peak areas, defined as the product of the peak area and the full-scale sensitivity, were calculated for each sample and the data subjected to linear regression analysis weighted through the origin (Table I). From these

TABLE I

LINEAR REGRESSION ANALYSIS OF STANDARD CURVES FOR THE QUANTIFICATION OF HYPOXANTHINE, THYMINE, OXYPURINOL, ALLOPURINOL AND THYMIDINE

r = correlation coefficient of line $y = a + bx$; a = intercept; b = slope; c = confidence limits of slope.

	Hypoxanthine	Thymine	Oxypurinol	Allopurinol	Thymidine
r	0.99991	0.991	0.99622	0.9961	0.9988
a	0.09761	−2.5488	−1.27071	−1.44377	0.0368
b	0.07559	0.05325	0.04412	0.04954	0.0309
c	0.00062	0.00285	0.00154	0.00176	0.0007

regression lines calibration factors were obtained allowing the accurate measurement of plasma nucleoside or base levels of 10^{-7} M and above.

RESULTS AND DISCUSSION

The separation of a mixture of standards on a μBondapak C-18 reversed-phase column is shown in Fig. 1a. The importance of using patients' samples in evaluating the usefulness of a separation system cannot be over-emphasised, as chromatograms obtained from healthy human plasma samples (Fig. 1b) differ from those obtained from patients' plasma samples (Fig. 1c).

From initial chromatograms of healthy human plasma it is possible to estimate directly the circulating levels of inosine (IR) and thymidine; other components are resolved when appropriate fractions are re-chromatographed. The differences between the patients' and healthy human plasma samples were not uniform: for example approximately half the patients appeared to have an unknown plasma component which co-eluted with thymidine. Fractions collected from patients' initial plasma runs were used to select conditions which would separate thymidine from its unknown contaminants (Fig. 6). This contaminant does not co-elute with a wide range of nucleosides or bases, including the following: adenine, 1-methyladenine, adenosine, 1-methyladenosine, deoxyadenosine, cytosine, 1-methylcytosine, 5-methylcytosine, cytidine, 3-methylcytidine, deoxycytidine, guanine, 7-methylguanine, N-methylguanine, 2-dimethylguanine, guanosine, 1-methylguanosine, 7-methylguanosine, N^2-methylguanosine, deoxyguanosine, thioguanine, 6-mercaptopurine, 6-mercaptopurine riboside, uric acid, xanthine, 1-methylhypoxanthine, 1-methylinosine, 7-methylinosine, uracil, 5-ethyluracil, 5-butyluracil, 5-hexyluracil, 5-fluorouracil, uridine, deoxyuridine, 5-ethyldeoxyuridine, 5-butyldeoxyuridine, 5-hexyldeoxyuridine, pseudouridine.

Circulating levels of inosine were not measured in patients' samples, since they received allopurinol and it is difficult to preclude the presence of allopurinol riboside (ApR, Fig. 1a) as a possible contaminant of the inosine fraction.

Fractions collected on each side of a peak of interest were assayed for any spillover which could occur in samples containing high nucleoside or base levels. This is illustrated by the presence of oxypurinol in the thymine (Fig. 3) and allopurinol (Fig. 5) fractions. As an indication of the sensitivity of the method the thymine and oxypurinol peaks in Fig. 3 and the allopurinol peak in Fig. 5 represent 1.9, 1.43 and 3.66 ng, respectively.

Fig. 7 shows the data obtained from a patient who received an infusion of 27.2 g of thymidine and 2.72 g of inosine over a 47-h period. The plasma from this patient was free of components which co-eluted with thymidine on the initial run, and the close agreement between estimations from the initial run and those obtained from re-running the fractions on the second chromatographic system indicates that this method is both accurate and sensitive (Table II).

The study has revealed features of interest in relation to nucleoside rescue. For example, inspection of Fig. 7 reveals that although high levels of thymidine are infused, only relatively low concentrations (< 3 μM) of this nucleoside are detected, while much higher levels of thymine (>10 μM) are achieved during

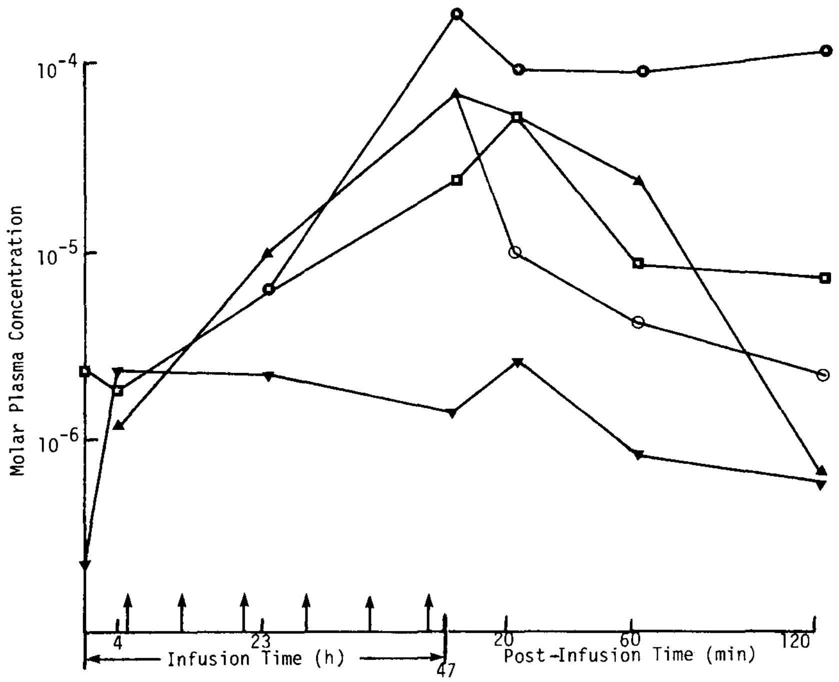

Fig. 7. Plasma concentrations of the nucleosides and bases of interest in a patient receiving an infusion of 2.72 g of inosine and 27.2 g of thymidine over a 47-h period together with 6 doses of allopurinol (200 mg orally at the times indicated by arrows). ■, Hypoxanthine; ▲, thymine; ●, oxypurinol; ○, allopurinol; ▼, thymidine.

TABLE II

COMPARISON OF THYMIDINE LEVELS ESTIMATED FROM INITIAL PLASMA RUNS (A) OR AFTER THE SECOND CHROMATOGRAPHIC SEPARATION (B)

Infusion time (h)	Post-infusion time (min)	Plasma TdR concentration (μM)	
		A	B
0		0.15	–
4		2.31	1.85
23		2.20	2.34
27		—	6.34
47	0	1.45	1.40
	20	2.67	2.50
	60	0.83	0.84
	120	0.60	0.35

the infusion. Clearly, considerable catabolism of thymidine occurs under these conditions, and our results confirm those of other investigators [32,33].

High levels of oxypurinol (10^{-4} M) circulate for considerable periods (>6 h) following the oral administration of allopurinol. The consequent inhibition of orotidylate decarboxylase [34] might alter significantly the efficacy of concomitantly administered antimetabolites such as 5-fluorouracil [35], azacytidine [36], N(phosphonacetyl)-L-aspartate [37].

Circulating plasma levels of nucleosides and bases were measured in pretreatment plasmas, and in samples obtained from healthy volunteers (Table III). Hypoxanthine levels in the patients' samples fell into two distinct groups, a high group (ca. 50 μM Hx) and a low group (ca. 2.5 μM Hx). Comparison of plasma Hx in the low group and in healthy volunteers (ca. 1.6 μM) shows a significant difference ($p = 0.006$).

TABLE III

CIRCULATING PLASMA LEVELS OF NUCLEOSIDES AND BASES IN HEALTHY HUMAN VOLUNTEERS AND PATIENTS PRIOR TO TREATMENT

	Nucleoside (base)	Number of samples	Mean ± SEM (μM)	Range (μM)
Healthy human plasma	Hx	13	1.2 ± 0.20	0.51—2.81
	T	14	<0.1	<0.1
	IR	14	0.68 ± 0.10	0.26—1.56
	TdR	12	0.43 ± 0.06	0.10—0.92
Patient plasma (pretreatment)	Hx (low)	20	2.62 ± 0.32	0.85—6.50
	Hx (high)	5	52.40 ± 2.30	49.80—64.20
	T	25	<0.1	<0.1
	TdR	12	0.17*	<0.1—2.05

*Median.

In addition to its use in monitoring circulating purines and pyrimidines in patients undergoing clinical study, this method has proved to be highly versatile. Perhaps its most useful minor application has been in the purification of radiolabelled compounds since it allows a simple one-step purification with only limited dilution. In this respect it was particularly useful with relatively unstable isotopes e.g. [^{3}H]thymidine; background counts could be reduced substantially and accurate specific activities determined.

The method is economical with respect to columns, several components being isolated from a single 500-μl injection of biological extract. Indeed the chromatograms shown in Fig. 1 were obtained from a column which had already been used for ten 500-μl plasma samples and was originally prepared using packing material obtained from old columns which had lost their resolution. In addition, as an aqueous isocratic system is used, baseline fluctuations are eliminated, even at very high recorder sensitivities and the fractions collected are of a constant composition lending themselves readily to lyophilisation as the buffers used are volatile.

ACKNOWLEDGEMENTS

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CHROMBIO. 626

THE DETERMINATION OF BIFUNCTIONAL COMPOUNDS

IX*. A SELECTIVE REACTION FOR THE DETERMINATION OF GUAIFENESIN IN PLASMA BY GAS CHROMATOGRAPHY

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SUMMARY

Guaifenesin after extraction from plasma with an organic solvent can be selectively derivatized with 2,4-dichlorobenzeneboronic acid and determined by gas chromatography with electron-capture detection. The detection limit for guaifenesin was 15 ng ml^{-1} for a 2.0-ml plasma sample. The mass spectra of the boronate derivatives of guaifenesin and mephenesin, used as internal standard, show good molecular ions with characteristic modes of fragmentation useful for their identification.

INTRODUCTION

Guaifenesin [guaiacol glyceryl ether, 3-(*o*-methoxyphenoxy)-1,2-propanediol] is a widely used expectorant and demulcent in many asthma- and cough-preventive medicines. Other uses are as a muscle relaxant [1], as a hypocholesteremic reagent [2,3], as an agent for reducing platelet adhesiveness [4] and as a general equine anesthetic [5—7].

Guaifenesin has been determined in pharmaceutical formulations by direct current polarography after nitration [8], by a colorimetric reaction with formaldehyde in sulfuric acid—methanol [9], by gas chromatography (GC) without derivatization [10] and as its acetate [11] and trimethylsilyl ether [12] derivatives and by high-performance liquid chromatography [13,14]. In cough syrups these methods provide adequate detection limits for the relatively high concentrations (e.g. 20 mg ml^{-1}) of drug in the formulations but lack the necessary sensitivity required to determine trace levels in plasma. The

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*For Part VIII, see ref. 19.

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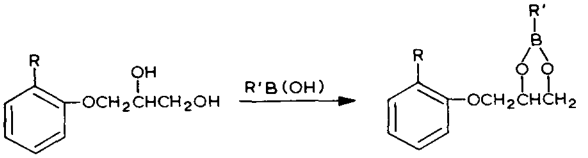
blood levels of guaifenesin in dogs [15] and rabbits [16] has been studied at rather high dose levels using relatively non-selective spectroscopic methods for the analysis. Maynard and Bruce [17] have described a sensitive method for the determination of guaifenesin in plasma by solvent extraction and derivatization of one of the hydroxyl groups of the propanediol side chain with heptafluorobutyric anhydride for analysis by GC with electron-capture detection. This method provided adequate sensitivity for monitoring the fate of the drug (plasma half-life 1.00 h) in volunteers given oral doses of 600 mg. However, the non-specific nature of the derivatization reaction produces many detected peaks in the plasma chromatogram often making quantitation difficult. Also, the anhydride must be freshly purified to avoid extraneous peaks, all traces of moisture must be absent during the derivatization reaction and all traces of the reagent must be removed prior to injection. The presence of excess reagent in the solution injected into the gas chromatograph causes a large disruption of the detector baseline in the region of the chromatogram where guaifenesin elutes.

One approach to simplifying the analysis of guaifenesin in biological fluids would be to take advantage of the bifunctional nature of the molecule and employ a chemically specific reagent to carry out the derivatization reaction. The boronic acids are suitable for this purpose as they form thermally stable derivatives with diols but do not produce derivatives stable to GC with monofunctional compounds [18]. Also if boronic acids containing electron-capturing groups are employed then advantage can be taken of the high sensitivity and selectivity of the electron-capture detector [19—24]. Thus by a combination of reagent specificity, chromatographic resolving power and detector selectivity it should prove possible to determine guaifenesin at therapeutic levels in a complex biological fluid like plasma, employing the minimum number of sample isolation and purification steps.

The method for the analysis of guaifenesin described in this paper uses mephenesin as internal standard and a simple extraction from plasma into an organic solvent with selective derivatization of the drug in the extract prior to gas chromatography with electron-capture detection.



R = OCH_3, guaifenesin
R = CH_3, mephenesin

R' = 2,4- dichlorobenzene
R' = 3,5- bis(trifluoromethyl) benzene
R' = 4- bromobenzene
R' = 4- iodobutane

EXPERIMENTAL

Reagents

Guaifenesin and mephenesin were obtained from Ciba-Geigy (Basle, Switzerland). 2,4-Dichlorobenzeneboronic acid, 3,5-bis(trifluoromethyl)benzenoboronic acid and 4-iodobutaneboronic acid were obtained from Lancaster Synthesis (St. Leonard Gate, Lancaster, Great Britain) or from the Alfa Products Division, Ventron Corp. (Danvers, MA, U.S.A.). 4-Bromobenzeneboronic acid was obtained from Aldrich (Milwaukee, WI, U.S.A.). Ethyl acetate and hexane were of nanograde quality and dichloromethane was certified ACS grade.

Standard solutions

Guaifenesin. Guaifenesin (25.00 mg) was dissolved in 50.00 ml of distilled water; 100 μl of this solution was diluted to 50.00 ml with water to give a final concentration of 5 μmole l^{-1}.

Mephenesin. Mephenesin (25.00 mg) was dissolved in 50.00 ml of distilled water; 185 μl of this solution was diluted to 10.00 ml with water to give a final concentration of 50 μmol l^{-1}.

2,4-Dichlorobenzeneboronic acid. 2,4-Dichlorobenzeneboronic acid (25.00 mg) was dissolved in 50.00 ml of ethyl acetate; 1.00 ml of this solution was diluted to 25.00 ml with ethyl acetate to give a final concentration of 105 μmole l^{-1}.

Buffer solution pH 6.5. The buffer solution was prepared by mixing 100.00 ml of 0.3 *M* Na_2HPO_4 with 29.00 ml of 1.0 *M* NaH_2PO_4.

Plasma samples. Plasma samples were obtained from the Texas Medical Center Blood Bank and fortified by making standard additions of the dilute aqueous guaifenesin standard. The plasma-containing drug samples were then divided into 2.00-ml aliquots and frozen at —20°C until used.

Extraction and derivatization

To a thawed 2.00-ml plasma sample in a 13.0 × 1.5 cm I.D. Pyrex PTFE-lined screw-capped culture tube was added 50 μl of internal standard (mephenesin, 50 μmole l^{-1}), 0.20 ml of phosphate buffer (pH 6.5) and 10.00 ml of hexane—dichloromethane (4:1). The tube was shaken mechanically for 10 min and centrifuged. A 5.00-ml aliquot of the organic phase was withdrawn by pipette and evaporated to a residue with a stream of nitrogen at approximately 50°C in a conical tipped culture tube. To the residue was added 1.00 ml of the 2,4-dichlorobenzeneboronic acid solution, the solution mixed thoroughly and the capped tube heated for approximately 5.0 min at 50°C. The solution was evaporated to dryness with nitrogen, 200 μl of hexane added and after mixing 1.0—2.0 μl was injected into the gas chromatograph.

Gas chromatography

For GC a Varian 3700 gas chromatograph equipped with a flame ionization and a pulse-modulated constant-current ^{63}Ni electron-capture detector was used. For analysis a 1.5 m × 0.2 cm I.D. glass column packed with 3% OV-17 on Gas-Chrom Q (100—120 mesh) and a nitrogen flow-rate of 30 ml min^{-1} was used. The oven temperature was 240°C isothermal, the injection temperature 270°C and the electron-capture detector temperature 350°C. For the calculation of Kovats indices a 100 cm × 0.2 cm I.D. nickel column packed with 3% SE-30 on Gas-Chrom Q (100—120 mesh) at 140°C and a nitrogen flow-rate of 60 ml min^{-1} was used.

For gas chromatography—mass spectrometry (GC—MS), a Hewlett-Packard

5992A mass spectrometer equipped with a single-stage glass-jet separator and a 6 ft. × 0.4 cm I.D. glass column packed with 3% OV-1 on Gas-Chrom Q (100—120 mesh) and operated with a helium flow-rate of 30 ml min^{-1} was used. Electron-impact mass spectra were recorded at an ionization potential of 70 eV.

RESULTS AND DISCUSSION

The solvent system hexane—dichloromethane (4:1) was selected for the extraction of guaifenesin and mephenesin as it is efficient while at the same time minimizing the amount of co-extractants and water which interfere in the chromatographic step. Guaifenesin and mephenesin are quantitatively derivatized by reaction with a slight excess of the boronic acid in ethyl acetate for either 15 min at room temperature or 5 min at 50°C. The Kovats indices for the boronate derivatives are given in Table I. All derivatives have good peak shape on GC and are stable in solution for at least two days at room temperature. The identity of the derivatives was confirmed by GC—MS. The electron-impact mass spectra of the 2,4-dichlorobenzeneboronate of guaifenesin (Fig. 1) and mephenesin (Fig. 2) are typical of all the derivatives studied. Characteristic modes of fragmentation are observed in all mass spectra with a prominent molecular ion and a base peak at *m/e* 108 for mephenesin and *m/e* 124 for guaifenesin (Fig. 3).

The plasma extracts show several peaks on GC in the area of the chromatogram where guaifenesin and mephenesin boronate derivatives elute. The exception is the 2,4-dichlorobenzeneboronate derivatives which elute in a relatively empty region of the chromatogram and are not interfered with. Fig. 4 shows a chromatogram for a plasma blank and a plasma sample containing approxi-

TABLE I

KOVATS INDICES ON SE-30 FOR THE BORONATE DERIVATIVES OF GUAIFENESIN AND MEPHENESIN

Boronate derivative	Kovats index	
	Guaifenesin	Mephenesin
2,4-Dichlorobenzeneboronate	2489	2420
3,5-Bis(trifluoromethyl)benzeneboronate	2081	1972
4-Bromobenzeneboronate	2455	2357
4-Iodobutaneboronate	2319	2210

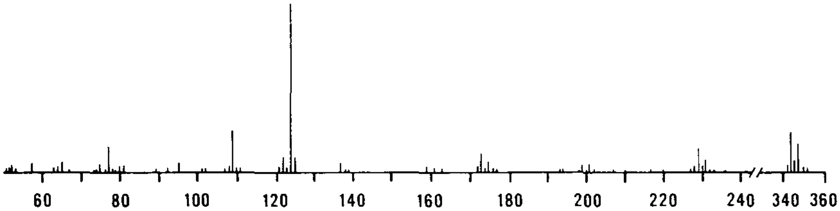


Fig. 1. Electron-impact mass spectrum of the 2,4-dichlorobenzeneboronate of guaifenesin at 70 eV.

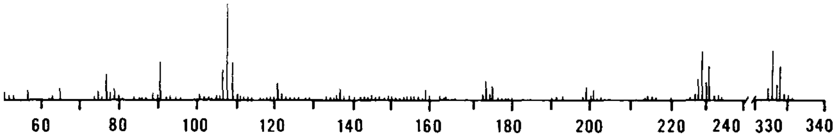


Fig. 2. Electron-impact mass spectrum of the 2,4-dichlorobenzeneboronate derivative of mephenesin at 70 eV.

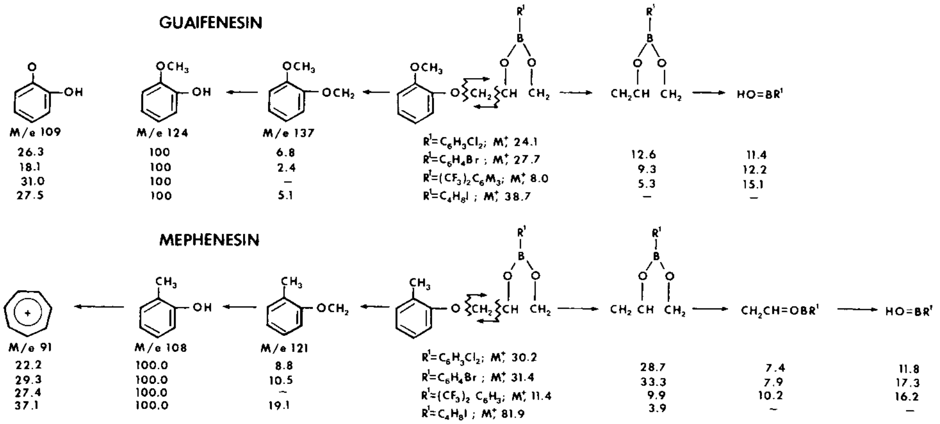
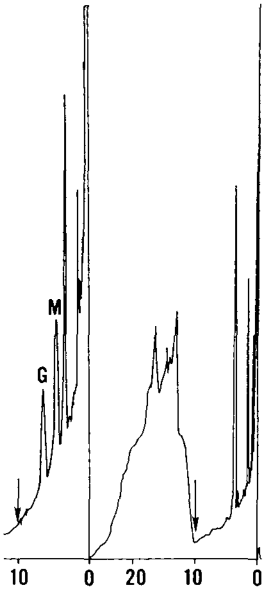

GUAIFENESIN
OCH3
OH
OCH2
O
CH2
CH
CH2
R'
B
HO=BR'
M/e 109
26.3
18.1
31.0
27.5
M/e 124
100
100
100
100
M/e 137
6.8
2.4
–
5.1
R¹=C6H3Cl2; M⁺ 24.1
R¹=C6H4Br ; M⁺ 27.7
R¹=(CF3)2C6M3; M⁺ 8.0
R¹=C4H8I ; M⁺ 38.7
12.6
9.3
5.3
–
11.4
12.2
15.1
–
MEPHENESIN
CH3
+
CH2CH=OBR'
M/e 91
22.2
29.3
27.4
37.1
M/e 108
100.0
100.0
100.0
100.0
M/e 121
8.8
10.5
–
19.1
R¹=C6H3Cl2; M⁺ 30.2
R¹=C6H4Br ; M⁺ 31.4
R¹=(CF3)2 C6H3; M⁺ 11.4
R¹=C4H8I ; M⁺ 81.9
28.7
33.3
9.9
3.9
7.4
7.9
10.2
–
11.8
17.3
16.2
–

Fig. 3. Characteristic modes of fragmentation under electron-impact of the 2,4-dichlorobenzeneboronate derivatives of guaifenesin and mephenesin.

mately 1.00 ng each of guaifenesin (G) and mephenesin (M) derivatives. The arrow marks the point where the column temperature was increased from 240°C to 290°C ballistically and maintained at the higher temperature for 10 min. This treatment was required to rapidly elute less volatile plasma constituents and excess reagent from the column before the next injection could be made. This treatment does not damage the column or adversely affect the long-term stability of the detector.

The response of the electron-capture detector is markedly temperature dependent, a phenomenon arising from the mechanism of the electron-capture process itself [25]. The 2,4-dichlorobenzeneboronate derivative of guaifenesin captures electrons by a dissociative mechanism giving its maximum response to the detector at high detector temperatures (Fig. 5, B). This should be compared to the 3,5-bis(trifluoromethyl)benzeneboronate derivative of guaifenesin (Fig. 5, C) which captures electrons by a non-dissociative mechanism with its maximum detector response being obtained with low detector temperatures. With a detector temperature of 375°C, the minimum detectable quantity of guaifenesin as its 2,4-dichlorobenzeneboronate derivative was $25 \cdot 10^{-12}$ g.

A linear calibration curve was obtained for guaifenesin added to plasma using mephenesin as internal standard over the investigated range 0.1—2.0 ng (Fig. 6). Each point is the average of five independent determinations and the bars on the graph represent one standard deviation. At the 0.1-ng level the relative standard deviation was 12% and at the 1.0-ng level it was 7%.



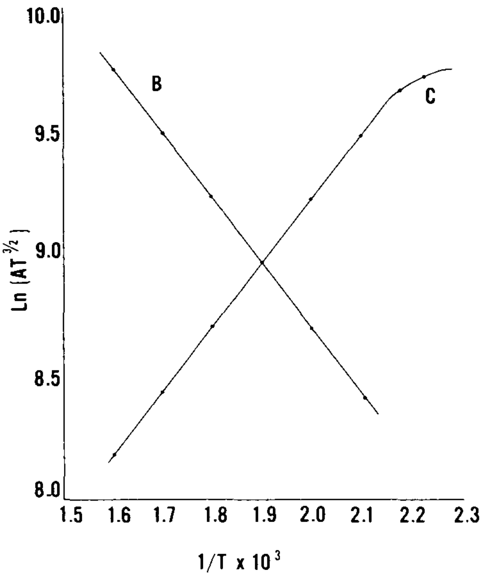


Fig. 4. Gas chromatogram with electron capture detection of a plasma blank and a plasma sample containing approximately 1.0 ng each of guaifenesin (G) and mephenesin (M) as their 2,4-dichlorobenzeneboronates.

Fig. 5. Temperature dependence of the electron-capture detector response for the 2,4-dichlorobenzeneboronate of guaifenesin (B) and the 3,5-bis(trifluoromethyl)benzeneboronate of guaifenesin (C). A = peak area for a fixed mass of derivative and T = detector temperature in °K.

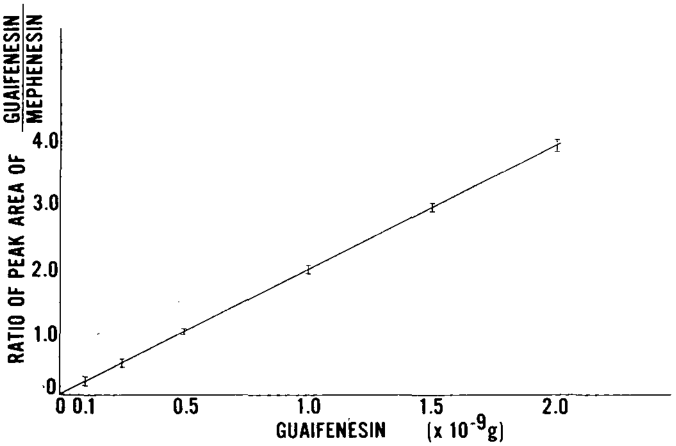


Fig. 6. Calibration curve for guaifenesin in plasma.

The detection limit corresponds to a concentration of approximately 15 ng ml^{-1} of guaifenesin in plasma assuming a 2.0-ml sample. This detection limit is adequate for pharmacological studies of this drug. However, lower detection limits could be obtained if desired by making appropriate adjustments to the experimental procedure described here.

ACKNOWLEDGEMENT

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CHROMBIO. 627

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC AND GAS—LIQUID CHROMATOGRAPHIC DETERMINATION OF DIAZEPAM AND NORDIAZEPAM IN PLASMA*

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SUMMARY

A one-step method for extraction of diazepam, nordiazepam, and internal standard into toluene is followed by chromatographic separation and detection with either dual-wavelength high-performance liquid chromatography or electron-capture gas—liquid chromatography. Agreement between the two methods was excellent for diazepam ($r = 0.99$, $n = 38$) and good for nordiazepam ($r = 0.96$, $n = 79$) over a concentration range that included subtherapeutic, therapeutic, and toxic plasma levels.

INTRODUCTION

The benzodiazepines are widely used as antianxiety, antispasmodic, and antiepileptic drugs [1]. Patients treated chronically with diazepam exhibit significant plasma concentrations of the active metabolite nordiazepam, as well as the parent drug. The pharmacological actions of two newer benzodiazepines, clorazepate and prazepam, are a result of their biotransformation to nordiazepam [2, 3]. Thus techniques which measure plasma levels of diazepam and nordiazepam are sufficient for plasma level monitoring in patients receiving diazepam, clorazepate, or prazepam. The minimum therapeutic level for diazepam in treatment of acute anxiety is reported to be 0.4 µg/ml [4]; the minimum therapeutic level for diazepam or nordiazepam in epileptic patients is 0.5 µg/ml [5, 6].

In this communication we report a simplified extraction procedure for diazepam, nordiazepam, and internal standard which yields extracts suitable for

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analysis by high-performance liquid chromatography (HPLC) with dual-wavelength monitoring, or by gas—liquid chromatography (GLC) with electron-capture (EC) detection. The two methods of chromatographic separation and detection are compared, and a case study with monitoring of diazepam levels, after intravenous injection of diazepam for control of status epilepticus, is presented.

EXPERIMENTAL

Reagents

Diazepam, nordiazepam, prazepam and nitrazepam were obtained from Hoffmann-LaRoche (Nutley, NJ, U.S.A.). Stock solutions of each drug were prepared in methanol or ethanol (1 mg/ml). Working standard solutions were prepared by appropriate dilutions of stock solutions.

Acetonitrile, glass distilled and of ultraviolet grade was purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). All other reagents used were analytical grade and solvents were nanograde.

HPLC mobile phase was prepared by adding 360 ml acetonitrile to 640 ml of 10 mmol/l phosphate buffer pH 6.0. The solution was filtered through a 0.22-μm filter (type GS, Millipore, Bedford, MA, U.S.A.).

Instrumentation

A Hewlett-Packard Model 5713 gas chromatograph equipped with 15 mCi ^{63}Ni electron-capture detector was used. A 1.8 m × 2 mm I.D. glass column pretreated with 5% dimethyldichlorosilane in toluene packed with 3% OV-17 on Chromosorb W HP 80—100 mesh (Supelco, Bellefonte, PA, U.S.A.) was found most suitable. The column was conditioned by heating at 325°C for 4 h without carrier gas flow, then at 265°C for 12 h with full carrier gas flow. The following gas chromatographic parameters were used: carrier gas methane—argon (5:95), flow-rate 30 ml/min; column temperature 265°C isothermal; inlet temperature 250°C; detector temperature 350°C and attenuation was set at 512. The EC detector was removed from the gas chromatograph at least once a month and cleaned by rinsing with 200 ml absolute methanol.

A Waters Model 204 high-performance liquid chromatograph was used. The flow cell of the Model 440 detector (254 nm) in this system was connected in series to Model 450 variable-wavelength detector (230 nm). A reversed-phase column μBondapak C_{18} (10 μm particle size) 30 cm × 4 mm I.D. was maintained at room temperature. A Waters guard column (3 cm) filled with Bondapak C_{18} Corasil (37—50 μm particle size) was connected to the inlet end of the column (all of the preceding components were obtained from Waters Assoc., Milford, MA, U.S.A.). The absorbance detectors were connected to a dual-pen recorder (Omniscribe, B-5000; Houston Instruments, Austin, TX, U.S.A.).

Procedure

Plasma (1.0 ml), internal standard (0.75 μg nitrazepam for HPLC, 1.00 μg prazepam for EC—GLC), 0.5 ml borate buffer pH 9.0, and toluene (5 ml for HPLC 3 ml for EC—GLC), were combined in glass-stoppered extraction tubes. The drugs were extracted by mechanical shaking of the tubes for 5 min. Following centrifugation, as much of the toluene phase as possible was transferred to a clean tube and evaporated to dryness. In the EC—GLC procedure, the residue was resuspended in 100 μl of acetone—hexane (20:80, v/v) and 5 μl were injected into the chromatograph. In the HPLC procedure, the residue was dissolved in 50 μl of methanol, and 5 μl were injected into the liquid chromatograph with a mobile phase flow-rate of 2.4 ml/min. The column effluent was monitored at 230 nm (0.01 a.u.f.s.) and 254 nm (0.005 a.u.f.s.).

RESULTS AND DISCUSSION

Chromatograms from our methods are presented in Figs. 1 and 2. Fig. 1A shows a serum blank carried through the procedure and analyzed by HPLC. The chromatogram in Fig. 1B is serum supplemented with diazepam, nordiazepam and nitrazepam (internal standard). Similar chromatograms are obtained by EC—GLC as is shown in Fig. 2.

The concentration of diazepam and nordiazepam in both methods was determined from standard curves using the peak height ratio method (height of the drug/height of internal standard, vs. drug concentration). In the HPLC method

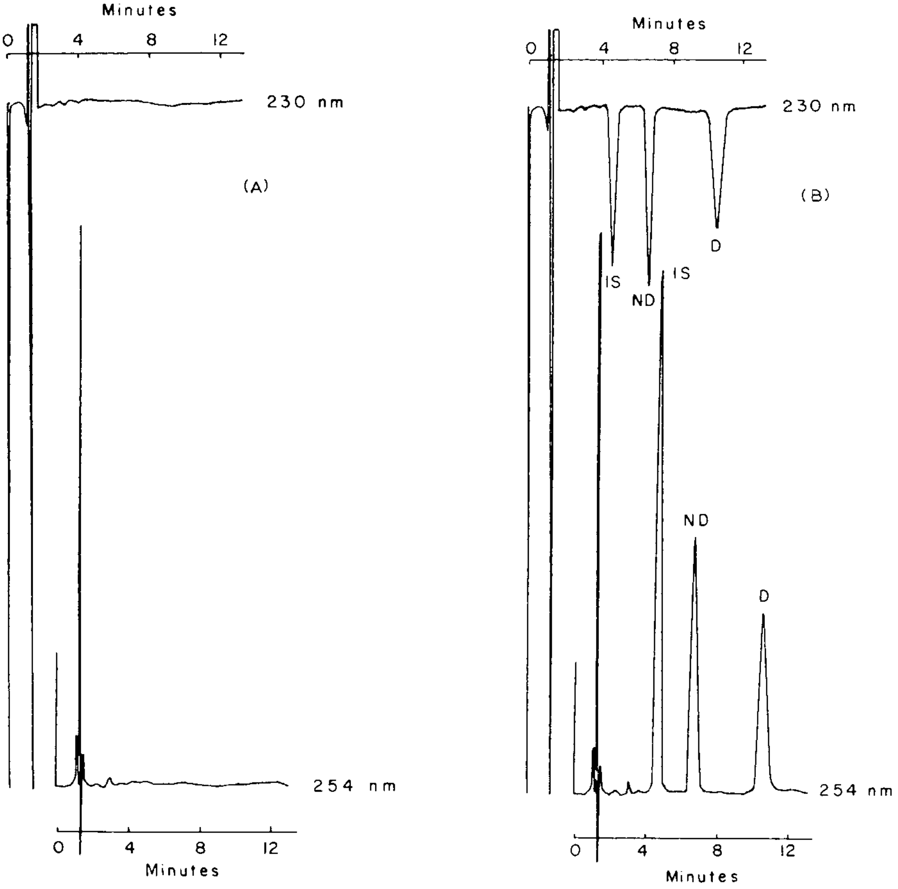

Fig. 1. HPLC chromatograms of plasma blank (A) and plasma extract (B) containing nitrazepam (IS), nordiazepam (ND), and diazepam (D).

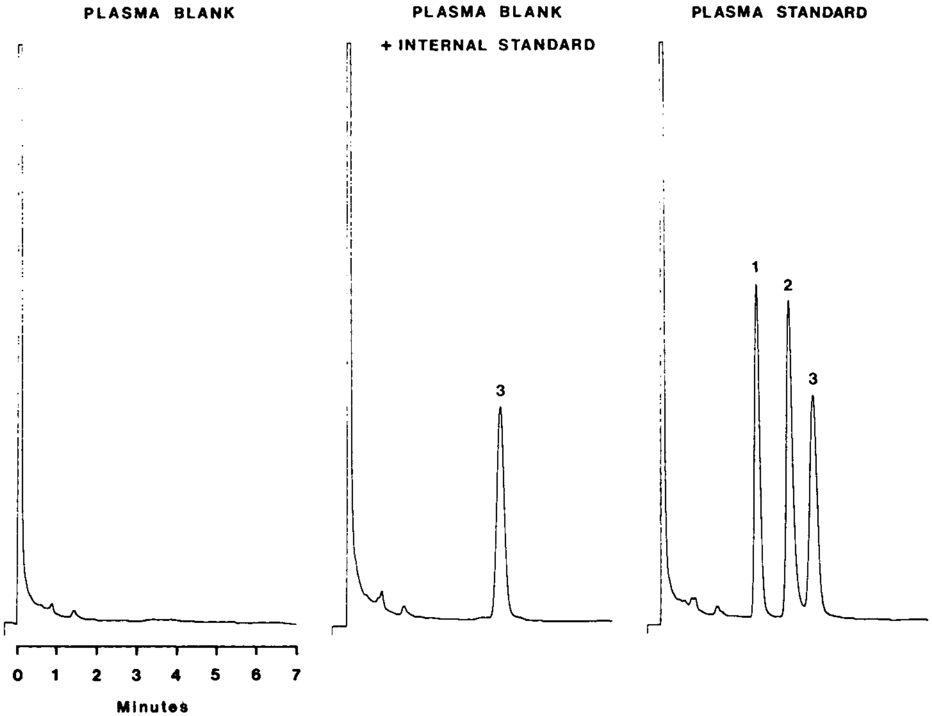

Fig. 2. GLC chromatograms of plasma blank, blank plus internal standard (prazepam) and standard containing diazepam (1), nordiazepam (2), and prazepam (3). Note that there is no apparent decomposition of prazepam to nordiazepam on column.

absorbance at 254 nm was used for quantitation. The peak height ratios are linearly related to drug concentration up to at least 5 μg/ml for each method.

After correction for aliquot loss, the recovery of each of the benzodiazepines studied was greater than 90% with the one-step toluene extraction in both methods.

The precision of the methods was evaluated both within-run and between-runs and was found to be acceptable. The within-run coefficient of variation (C.V.) by the HPLC method was 1.6% for both diazepam and nordiazepam with a mean concentration 2.5 μg/ml (n = 10). At a concentration of 0.7 μg/ml the C.V. was 1.5% and 7.1% for nordiazepam and diazepam respectively (n = 6). The C.V. by GLC was 1.4% for nordiazepam ($\bar{X}$ = 0.5 μg/ml) and 0.4% for diazepam ($\bar{X}$ = 0.5 μg/ml, n = 6). The between-run precision by HPLC was 4.3% for both nordiazepam and diazepam (n = 15) at a mean concentration of 0.7 μg/ml. At the concentration of 2.5 μg/ml the C.V. was 2.6% for nordiazepam and 3.6% for diazepam (n = 21). The between-run precision by GLC was 6.5% for diazepam at a concentration of 0.37 μg/ml and C.V. of 5.7% for nordiazepam at a concentration of 0.76 μg/ml (n = 16).

Correlation studies between the two methods using patient specimens (n = 38, diazepam and n = 79, nordiazepam) showed good agreement. The linear regression line was $y = 0.006 + 1.07\,X$ for nordiazepam with correlation coefficient r = 0.96 and standard error of estimate of 0.240 (Fig. 3). The linear

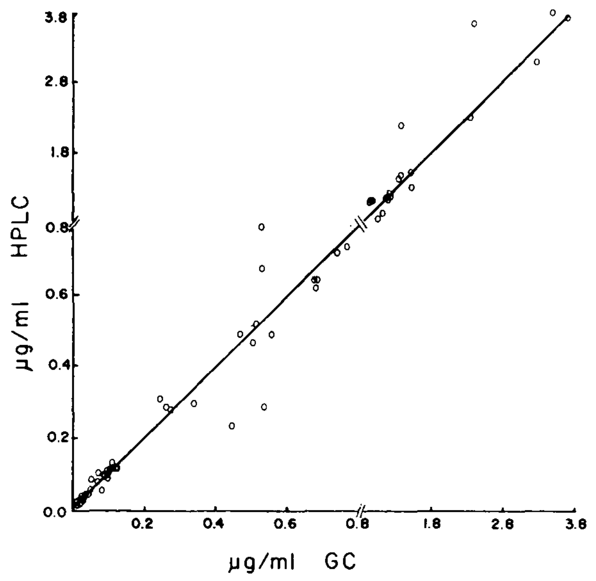

Fig. 3. Comparison of GLC and HPLC results for 79 nordiazepam samples.

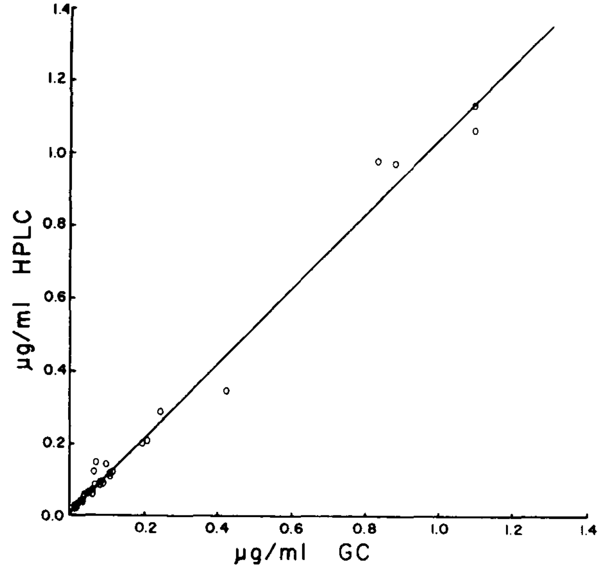

Fig. 4. Comparison of GLC and HPLC results for 38 diazepam samples.

regression line for diazepam was $y = 0.010 + 1.03\ X$ with correlation coefficient of 0.99 and standard error of estimate of 0.036 (Fig. 4).

In the HPLC method we monitored the column effluent at two different wavelengths, 230 nm and 254 nm. The peak height ratio (230/254) was calculated for each drug and internal standard. The ratio is characteristic for each drug and is independent of concentration. Deviation from the established ratio even though the peaks are symmetrical indicates an interfering substance is being co-eluted. Such samples should be reanalyzed by another method or the mobile phase should be modified to separate the interfering substance. The mean and standard deviation of ratios for diazepam, nordiazepam and internal standard are shown in Table I. There was some variation in the ratio from day to day and the ratio should be established daily from the standards. When the ratio for the drugs or internal standard deviates by 5% or more an interference from another substance is suspected.

TABLE I

ABSORBANCE RATIO MONITORING

n	Drug	Peak height ratio (230 nm/254 nm) ± S.D.
7	diazepam	1.67 ± 0.01
7	nordiazepam	1.74 ± 0.01
9	internal standard	0.84 ± 0.02

In addition to diazepam and nordiazepam, serum chlordiazepoxide concentrations can be determined using the proposed HPLC method. The precision of chlordiazepoxide determination was evaluated and the between-run C.V. was found to be 5.8% at a mean concentration of 0.77 μg/ml ($n = 15$).

None of the commonly prescribed anticonvulsant drugs interfered in the two methods. We have not encountered any interfering substances in the HPLC method by monitoring the two-wavelength ratio. The only drug interference observed in the EC—GLC method was caused by chlorpromazine, which has a retention time of 0.935 relative to diazepam, and is not fully resolved from diazepam in chromatograms where both compounds are present [7]. Use of prazepam as the internal standard in the EC—GLC procedure for determining nordiazepam levels in patients receiving prazepam might lead to an erroneous elevation in the internal standard peak height, and incorrectly low nordiazepam concentrations. However, no prazepam was detected in plasma of subjects receiving single doses of the compound, indicating that the biotransformation to nordiazepam is very rapid [3]. Several other unmarketed benzodiazepines are available from their manufacturers as alternative internal standards [8, 9].

Fig. 5 shows the serum concentration—time curve for a patient who received 5 mg diazepam intravenously for treatment of absence status epilepticus. Diazepam levels were determined by EC—GLC and verified by HPLC. After the injection the patient's seizures stopped and the electroencephalogram normalized for approximately 30 min, when behavioral and electroencephalographic seizures recurred at a diazepam level of 0.116 μg/ml (0.149 μg/ml nordiazepam was also present at this time, primarily due to administration of chlorazepate on the previous day). The dramatic effectiveness and brevity of action of diazepam in status epilepticus are substantiated by its serum level profile after intravenous injection [10].

Many benzodiazepine procedures have used benzene, or a combination of benzene and a more polar solvent, as extraction solvents [8, 9, 11—13]. We have substituted toluene for benzene in this procedure because it is equally efficient in extracting the compounds of interest, and poses a lower risk of chronic poisoning to technologists who are regularly exposed to its vapors [14].

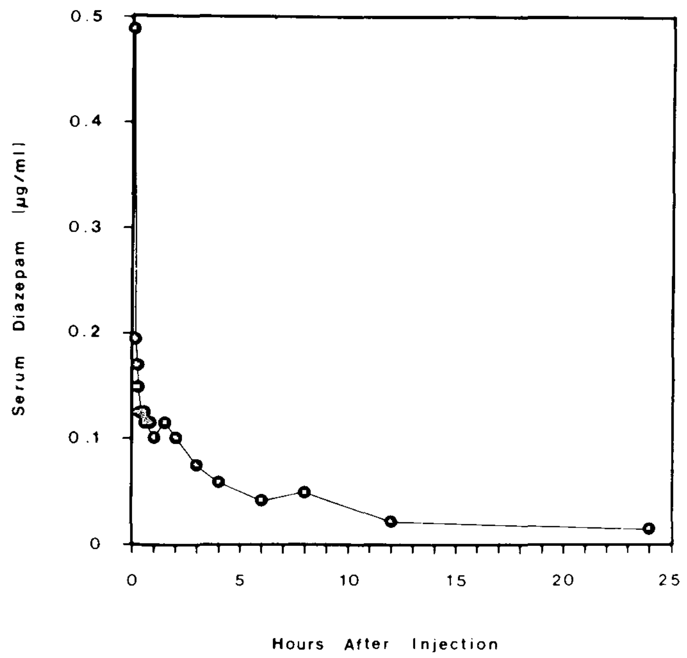

Fig. 5. Serum concentration—time profile for diazepam (5 mg intravenously in an epileptic patient (35 years old, male).

Toluene was used alone, since addition of more polar solvents did not improve the extraction yield of benzodiazepines, but resulted in more rapid contamination of the EC detector (presumably because the extraction residue contained more plasma lipids), which necessitated more frequent detector cleaning and maintenance.

The EC—GLC method described in this paper has been in routine use for two years, and has been adapted, with minor modifications, for the determination of clonazepam in serum [15]. The HPLC method has been in routine use for one year and continuing comparisons of HPLC and EC—GLC results have demonstrated reliable agreement between the two methods, over a range of concentrations that include subtherapeutic, therapeutic, and toxic plasma levels.

ACKNOWLEDGEMENTS

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CHROMBIO. 625

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF CYTOSINE ARABINOSIDE AND METABOLITES IN BIOLOGICAL SAMPLES

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SUMMARY

A rapid, non-radioactive method to quantitate therapeutically realistic levels of 1-β-D-arabinofuranosylcytosine (Ara-C) and its metabolites would be useful both in the clinic, for monitoring drug levels, and in the laboratory for correlating drug levels with cellular and molecular perturbations. Liquid chromatographic analysis of arabinose-nucleoside analogs in biological samples is complicated by the presence of interfering nucleosides and nucleotides. We report the development of two analytic procedures to measure Ara-C and metabolite levels in biological samples. One method uses a quaternary ammonium type anion-exchange resin to achieve isocratic separation in less than one hour. The second method utilizes a boronate-derivatized polyacrylamide column which binds *cis*-diols to selectively retain cytosine and uridine, while arabinose compounds are eluted with recovery approaching 100%. The eluted compounds are then easily quantitated on a reversed-phase C_{18} column. The sensitivity of both procedures was sufficient to obtain pharmacokinetic data on Ara-C and uracil-arabinose levels in serum and urine and on Ara-C triphosphate levels in tumor cells.

INTRODUCTION

The potential of pharmacokinetic parameters as predictors of response to cancer chemotherapeutic agents is becoming more apparent [1—4]. Information about drug levels, tissue distribution, and the persistence of drug metabolites in biological samples can be used to monitor drug effects at both the clinical and experimental level. Clinically, variation in drug metabolism and excretion patterns is believed to be one of the factors responsible for differences in drug response and toxicity between individual patients. In addition, since it has been shown that drug metabolism may change during a multiple dose therapeutic schedule [5], which is the common treatment regime for many cancers,

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it is important to be able to monitor drug levels during a course of therapy. Such information would allow drug dose adjustments to be made to accommodate inter- and intra-patient variation. Experimentally, pharmacokinetic information is needed to better understand the extent and duration of drug-induced biochemical and cellular perturbations.

1-β-D-Arabinofuranosylcytosine (Ara-C) is a pyrimidine nucleoside analog which is currently a component of several therapeutic drug combination protocols. It is metabolized to an active metabolite, Ara-C triphosphate (Ara-CTP) and to an inactive compound, 1-β-D-uracil arabinoside (Ara-U). High-performance liquid chromatography (HPLC) offers a rapid method to monitor levels of Ara-C and metabolites in tissues. However, chromatographic analysis of arabinose-nucleoside analogs in biological samples is complicated by interference of naturally occurring nucleosides and nucleotides. Most of the previously described chromatographic procedures cannot separate arabinose analogs from the ribose or deoxyribose compounds of cytosine and/or uridine [1, 6–9]. Therefore, in the past it has been necessary to use radioactively-labeled Ara-C, chromatography and subsequent liquid scintillation counting to separate and quantitate drug levels in biological samples. Rustum [6] described an HPLC technique to separate arabinose nucleosides; however, baseline separation was not achieved with all potentially contaminating compounds. Rustum and Preisler [3] used a different HPLC column to separate arabinose and ribose metabolites in biological samples; however, their technique still required a radioactively-labeled precursor since several of the separated compounds appeared as shoulders on major peaks, and the baseline showed considerable upward drift. These techniques tend to be time consuming and the expense and radioactivity of radiolabeled Ara-C limits their usefulness in obtaining pharmacokinetic information in animals and patients.

We have developed two methods to separate and quantitate Ara-C and its metabolites in biological tissues using HPLC. One method utilizes a quaternary ammonium anion-exchange resin (Aminex) to separate isocratically arabinose-containing analogs from all other interfering compounds. The usefulness of Aminex columns in nucleoside and nucleotide quantitation has been discussed in detail by Khym [10]. The second method utilizes a boronate-derivatized polyacrylamide resin to selectively retain cytosine, uridine and other *cis*-diols, while the arabinose-containing analogs are eluted. The eluted compounds are then easily quantitated on a reversed-phase C_{18} column. These methods were used to measure drug levels in mouse serum and urine and in tumor cells.

MATERIALS AND METHODS

Reagents and standards

Ara-C was purchased from Upjohn (Kalamazoo, MI, U.S.A.). Other arabinose standards, Ara-U and Ara-CTP, were obtained from Sigma (St. Louis, MO, U.S.A.). Cytidine, uridine and their triphosphate derivatives were obtained from P. & L. Biochemicals (Milwaukee, WI, U.S.A.). Ammonium acetate (Mallinckrodt, St. Louis, MO, U.S.A.), formic acid (J.T. Baker, Phillipsburg, NJ, U.S.A.), sodium citrate (Mallinckrodt) and sodium tetraborate decahydrate (Matheson, Cole and Bell, Norwood, OH, U.S.A.) were used in preparing buffers and aqueous solutions. All solutions used in the HPLC system were filtered through a membrane filter (0.45 μm) prior to use.

Instrumentation and HPLC columns

We used a Beckman chromatographic unit (Model 330) with a variable-wavelength ultraviolet detector (Model 450, Waters Assoc., Milford, MA, U.S.A.) set at 270 nm to detect Ara-C and metabolites. Aminex A-27 or A-29 (particle size 13.5 μm and 9.0 μm, respectively), strong anion-exchange resins (Bio-Rad Labs., Richmond, CA, U.S.A.) were used for isocratic separation of Ara-C and its metabolites from naturally occurring compounds. For nucleoside quantitation the A-27 material was slurry-packed at 140 bar into a stainless-steel column (500 mm × 4 mm I.D.) and nucleosides were eluted with a 0.025 *M* sodium citrate and 0.08 *M* sodium tetraborate buffer, pH 9.3 at a pressure of 105 bar, a flow-rate of 0.7 ml/min and at a temperature of 65°C. Nucleosides can also be eluted on a reversed-phase μBondapak (10 μm) C_{18} column (300 mm × 3.9 mm I.D.) (Waters Assoc.) with 0.01 *M* potassium phosphate, pH 5.6 at a flow-rate of 0.6 ml/min. A guard column (7 cm) packed with Co:pell ODS (Whatman, Clifton, NJ, U.S.A.) was used when analyzing biological samples to protect the analytical column from absorbing compounds. Nucleotides were eluted from a slurry-packed Aminex A-29 resin (200 mm × 4 mm I.D.) (see above) using 0.25 *M* sodium citrate, pH 8.2 at a flow-rate of 0.7 ml/min and at a temperature of 65°C.

Boronate affinity gel

In order to quantitate Ara-C and its metabolites on a reversed-phase C_{18} column, we found it necessary to use Affi-gel 601 to separate arabinose compounds from naturally occurring nucleosides. Affi-gel 601 (1.0 mequiv./g) was obtained from BioRad Labs. A slurry was made in 0.1 *M* sodium chloride and the resin (approximately 0.2 g) placed in a glass column (5 mm I.D.) to a height of 30 mm. The resin was then rinsed with 20 ml of 0.25 *M* ammonium acetate (pH 8.8) followed by 0.1 *M* formic acid, as described by Gehrke et al. [11]. Prior to application of the sample, the column was re-equilibrated with the ammonium acetate buffer. After the sample was applied to the resin, the arabinose-containing compounds were eluted with 8.0 ml of ammonium acetate. To remove the bound *cis*-diols, the column was washed with 8.0 ml of formic acid.

Biological samples

Arabinose-nucleoside levels were measured in mouse urine and plasma at various time intervals after administration of Ara-C in vivo. Ara-C (10 mg/ml) was dissolved in sterile saline and injected intraperitoneally into female C3H mice (8—12 weeks) at a dose of 100 mg/kg. Samples from 3—5 animals were pooled for each analysis. Urine samples were collected from ether-anaesthesized animals by bladder puncture and blood was withdrawn from the abdominal aorta with a heparinized syringe with care being taken to minimize red blood cell lysis. The plasma was removed from blood by centrifugation at 1000 *g* for 10 min at 4°C. Urine and plasma samples were stored at 0°C until subsequent cleanup procedures and analysis.

Prior to HPLC analysis, urine samples (200 μl) were passed through the Affi-gel column to remove the *cis*-diols. The ammonium acetate urine eluate was then injected directly onto the C_{18} reversed-phase column. Plasma samples were deproteinized by ultracentrifugation (Amicon conical ultrafilters, 2500 GE, Amicon, Lexington, MA, U.S.A.) and the ultrafiltrate then analyzed on the Aminex anion-exchange column (A-27).

Nucleotide levels were measured in KHT tumor cells exposed to Ara-C in vitro. These mammalian cells were derived from the solid murine KHT tumor [12] and adapted in our laboratory to grow in monolayers. Ara-C was added to KHT cells (approximately $2 \cdot 10^7$ cells) in exponential growth phase and aliquots of the culture removed at specified intervals after drug administration. These samples were washed twice with cold phosphate-buffered saline, pH 7.2 and protein precipitated overnight in 60% methanol at 0°C. After centrifugation (700 *g*) the supernatant was evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 100 μl of 0.25 *M* sodium citrate buffer, pH 8.2. Ara-CTP levels were then quantitated on the Aminex (A-29) column.

Concentrations of standards and drugs in biological samples were quantitated using peak height measurements. Maximum sensitivity for peak height quantitation was considered to be five times the background noise level. Samples were analyzed at 0.10 a.u.f.s.

RESULTS

Analytical conditions

Fig. 1a illustrates the separation of a standard pyrimidine nucleoside mixture achieved in less than one hour by isocratic elution from the A-27 anion-exchange column. Aproximately 2.5 ng Ara-C and 10 ng Ara-U can be detected minimally in this system. Ara-C and Ara-U are also separated on a reversed-phase C_{18} column in less than 20 min as shown in Fig. 1b. Although the C_{18} column is unable to resolve Ara-C and Ara-U from cytosine and uridine, respec-

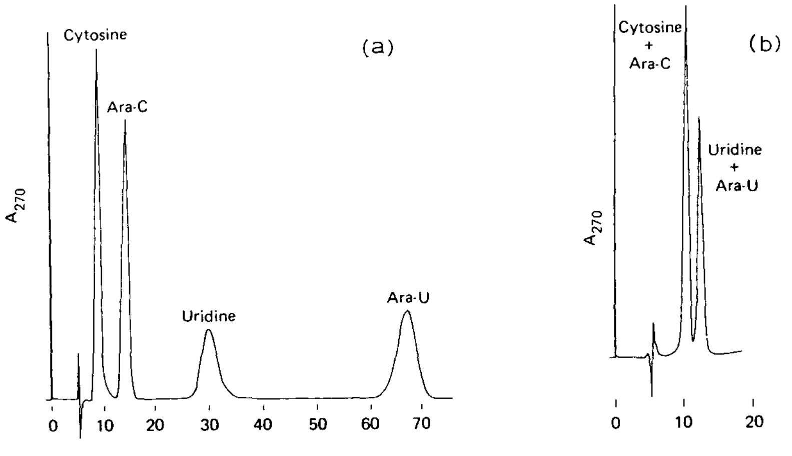

Fig. 1. Chromatogram of a nucleoside mixture containing Ara-C, Ara-U, cytosine and uridine on the ion-exchange (A-27) resin (a) and on the reversed-phase C_{18} column (b). Column operating conditions are described in Materials and methods.

tively, this problem is circumvented by prior treatment of the sample with a boronate affinity gel, as described below. The minimum detection limit of Ara-C and Ara-U on the C_{18} column is 0.32 and 0.44 ng, respectively. The reproducibility of sample injection was greater than 97%. Both analytical HPLC methods gave linear response curves for all nucleosides over a 1000-fold nmole range. This wide range of linearity is sufficient for quantitation of Ara-C and Ara-U levels in biological samples.

Fig. 2 shows the separation of nucleoside triphosphates on the A-29 anion-exchange column. Pyrimidine nucleotide triphosphates were eluted isocratically with sodium citrate buffer in less than 40 min. Ara-CTP is well separated from CTP and UTP and approximately 10 ng Ara-CTP and 20 ng Ara-UTP can be detected minimally at 270 nm.

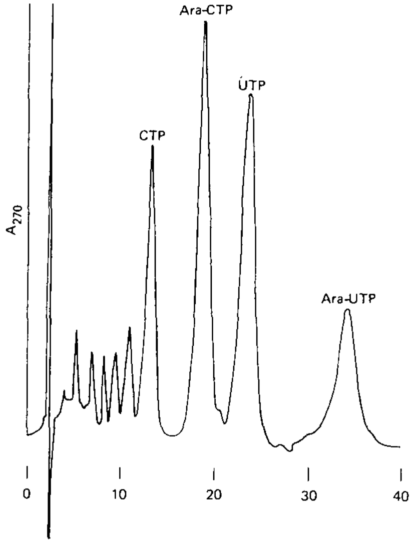

Fig. 2. Chromatogram of a nucleotide mixture of Ara-CTP, Ara-UTP, CTP and UTP on the ion-exchange (A-29) column. Temperature 65°C; flow-rate 0.7 ml/min.

Boronate affinity resin

We used a boronate affinity gel (Affi-gel 601) to selectively bind *cis*-diols as an aid to separating ribose and arabinose compounds. A mixture of nucleoside standards, Ara-C, Ara-U, cytosine and uridine was applied to the Affi-gel 601 column. The column was washed with ammonium acetate and formic acid as described in Materials and methods. The eluates were then analyzed on the A-27 ion-exchange resin (Fig. 3). Ara-C and Ara-U were found only in the ammonium acetate wash, whereas cytosine and uridine were found in the formic acid eluate. Recovery of the eluted compounds was greater than 98%. Thus, pretreatment of samples with Affi-gel allows quantitative measurements of Ara-C and Ara-U levels with the commonly available C_{18} column.

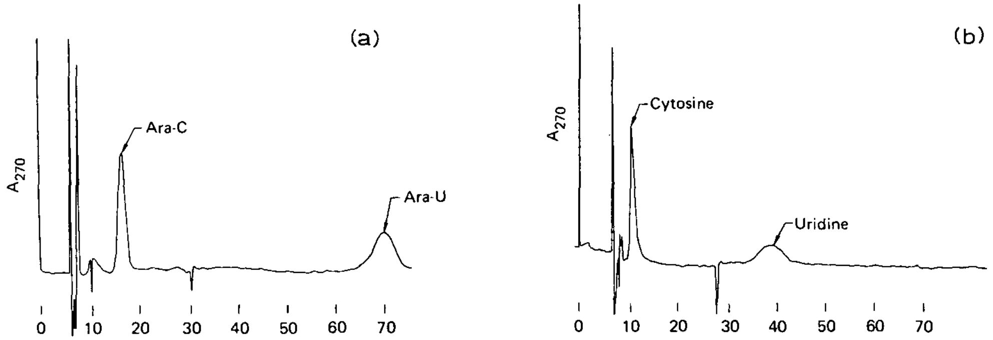

Fig. 3. Profiles of ribose and arabinose compounds after elution from Affi-gel 601. A standard pyrimidine nucleoside mixture (Ara-C, Ara-U, cytosine and uridine) was passed through Affi-gel 601 and the ammonium acetate eluate (a) and formic acid eluate (b) analyzed on the A-27 column.

Biological samples

As was discussed previously, one of the major problems in analyzing biological tissues using HPLC is that naturally occurring compounds may coelute with the compound of interest. Therefore, with each analytical system, it is necessary to obtain nucleoside and nucleotide profiles of the tissues to be analyzed to determine if the region of interest is free of contaminating peaks. Urine and plasma from untreated animals were analyzed on both the anion-exchange column (A-27) and on the reversed-phase C_{18} column. KHT tumor cell extracts were analyzed on the A-29 Aminex resin. These chromatograms, as well as those obtained from drug-treated animals are shown in Fig. 4. It is evident that at the attenuation (0.10 a.u.f.s.) necessary to analyze drug levels there are no major peaks which interfere with the quantitation of Ara-C and Ara-U in either urine or plasma.

We measured Ara-C and Ara-U levels in plasma and urine at various times after administration of Ara-C (100 mg/kg). This dose level of Ara-C was chosen since it is minimally toxic to C3H mice and previous studies in our laboratory indicated that it induces marked cell cycle kinetic perturbations in solid tumors (unpublished results). Ara-C was injected intraperitoneally and samples were collected at 0.5—24 h after drug administration. Fig. 5a illustrates Ara-C and Ara-U levels in plasma ultrafiltrates. The disappearance curve of Ara-C was biphasic with a half-time ($t_{1/2}$) of 20 min, as estimated from the first phase of the decay curve. Ara-U reached a maximum level of 12 ng/μl over a period of 30—60 min and decayed exponentially thereafter. Both Ara-C and Ara-U were excreted in the urine (Fig. 5b). Data in Fig. 5b are expressed as the ratio of Ara-C and Ara-U concentration in order to normalize variation in urinary output between individual animals. The ratio value of 1.0 is equivalent to 186 ng nucleoside per μl urine. These urinary data indicate that initially Ara-C was the major arabinose analog excreted; however, at two hours, equivalent amounts of Ara-C and Ara-U were eliminated. At later times after Ara-C administration a large percentage of the excreted arabinose nucleosides was due to Ara-U. Neither Ara-C or Ara-U were detected in the urine and plasma 24 h after drug injection. To verify that Ara-U was not simply a result of chemical breakdown

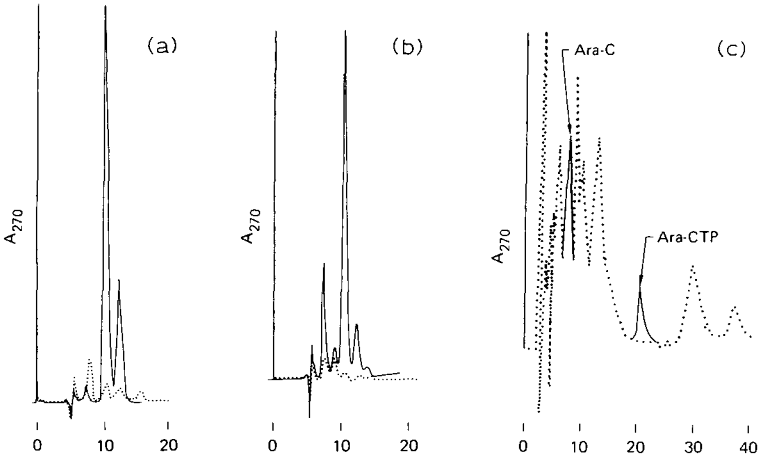

Fig. 4. Representative chromatograms of biological samples obtained from untreated (. . .) and treated (—) animals. Identical attenuation settings were used for each set of control and drug-treated samples. (a) Urine was obtained from mice 30 min after injection of Ara-C (100 mg/kg) or saline. Samples were passed through the Affi-gel resin prior to analysis on the reversed-phase C_{18} column. (b) Plasma was obtained from drug-treated and saline-treated animals 30 min after injection. Samples were analyzed on the ion-exchange (A-27) column. (c) Location of Ara-CTP peak in KHT tumor cells extracts. Samples were analyzed on the ion-exchange (A-29) column.

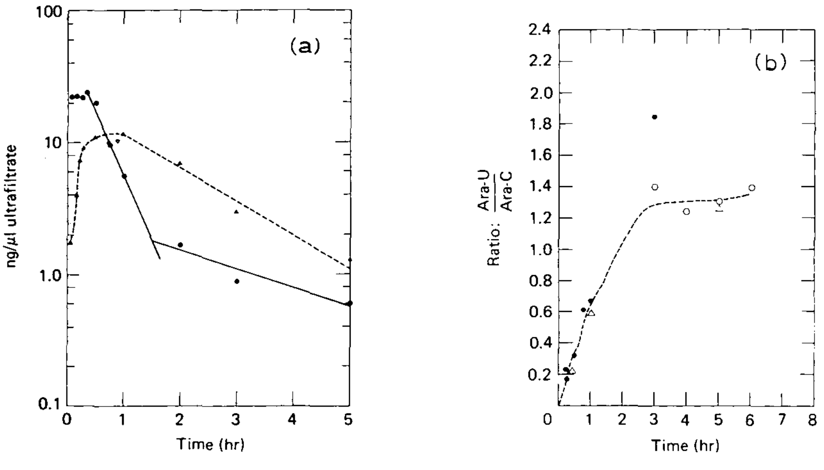

Fig. 5. Arabinose-nucleoside levels in plasma ultrafiltrates (a) (●, Ara-C; ▲, Ara-U) and urine pretreated with Affigel (b). Animals received Ara-C (100 mg/kg) and samples were collected at specific intervals thereafter. Plasma samples were analyzed on the ion-exchange (A-27) column and urine samples on the reversed-phase C_{18} column. In (b) each symbol represents an individual experiment.

of Ara-C, the parent drug was added to both urine and plasma ultrafiltrates and stored at room temperature for 18 h. These samples were analyzed on the reversed-phase C_{18} column. During this period, Ara-U was not detected in either sample, indicating that catalytic breakdown occurring after sample collection was not responsible for the observed Ara-U levels.

We also measured Ara-CTP levels in KHT tumor cells exposed to Ara-C ($2 \cdot 10^{-6}$ *M*) in vitro. A 50% cell kill is achieved during a 30-min exposure to this dose level of Ara-C (data not shown). Ara-C was added to cultures of KHT cells and aliquots of the suspension taken at 30-min intervals for a 2-h period. The cells were washed twice, protein precipitated with 60% methanol, and after evaporation the extract was reconstituted in 100 μl buffer and analyzed by HPLC using the ion-exchange column (A-29). These data, shown in Table I, are expressed as pmoles Ara-CTP per 10^7 cells. Ara-CTP levels ranged from 80—320 cells and from 60—410 pmoles per 10^7 cells in two experiments.

TABLE I

ARA-CTP LEVELS IN KHT CELLS EXPOSED TO ARA-C IN VITRO

KHT cells were incubated for various time intervals at 37°C in α-media plus Ara-C ($2 \cdot 10^{-6}$ *M*)

Time (min) in Ara-C	Ara-CTP (pmole/10^7 cells)	
	Exp. 1	Exp. 2
30	80	60
60	240	310
90	260	410
120	320	160
150	80	140

DISCUSSION

We have described two HPLC analytical methods to separate and identify arabinose-containing pyrimidine analogs from naturally occurring compounds. Both separation methods are isocratic, thereby avoiding the drift in baseline observed with gradient elution, and can detect nanogram quantities of Ara-C and its metabolites. The anion-exchange resins (A-27 and A-29) were used to quantitate Ara-C, Ara-U and Ara-CTP levels directly from biological tissues in less than 40 min. Ara-C and Ara-U were also quantitated on a reversed-phase C_{18} column after pre-treatment of samples with a boronate affinity gel. The use of the C_{18} column for drug quantitation offers the advantages of speed (Ara-C and Ara-U are eluted in approximately 20 min), less expense, greater sensitivity and is more common in clinical laboratories than the ion-exchange resin.

The use of a boronate affinity gel for HPLC analysis of nucleosides in biological materials has previously been described [11, 13]. These workers used the affinity gel as a sample clean-up procedure to remove deoxyribonucleosides and other contaminating material prior to analysis of ribonucleosides. Their biological sample was placed on the column and the column washed with ammonium acetate and formic acid buffers. The formic acid eluate containing *cis*-diols was then analyzed by HPLC. We used the capabilities of affinity gel in an alternative manner to achieve separation of arabinose and ribose nucleosides; we collected and analyzed the ammonium acetate eluate instead of the formic acid eluate. Thus, it was possible to eliminate contamination with the interfering *cis*-diols, cytosine and uridine, which are not separated from arabinose-containing nucleosides on the reversed-phase C_{18} column. The recovery of the arabinose nucleoside compounds from the affinity gel was excellent and biological levels were sufficiently high that drug and metabolite dilution with the ammonium acetate buffer did not pose problems of detection. However, if lower drug levels are to be measured, the ammonium acetate buffer can easily be evaporated or lyophilized with no detrimental effects on drug recovery.

We applied both the ion-exchange and the reversed-phase C_{18} methods to the measurement of Ara-C and Ara-U levels in plasma and urine. The plasma disappearance curve of Ara-C was biphasic and the $t_{1/2}$ was estimated to be 20 min. This value is in agreement with estimates obtained by other techniques [14]. Plasma Ara-U levels increased slowly over a 60-min period after Ara-C administration and disappeared exponentially thereafter. Approximately equivalent amounts of Ara-C and Ara-U were excreted at 2 h after drug administration (see Fig. 5b). These data are similar to those reported by Creasey et al. [15] using radioactively-labeled Ara-C.

The measurement of Ara-CTP levels in biological samples is somewhat more difficult than quantitating arabinose-nucleoside analogs. Ara-CTP, the active metabolite of Ara-C, is present in extremely low levels. In addition, the lability of nucleotide triphosphates in extraction procedures [16] requires careful experimental controls to ensure that measurements are indeed accurate. We found that extraction of nucleotides after protein precipitation with 60% methanol yielded good recoveries of Ara-CTP. This extraction procedure was used to recover nucleotides and nucleosides after exposure of KHT tumor cells to Ara-C in vitro (Table I). Ara-CTP concentrations ranged from 60—410 pmoles per 10^7 cells during a 150-min incubation period. These levels are similar to those reported by Rustum and Preisler [3] in leukemic cells using the radioactive form of Ara-C.

The rates and extent of phosphorylation and deamination of Ara-C in vitro to active and inactive metabolites, respectively, are believed to correlate with drug response [1, 4, 9, 17]. However, the tedious nature and expense of the studies using the radiochemical form of Ara-C has limited the detailed investigation of Ara-C pharmacokinetics in animals and patients. Such studies are needed to determine patient and tumor variation in drug metabolism and to better understand the extent and duration of drug effects at the cellular and molecular levels in both normal and tumor tissues.

We are applying the described analytical procedures to analyze Ara-C and metabolite levels in vivo in solid tumors and normal tissue. These pharmacokinetic data can then be related to observed drug-induced biochemical and cellular perturbations in an effort to rationally design multiple treatment schedules of Ara-C with improved therapeutic indices.

ACKNOWLEDGEMENTS

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CHROMBIO. 630

DETERMINATION OF ORG NC 45 (A MYONEURAL BLOCKING AGENT) IN HUMAN PLASMA USING HIGH-PERFORMANCE NORMAL-PHASE LIQUID CHROMATOGRAPHY

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SUMMARY

The assay for the quantification of Org NC 45 in human plasma is described in detail. It comprises ion-pair extraction and normal-phase liquid chromatography in conjunction with UV detection. An analogue, 17β-deacetyl-Org NC 45, is used as standard in the assay procedure. The accuracy and precision of the assay at 400 ng of Org NC 45 per ml of plasma are 0.7% and 2.8%, respectively. The detection limit is approx. 50 ng Org NC 45 per ml of plasma. The assay can be used for the pharmacokinetic evaluation of Org NC 45 in man.

INTRODUCTION

Org NC 45 (1-[(2β,3α,5α,16β,17β)-3,17-bis(acetyloxy)-2-(1-piperidinyl)-androstan-16-yl]-1-methylpiperidinium bromide) is a non-depolarizing neuromuscular blocking agent of the pancuronium type with a wide margin of cardiovascular safety [1—4]. Clinical data indicate a faster onset, shorter duration and more rapid offset of action than with equivalent doses of pancuronium (Pavulon®). For the latter compound a fluorimetric assay method [5] has been described where the sum of pancuronium and its biotransformation products is determined in human plasma non-selectively. Thin-layer chromatography (TLC) allowed complete separation of pancuronium from its biotransformation products [6]; however, quantification by TLC is semi-quantitative. A similar batch procedure [7] was developed for the quantification of Org NC 45 in plasma. Direct extraction from plasma with the fluorescent dye rose bengal was not feasible; therefore, a sequence of extractions was used. The latter procedure does not separate Org NC 45 from its theoretically probable biotransformation products (Fig. 1) and it is subject to large variations.

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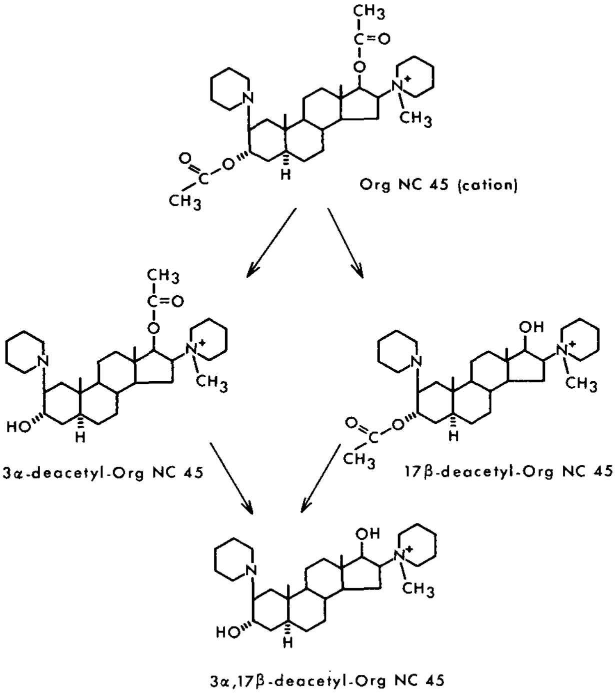

Fig. 1. Structural formulae of Org NC 45 and its theoretically probable biotransformation products.

A specific and sensitive assay method is required for the assessment of the pharmacokinetic parameters of Org NC 45 in man. De Zeeuw et al. [8] and Greving et al. [9] advocated the use of normal-phase ion-pair liquid adsorption chromatography for the determination of quaternary ammonium compounds. This report describes the assay for selective quantification of nanogram amounts of Org NC 45 in human plasma using ion-pair extraction and normal-phase liquid chromatography.

MATERIALS AND METHODS

Chemicals and glassware

Org NC 45 and its deacetylated products were obtained from Organon (Newhouse, Great Britain). Methanol and 1,2-dichloroethane (DCE) were of crude quality [Diosynth (Oss, The Netherlands) quality grade B] and distilled prior to use. All other chemicals were of analytical grade quality and purchased from Merck (Darmstadt, G.F.R.). The Glycine stock solution contained 750.5 mg of Gly and 585 mg of NaCl per 100 ml of water. The KI—Gly buffer solution was prepared freshly each day of analysis by mixing 4 ml of 0.1 *N* NaOH with 6 ml of Gly stock solution and 6,4 g of KI. All glassware was cleaned prior to use with a detergent (Alconox) by immersion in an ultrasonic tank and washing thoroughly with dilute hydrochloric acid and distilled water.

Acidification and storage of plasma samples

Sodium citrate was used as the anti-coagulant. After blood sampling in a clinical study, plasma samples were adjusted immediately to approx. pH 6 by addition of approx. 150 μl of 1 M NaH_2PO_4 per ml of plasma. This prevented the hydrolysis of Org NC 45 (Fig. 1). Plasma samples were stored at −20°C until required for use.

Sample processing

The assay standard, 17β-deacetyl-Org NC 45 (dissolved in 0.01 N HCl) was added to the mixture of 2 ml of acidified (pH 6) human plasma, 1 ml of KI—Gly solution and 7 ml of DCE in a 30-ml glass-stoppered centrifuge tube. Mild extraction was attained by gently rotating the tubes on a rotary disc for 30 min. After centrifugation for 10 min at 1300 *g*, the upper (aqueous) layer was carefully aspirated. The organic layer was then removed using a 10-ml syringe. The organic phase was concentrated by solvent evaporation under a gentle stream of nitrogen (waterbath, 40°C). The residue was dissolved in 70 μl of methanol by ultrasonification for 1 min and injected into the high-performance liquid chromatographic (HPLC) system.

HPLC apparatus and phase system

The HPLC set-up consisted of a Waters Model 6000A pump, a Waters U6K universal injector and a Pye Unicam LC 3-UV variable-wavelength detector (detection at 215 nm). All chromatograms were obtained using a Kipp BD8 multi-range recorder. A prepacked LiChrosorb Si-60 (particle size 7 μm) column (Chrompack, Middelburg, The Netherlands) of 25 cm × 4.6 mm I.D. was used. The mobile phase consisted of methanol, 1% of a 25% aqueous ammonia solution in water and 60 mM ammonium chloride and was prepared freshly each day of analysis. The eluent was ultrasonified and filtered through a Millipore filter (0.2 μm) prior to use. The eluent flow-rate was 2 ml/min. All experiments were performed at ambient temperature.

Calibration and quantification

For calibration purposes, 2 ml of human plasma free of drug (blank) was processed, spiked with 1 μg of Org NC 45 (0.01 N HCl) and various amounts of standard in a ratio of Org NC 45 to the standard ranging from 2.5 to 0.33. Peak heights of Org NC 45 and the standard were measured. The Org NC 45 plasma concentration was calculated using a calibration curve based on a BASIC programme with DEC PDP-11 RSTS computer facilities.

RESULTS

Selectivity

For pharmacokinetic evaluation Org NC 45 should be separated from its probable biotransformation products and endogenous plasma components. The chromatogram showing the separation of Org NC 45 from its theoretically probable biotransformation products is shown in Fig. 2. Fig. 3 shows characteristic chromatograms of blank human plasma, spiked human plasma and a plas-

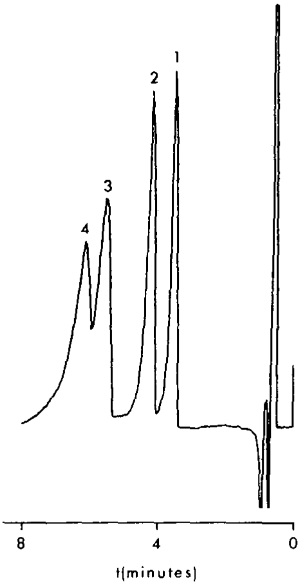

Fig. 2. Separation of Org NC 45 from its probable hydrolysis products. Stationary phase, LiChrosorb Si-60 (7 μm); mobile phase; methanol, 1% of a 25% aqueous ammonia solution and 60 m*M* ammonium chloride; ambient temperature; flow-rate, 2 ml/min. Peaks: 1 = 17β-deacetyl-Org NC 45; 2 = Org NC 45; 3 = 3α, 17β-deacetyl-Org NC 45; 4 = 3α-deacetyl-Org NC 45.

ma sample derived from a clinical study. It can be seen that the assay method offers ample selectivity for the determination of Org NC 45.

Retention and column performance

Retention of standard and Org NC 45 was subject to slight changes in the course of routine application. In addition, retention varied slightly from column to column. In principle, retention can be enhanced either by decreasing the ammonium chloride concentration or by adding more of the ammonia solution. For adjusting the retention time, a change in the ammonium chloride concentration proved to be the more appropriate method. A twofold increase in the ammonium chloride concentration will result in approximately a twofold decrease in retention time. At a flow-rate of 2 ml/min, the retention time for Org NC 45 should not exceed 10 min, since peak tailing will result in a considerable loss in accuracy and precision when measuring peak heights.

A gradual decrease in column performance was observed during routine application. Washing the column with 50 ml of methanol, 50 ml of acetone, 50 ml of hexane and then the same solvents in the reverse order, and re-equilibrating the column with the eluent, partly restored the performance. Renewing the top of the column packing by manual refilling was also beneficial. When not in use, the HPLC system was flushed thoroughly with methanol. Overnight, the eluent was recirculated at a solvent flow-rate of 0.5 ml/min.

Assay linearity, accuracy and precision

The calibration curve was fitted using polynomial regression analysis accord-

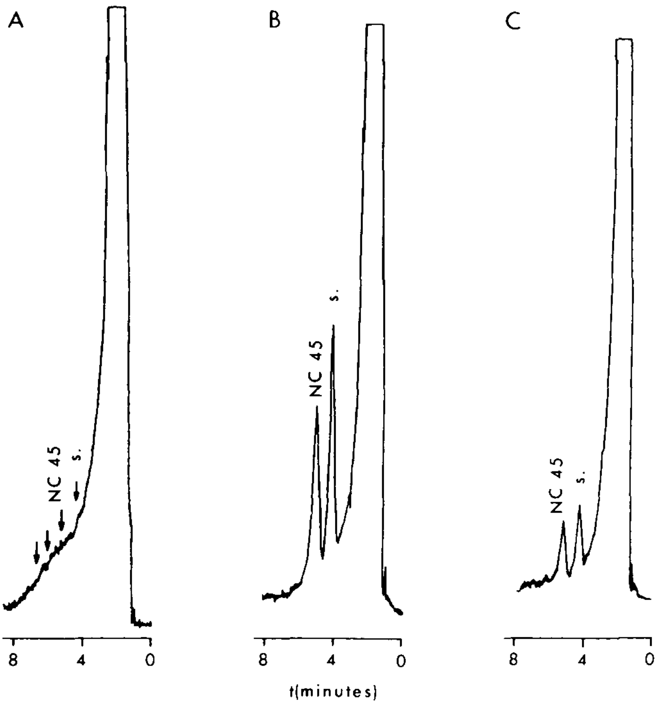

Fig. 3. Chromatograms of blank human plasma, spiked human plasma and human plasma sample from a clinical study. Stationary phase, LiChrosorb Si-60 (7 μm); mobile phase, methanol, 1% of a 25% aqueous ammonia solution and 60 m*M* ammonium chloride; ambient temperature; flow-rate, 2 ml/min. (A) Blank human plasma; the expected positions of the standard (s.), Org NC 45 and other probable biotransformation products (see Fig. 2) are indicated by arrows. (B) Human plasma, spiked with 1 μg of Org NC 45 and 1 μg of standard (s.) per 2 ml of plasma. (C) Clinical plasma sample; 3 ml of plasma were processed after spiking with 300 ng of standard (s.). The Org NC 45 concentration was calculated to be 145 ng per ml of plasma.

ing to $y = ax^2 + bx + c$, where typically $a = 1.88 \times 10^{-2}$, $b = 0.78$, and $c = -3.86 \times 10^{-2}$. The deviation from linearity is small. The peak height ratios proved to be independent of the amount of Org NC 45 at different ratios of Org NC 45 and standard. The assay accuracy [= relative difference (%) between actual and observed mean values] and precision [= relative (%) standard deviation] were determined by ninefold processing of 800 ng of Org NC 45, added to 2 ml of blank human plasma and spiked with 800 ng of standard. The within-day assay accuracy was 0.7% and the precision 2.8%.

Detection limit

The detection limit was arbitrarily defined as equivalent to three times the peak-to-peak noise level. In routine analysis 2 ml of plasma were processed. The detection limit was approx. 200 ng of Org NC 45 per 2 ml of plasma. Since 4 ml can be processed with adapted amounts of extractants without influencing the noise level, the detection limit ultimately amounted to approx. 50 ng of Org NC 45 per ml of plasma.

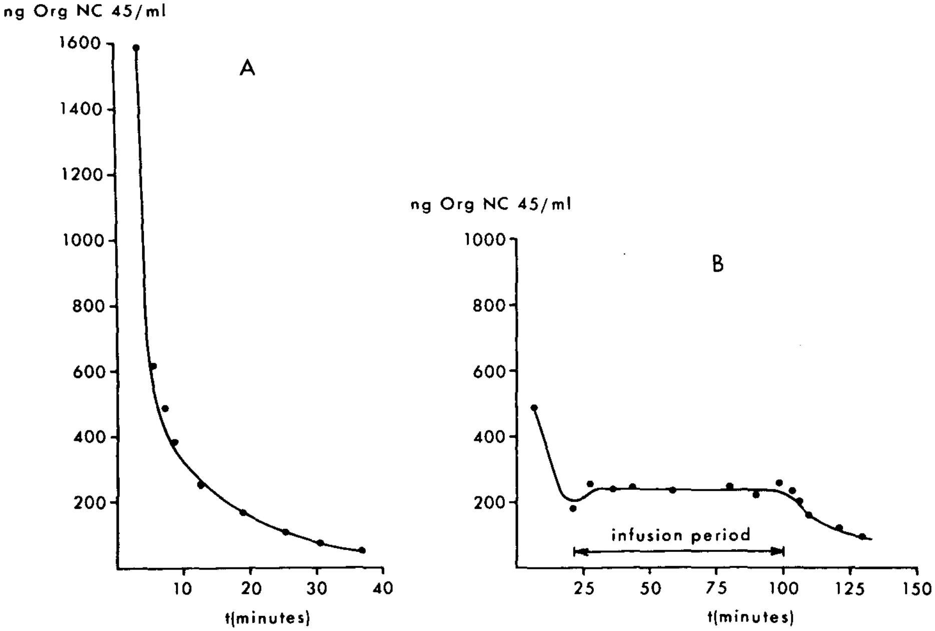

Fig. 4. Profiles of Org NC 45 plasma concentration versus time. (A) Intravenous bolus dose (0.11 mg/kg). (B) Intravenous bolus dose (0.11 mg/kg); intravenous infusion rate = 1.51 μg/kg per min. Infusion period was as indicated.

Quality control

Concurrently, on each day of analysis, human plasma samples (free of drug) spiked with a known amount of Org NC 45 at different levels were processed and quantified. When there were significant differences between the actual and true value for the Org NC 45 concentration, all samples (clinical, calibration, test) were reprocessed. This continuous quality control of the assay guarantees the acquirement of accurate data, also around the detection limit.

Routine application

The pharmacokinetics and pharmacodynamics of Org NC 45 have been determined with patients undergoing elective facial or oral surgery by concurrent plasma level determination of Org NC 45 and monitoring the twitch response of the adductor pollicis brevis muscle as a parameter for the degree of muscular block [10]. Typical examples of the time course curve of Org NC 45 plasma levels after intravenous administration and/or intravenous infusion are shown in Fig. 4.

DISCUSSION

It should be noted that the standard, 17β-deacetyl-Org NC 45, is a probable hydrolysis and/or biotransformation product derived from Org NC 45 (Fig. 1). However, in all experiments performed either with blank human plasma samples spiked with Org NC 45 in the framework of a time—course hydrolysis experiment or with human plasma samples derived from a clinical study, the presence of 17β-deacetyl-Org NC 45 could never be demonstrated. Thus, the use of 17β-deacetyl-Org NC 45 as standard for the Org NC 45 assay was justified. In the course of the assay development, addition of the standard to plasma gave rise to inaccurate and irreproducible results. Although only conjectures (drug—plasma protein binding) can be made about the explanation of this phenomenon, the problem was circumvented by the addition of the standard to the incubation mixture of plasma and extractants, just prior to extraction. It should be stressed that, in the course of the sample processing, the residue after concentration should not be dissolved in the HPLC eluent, since this will lead to erroneous results.

Org NC 45 is liable to hydrolyze under alkaline and neutral conditions (plasma, water). However, under the "alkaline" conditions for elution, no on-column hydrolysis of Org NC 45 was observed. There are indications (decrease in retention with increasing ammonium chloride concentration) that the retention mechanism is governed by ion-pair adsorption. However, the composition of the mobile phase was not changed systematically as to verify these indications quantitatively [9]. These aspects were judged to fall beyond the scope of this paper.

CONCLUSION

The assay method developed allows the selective determination of Org NC 45 in human plasma with good accuracv and precision. It has been shown to be applicable [10] for the assessment of the pharmacokinetic parameters of Org NC 45 in man.

ACKNOWLEDGEMENT

The authors would like to thank Mrs. Helga Wingens for her skilful practical assistance in the routine application.

NOTE ADDED IN PROOF

Occasionally unacceptable noise and baseline drifts were observed even after excessive ultrasonic degassing of the eluent before chromatography. This appeared to be dependent upon the reabsorption of oxygen into the eluent during chromatography. Continuous helium degassing solved the problem. The role of dissolved gases has been discussed before [11]. Recently, results of a systematic study were presented on solvent degassing and other factors affecting LC detector stability at low UV wavelengths [12].

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CHROMBIO. 639

DETERMINATION OF GLICLAZIDE IN HUMAN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING AN ANION-EXCHANGE RESIN

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SUMMARY

A method for the routine clinical examination of serum gliclazide by high-performance liquid chromatography (HPLC) on a column packed with a macroporous anion-exchange resin, Diaion CDR-10, was developed. The elution was performed with acetonitrile—methyl alcohol—1.2 *M* ammonium perchlorate (4 : 3 : 7, v/v/v) at a flow-rate of 0.4 ml/min. The retention time of gliclazide was 15 min. It seems that the retention mechanism of gliclazide under the HPLC conditions described is not only ion-exchange mode but reversed-phase mode between the anion-exchange resin and the mobile phase. The detection limit of gliclazide was 0.2 μg/ml in plasma. The coefficient of variation for the within-day assay was 5.0% (0.2 μg/ml, $n=8$). The decay curve of serum gliclazide in diabetic patients was determined.

INTRODUCTION

Gliclazide, N-(4-methylphenylsulfonyl)-N′-(3-azabicyclo[3,3,0]octyl)urea, is one of a series of sulfonylureas recently evaluated as a potential oral hypoglycemic drug. A simple efficient method for the determination of serum gliclazide was required for clinical monitoring of the serum drug levels in diabetic patients. This method had to be quick and highly sensitive. Conventional methods for the determination of serum sulfonylureas, such as tolbutamide, chlorpropamide and acetohexamide, have been based on colorimetry [1], gas—liquid chromatography (GLC) [2—8] or high-performance liquid

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chromatography (HPLC) [9]. Highly sensitive methods for the determination of gliclazide in human serum by GLC with electron-capture detection (ECD) [10] and by reversed-phase HPLC [11] have been developed; however, these methods present the following problems: the gas—liquid chromatographic method uses extraction and derivatization steps which are time-consuming, and the reversed-phase packing (chemical bond silica) used in the HPLC method tends to deactivate through routine assays of many samples.

In the present work, a more suitable method for the routine clinical examination of serum gliclazide by HPLC with the use of a macroporous anion-exchange resin, which is chemically stable, was developed.

EXPERIMENTAL

Reagents

A pure reference sample of gliclazide was obtained from Dainippon Pharmaceutical (Osaka, Japan). Methyl benzoate, methyl alcohol, acetic acid, sodium acetate, chloroform, acetonitrile and ammonium perchlorate (analytical reagent grade) were obtained from Wako Pure Chemicals (Osaka, Japan). All other chemicals were of analytical reagent grade. All aqueous solutions containing reagents were passed through a filter (Millipore type CS, 0.22 μm, Millipore, Bedford, MA, U.S.A.) before use.

Apparatus

A high-performance liquid chromatograph, Model Tri Rotar, equipped with a variable-wavelength spectrometric detector, Model UVIDEC—100 II (Japan Spectroscopic, Tokyo, Japan) was used. The packed column (250 mm × 4.6 mm I.D.) of Diaion CDR-10, a macroporous anion-exchange resin particle (size 7 μm) was obtained from Mitsubishi Chemicals (Tokyo, Japan). The following conditions were used: column temperature, ambient (25—27°C); detection wavelength, 227 nm; mobile phase flow-rate, 0.4 ml/min; sample volume injected, 10 μl; mobile phase, acetonitrile—methyl alcohol—1.2 *M* ammonium perchlorate (4:3:7, v/v/v).

Procedure for extraction of gliclazide from serum

A mixture of 250 μl of human serum and 1 ml of 0.5 *M* phosphate buffer (pH 7.0) was shaken vigorously for 15 min with 4 ml of chloroform. The mixture was centrifuged for 15 min at 1400 *g*, then 3 ml of the chloroform layer withdrawn, mixed with 2 ml of 1 *N* sodium hydroxide, and shaken for 10 min. After centrifugation of the mixture for 5 min, 1.5 ml of the aqueous layer was added to a mixture of 0.5 ml of 3 *N* hydrochloric acid, 2.5 ml each of 0.5 *M* phosphate buffer (pH 7.0) and chloroform, followed by shaking for 10 min. After centrifugation for 5 min, 2 ml of the chloroform layer was evaporated to dryness under a stream of nitrogen at 50°C. The residue was redissolved in 50 μl of methyl alcohol containing the internal standard (IS), methyl benzoate, 0.4 μg/50 μl, and a 10-μl aliquot was injected onto the HPLC column. The extractability of gliclazide from serum using the present procedure was maintained at over 96% down to pH 7.0. The methyl alcohol solution containing the extracted drug was stored at 2—4°C, and assayed within 3 days.

Preparation of calibration curve

Serum was obtained from the blood of a normal male. De-ionized water and serum found to have no drugs detectable by the present method were used to make up a solution ranging in concentration from 0.3 to 10 μg/ml of gliclazide. The calibration curve was constructed from peak height ratios of gliclazide to IS. De-ionized water and serum blanks were analyzed with each set of standard solutions. Serum samples from gliclazide-administered diabetic patients were extracted with chloroform and chromatographed in the same manner.

RESULTS AND DISCUSSION

A highly sensitive method for the determination of serum gliclazide was required because of the small drug dosage, resulting in low serum levels. At the same time, separation of the drug from several endogenous serum components could also be an important problem when using HPLC. Therefore, the procedure for the extraction of serum gliclazide was studied.

Conventionally, the extraction of sulfonylureas with chloroform from serum has been performed by using acetate buffer (pH 3.95, Walpole's buffer). Since the pK_a values of gliclazide are 5.98 and 2.21, the possibility of extraction with chloroform at around pH 7 was considered. The extractability of gliclazide from serum with chloroform at different pH values in the range 1.0 to 10.0 was examined. As shown in Fig. 1, gliclazide almost quantitatively passed into

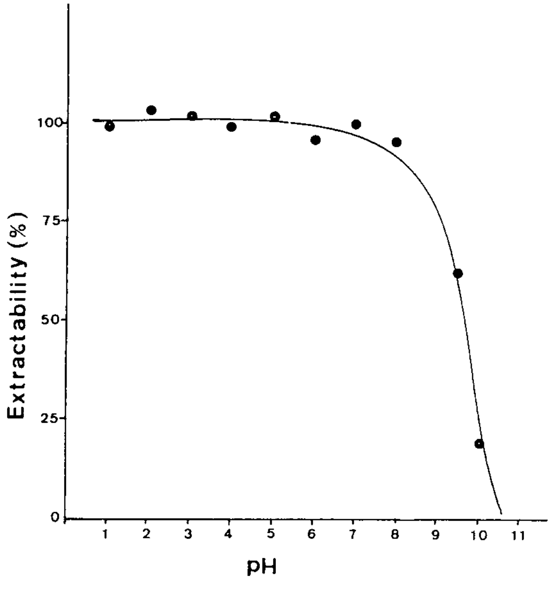

Fig. 1. Effect of pH on extractability of serum gliclazide. The same serum used for the preparation of the calibration curve was also used for the examination of drug extractability. The serum contained 5 μg/ml of the drug and extraction was carried out as described in Experimental. The extractability was calculated from the peak height ratio of gliclazide to IS (methyl benzoate). Each point represents the mean value of three separate experiments.

the chloroform layer from the mixture of serum and phosphate buffer below pH 7.0, while it was extracted with only 20% yield at pH 10.0. The present extraction procedure including a back-extraction may be tedious; however, a highly sensitive analysis is required to minimize some of the endogenous serum components or other drugs in the extracted sample.

Fig. 2 shows two HPLC chromatograms of endogenous serum components back-extracted with chloroform from serum at pH 7.0 and 3.95. As shown in Fig. 2A, six serum component peaks were detected at retention times in the range 7—24 min. In the case of extraction at pH 7.0, only two serum component peaks were detected at retention times in the range 7—12 min (Fig. 2B). Thus, the extraction using phosphate buffer at pH 7.0 seems to be more advantageous than when using acetate buffer (pH 3.95) for the determination of serum gliclazide by HPLC. Fig. 3 shows a typical chromatogram of gliclazide and IS (methyl benzoate) in serum. The gliclazide and IS peaks were detected at retention times of 15 and 19 min, respectively, whereas the serum component peaks were detected earlier (within 12 min).

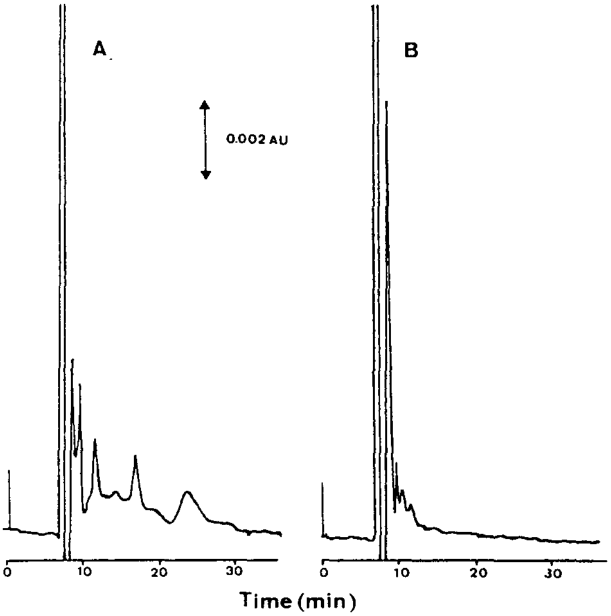

Fig. 2. Chromatograms of serum extracts: (A) extracted at pH 3.95 and (B) extracted at pH 7.0.

Recently, Diaion CDR-10, a macroporous anion-exchange column was developed for the HPLC analysis of nucleotides, carbohydrates and fatty acids. It is known that the macroporous resins have a relatively higher cross-linkage, larger surface area, and larger porosity than the microporous resins [12]. Usually, 0.1—6.0 *M* acetate buffer (pH 4.0) is recommended to be used in the mobile phase when using this resin. But an attempt to use this mobile phase failed because of gliclazide is hard to dissolve. Furthermore, the use of an alkaline buffer of high ionic strength did not give good separation. In order to overcome

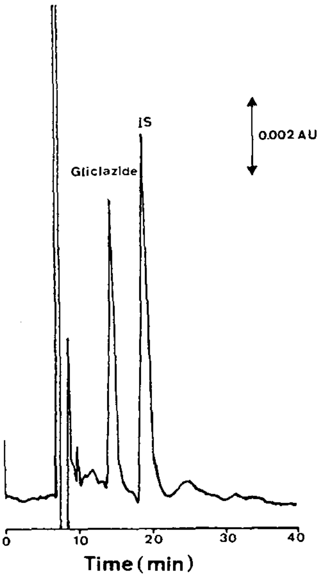

Fig. 3. Chromatogram of control human serum plus gliclazide and IS (methyl benzoate).

these difficulties, it was found that the addition of acetonitrile and methyl alcohol to 1.2 *M* ammonium perchlorate in the mobile phase gave sharper peaks and better separation. It seems that the retention mechanism of a weak acidic and hydrophobic compound (gliclazide) under the HPLC conditions described is not only ion-exchange mode but also reversed-phase mode between the styrene—divinylbenzene skeleton of the anion-exchange resin and the mobile phase. Therefore, this column was tried for the determination of serum gliclazide.

The calibration curve was linear (correlation coefficient, r = 0.99) over a range of 0.3—10 μg/ml of gliclazide, and the detection limit of gliclazide was estimated to be 0.2 μg/ml, using an injection volume of 10 μl. The sensitivity of the spectrophotometer was kept at 0.02 a.u.f.s. throughout.

Within-run precision and recoveries were determined for various sera concentrations of gliclazide (Table I). The coefficients of variation (C.V.) for the

TABLE I

WITHIN-DAY PRECISION OF GLICLAZIDE ASSAY

Added (μg/ml)	Found (mean ± S.D., μg/ml)	C.V. (%)	Recovery (mean ± S.D., %)	*n*
0.2	0.2 ± 0.01	5.0	100 ± 5.0	8
0.4	0.4 ± 0.02	5.0	100 ± 5.0	8
2.0	2.1 ± 0.10	4.8	100 ± 1.7	6
5.0	5.0 ± 0.14	2.8	101 ± 1.7	6
8.0	7.9 ± 0.28	3.5	96 ± 3.5	5

pooled sera containing 0.2, 0.4, 2, 5 and 8 μg/ml gliclazide were 5.0, 5.0, 4.8, 2.8 and 3.5% (n = 5—8), respectively. The recovery of the drug ranged from 96 to 101%. The coefficient variation for the day-to-day assay was 2.7% from the results measured nine times during three weeks.

To show the applicability of the proposed method, typical decay curves of serum gliclazide in a diabetic patient given 80 mg of the drug orally were determined by the present method and by GLC with electron-capture detection [10] (Fig. 4). The data show that the two methods gave the same results. The serum level of gliclazide rapidly reached a maximum (ca. 7 μg/ml) about 2 h after administration of the drug; it subsequently declined to the pre-administration level within 48 h.

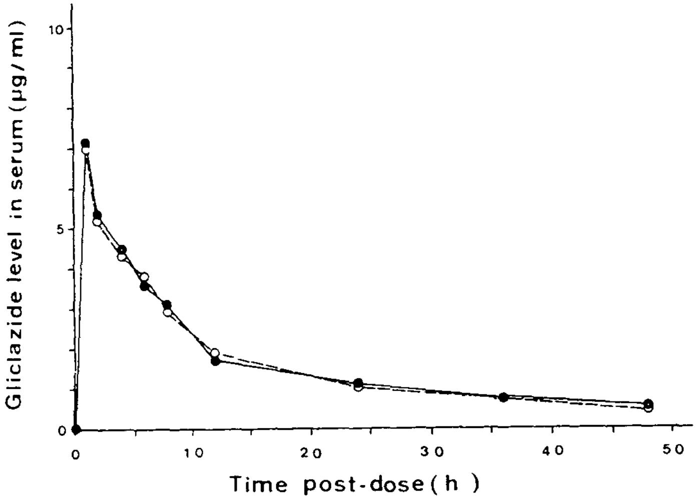

Fig. 4. Decay curves of serum gliclazide levels after oral administration of 80 mg to a diabetic patient. Each point represents the mean value of duplicate determinations. The same sample was determined by the present HPLC method (●) and by GLC with electron-capture detection [10] (○).

The therapeutic oral dose of gliclazide, which is a more potent hypoglycemic than other sulfonylureas, was not more than 1/5 of that of tolbutamide. However, the detection limits of the conventional methods for assay of serum sulfonylureas by colorimetry and by pyrolysis—GLC are 10 and 5 μg/ml [13], respectively. These methods are not satisfactory for the determination of serum gliclazide. On the other hand, the detection limit of GLC with electron-capture detection is 0.1 μg/ml of gliclazide [10] which would seem to be useful for the clinical examination of serum gliclazide, but this method usually requires tedious procedures, such as acylation of the extracted sample, and gives lower reproducibility (C.V. = 6.4%, n = 11) [10]. Neither co-administered drugs (e.g. vitamins or antibiotics) nor possible metabolites were detected in the present analytical system. Probably, they were removed through the extraction procedure. Recently, a high-potent hypoglycemic drug, glipizide was determined in blood with high sensitivity (detection limit 10 ng/ml) by HPLC using a μBondapak C_{18} column [14].

CONCLUSION

The present method for the determination of serum gliclazide by HPLC with use of an anion-exchange resin is comparable to GLC with electron-capture detection in sensitivity (0.2 μg/ml), specificity and speed (shorter retention time). Diaion CDR-10 resin is mechanically stable when operated at high pressures, and is easy to handle. The present method is preferred for routine clinical examination because of its simplicity and high reproducibility.

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CHROMBIO. 637

RELIABLE ROUTINE METHOD FOR DETERMINATION OF PERAZINE IN SERUM BY THIN-LAYER CHROMATOGRAPHY WITH AN INTERNAL STANDARD

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(First received February 22nd, 1980; revised manuscript received May 21st, 1980)

SUMMARY

The use of a high-performance thin-layer chromatography linear chamber and of thioridazine as internal standard increases the performance of thin-layer chromatography (TLC) with direct densitometric scanning, and allows the rapid determination of serum levels of the neuroleptic drug perazine under usual therapeutic conditions. TLC is superior to gas—liquid chromatography in so far as the main metabolite desmethylperazine can be easily separated and detected by the same procedure.

INTRODUCTION

In 1976 two methods for the quantitative determination of perazine (P) in human sera were published, which greatly advanced the search for a practical and precise analytical procedure at reasonable costs. One of the methods used was gas—liquid chromatography (GLC) [1], the other thin-layer chromatography (TLC) [2].

As the GLC method was not fully optimized and not sensitive enough for clinical conditions, an improved method was developed in our laboratory [3,4]. The latter procedure proved to be very satisfactory with regard to specificity and sensitivity, but had the disadvantage that the main metabolite desmethylperazine could not be assessed together with perazine in human sera. To overcome this difficulty and to have an independent method for an internal comparative control in our laboratory, we adopted the TLC method by Breyer and Villumsen [2]. This method was easily reproducible but was too time-consuming and sophisticated for clinical routine procedures.

However, it turned out that a faster and simpler modification of this TLC method could be developed, which is described in this paper.

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MATERIALS AND METHODS

Reference compounds and reagents

Reference compounds were gifts from the following companies: perazine base from Chem. Fabrik Promonta (Hamburg, G.F.R.; thioridazine from Ciba (Basle, Switzerland). Desmethylperazine (DMP) was prepared by reacting phenothiazine with 1-bromo-3-chloropropane followed by reaction with piperazine in methanolic solution, and perazine sulphoxide (PSO) was prepared according to the method of Breyer [5].

Reagents and solvents were Merck p.a. quality (Merck, Darmstadt, G.F.R.). Sodiumdioxycholate was obtained from Serva (Heidelberg, G.F.R.).

Stock solutions

Stock solutions of perazine (P), desmethylperazine (DMP), and thioridazine (THIO) were prepared by dissolving 10 mg of each compound in 10 ml of ethanol. These three solutions were mixed and further diluted with ethanol to obtain standard solutions of 5—50 mg/l of P and DMP, and 30 mg/l of THIO, respectively. The solutions were stored at 4°C. They were found to be stable for at least one month.

Plasma samples

Patients. Blood samples from perazine-treated patients were obtained within the context of a double-blind study described elsewhere [6].

Blank plasma. For recovery experiments we used plasma from blood donors. Blood (400 ml) was collected into a glass bottle containing 100 ml of ACD (acid—citrate—dextrose) solution as anticoagulant and preservative. The blood was centrifuged at 1930 *g* for 10 min, and the plasma stored in 10-ml portions at −20°C. The plasma was used for extraction within a period of two months.

Both the patients' and the blank plasma were thawed shortly before processing.

Extraction procedure

To 5 ml of blank plasma were added 50 µl of one of the standard solutions. To patients' plasma or serum (generally 5 ml) 1500 ng THIO as internal standard were added. Extraction was carried out according to Breyer's method [2] with minor modifications: *n*-heptane was used instead of benzene as solvent; THIO was added as the internal standard; after evaporation of the *n*-heptane phase, two portions of ethanol (100 µl) were used to transfer the residue into a polyethylene microtube (Eppendorf 3810 Reaktionsgefässe) taking care to rinse the sides of the glass vessels, and stirring carefully with a spatula to ensure complete dissolution of the residue. After evaporating the ethanol phase, the residue was dissolved in 50 µl of ethanol. A 10-µl aliquot of this solution was spotted onto the TLC plates.

Thin-layer chromatography

Plates. Precoated silica gel 60 high-performance thin-layer chromatography (HPTLC) plates (10 × 20 cm) with a layer thickness of 0.25 mm without fluorescent indicator were used (Merck).

Chromatography was performed in a HPTLC linear chamber 28520 (Camag, Muttenz, Switzerland) using the horizontal technique at room temperature with protection from daylight.

Treatment of the HPTLC plate. Plates were prerun twice with methanol—ammonia (18:1) and stored in a closed box. Prior to use, they were activated at 100°C for 10 min.

Spotting the plates. Ethanolic solution of extracts or standards were applied with disposable micro-pipettes (10 μl) (Brand, Wertheim, G.F.R.) in lines 10 mm long. Extracts were applied twice, four on each plate. Together with six standards, 14 spots were applied per plate.

TLC developing solvent. The solvent was freshly prepared before use and consisted of isopropanol—ethyl acetate—chloroform—methanol—25% ammonia (25:10:6:2.4). The plates were removed from the chamber 10 min after the two solvent fronts had met in the middle of the plate (45 mm). Only 6 ml of solvent per run were required.

Photometry. The Chromatogramm-Spektralphotometer KM3 (Carl Zeiss, Oberkochen, G.F.R.) was used in remission mode at the UV absorption maximum of perazine (250 nm). Peak areas were evaluated by means of a Summagraphics digitizer (HW-TT) attached to a HP 9825 desk computer.

Recovery experiments. For recovery experiments, standards and extracts were applied in alternating spots (10 μl each, in ethanolic solution). Using this method, a comparison between the recovery and the internal standard method was possible on the same plates.

Preparation of glass vessels. All glass tubes were cleaned with dichromate—sulfuric acid. Silanization was omitted because it produced no better results.

RESULTS

Calibration curves with ethanolic standard solutions

Calibration curves were calculated and plotted with a linear regression program (Hewlett-Packard) on an HP 9825 calculator. Calibration curves with reference compounds were linear over a range of 50—500 ng. There was no loss

TABLE I

PLATE-TO-PLATE VARIABILITY OF LINEAR REGRESSION PARAMETERS: ETHANOLIC STANDARD SOLUTIONS ON SIX DIFFERENT HPTLC PLATES

Data are given as means ± S.D. I = Area (mm^2) as a function of amount of substance (ng). II = (P/THIO or DMP/THIO) × 100 as a function of amount of substance (ng). A = Intercept; B = slope; r = correlation coefficient; C.V. = coefficient of variation.

Function	A	C.V.	B	C.V.	*r*	C.V.
Perazine						
I	83.5 ± 14.2	17	1.40 ± 0.05	4	0.99 ± 0.00	0.0
II	−12.9 ± 3.0	24	0.60 ± 0.04	7	1.00 ± 0.00	0.0
Desmethylperazine						
I	77.1 ± 8.1	11	1.30 ± 0.04	3	0.98 ± 0.01	1.0
II	−9.6 ± 1.3	13	0.50 ± 0.00	0.0	1.00 ± 0.00	0.0

of linearity when the ratio DMP/THIO or P/THIO was used instead of area (Table I).

Effect of the time interval between development and scanning of the plates on UV absorption of DMP and THIO

Time intervals between development and scanning considerably influence the absorption of P, DMP, and THIO. This is illustrated by the peak-height ratios of P and DMP to THIO, determined in 10- and 20-min intervals over a total period of 220 min. Table II records some selected ratios. The decreases in peak-height ratios were due to a faster reduction of absorption of P and DMP than of THIO, which might be caused by oxidative decomposition of P and DMP. The interval between development and scanning must, therefore, be kept constant, e.g. 30 min.

TABLE II

EFFECT OF THE TIME INTERVAL BETWEEN PLATE DEVELOPMENT AND SCANNING ON THE ABSORPTION RATE AT 250 mm

Time (min)	P/THIO	DMP/THIO
30	1.5	1.4
40	1.4	1.4
50	1.4	1.2
60	1.2	1.1
80	1.2	0.9
100	1.1	0.9
160	1.1	0.9
220	1.0	0.8

Separation

No significant differences between normal and HPTLC plates regarding the R_F values of DMP, P, PSO, and THIO could be observed. (One normal plate was cut into two pieces of 20 × 10 cm and processed in the same way as a HPTLC plate.)

To improve separation, Breyer's solvent [2] had to be modified. R_F values were kept low to avoid interference from endogeneous material. With this solvent it was not possible, however, to separate PSO from DMP within the short migration distance (45 mm) in the HPTLC chamber. For complete separation of PSO runs of at least 80 mm on both types of plates are necessary. Examples of chromatogram scans are shown in Fig. 1.

Identification and specificity

R_F values for DMP, P, THIO, and other psychoactive drugs are listed in Table III. Due to the short run of only 45 mm, there is considerable interference from other drugs, particularly with THIO.

It should be noted that for complete separation on a HPTLC plate a difference in peak distance of at least 4 mm is needed, i.e. the difference in $R_F \times 100$ values must be at least 8. Endogenous material (e.g. lipids) moved to the upper third of the migration distance without interfering with the drugs. Thus, there

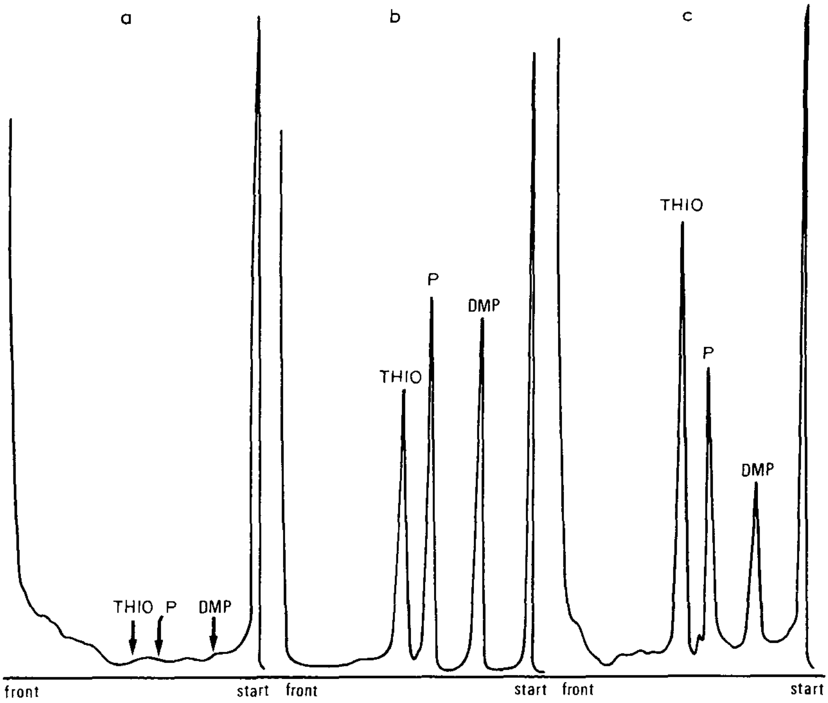

Fig. 1. Examples of chromatogram scans. (a) Blank patient serum; (b) ethanolic standard solutions: THIO, 300 ng; P, 100 ng; DMP, 100 ng; (c) serum from a patient treated with perazine (200 mg t.i.d., orally).

TABLE III

R_F VALUES OF SOME DRUGS ON HPTLC PLATES

Compound	R_F
Desmethylperazine	0.13
Desipramine	0.22
Perazine	0.30
Nortriptyline	0.36
Imipramine	0.40
Thioridazine	0.44
Trifluoperazine	0.44
Perphenazine	0.47
Amitriptyline	0.49
Prochlorperazine	0.53
Biperidene	0.85

was no need for running the plates with chloroform—isopropanol (20:2) before development, as recommended by Breyer and Villumsen [2]. Besides, considerably less interferences from endogenous material occurred due to extraction with *n*-heptane [3]. The homogeneity of the spots was proved in some samples by two-dimensional chromatography [2].

Sensitivity

Amounts of at least 20 ng/ml of P and DMP were detectable on the TLC plates with moderate gain of the amplifiers. As to sera spiked with perazine, the sensitivity was slightly lower (approximately 30 ng/ml) due to an increased variability of the recoveries with smaller amounts of the compounds (Table IV). Sensitivity can be increased up to 10 ng/ml by applying 90 or 100 μl of the extract per spot.

Recovery and reproducibility

Recovery rates are listed in Table IV. Every extract was chromatographed in duplicate, and the mean area was transformed into (ng/spot) on the basis of the corresponding standard curve obtained from the same plate.

Table V presents a comparison of the linear regressions of areas obtained with standard solutions vs. areas obtained with spiked sera, and of the corresponding peak-area ratios when the internal standard is used.

TABLE IV

RECOVERY OF P AND DMP FROM SPIKED HUMAN SERA

Recovered amounts are given as ng/ml (mean ± S.D.); N = number of extractions; R = recovery; C.V.$_1$ = Coefficient of variation and C.V.$_2$ = Coefficient of variation of the ratio DMP/THIO or P/THIO.

Concentration (ng/ml)	N	(ng/ml)	R (%)	C.V.$_1$ (%)	C.V.$_2$ (%)
Perazine					
50	8	49.0 ± 8.5	98.0	17	7
100	17	84.5 ± 8.7	84.5	8	8
200	8	167.8 ± 13.6	84.0	8	6
500	4	366.0 ± 22.3	73.0	6	2
Desmethylperazine					
50	8	42.2 ± 5.4	84.0	13	30
100	17	82.2 ± 6.8	82.2	8	7
200	8	150.0 ± 17.9	75.0	12	7
500	4	337.0 ± 62.4	67.0	19	3

TABLE V

LINEAR REGRESSION RESULTS

Obtained by correlating: I, areas calculated for spiked sera with areas for standard solutions and II, ratios of (P and DMP/THIO) × 100 for spiked sera with ratios × 100 calculated for standard solutions. A = Intercept; B = slope; r = correlation coefficient. Data from 4 plates.

Function	A	B	r
Perazine			
I	−3.9	0.86	0.99
II	22.8	0.84	0.97
Desmethylperazine			
I	39.7	0.72	0.99
II	27.6	0.75	0.99

DISCUSSION

The reason for modifying Breyer's method [2] was to render it more suitable to the demands of the clinical laboratory, i.e. to achieve a facilitated and faster performance without any loss of specificity and sensitivity.

The introduction of an internal standard offers the following advantages: (i) the performance can be greatly augmented since there is no need for running standards together with the extracts; (ii) it appears to be much more suitable to handle relative rather than absolute data, e.g. in our laboratory we worked with two different recorders and it was not possible to compare areas recorded on the one with areas recorded on the other — using ratios instead of absolute areas solved the problem; (iii) a simple and fast daily recovery control is achieved by running two standards per plate [7,8]; if the then computable recovery for THIO is within an acceptable range, one can be quite sure that the recovery for P and DMP is under control too.

The use of an internal standard presents many problems known from GLC [7—10]: (i) the chemical structure of the internal standard must not be too different from the compound to be determined so that analogous protein-binding and extraction properties can be assumed; (ii) it must be different enough from the compound to be determined so that it can be separated easily from it; (iii) it should have an absorbance maximum close to the one of the drug to be determined (THIO, 265 nm; P and DMP, 250 nm).

Considering these aspects, THIO appears to be a satisfactory compound. The correlation of quotients with the amount of P and DMP per spot is as good as when absolute areas are computed (Table I).

As to serum extracts, the variability of the peak area ratios is equal or even less than that of absolute areas (Table IV).

In Table V is shown to what extent the absolute areas and the ratios of standards are modified by the extraction procedure: the correlation coefficient for both substances and functions was excellent; the slope coincides well with the mean recovery for both substances (Table IV) (P: ϕR = 84.9, slopes = 0.86 and 0.84; and DMP: ϕR = 77.2, slopes = 0.72 and 0.75); and the intercept can most likely be interpreted as interference from endogeneous material. This interference seems to influence mainly DMP and frequently led to a broadening of the extraction peaks, which might be due to the low R_F value of DMP.

The usefulness of thioridazine as internal standard and the preciseness of the method was also proven by the analysis of 30 plasma samples from perazine-treated patients. These 30 plasma samples were each analysed twice, by a GLC method [4] and by the TLC method described above, resulting in an excellent correlation between both methods [12].

As thioridazine is a very common drug in psychiatry, it is easily available without the need of any synthetic procedures. On the other hand, as it can be separated from perazine, and can be extracted by the same procedure, it can be assayed with perazine as internal standard using the same method.

The use of the Camag HPTLC chamber reduced the time required for the performance of Breyer and Villumsen's [2] original method by 1 h because only 30 min were required to obtain values for 14 extracts. Another 30 min were saved by the use of *n*-heptane as the solvent for extraction, because predevelopment of the plates proved to be unnecessary. Besides, *n*-heptane is less toxic than benzene. A drawback to using *n*-heptane is the decreased recovery of DMP. The low solubility of DMP in *n*-heptane may lead to variable and erroneous results when small volumes of *n*-heptane are used for dissolution of the final residue; ethanol was used instead, therefore.

In contrast, perazine sulphoxide can only be extracted from serum with *n*-heptane if concentrations greater than 1000 ng/ml are present. Thus, PSO in patients' sera will not interfere with the determination of DMP (from which it cannot be separated satisfactorily).

Due to the short migration distance of only 45 mm interferences from other drugs cannot be excluded. If such interferences are to be expected from the therapeutic strategy in an individual patient, then the use of alternate methods such as GLC might become necessary to overcome this difficulty.

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Note

Comparison of liquid chromatography—electrochemical and gas chromatography—mass spectrometry methods for brain dopamine and serotonin

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Among the analytical methods for determination of catecholamines and serotonin (5-HT) in biological tissues, gas chromatography—mass spectrometric (GC—MS) techniques are regarded as the most accurate, specific and sensitive [1,2]. With the advent of the high-performance liquid chromatography with electrochemical detection (LC—EC) (3—5), alternative high-sensitivity assays of these compounds are available. However, whether the accuracy of LC—EC procedures compares favourable with GC—MS techniques has not been established. In the present paper, we report on the direct comparison of LC—EC assay with GC—MS for the simultaneous measurement of dopamine (DA) and 5-HT using rat hypothalamus as a representative brain sample.

EXPERIMENTAL

Procedure

Male Wistar rats (High Oak Ranch, Goodwood, Canada) weighing 300—350 g were housed communally in a temperature (22° C) and light (lights on 08.00—20.00 h) controlled environment for 2 weeks before the experiment. Food and water were given ad libitum. Animals were killed at 13.00 h by decapitation, the brains rapidly removed from the crania and the hypothalami quickly dissected over dry ice using the method described by Holman et al. [6]. The hypothalami were frozen on dry ice and stored at —70°C (less than 24 h). The wet weights of the hypothalami were 40.0 ± 1.9 mg ($N = 11$).

Each hypothalamus was homogenized in 1 ml of 80% ethanol in a polycarbonate centrifuge tube. Homogenization was performed in an ice bath for 1

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min using a Biosonik ultrasonic probe homogenizer (Bronwill Scientific, Rochester, NY, U.S.A.) at a setting of 5. The homogenate was centrifuged (12,300 *g*) at 4°C for 30 min. Epinine (4 ng) (Aldrich, Milwaukee, WI, U.S.A.), an internal standard for the LC—EC assay, was added to a 200-μl portion of the supernatant, while 20 ng each of α,β-[$^2H_4$]DA and α,β-[$^2H_4$]5-HT (Merck, Sharp & Dohme, Pointe Claire, Canada) were mixed with the remaining 800 μl for GC—MS assay. These supernatant aliquots were then passed through small columns of Amberlite CG-50 as previously reported [5] and the column eluates processed by LC—EC or GC—MS as described below. Tissue pellets were solubilized in 0.3 *M* sodium hydroxide for protein determination [7].

LC—EC and GC—MS assays

The procedure of Warsh et al. [5] was used for the simultaneous LC—EC assay of DA and 5-HT. The Amberlite eluates were evaporated to dryness and the residues reconstituted in 400 μl citrate—acetate buffer (pH 5.2). A 100-μl aliquot was injected into the LC—EC instrument (BAS Model LC-2A equipped with a TL 3 carbon paste electrochemical detector; Bioanalytical Systems, West Lafayette, IN, U.S.A.). Separation of DA and 5-HT was achieved on a 500 × 2.1 mm I.D. glass column packed with Zipax SCX resin (25—37 μm; Dupont Instruments, Wilmington, DE, U.S.A.). The mobile phase consisted of citrate—acetate buffer, pH 5.2 [4] at a flow-rate of 0.5 ml/min. The working electrode potential of the detector was maintained constant at +0.65 V vs. the Ag/AgCl reference electrode.

The GC—MS assays for hypothalamic DA and 5-HT were performed by the methods of Koslow et al. [1] and Warsh et al. [2], respectively, with minor modifications. Briefly, the formic acid—ethanol eluates containing the amines were evaporated to dryness in 3-ml reacti-vials and the residues reacted with 120 μl pentafluoropropionic anhydride (Pierce, Rockford, IL, U.S.A.) in ethyl acetate (5:1, v/v). After removal of the reagents under a stream of nitrogen, the reaction products were reconstituted in 15 μl ethyl acetate and a 2-μl aliquot analyzed by GC—MS using a Finnigan 3200 GC—MS system equipped with multiple programmable ion monitors.

The ion fragments (*m/e*) selected for minitoring were 428 for DA (431 for deuterated-DA) and 438 and 451 for 5-HT (440 and 454 for deuterated 5-HT). The ion fragment ratios at 438/451 for the hypothalamic extracts were identical to those for authentic 5-HT standards, thus providing positive identification for 5-HT; interference from unknown compounds prevented monitoring of a second ion fragment (*m/e* 281) for DA in the tissue samples. For both GC—MS and LC—EC assays, quantitation of DA and 5-HT was performed using calibration curves of the peak height ratios of standard to internal standard vs. the concentration of standard.

Under these conditions, the limits of sensitivity for DA and 5-HT in tissues are 500 pg by the GC—MS method and 100 pg by the LC—EC assay.

RESULTS AND DISCUSSION

Comparison of methods for the determination of brain biogenic amines cannot be done reliably using data derived from different laboratories since a variety of factors such as differences in animal strain, housing conditions, diet, age and post-mortem handling of tissues may affect the tissue levels of these compounds. Valid direct comparison of methods requires the simultaneous determination of the substance in question in the same tissue sample with each method. This has been achieved in the present report for the comparison of LC—EC with GC—MS methods for DA and 5-HT.

Table I demonstrates that hypothalamic 5-HT concentrations determined by LC—EC are virtually the same as the values obtained by highly specific GC—MS assay (paired $t = 1.65; p > 0.2$). The intra-assay coefficients of variation (C.V.) for the LC—EC assay of 5-HT in replicate (N=6) samples from a pooled standard and tissue extract were 2.8% and 1.4%, respectively. These C.V. values agree closely with the values obtained by GC—MS assay (3.8% and 2.2%, respectively). Although the DA concentrations determined by both methods correlated significantly ($r = 0.8$; $p < 0.01$, Pearson Product—Moment correlation) the paired t-test showed the values measured by LC—EC to be slightly (9%) but significantly higher than GC—MS levels (paired $t = 3.17$; $p < 0.01$). However, as for 5-HT, the intra-assay C.V. values for replicate (N=6) samples of DA from a pooled standard and a tissue extract determined by LC—EC (3.1% and 1.6%, respectively) agreed closely with the C.V. values for DA determined by GC—MS (4.3% and 1.2%, respectively).

TABLE I

COMPARISON OF LC—EC AND GC—MS ASSAYS OF RAT HYPOTHALAMIC DA AND 5-HT

Animal No.	DA (ng/mg protein)		Difference (%)	5-HT (ng/mg protein)		Difference (%)
	LC—EC	GC—MS		LC—EC	GC—MS	
1	3.28	3.07	+ 6.84	8.43	7.52	+12.10
2	1.73	1.73	0	7.51	7.58	− 0.92
3	3.09	2.88	+ 7.29	8.15	7.84	+ 3.95
4	3.24	2.89	+12.11	8.35	7.68	+ 8.72
5	3.46	3.11	+11.25	8.23	8.42	− 2.25
6	3.43	2.95	+16.27	7.56	7.57	− 0.13
7	3.01	2.65	+13.58	8.73	7.67	+13.83
8	2.85	2.66	+ 7.14	8.25	7.91	+ 4.29
9	2.97	3.27	− 9.17	9.20	8.99	+ 2.33
10	2.84	2.75	+ 3.27	6.96	7.70	− 9.61
11	2.83	2.49	+13.65	7.64	7.32	+ 4.37
$\bar{X}$	2.98*	2.77	9.14	8.09	7.84	5.68
S.D.	0.47	0.41	4.84	0.63	0.47	4.64

*$p < 0.01$; paired t-test.

The basis for the positive bias in DA values determined by LC—EC vs. GC—MS is not clear although several possibilities are likely. Firstly, since we were unable to accurately quantify a second ion fragment in the GC—MS assay of tissue DA, we cannot conclude with complete confidence that the GC—MS assay achieved the highest degree of specificity. However, if the specificity of the GC—MS assay using the ion fragment at m/e 428 were compromised in any way this would have resulted in elevated values compared to the true tissue concentration. Since the LC—EC values for DA exceed those determined by GC—MS, it is more likely that factors affecting either the specificity and/or accuracy of the LC—EC assay account for the small difference. Although epinine was employed as an internal standard in the LC—EC assay, this compound may be sufficiently different from DA in its chemical characteristics such that it does not provide a complete control for all DA recovery losses in the method. In contrast, deuterated DA used as an internal standard in the GC—MS assay is an ideal internal standard, being only isotopically different from DA. However, epinine appears to be quite satisfactory for use in quantitation of 5-HT by LC—EC.

The higher DA values determined by LC—EC might alternatively arise from a lower specificity of this procedure compared to GC—MS. Since the electrochemical detector is not compound specific, the specificity of the LC—EC procedure is primarily dependent upon the separation and resolution of the compounds chromatographed on the LC column. In the LC—EC assay used here, DA and 5-HT were separated by cation exchange high-performance LC. This column separation may not be optimal for DA determination. In this regard, reversed-phase LC provides superior separations of biogenic amines [8, 9] and may allow even better accuracy in the LC—EC assay.

In summary, quantitation of 5-HT and DA by LC—EC using a cation-exchange column compares very favourably with the GC—MS assay of these compounds although the values obtained for DA are slightly higher. However, the sensitivity of this LC—EC procedure is comparable to that of GC—MS and obviates the need for derivative formation and complex instrumentation in GC—MS assays.

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Note

The estimation of α-tocopherol in biological material by gas chromatography

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α-Tocopherol, the biologically most active of the E-vitamins, is a natural scavenger of free radicals and protects the organism from non-enzymatic attack by molecular oxygen on poly-unsaturated fatty acids. The normal range for free vitamin E concentrations in human plasma is 5–20 μg/ml. According to Horwitt [1] levels of at least 5 μg/ml are essential to prevent peroxide-induced hemolysis of red blood cells. Levels lower than 5 μg/ml are considered to indicate vitamin E deficiency.

The connection between α-tocopherol and cardiac toxicity of iron in a case with extremely low levels of α-tocopherol in serum has been investigated by Lindvall [2]. For this study a specific and sensitive gas chromatographic (GC) method for determination of α-tocopherol in plasma was required.

A large number of methods already exists for determination of tocopherols. A review by Bunnell [3] summarizes the different types of methods reported up to 1971. Many of these methods are based on indirect measurements of α-tocopherol and do not differentiate between the tocopherol isomers, and other reducing substances may also interfere.

Sheppard et al. [4] reviews GC assays for vitamin E in plants, animal tissues and food materials up to 1972. In recent years some gas and liquid chromatographic methods for estimation of plasma levels of α-tocopherol have been described [5–11]. The main problem to be overcome in the GC determination is interference by cholesterol present in plasma. Prior separation of α-tocopherol and cholesterol using techniques such as thin-layer chromatography [5,8] or digitonide precipitation of cholesterol [12] is necessary and this may be time-consuming. Even if the separation can be performed directly on the GC column it will take a rather long time [6,7].

The method given below is a fast and accurate GC method to determine non-esterified α-tocopherol in plasma. Even now the cholesterol must be removed in advance, but this is rapidly and easily done on a small digitonin–celite column, a modification of a method described by Christie et al. [12]. The α-tocopherol is chromatographed as tocopheryl acetate and is eluted with a retention

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time of about 6 min. The method requires 0.1 to 1.0 ml of plasma and the detection limit of tocopherol is about 0.2 μg/ml.

EXPERIMENTAL

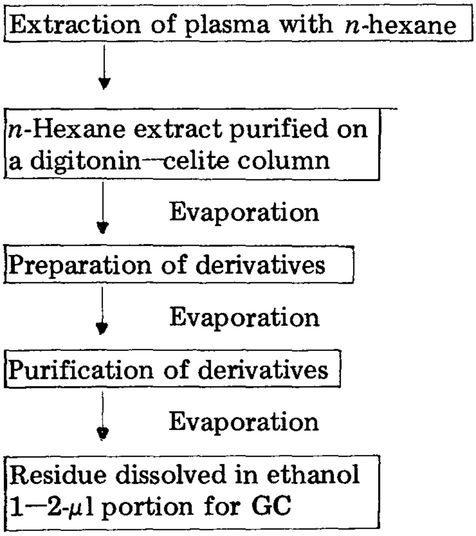
Reagents

α-Tocopherol and digitonin were obtained from Merck (Darmstadt, G.F.R.). *n*-Hexane, ethanol, pyridine and acetic anhydride were all of analytical grade quality and used without further purification. Celite 45 was standardized kieselguhr with a particle size of 20—45 μm. Octacosane, also analytical grade, was used as internal standard.

Instrumental conditions

A Perkin-Elmer gas chromatograph Model F 11 equipped with a flame ionization detector was used. The electrometer was connected to a Perkin-Elmer 159 1-mV recorder and the chart speed was 60 cm/h. The column used was a silanized glass column (180 cm × 2 mm I.D.) packed with 3% JXR (methyl silicone, Applied Science Europe, Oud-Beijerland, The Netherlands) on Gas-Chrom Q (100—120 mesh). The temperature of the oven was 260°C and that of the injector and detector 280°C. The flow-rate of the carrier gas (nitrogen) was 30 ml/min.

General procedure


Extraction of plasma with *n*-hexane
n-Hexane extract purified on a digitonin—celite column
Evaporation
Preparation of derivatives
Evaporation
Purification of derivatives
Evaporation
Residue dissolved in ethanol 1—2-μl portion for GC

Extraction

A 0.1—1.0-ml plasma sample (if less than 1.0 ml plasma, add distilled water up to 1.0 ml) was mixed with 1.0 ml absolute ethanol in a 10-ml test tube. *n*-Hexane (2.0 ml) containing the internal standard octacosane (4 μg/ml) was added and the tube was shaken mechanically in a rotary mixer for 15 min. After centrifugation for about 10 min at 3000 *g* as much as possible of the organic phase was transferred to the digitonin—celite 545 column described below in order to purify the extract from cholesterol.

Removal of cholesterol

Digitonin (300 mg) was dissolved by heating in 5 ml of water. The solution was well mixed with 10 g celite 545, which had been dried overnight at 110°C [12]. About 250—300 mg of the mixture was dry packed in a pasteur pipette containing a glass wool plug. The hexane extract was added to the column and allowed to pass at a rate of approximately 1 drop/sec. About 2 ml *n*-hexane was added to wash the column. The eluate was then evaporated to dryness in a 3-ml test-tube at a temperature of 50°C under a stream of nitrogen.

Acylation and purification of derivatives

To the residue 20 μl dry pyridine and 100 μl acetic anhydride were added. The tube was stoppered and incubated in a heating block at 50°C for 15 min. The excess of reagents was evaporated with a stream of nitrogen at a temperature of 50°C. The residue was dissolved in about 1.5 ml of *n*-hexane; 1.0 ml of water was added and the tube was shaken vigorously on a whirlimixer for 30 sec. After centrifugation for 5 min, the *n*-hexane extract was evaporated to dryness in the same way as above. The residue was dissolved in 200 μl absolute ethanol and 1—2 μl was injected into the chromatograph.

Calibration curve

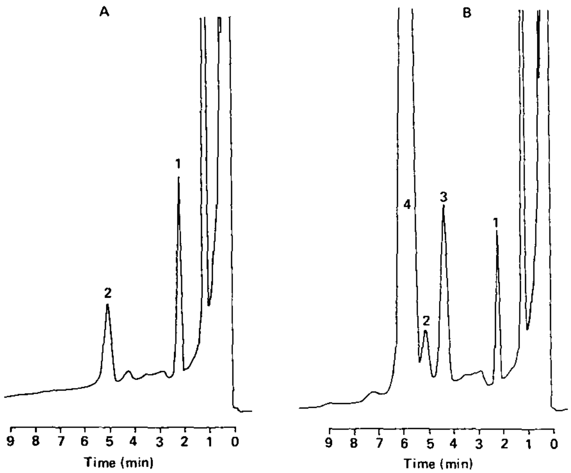
Ethanolic solutions of α-tocopherol ranging from about 0.4 to 17.0 μg/ml were used as standards. From each of these solutions 1.0 ml was evaporated to dryness together with the internal standard and acylated as above without the purification steps. A calibration curve was plotted from the ratio of the peak heights of α-tocopherol and octacosane against the α-tocopherol concentration (Fig. 3B).

RESULTS AND DISCUSSION

A typical chromatogram is shown in Fig. 1A. The retention time for α-tocopherol as tocopheryl acetate was about 5.5 min and for octacosane about 2.5 min. Low levels of δ-, β- and γ-tocopheryl acetates were also shown by the chromatogram (retention time for δ-tocopheryl acetate about 3.5 min, for β- and γ-tocopheryl acetate about 4.5 min). No attempt was made to determine these tocopherols.

Cholesterol is completely removed by a digitonin—celite mixture, easily packed as a very small column (about 2 cm high) into a pasteur pipette. Only 250—300 mg of the mixture was necessary. Such a small column usually gave a drop rate of about 1 drop/sec which means that the whole purification is performed in a few minutes. Fig. 1 shows the effect of such a purification step (chromatogram A) while chromatogram B was run without this treatment.

It is also necessary to purify the derivatives after acylation, otherwise unidentified interferences occur as in the chromatogram in Fig. 2 (note the large asymmetrical peak between the internal standard and tocopheryl acetate). This purification involves an evaporation to remove the derivatisation reagents and



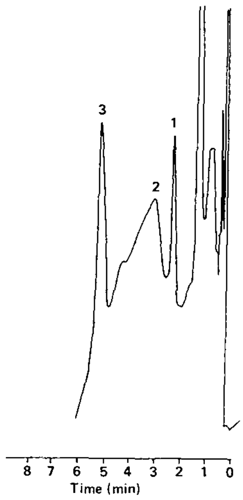


Fig. 1. Chromatograms of an extract of normal plasma (A) analyzed according to the method and (B) not treated on the digitonin—celite column. Peaks: 1, octacosane; 2, α-tocopheryl acetate; 3, cholesterol and 4, cholesteryl acetate.

Fig. 2. Chromatogram of a normal plasma extract analyzed according to the method but without purification of the derivatives. Peaks: 1, octacosane; 2, unidentified impurities and 3, α-tocopheryl acetate.

an aqueous extraction of the hexane solution of the residue, both steps being very important for getting a clean extract. Without this purification the injection part of the column as well as the detector are rapidly contaminated making further chromatography impossible.

Fig. 3 shows two calibration curves where the ratio of the peak heights of α-tocopherol and octacosane is plotted against the α-tocopherol concentration. Curve A is obtained after analysis of plasma samples spiked with known amounts of α-tocopherol (0, 0.81, 4.25 and 17.0 μg/ml). In curve B ethanolic solutions of α-tocopherol in a concentration ranging from 0.4 to 17.0 μg/ml were used. The two curves are parallel with correlation coefficients of 0.999 in both cases; therefore ethanolic solutions can be used as standards for determination of plasma levels of α-tocopherol. The parallel displacement between the two curves is due to the basic level of α-tocopherol in the pooled human plasma used.

The reproducibility of the method was tested with pooled human plasma analyzed with regard to the basic α-tocopherol level. For a series of five analyses of the same sample the mean value was 6.81 μg/ml with a coefficient of variation of 3.2%. The validity of this value was confirmed by gas chromatographic—mass spectrometric analysis showing that the registered peak contained only α-tocopheryl acetate. The recovery and reproducibility were also investigated with spiked plasma at three different levels (0.81, 4.25 and 17.0 μg/ml). Five samples at each level were analyzed according to the procedure described. The results are given in Table I. The recovery ranged from 100 to 104%, with a mean value of 101%.

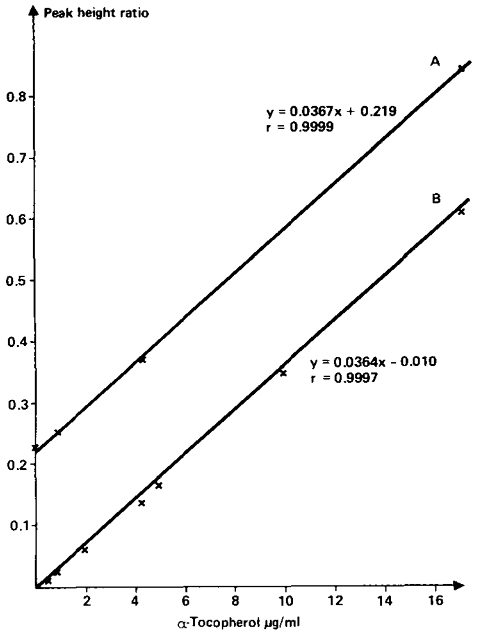


Fig. 3. Calibration curves. (A) Plasma solutions supplemented with known amounts of α-tocopherol. Each point is the mean of the analysis of five samples. (B) Ethanolic solutions of α-tocopherol.

TABLE I

REPRODUCIBILITY AND RECOVERY OF α-TOCOPHEROL ADDED TO PLASMA CONTAINING A BASIC α-TOCOPHEROL LEVEL OF 6.81 μg/ml

Each level is analyzed five times.

Initial concn. (μg/ml)	Added (μg/ml)	Total level (μg/ml)	Found (μg/ml) (Mean, C.V.%)		Recovery (%)
6.81	0.85	7.66	7.72	1.2	100.8
6.81	4.25	11.06	11.03	3.2	99.7
6.81	17.02	23.83	24.67	1.3	103.5
					Mean 101.3 ± 2.7 (S.D.)

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Note

High-performance liquid chromatographic determination of pyridoxine and congeners in biological fluids of man after high-dose therapy

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Pyridoxine, a vitamer of vitamin B_6, is used alone and in combination with other agents such as tryptophan in the treatment of depressive illness [1, 2]. For this purpose the usual dose ranges from 0.1 to 1 g a day and greatly exceeds the recommended daily intake of the compound as a vitamin (2 mg [3]). As part of research into the clinical applications of pyridoxine in psychiatry, it was necessary to investigate the pharmacokinetics of the drug and metabolites at the dose levels used. To obtain this information a simple direct assay for pyridoxine and several of its metabolites has been developed using high-performance liquid chromatography (HPLC).

Assays available for the compound in biological material involve enzymatic, microbiological and chemical techniques [4—6]. The enzymatic procedures usually make use of the co-enzyme function of pyridoxine and some congeners,

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particularly pyridoxal [7]. These methods are adapted to the determination of sub-microgram quantities of material and are not readily applicable to routine pharmacokinetic studies.

The microbiological assays are tedious and somewhat inaccurate for pharmacokinetic studies and are generally non-specific for pyridoxine metabolites [8]. Many of the chemical methods involve lengthy separation procedures [9—12] or electrophoresis [13, 14]. Other chemical procedures require selective oxidation of the vitamers to 4-pyridoxic acid lactone and determination of this compound fluorimetrically [15—17]. Some assays have been developed for analysis of pyridoxine and some vitamers in food and drug analysis [9, 18, 19]. The gas chromatographic separation of the non-phosphorylated vitamers has been published [20], but the technique requires derivatization.

Stewart et al. [18] have proposed an HPLC method for pyridoxine in pharmaceutical products using an ion-pair development method. Other HPLC methods allow the determination of vitamin B_6 components in food products and biological fluids of man using fluoromonitor detectors [19, 21], but column separation may take up to 2 h and therefore limits the number of samples assayed per day.

The assay described here permits the rapid and specific determination of pyridoxine and some of its metabolites, pyridoxal and 4-pyridoxic acid, in human plasma and urine after oral administration of the drug at psychiatric dose levels.

MATERIALS AND METHODS

Apparatus

A Spectra Physics 3500B high-performance liquid chromatograph was used, equipped with a variable-wavelength spectrophotometric detector (Model 700). The detector was connected to a 1-mV recorder (BD 7; Kipp and Zonen, Emmen, The Netherlands). The stainless-steel column, 25 cm × 4.6 mm I.D., was commercially packed with Partisil 10-ODS, particle size 10 μm (Chrompack, Middelburg, The Netherlands). The injection loop was 100 μl size. Detection of pyridoxine and metabolites was effected at 291 nm.

Solvent

The solvent used was a 0.067 *M* potassium dihydrogen phosphate solution in double-distilled water, to which 10 ml of a phosphoric acid solution (40%) were added per liter. The final pH of this mixture was 2.6 and the flow-rate used was 1.6 ml/min at a pressure of 155 atm.

Drugs

Pyridoxine hydrochloride, pyridoxal hydrochloride, 4-pyridoxic acid and pyridoxamine hydrochloride were obtained from Sigma (St. Louis, MO, U.S.A.) and were chromatographically pure in all systems used. Pyridoxal-5-phosphate and pyridoxamine-5-phosphate (Sigma) exhibited minor contaminating peaks (< 0.5%) due to parent compounds in the system described. To prepare standard solutions the compounds were dissolved in water (1 mg/ml). In the case of 4-pyridoxic acid a small amount of 1 *N* sodium hydroxide solution was added to the acid solution. Standard solutions were kept in a refrigerator. All were stable except pyridoxal, which was renewed within three weeks, and the phosphate derivative solutions, which were freshly prepared.

Subjects and clinical sampling

Pyridoxine was administered to Caucasian volunteers from the Department of Clinical Pharmacy and to patients in the Departments of Psychiatry of the St. Radboud Hospital (Nijmegen, The Netherlands) and of the Bethesda Hospital (Tiel, The Netherlands). The drug was administered orally as tablets of 250 mg.

From volunteers, blood samples of 0.5 ml were collected at scheduled time intervals by fingertip puncture (Microlance No. 433, Becton and Dickinson) in 2-ml Eppendorf vials, in which 0.1 mg of solid calcium heparin (Organon, Oss, The Netherlands) was used as anticoagulant (strength ±150.000 I.E./g). Spontaneously voided urine samples were collected over 24 h. In patients blood samples were obtained by venapuncture (5 ml) at scheduled time intervals, while urine collection was made over 24 h. Blood samples were centrifuged soon after collection (2500 *g* for 10 min). All samples (plasma and urine) were frozen after collection and assayed within 72 h.

Sample preparation

Plasma. A 0.2-ml sample of plasma was added to 0.3 ml of perchloric acid (0.33 *M*) and mixed by inversion. The mixture was allowed to stand for 5 min and then centrifuged for 5 min at 2500 *g*. A 100-μl aliquot of the clear supernatant was injected onto the column.

Urine. Ten microliters of urine were mixed with 0.3 ml of perchloric acid (0.33 *M*) on a vortex mixer. A 100-μl aliquot of the clear mixture was injected onto the column.

Calibration

A calibration curve was made by adding known concentrations of pyridoxine, pyridoxal, 4-pyridoxic acid and pyridoxamine to blank human plasma and urine. A calibration curve was constructed by plotting peak height versus concentration. Three to five standards were included as a control with each series of determinations.

RESULTS

Pyridoxine and its vitamers were well separated from endogenous compounds in plasma and urine by this HPLC technique (Fig. 1). The relative retention times of the vitamers and the major endogenous peaks, which were the same in plasma and urine, are shown in Table I. In Plasma, a linear response was obtained over a range of 0.3—25 μg/ml and in urine the curve was linear from 0.5 to 125 μg/ml (Table II). The correlation coefficients indicate the excellent linearity of the response. Recovery of pyridoxine added to human plasma in the concentration range of 1—30 μg/ml was found to be 100 ± 5%. The lowest concentration which can be measured accurately is 0.3 μg/ml for pyridoxine, pyridoxal and 4-pyridoxic acid, and 1.0 μg/ml for pyridoxamine. The total

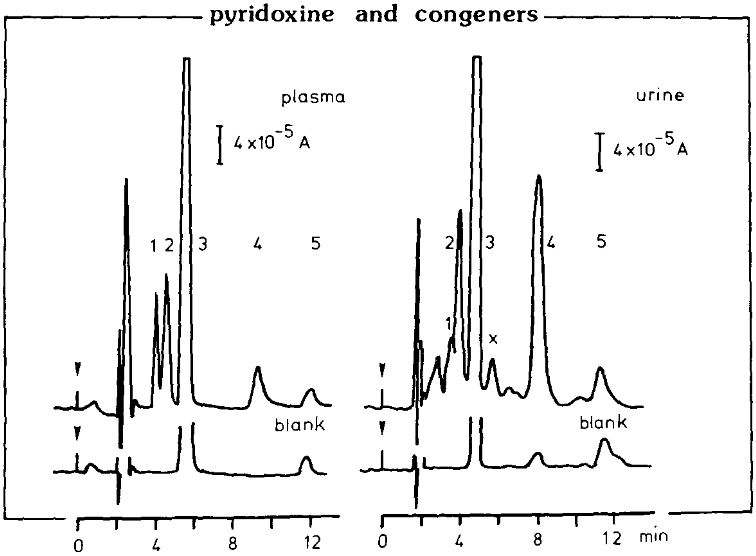

Fig. 1. HPLC chromatograms of pyridoxine and congeners in plasma and urine samples obtained from volunteers after the intake of 750 mg of pyridoxine and their respective blanks, sampled prior to drug intake. Peaks: 1 = pyridoxal; 2 = pyridoxine; 4 = 4-pyridoxic acid; 3 and 5 are unknown plasma and urine compounds; x represents an unknown metabolite in urine.

TABLE I

RELATIVE RETENTION TIME OF PYRIDOXINE AND CONGENERS

Compound	Relative retention time
Pyridoxamine	0.30
Pyridoxamine-5-phosphate	0.46
Pyridoxal	0.82
Pyridoxal-5-phosphate	1.25
Pyridoxine	1.46
Unknown plasma and urine peak	1.80
Unknown metabolite	2.36
4-Pyridoxic acid	3.75

time required for an analysis is 15 min. A series of assays was carried out to determine the reproducibility of the assay. In plasma it appeared to be 100 ± 2%; in urine at high levels (575 μg/ml) it was 100 ± 1% and at low levels (7.5 μg/ml) 100 ± 3.5% (n = 10).

When the method was applied in preliminary pharmacokinetic studies of pyridoxine, a number of clear-cut results were obtained. The measurements showed that this drug was rapidly absorbed with a peak plasma level appearing in less than an hour after oral administration (Fig. 2), and indicated an elimination half-life for the parent compound as well as for the metabolites of 1—1.6 h.

In urine, high levels of the vitamers, particularly 4-pyridoxic acid, were observed, up to 500 μg/ml in some samples. The limit of sensitivity in urine was about 0.5 μg/ml.

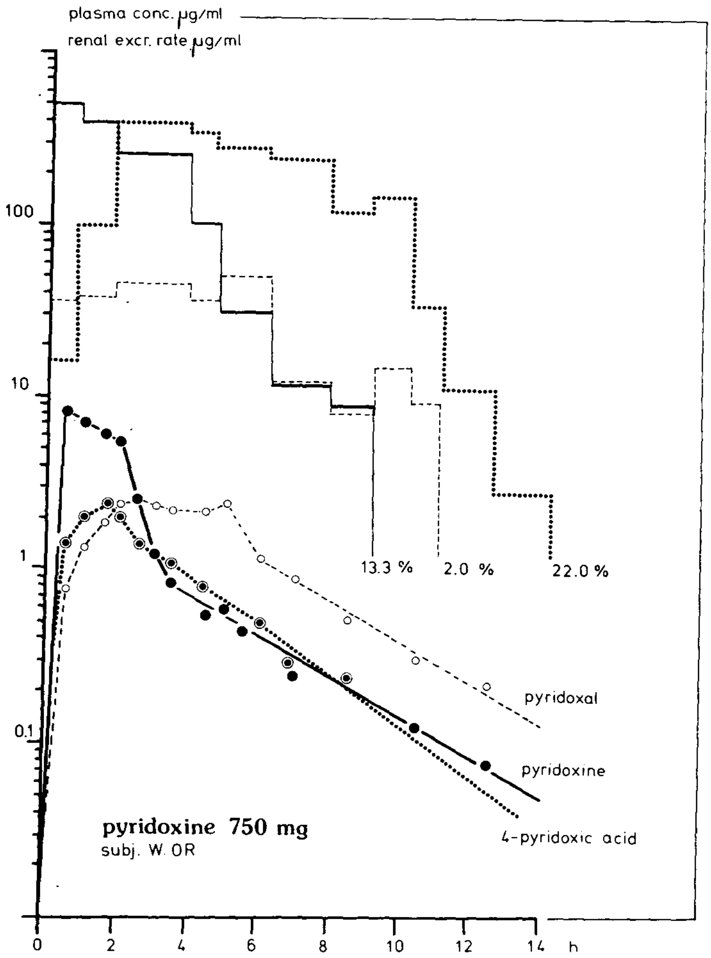

Fig. 2. Pharmacokinetics of pyridoxine and congeners (pyridoxal and 4-pyridoxic acid) in man after the intake of 750 mg of pyridoxine.

TABLE II

STANDARD CURVE DATA FOR PYRIDOXINE AND CONGENERS

The standard curve was constructed from peak height versus plasma or urine concentration (µg/ml).

Compound	Plasma*			Urine**		
	Slope	Intercept with *y*-axis	Corr. coeff.	Slope	Intercept with *y*-axis	Corr. coeff.
Pyridoxine	6.78	1.12	0.99	0.57	0.08	0.98
Pyridoxal	5.49	0.40	0.99	0.41	−0.50	0.99
4-Pyridoxic acid	1.85	0.10	0.99	0.17	0.80	0.98

*Concentration range 0—25 µg/ml.
**Concentration range 0—125 µg/ml.

The major metabolic products of pyridoxine were pyridoxal and 4-pyridoxic acid (Fig. 2) the latter being the predominant urinary product (Table III). The total amount of recovered pyridoxine, as free drug and metabolites, represents approximately 35% of the administered dose, so there must be other unidentified metabolites or pathways yet to be elucidated (Table III). One unidentified metabolite was observed in all subjects (Table I) and appeared to exhibit a similar pharmacokinetic profile to pyridoxine and 4-pyridoxic acid. More extensive studies in pyridoxine pharmacokinetics will be carried out in order to elucidate the structure of this unknown metabolite.

TABLE III

PHARMACOKINETIC PARAMETERS OF PYRIDOXINE AND CONGENERS AFTER THE ORAL ADMINISTRATION OF 750 mg OF PYRIDOXINE TO FOUR VOLUNTEERS

Values are expressed as mean ± S.D.

	Pyridoxine	Pyridoxal	4-Pyridoxic acid	Total
Half-life (h)	1.7 ±0.4	1.8 ±0.3	1.8 ±0.4	—
Percentage of the dose excreted in urine over 24 h calculated as percentage of free pyridoxine	13.61±2.56	1.92±0.62	20.05±2.32	35.58±2.65

DISCUSSION

In these studies, the only vitamers identified in plasma and urine after high-dose therapy were pyridoxine, pyridoxal and 4-pyridoxic acid. Therefore discussion of the assay will be limited to these compounds. The results indicate that pyridoxamine and probably the phosphorylated congeners could be quantitated by a modification of the method described, but since these compounds were not observed, their estimation was not further investigated.

The method described permits the rapid assay of pyridoxine, pyridoxal and 4-pyridoxic acid in plasma and urine in the concentration achieved by high-dose therapy. The day-to-day reproducibility of the assay was constantly checked by the inclusion of standards in all series and was excellent. In contrast to the available enzymatic and microbiological methods [4—8], the technique is sufficiently sensitive and highly specific for the non-phosphorylated vitamers. An assay requires only 15 min per sample compared to lengthy ion-exchange procedures [10], gel filtration [9] or thin-layer chromatographic methods [13]. No complicated oxidative procedures are required as in the technique of Fujita and co-workers [15, 16]. The HPLC assay developed by Stewart et al. [18] was designed to assay simultaneously pyridoxine and isoniazid in pharmaceutical products. This technique involves the use of an ion-pair reagent (sodium dioctyl sulphosuccinate) on a reversed-phase column. In our hands this method gave inadequate separation of the three vitamers, pyridoxine, pyridoxal and 4-pyridoxic acid. The gas—liquid chromatographic method of Williams [20] gives a slightly lower sensitivity than the present assay but has the disadvantage of requiring derivatization of the polar and non-volatile vitamers. Moreover, it requires considerably more time.

Greater sensitivity, however, would permit the assay of normal plasma levels of the compounds (normal vitamin B_6 activity is 15–35 ng/ml [3]). The endogenous concentrations obtained in urine by back projection of the standard curve in one subject gave a total daily urinary output of the vitamers as 2.6 mg. This is close to the recommended daily intake [3], which probably reflects the vitamin B_6 content of an average diet. If lower levels of the compound were to be quantitated, extraction or concentration procedures may be required. Because the compounds are extremely polar, attempts at solvent extraction from plasma and urine were unsuccessful. The use of other detection methods and suitable pretreatment could increase the sensitivity of the present assay and might permit the direct determination of clinically normal and deficient concentrations of vitamin B_6.

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Note

Identification of adenosine and eight modified adenine nucleosides using reversed-phase high-performance liquid chromatography and enzymatic peak shift with adenosine deaminase

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The modified nucleosides have been increasingly studied by high-performance liquid chromatography (HPLC) [1—17]. They are of interest as indicators of RNA metabolism [1—3, 17] and because some are elevated in the urines of patients with cancer [4, 5, 18]. It is the purpose of this report to show that the naturally occurring modified adenine nucleosides (and 3′-O-methyladenosine) can be identified by high-performance reversed-phase chromatography which has been optimized for the separation of most nucleosides [9, 13]. The basis for this identification is the enzyme peak shift in which the retention times are determined, both of the sample and of the N^6-deaminated product, after incubation with adenosine deaminase (ADA) [7, 8].

MATERIALS

Adenosine deaminase was prepared from chicken liver by the method of Ma and Fisher [19] with the following modifications: A Sephacryl S-200 column was used for the initial chromatography instead of a Sephadex G-150 column. Additionally, the 35,000 molecular weight ADA fractions obtained were concentrated by ammonium sulphate precipitation, passed through a Sephadex G-75 column and fractions containing 35,000 molecular weight ADA reconcentrated. This 35,000 molecular weight ADA, used for the HPLC enzymatic

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analysis, had no detectable contaminating purine nucleoside phosphorylase activity as determined spectrophotometrically.

1-Methyladenosine, 3′-O-methyladenosine, N^6,N^6-dimethyladenosine, N^6-isopentenyladenosine, N^6-2′-O-dimethyladenosine, and 1-methylinosine were purchased from P & L Biochemicals (Milwaukee, WI, U.S.A.). Adenosine, 2′-deoxyadenosine, inosine, and 2′-deoxyinosine were purchased from Sigma (St. Louis, MO, U.S.A.). Other reagents used were the highest grade obtainable: monobasic ammonium dihydrogen phosphate, Fisher Scientific (Fairlawn, NJ, U.S.A.), methyl alcohol, Burdick and Jackson Labs. (Muskegon, MI, U.S.A.) and Tris (Base) Ultra Pure, Schwarz/Mann (New York, NY, U.S.A.). Water used was passed through an Ultra Pure waters system, Hydro Service and Supplies (Durham, NC, U.S.A.). Type 1 calf intestinal ADA was obtained from Sigma.

METHODS

Standard compounds were dissolved in 0.01 *M* Tris—HCl buffer, pH 7.0, at a concentration of 10^{-4} *M*. Incubation of nucleosides with chicken liver ADA varied from 15 min to 24 h at 37°C. Aliquots were removed at different time intervals until there was 95—100% conversion of the substrate (except for N^6,N^6-dimethyladenosine, which was a very poor substrate). In order to analyze a complex mixture, nucleosides can be incubated with 50 times the amount of enzyme necessary to totally convert adenosine in one hour. Similar results can be obtained with commercially available calf intestinal ADA (Sigma) using 17.4 U/1.0 ml for 1 h at 37°C.

Analyses were performed on a reversed-phase column [μBondapak C_{18}, Waters Assoc. (Milford, MA, U.S.A.)] at room temperature using a modification of published methods [9, 15]. Two single-piston high-pressure pumps (Constametric I and II, Laboratory Data Control, Riviera Beach, FL, U.S.A.) delivered solvents in a linear gradient from 0—40% methanol in 0.01 *F* $NH_4H_2PO_4$, pH 5.5, during 60 min at a final constant flow-rate of 1.5 ml/min. Samples (25—100 μl) were injected (Rheodyne 7120 syringe loading sample injection valve, Laboratory Data Control) and the effluent monitored with a variable-wavelength ultraviolet detector at 260 nm with a 10-μl flow cell (modified Spectromonitor 1, Laboratory Data Control).

RESULTS AND DISCUSSION

In preliminary experiments, when the various adenine nucleosides were incubated with a relatively crude preparation of ADA in the presence of phosphate buffer, two different products were often generated. These were thought to result from the action of contaminating nucleoside phosphorylase, which can further metabolize the deaminated nucleosides to the respective base and ribose-1-phosphate or to a kinase yielding a phosphorylated derivative. Both of these reactions require phosphate [20]. Therefore the ADA was further purified and incubations with ADA were carried out in Tris buffer. Under these conditions a single product was formed for each compound investigated.

Chicken ADA (molecular weight 35,000) has a broad spectrum of activity in its ability to N^6-deaminate adenine nucleosides with modifications in the base or sugar moieties [21—23]. All eight modified nucleosides served as substrates for chicken liver ADA as assayed by HPLC [23]. Table I lists the retention times of adenosine, seven naturally occurring adenine nucleosides (and 3′-O-methyladenosine) and their corresponding inosine products after treatment with ADA. All of the modified adenine nucleosides eluted separately except for 3′-O-methyladenosine and N^6-methyladenosine, which co-eluted. Incubation of either adenosine, N^6-methyladenosine, N^6,N^6-dimethyladenosine or N^6-isopentenyladenosine with ADA all resulted in the appearance of the same product, which co-eluted with authentic inosine. Incubation of 1-methyladenosine gave rise to 1-methylinosine and of 2′-deoxyadenosine to 2′-deoxyinosine, as determined by co-elution with authentic 1-methylinosine and 2′-deoxyinosine. The N^6-2′-O-dimethyladenosine and 2′-O-methyladenosine both gave rise to a product with the same retention time. It was presumed that 2′-O-methylinosine was their single corresponding N^6-deaminated product. Similarly, 3′-O-methyladenosine was presumed to yield 3′-O-methylinosine. In support of this conclusion, the area of the peaks of the products generated was approximately one half that of the substrate, similar to the absorption ratios of adenosine and inosine at 260 nm. Thus, under these conditions, all of the N^6-deaminated products (inosine, 2′-deoxyinosine, 1-methylinosine, 2′-O-methylinosine and 3′-O-methylinosine) had characteristic, non-overlapping retention times. (It should be noted that 3′-O-methyladenosine stored at −20°C for several months in water contained trace amounts of an unidentified contaminant that eluted at 30.0 min and was not sensitive to treatment by ADA.)

Six of the eight compounds tested each had unique retention times. While N^6-methyladenosine and 3′-O-methyladenosine had identical retention times, their respective deaminated reaction products, inosine and 3′-O-methylinosine were quite widely separated. However, in biological samples other compounds could have similar elution times. Therefore, application of the peak shift method [7, 9] using ADA allows for identification of an ADA-sensitive compound and of the deaminated product.

TABLE I

RETENTION TIMES OF ADENOSINE AND EIGHT MODIFIED ADENINE NUCLEOSIDES AND OF THEIR SINGLE PRODUCTS GENERATED AFTER INCUBATION WITH ADA

See Materials and Methods for preparation of chicken liver adenosine deaminase (molecular weight 35,000), for enzymatic assay conditions and for HPLC system used.

Substrate	Retention time (min)	Product	Retention time (min)
1-Methyladenosine	6.7	1-Methylinosine	12.0
Adenosine	16.1	Inosine	8.6
2′-Deoxyadenosine	17.7	2′-Deoxyinosine	10.4
2′-O-Methyladenosine	21.4	2′-O-Methylinosine	13.2
3′-O-Methyladenosine	24.2	3′-O-Methylinosine	14.8
N^6-Methyladenosine	24.2	Inosine	8.6
N^6-2′-O-Dimethyladenosine	28.8	2′-O-Methylinosine	13.2
N^6,N^6-Dimethyladenosine	33.9	Inosine	8.6
N^6-Isopentenyladenosine	55.5	Inosine	8.6

The applicability of the enzyme peak shift technique to detection of modified adenine nucleosides in biological samples is illustrated in Fig. 1. Peaks with retention times of 1-methyladenosine and 2′-deoxyadenosine are seen in this chromatogram of urine from a neonatal mouse which had been injected with deoxycoformycin. Following incubation of the urine sample with ADA (lower panel) the 1-methyladenosine and 2′-deoxyadenosine peaks are no longer visible but two new peaks have appeared with the retention times of 1-methylinosine and 2′-deoxyinosine.

Application of this method to identification of all of the compounds in biological samples may require preliminary separative procedures such as anion-exchange [24] or boronate columns [5]. Unambiguous determination may on occasion require collection of relevant substrate peak, concentration, treatment of this isolated peak with ADA, and rechromatographing to determine product

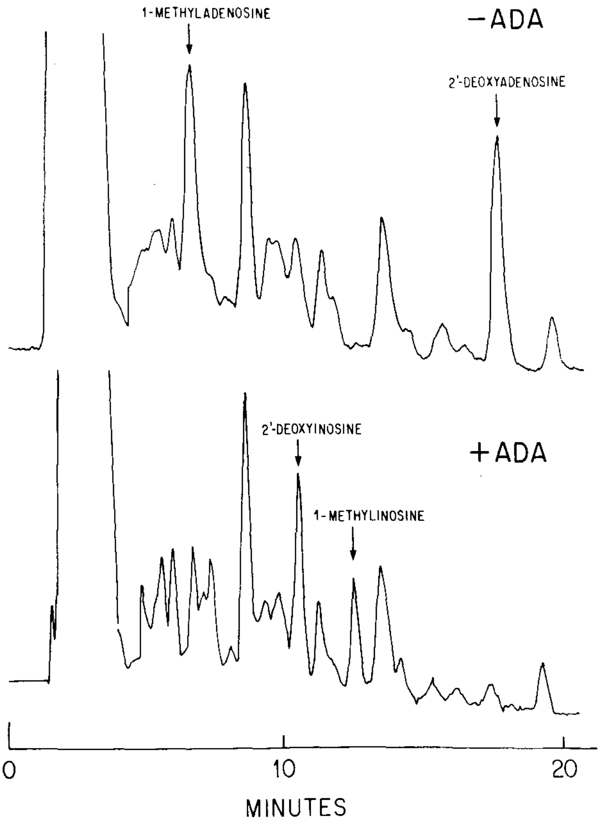

Fig. 1. Demonstration of enzymatic peak shift technique for identification of 1-methyladenosine and 2′-deoxyadenosine in whole neonatal mouse urine. The mouse had been treated with deoxycoformycin, an ADA inhibitor, 24 h prior to collection of the urine. Two peaks, eluting at 6.4 and 17.7 min, the retention times of 1-methyladenosine and 2′-deoxyadenosine respectively, are seen in the upper panel but not following treatment of the urine with ADA (lower panel). Two peaks eluting at 12.5 and 10.1 min, the retention times of 1-methylinosine and 2′-deoxyinosine respectively, appear in the lower chromatogram following treatment of the urine with ADA but are not seen in the untreated urine (upper panel).

retention time without the presence of interfering compounds that might have been present in the original complex sample mixture.

Several modified and unmodified adenine nucleosides and bases have previously been analysed using HPLC (Table II). We have identified all of these modified nucleosides, except for N^6-isopentenyl-2-methylthioadenosine in the system used here, and additionally have identified 2′-O-methyladenosine, 3′-O-methyladenosine and N^6-2-O-dimethyladenosine and their respective deaminated products. Analysis of modified adenine nucleosides using reversed-phase columns would appear to be most advantageous for application to biological materials, since these compounds (except for 1-methyladenosine) are relatively retarded and therefore elute after most of the other UV absorbing materials. As more attention is directed to analyzing tRNA metabolism and the modified nucleosides found in the urine of patients with neoplastic and metabolic diseases the importance of identifying members of this class of compounds will increase.

TABLE II

ADENINE NUCLEOSIDES ANALYZED BY HPLC

Compound	Column	Reference
1-Methyladenosine	Cation	6
	Reversed phase	5,*
Adenosine	Cation	6
	Reversed phase	5, 7–9, 11, 13, 14,*
2′-Deoxyadenosine	Cation	22
	Reversed phase	14,*
N^6-Methyladenosine	Cation	6
	Reversed phase	13,*
N^6-2′-O-Dimethyladenosine	Reversed phase	*
N^6,N^6-Dimethyladenosine	Cation	6
	Reversed phase	*
N^6-Isopentenyladenosine	Cation	15
	Reversed phase	10,*
N^6-Isopentenyl-2-methylthioadenosine	Cation	15
	Reversed phase	10,*
2′-O-Methyladenosine	Reversed phase	*
3′-O-Methyladenosine	Reversed phase	*

*This paper.

In conclusion, identification of the modified adenine nucleosides has been demonstrated on reversed-phase chromatography on the basis of the differential retention times of the nucleosides and/or the reaction products generated after treating the nucleoside sample mixture with ADA. This extends the usefulness of the previously described enzymatic peak shift technique for identifying adenosine [7, 9]. It should prove of value in further identifying the modified adenine nucleosides in polynucleotide metabolism and in complex biological tissue and fluid samples. Preliminary investigations suggest that this method can indeed be applied to body fluids.

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CHROMBIO. 623

Note

Rapid method for simultaneous determination of phenobarbital, diphenylhydantoin and their main hydroxylated metabolites by nitrogen-selective gas chromatography

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Phenobarbital (PB) and diphenylhydantoin (DPH) remain the drugs of choice in epilepsy characterized by general convulsive seizures [1].

Some years ago a gas chromatographic method was developed and employed in this laboratory to assay PB and DPH plasma levels in epileptic patients [2]. The introduction of a nitrogen-selective detector to gas chromatography made this method simpler, quicker and highly precise for use in pharmacokinetic studies [3].

The principal metabolic route for both PB and DPH is the aromatic hydroxylation to *p*-hydroxy compounds, by the hepatic microsomal enzymes [4, 5], which are subsequently conjugated with glucuronic acid before being excreted into the urine [6, 7]. Urinary and also biliary excretion data provide precise information about the overall elimination constants for the loss of drug from the body as well as the rate constants for drug metabolism [8]. As the two drugs are very often administered together and as possible drug—drug interaction is expected in the level of urinary excretion and metabolism, the analytical systems applicable to the determination of PB and DPH in biological samples should permit simultaneous quantitation of both unchanged drugs and their metabolites.

To separate the two drugs from their *p*-hydroxylated metabolites several techniques have been published including spectrophotometry [4], paper chromatography [9], thin-layer chromatography [10], gas—liquid chromatography [11, 12] and liquid chromatography [13]. Most of the published methods present either problems of interference [4] or time consuming procedures [11—13]. In addition, amongst these methods only a few considered the importance of determining the two drugs and their hydroxylated metabolites in the same sample [13].

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In this report we describe a simple gas—liquid chromatographic method using nitrogen selective detection to assay PB, DPH, *p*-hydroxyphenobarbital (*p*-OHPB) and *p*-hydroxyphenylphenylhydantoin (*p*-HPPH), simultaneously in biological specimens, including urine and bile. The chromatographic analysis was realized in less than 10 min without the need for temperature programming.

MATERIALS AND METHODS

Instruments

A Packard Model 429 gas chromatograph equipped with nitrogen—phosphorus (N—P) selective detector and a 1.8 m × 2 mm I.D. spiral glass column packed with 3% OV-1 on 100—120 mesh Gas-Chrom Q, was used for the gas chromatographic analysis.

The column was silanized with 5% dimethylchlorosilane (Eastman-Kodak, Rochester, NY, U.S.A.) and conditioned overnight at 250°C before use. Column, injector and detector temperatures were fixed at 210, 240 and 250°C, respectively. Nitrogen was used as the carrier gas at a flow-rate of 60 ml/min. The chemical structures of the metabolites in the urine extract were confirmed by using an LKB 2091 mass spectrometer coupled to a gas chromatograph.

Reagents

p-Tolylphenylhydantoin (*p*-MPPH), *p*-OHPB and *p*-HPPH were purchased from Aldrich-Europe (Beerse, Belgium). DPH was obtained from Fluka (Buchs, Switzerland). Trimethylaniliniumhydroxide (TMPAH) was obtained from Eastman-Kodak. PB and all organic solvents of analytical grade were obtained from Merck (Darmstadt, G.F.R.).

A stock solution of the four tested compounds was prepared in pure methanol at a concentration of 1 mg/ml, the internal standard was made separately in solution at a concentration of 1 mg/ml. The two solutions were kept at +4°C when not in use. Prior to the assay, aliquots of the mother solution were diluted to the desired concentrations in blank samples.

Extraction procedure

To a 40-ml glass centrifuge tube, were added 0.1 ml of the internal standard (400 μg/ml), 0.5 ml of urine sample, 1.5 ml of phosphate buffer pH 6.8 and 10 ml of diethyl ether. After mixing for 10 min on a mechanical shaker and centrifuging at 1340 *g*, the diethyl ether extract was separated and filtered through filter paper. The filter paper was then washed with 3 ml of diethyl ether. The combined organic extract and washing were evaporated to dryness under vacuum in a water bath at 45°C. The dry residue was transferred into a small vial (1.5 ml) by dissolving it into 0.6 ml ethanol, twice. The ethanol was evaporated off on a sand bath at moderate temperature under a slight stream of nitrogen. The residue was then dissolved in 0.1 ml of 0.2 *M* TMPAH and 0.5—1 μl was injected into the chromatograph.

Conjugated metabolites

The conjugated metabolites were hydrolyzed with concentrated hydrochloric acid prior to the extraction. Urine (0.5 ml) was mixed with 0.5 ml 12 *N*

HCl in the centrifuge tube containing the internal standard and placed in a water bath at 90°C for 60 min. After cooling the mixture was carefully neutralized with 0.5 ml 12 *M* NaOH followed by the addition of 1.5 ml phosphate buffer pH 6.8. The sample was then processed as described above. This drastic hydrolysis liberated all the conjugated metabolites, either sulphates or glucuronides.

In order to determine both free and conjugated hydroxy metabolites the urine samples were analyzed before and after hydrolysis. The amounts of conjugated metabolites were then calculated from the difference between the total and the free compounds.

Calibration curves

Calibration curves were prepared by adding different amounts of the four compounds to drug-free urine; then the urine samples were assayed as described before. The peak height ratios of each compound to the internal standard were plotted against concentration in the range of 5—100 μg/ml.

RESULTS

Table I shows the within-run precision, at two different concentrations (n = 10) for each of the tested compounds. The coefficient of variation did not exceed 7.2% for *p*-OHPB and was less for the other three compounds.

TABLE I

WITHIN-RUN ESTIMATES OF THE PRECISION OF THE ANALYSIS OF PB, DPH, *p*-OHPB AND *p*-HPPH

Results are based on ten analyses for each of two concentrations of PB, DPH, *p*-OHPB and *p*-HPPH. The internal standard is *p*-tolylphenylhydantoin. Chromatography on a 1.8-m column containing 3% OV-1 on 100—120 mesh Gas-Chrom Q.

Drug	Concn.	Mean	± S.D.	C.V. (%)
PB	20	19.18	0.89	1.7
	40	37.90	0.91	
DPH	20	21.50	0.46	2.2
	40	40.50	1.10	2.8
p-OHPB	10	10.04	0.72	7.2
	40	38.80	2.20	5.8
p-HPPH	20	18.20	0.95	5.2
	40	42.40	1.10	2.5

Standard curves were constructed by plotting peak height ratios (drug/internal standard) versus drug concentrations (μg/ml). The instrument responses and the concentrations were linearly related for all compounds (Fig. 1) over a range of 5—100 μg/ml. The correlation coefficient of the linear regression approaches 0.99 for each of the four drugs.

Extraction efficiencies of urine samples containing known amounts of PB, DPH, *p*-OHPB and *p*-HPPH were 88, 97, 84 and 107% respectively.

Fig. 2 depicts chromatograms of rat urine (animal dosed with PB and DPH), before and after hydrolysis, compared to a chromatogram of the pure compounds in methanol. The retention times for PB, DPH, *p*-OHPB, internal stan-

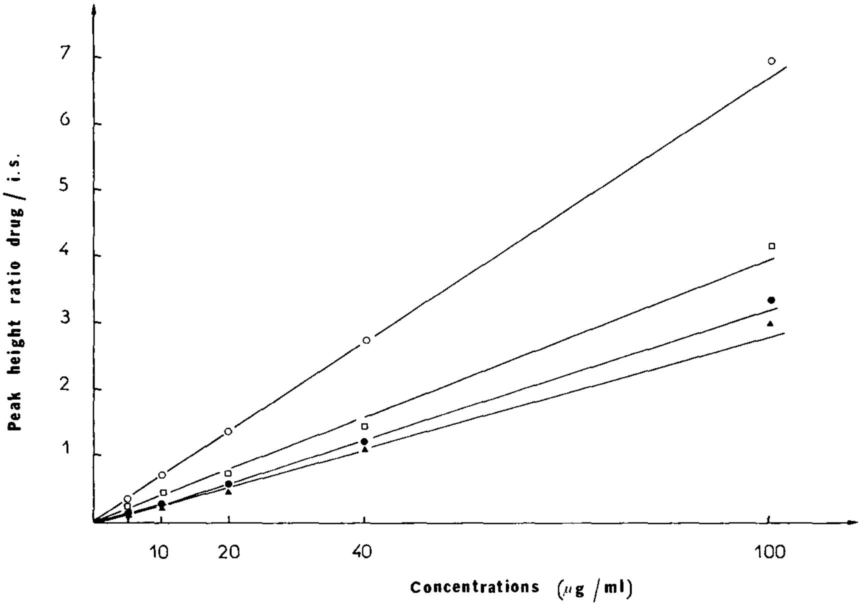

Fig. 1. Standard curves for determination of PB (○), *p*-OHPB (●), DPH, (□) and *p*-HPPH (▲). Internal standard, *p*-tolylphenylhydantoin.

dard and *p*-HPPH were 1, 3, 4, 6 and 9 min, respectively. No interference from urine components was observed and 100 μl of 0.2 *M* TMAH was enough to give complete methylation.

The quantification of the DPH in urine can be achieved by injecting a larger sample volume, as less than 5% of this drug is excreted in urine in an unchanged form.

The mass spectrum of the urine extract showed the formation of N,N,O-trimethyl derivatives of the two hydroxylated metabolites. The values of the more intense ions were 261 (100), 148 (83), 290 ($M^{+\cdot}$) (75) and 262 (50) for the *p*-OHPB-trimethyl derivative and 233 (100), 148 (53), 310 ($M^{+\cdot}$) (47) 224 (31), 118 (28) and 77 (27) for the *p*-HPPH-trimethyl derivative.

DISCUSSION

The proposed method possesses the important characteristics of precision, sensitivity and specificity over a wide range of PB, DPH, *p*-OHPB, and *p*-HPPH concentrations, in addition to extreme rapidity. The retention times for the four compounds and the internal standards are very satisfactory and the five peaks are very well separated.

The extraction procedure is efficient and using a pH of 6.8 to buffer the medium minimizes the extraction of the undesirable urine components. The use of the nitrogen-selective detector makes it possible to reduce sample size and to eliminate the sophisticated purification and clean-up steps. The limit of detection by the N—P detector is very satisfactory and superior to that reported for liquid chromatography [13]. The advantage in using such a detector is its relative insensitivity to hydrocarbons [14].

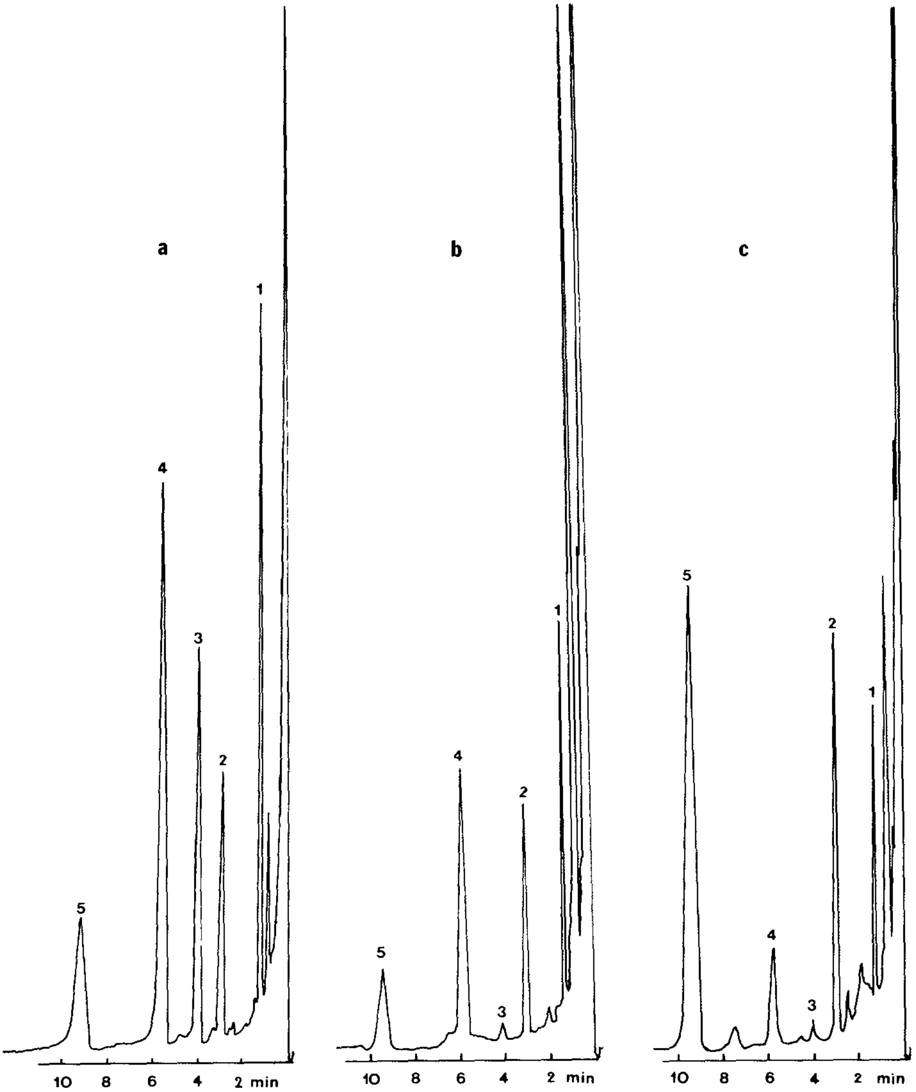

Fig. 2. Gas chromatograms of (a) pure solution of PB (1), *p*-OHPB (2), DPH (3), internal standard (4) and *p*-HPPH (5); (b) and (c) extracts of 0.25 and 0.5 ml of urine of rat pretreated with PB and DPH before and after hydrolysis respectively. Peaks as in a.

Most of the conjugated *p*-OHPB and *p*-HPPH excreted in the urine are in the form of glucuronides [12, 15], so acid hydrolysis can give a clear picture of the amount of glucuronides formed. But acid treatment of urine samples containing metabolites of PB and DPH in order to liberate the phenolic metabolites may transform the dihydrodiol metabolites into *m*- and *p*-hydroxy metabolites [16]. This can produce an error of about 10% in the case of *p*-HPPH. Therefore it might be preferable in precise metabolic studies to employ specific enzymatic cleavage. However with conjugated *p*-OHPB the values obtained with acid hydrolysis and β-glucuronidase treatment were very close

[12]. Diol metabolites can be removed from the medium by pre-extraction with isoamyl alcohol prior to hydrolysis [15], but since our aim was to describe a chromatographic method capable of the simultaneous separation of both parent drugs and their major metabolites, no attempts to remove the diol metabolites were made.

Investigations using the simultaneous quantitation of these compounds in urine and bile permitted the evaluation of the effects of the administration of one drug on the pharmacokinetics of the other [3]; in addition, more information about the factors affecting the *para*-hydroxylation in subjects under simultaneous DPH and PB treatment can be provided.

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CHROMBIO. 635

Note

Quantitative determination of benzilonium bromide in plasma by gas chromatography—mass spectrometry after oxidation to benzophenone

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Benzilonium bromide is a quaternary ammonium compound and an anticholinergic agent. While studying its pharmacological effects on urinary bladder function [1] we developed a method to quantify benzilonium bromide down to 5 ng/ml plasma; the method is described in this paper.

EXPERIMENTAL

Instrumental

A Finnigan 4000 gas chromatograph—mass spectrometer equipped with a Promim device was used (Finnigan, Sunnyvale, CA, U.S.A.). The column was a 3% OV-225 (1.4 m × 2 mm I.D.) operated at 175°C and directly interfaced with the ion source. Methane was used as the carrier gas (flow-rate 20 ml/min).

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Under these conditions benzophenone had a retention time of 1.8 min. Injection temperature was 200°C and the ion-source temperature was 250°C. The electron energy was 70 eV. The mass spectrometer was focused to monitor the ions *m/e* 183 and 188.

Internal standard

The internal standard, benzilonium-d_5 bromide, was synthesised by a Grignard reaction of pentadeuterobromobenzene with methylphenylglyoxylate [2], and by reacting the obtained methyl pentadeuterobenzilate with 1-ethyl-3-hydroxypyrrolidine, followed by quaternisation with ethyl bromide [3].

Reagents

Carbonate buffer solution (pH 9.0, 0.1 *M*) containing 1 mg/ml sodium 2-hydroxy-3,5-di-*tert*-butylbenzene sulphonate (Bofors Nobel Kemi S-690 20, Bofors, Sweden); saturated potassium permanganate solution (ca. 0.4 *M*); potassium hydroxide solution (5.7 *M*); aqueous solution of benzilonium-d_5 bromide (200 ng/ml).

Sample preparation

To a screw-capped 15-ml tube containing a plasma sample (2 ml) were added internal standard solution (100 µl), carbonate buffer solution (2 ml) and dichloromethane (6 ml). The mixture was shaken for 15 min and then centrifuged (5 min, 500 *g*). The aqueous phase was removed and discarded. After addition of anhydrous sodium sulphate (0.5 g) the tube was shaken for a short while and then centrifuged. The dry dichloromethane phase was removed and filtered through a Pasteur pipette, stoppered with glass wool, into a new screw-capped tube. The solvent was evaporated in a stream of nitrogen. Potassium hydroxide solution (3 ml), potassium permanganate solution (2 ml) and hexane (2 ml) were then added to the tube. The mixture was shaken in a water bath at 70°C for 1 h. After cooling, the tube was centrifuged and the hexane phase was transferred to a conical tube (5 ml).

Removal of the solvent was done by evaporation in a stream of nitrogen. The residue was dissolved in ethyl acetate (20 µl) and an aliquot was injected on to the column.

RESULTS

Extraction

Isolation of the "benzilonium ion" from plasma was accomplished by the addition of sodium 2-hydroxy-3,5-di-*tert.*-butylbenzene sulphonate and extraction with dichloromethane at pH 9. The sulphonate ion used is strongly lipophilic but still water-soluble, which makes it a good counter-ion for ion-pair extraction of quaternary ammonium compounds [4]. The recovery of the "benzilonium ion" was determined gravimetrically and found to be at least 98%. The recovery of benzilonium (determined as benzophenone) through the method was estimated to be about 85% at the 50 ng/ml level. This was accomplished by comparing the molecular ion intensity of benzophenone, generated from benzilonium by the method described, with that of a standard sample of benzophenone.

Oxidation

Oxidation of the "benzilonium ion" to benzophenone was performed according to a general procedure described by Hartvig et al. [5]. The benzophenone yield was 90%. When blank plasma samples were analysed, a peak appeared in the gas chromatograms corresponding to 0.5—1.5 ng/ml benzophenone. In plasma samples from the same patient, however, the background did not show any significant variation when the analyses were performed in one series using the same batches of reagents, solvents, pipettes, etc. In an attempt to minimize background disturbance, oxidation to benzophenone was performed by using barium peroxide in acidic solution. This procedure has been shown to give low reagent blanks [6]. However, on using this procedure, the yield of benzophenone from "benzilonium ion" was less than 10%.

Mass spectrometry

Chemical ionisation was preferred to electron impact since it was possible to achieve much higher sensitivity when analysing benzophenone with this mode of ionisation.

Sensitivity and precision of the method

The coefficient of variation obtained when analysing two series of plasma samples, nine samples in each series, to which had been added 5 and 50 ng of benzilonium bromide per ml, were 6% and 1.7%, respectively.

Application to biological samples

Five minutes after intravenous injection of 1 mg of benzilonium bromide in healthy adults the mean plasma concentration (five individuals) was 100 ng/ml. In the same individuals 10 mg taken orally two times daily for one week resulted in a plasma concentration of 1—4 ng/ml measured before taking the morning dose on the 8th day; and 2—3 h after the morning dose on that day the plasma concentration was 3—4 ng/ml. Since the obtained levels of benzilonium ion after the described oral administration were equal to or slightly above the detection limit of the method only a rough estimation of the plasma concentrations was possible in this case.

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CHROMBIO. 629

Note

An integrated approach to measurements of quinidine and metabolites in biological fluids

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Several procedures to quantitate quinidine in plasma by isocratic reversed-phase high-performance liquid chromatography (HPLC) have been described in the literature [1—6], but only a few of them allow simultaneous, separate quantitation of some of the known major metabolites [4—6]. We recently reported a method for determination of quinidine and its metabolites in urine by reversed-phase HPLC [7]. This analytical technique has now been modified and expanded so that one procedure allows determination in plasma or urine of quinidine, dihydroquinidine and the metabolites 3-hydroxyquinidine, an N-oxide of quinidine and 2'-quinidinone.

EXPERIMENTAL

Materials

The solvents used (acetonitrile and tetrahydrofuran, UV grade; all others, analytical grade) were obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.) except dichloromethane and isopropanol, which were supplied by Mallinckrodt (St. Louis, MO, U.S.A.).

Dihydroquinidine-free quinidine was prepared from commercially available quinidine (J.T. Baker, Phillipsburg, NJ, U.S.A.) according to the method described by Thron and Dirscherl [8]. The quinidine metabolites 2'-quinidinone and 3-hydroxyquinidine were kindly supplied by Dr. Irwin Carroll, Triangle Research Institute, O-desmethylquinidine by Syva Labs. (Palo Alto, CA,

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U.S.A.) and the N-oxide was prepared as described elsewhere [9]. Pronethalol was obtained from ICI (Macclesfield, Great Britain).

A Varian Model 8500 high-performance liquid chromatograph (Varian, Palo Alto, CA, U.S.A.) was used, equipped with either a Varian ultraviolet detector set at 230 nm or fluorescence detector (Schoeffel Instrument Corp., Westwood, NJ, U.S.A.), excitation at 245 nm, emission at 340 nm (cutoff filter) and an alkyl phenyl μBondapak column (particle size, 10 μm) of 30 cm × 3.9 mm I.D. (Waters Assoc., Milford, MA, U.S.A.). The mobile phase was 0.05 *M* phosphate buffer pH 4.75—acetonitrile—tetrahydrofuran (80:15:5, v/v) with a flow-rate of 1.5 ml/min.

Procedure

Plasma or urine (200 μl) is mixed with 200 μl or 400 μl respectively of an aqueous solution containing 200 μg/ml of pronethalol (internal standard). A 200-μl aliquot of 0.6 *M* borate buffer pH 9.0 is added followed by 10 ml of dichloromethane—isopropanol (4:1, v/v). After 1 min vortexing and centrifugation for 5 min at 540—1200 *g*, the organic layer is transferred to a separate test-tube and evaporated to dryness. The residue is reconstituted in 200 μl of eluent and a 50-μl aliquot injected onto the high-performance liquid chromatography column. Detection was achieved using the fluorescence detector for plasma extracts and the ultraviolet detector for urine extracts.

For low plasma levels of quinidine and metabolites the volume of plasma taken for assay and of buffer added can be increased from 200 μl to 1.0 ml.

RESULTS

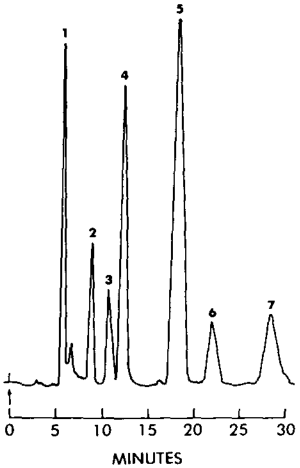
Fig. 1 shows a chromatogram from a plasma sample spiked with quinidine and metabolites. The retention times of 2′-quinidinone, 3-hydroxyquinidine, O-desmethylquinidine, pronethalol, quinidine, dihydroquinidine and an N-oxide of quinidine [9] are 5.7, 8.8, 10.7, 12.1, 18.1, 22.0 and 28.4 min respec-

TABLE I

PRECISION AND ACCURACY OF REVERSED-PHASE ASSAY OF SPIKED URINE SAMPLES

A 1-ml aliquot of urine was taken for assay.

	Spiked concn. (μg/ml)	Measured concn. (μg/ml)					Precision (C.V., %)	Bias (%)
		1	2	3	4	Mean		
Quinidine	0.570	0.565	0.557	0.567	0.596	0.571	3.0	+0.2
	3.80	3.80	3.61	4.09	3.59	3.77	6.1	−0.8
	11.40	12.50	11.07	11.75	12.17	11.87	5.2	+4.1
3-Hydroxyquinidine	0.374	0.388	0.394	0.389	0.398	0.392	1.2	+4.8
	0.934	0.921	0.942	0.938	0.902	0.926	2.0	−0.9
	2.24	2.31	2.27	2.23	2.29	2.28	1.5	+1.8
Quinidine-N-oxide	0.337	0.339	0.337	0.303	0.345	0.331	5.7	−1.8
	0.984	1.03	1.03	1.01	0.991	1.02	1.8	+3.7
	1.69	1.71	1.61	1.62	1.64	1.65	2.7	−2.4
2′-Quinidinone	0.302	0.303	0.301	0.303	0.309	0.304	1.1	+0.7
	0.503	0.494	0.511	0.497	0.489	0.498	1.9	−1.0
	1.21	1.20	1.18	1.17	1.19	1.19	1.1	−1.7



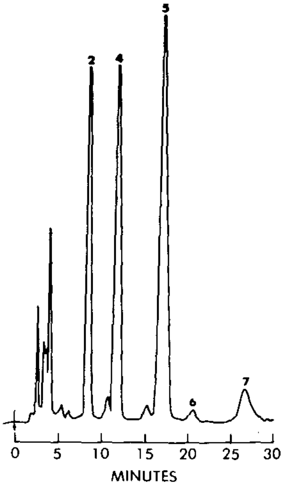


Fig. 1. Chromatogram of plasma spiked with quinidine and metabolites. Peaks: 1, 2'-quinidinone; 2, 3-hydroxyquinidine; 3, O-desmethylquinidine; 4, pronethalol (internal standard); 5, quinidine; 6, dihydroquinidine; 7, N-oxide of quinidine.

Fig. 2. Chromatogram of plasma sample from a patient after multiple doses of quinidine. Peaks: 2, 3-hydroxyquinidine; 4, pronethalol (internal standard); 5, quinidine; 6, dihydroquinidine; 7, N-oxide of quinidine.

tively. Fig. 2 shows a chromatogram of a plasma sample from a cardiac patient after multiple doses of quinidine. No interfering endogenous compounds could be detected in the blank plasma taken before the start of quinidine therapy in this patient. Not all metabolites are found in samples from each individual.

Results from accuracy studies with urine samples spiked at three different concentration levels unknown to the analyst are shown in Table I, where the precision of the assay for each of the compounds is expressed as a coefficient of variation (C.V.) after repeated measurement (n = 4) of the sample. The precision and accuracy are similar to that reported before for this assay using a different internal standard.

Plasma standard curves (Fig. 3) covering the concentration range of the samples to be analyzed were prepared by spiking blank plasma. They were linear for all the compounds, the coefficients of variation for concentration-normalized peak height ratios being 3.4, 7.8, 2.9 and 2.7% for quinidine, 2'-quinidinone, 3-hydroxyquinidine and the N-oxide respectively. Reproducibility and bias specifications for the plasma assay using fluorescence detection are given in Table II.

When taking 1 ml of sample the limit of detection in the assay described is 10 ng/ml for quinidine and 3-hydroxyquinidine, 15 ng/ml for the N-oxide and 20 ng/ml for 2'-quinidinone. The recently identified metabolite O-desmethylquinidine [10] could be detected in spiked plasma at levels exceeding 200 ng/ml.

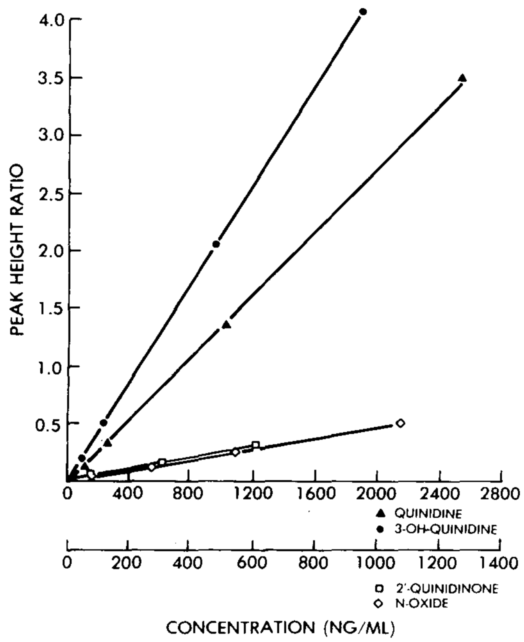


Fig. 3. Standard curves for quinidine and metabolites in plasma.

TABLE II

PRECISION AND ACCURACY OF REVERSED-PHASE ASSAY OF SPIKED PLASMA SAMPLES

A 200-μl aliquot of plasma was taken for assay.

	Spiked concn. (ng/ml)	Measured concn. (ng/ml)					Precision (C.V., %)	Bias (%)
		1	2	3	4	Mean		
Quinidine	285	285	284	281	278	282	1.1	−1.1
	980	981	994	970	968	978	1.2	−0.2
	1960	1870	1883	1851	1847	1863	0.9	−4.9
3-Hydroxyquinidine	93	93	94	93	92	93	0.9	0.0
	458	469	467	459	462	464	1.0	+1.3
	1220	1212	1218	1201	1156	1197	2.3	−1.9
Quinidine-N-oxide	105	101	110	101	108	105	4.5	0.0
	295	284	290	280	301	289	3.2	−2.0
	699	707	712	701	663	696	3.2	−0.4
2'-Quinidinone	74	73	74	71	76	74	2.8	0.0
	147	156	152	144	146	150	3.7	+2.0
	189	186	194	191	186	189	2.1	0.0

DISCUSSION

The procedure described above enables assay of both urine and plasma samples using the same extraction procedure and chromatographic system. Different detection methods are, however, required. Interfering endogenous compounds do not allow fluorescence detection of urine extracts without extensive work-up, but fortunately drug and metabolite levels in urine are high enough to be measured conveniently with ultraviolet detection. Plasma samples, in contrast, are often available in only limited volume or may contain levels in only the lower nanogram range. Fluorescence detection, with its greater sensitivity, can, however, be used for plasma extracts.

Several charges had to be made to our previously reported method to allow assay in both plasma and urine. The internal standard pronethalol has been adopted because it can be traced with both ultraviolet and fluorescence detectors. To achieve better separation between the compounds the pH of the eluent was adjusted to 4.75.

Adjustment of the plasma or urine pH to a value of 9 by addition of borate buffer is important to avoid the reduced extraction of O-desmethylquinidine at higher pH. Below pH 9 polar endogenous compounds are extracted and interfere with the 2′-quinidinone peak.

The above modifications to the pre-existing urine assay [7] thus allow analysis of either plasma or urine with a coefficient of variation of less than 6%. No appreciable bias is introduced (Tables I and II). All known quinidine metabolites can be separated (Fig. 1) and the assay is sufficiently sensitive for use in pharmacokinetic or clinical studies if quantitation of any of the metabolites other than O-desmethylquinidine is important. O-Desmethylquinidine appears in much lower concentration in plasma or urine than the other metabolites [11].

The superiority of assay procedures, where metabolites are separated from parent drug, over still widely used fluorescence techniques has been previously discussed [5, 7, 12, 13]. A lower but more accurate estimate of quinidine levels results. To gain full advantage of these more accurate levels, reassessment of the therapeutic range of quinidine plasma concentrations is necessary.

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