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edited by Z. DEYL, *Czechoslovak Academy of Sciences, Prague*

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CHROMBIO. 1734

GAS—LIQUID CHROMATOGRAPHIC DETERMINATION OF FATTY ACID COMPOSITION OF CHOLESTERYL ESTERS IN HUMAN SERUM USING SILICA SEP-PAK CARTRIDGES

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(Received January 19th, 1983)

SUMMARY

A simple and fast analytical procedure for separation and purification of cholesteryl esters of human serum is described. A single lipid extract, together with spiked cholesteryl pentadecanoate, as an internal standard, was passed through a Silica Sep-Pak cartridge. 1.5% diethyl ether in light petroleum was used to elute cholesteryl esters from the column. The separation was verified with thin-layer chromatography on silica gel using light petroleum—diethyl ether—glacial acetic acid (80:20:1) as a solvent. A very clean thin-layer chromatogram of cholesteryl esters without any additional spots of other lipids was obtained. The cholesteryl esters were quantitated by analyzing their fatty acid composition as methyl esters by gas—liquid chromatography. The coefficients of variation were 0.8—4.9% for the major fatty acids ($C_{16:0}$, $C_{16:1}$, $C_{18:1}$, $C_{18:2}$, $C_{20:4}$) and 6.7—30.8% for the minor fatty acids ($C_{18:0}$ and $C_{20:0}$). The recoveries for cholesteryl palmitate, cholesteryl oleate and cholesteryl linoleate were 90.7, 92.3 and 91.0%, respectively.

INTRODUCTION

Cholesteryl esters are normal constituents of lipoproteins in the blood and are the principal lipids to accumulate in the arterial wall during the progress of atherosclerosis [1]. They represent 60—75% of total cholesterol in the plasma in a normal healthy person. While the measurement of serum total cholesterol is one of the most frequently performed assays in the clinical laboratory, the analysis of cholesteryl esters, for their fatty acid composition, is not as widespread. The determination of the fatty acid composition of cholesteryl esters in plasma or serum is conventionally done by gas—liquid chromatography (GLC) after the lipids are extracted and the individual fractions separated by thin-layer chromatography (TLC) [2] or column chromatography [3, 4].

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There are drawbacks of both methods: The TLC procedure, which involves separation and extraction of the scraped-off fractions, is lengthy and cumbersome, and the column chromatography requires a large amount of sample. Recently Waters Assoc. have introduced a variety of small Sep-Pak cartridges for sample clean-up. These Sep-Pak cartridges are particularly useful in the clinical laboratory since they require only a small sample size. We are reporting here the investigation of the use of the silica Sep-Pak cartridges in combination with GLC for the determination of fatty acid composition of cholesteryl esters.

EXPERIMENTAL

Materials

The silica Sep-Pak cartridges were purchased from Waters Assoc. (Mississauga, Canada). Fatty acid methyl esters of PolyScience (Niles, IL, U.S.A.) and boron trifluoride—methanol of Pierce (Rockford, IL, U.S.A.) were obtained through Chromatographic Specialties (Brockville, Canada). Pentadecanoic acid methyl ester, cholesteryl pentadecanoate and cholesteryl esters were obtained from Sigma (St. Louis, MO, U.S.A.). The GLC glass column, packed with 10% SP-2330 on Chromosorb W AW 100—200 mesh, and the AOCS oil reference mixture of RM-3, were purchased from Supelco (Bellefonte, PA, U.S.A.). The E. Merck precoated TLC plates, silica gel 60, were supplied by BDH (Toronto, Canada), and Rhodamine 6G was obtained from Fisher Scientific (Toronto, Canada). All solvents were of analytical grade.

Lipid extraction

To 0.1 ml of serum and 10 μg of cholesteryl pentadecanoate as an internal standard, dissolved in 20 μl of chloroform, was added a mixture of 2 ml of isopropranol and 1 ml of water,. The sample was mixed on a Vortex mixer for 30 sec and then 2 ml of *n*-octane were added; this mixture was vortexed for another 30 sec. Brief centrifugation separated the upper octane layer which was then removed and evaporated to dryness at 37°C in a stream of nitrogen. The method of Folch et al. [5] was also used to extract 0.1 ml of serum with 5 ml of a chloroform—methanol (2:1) mixture. After phase separation the upper water—methanol layer was aspirated and the lower layer was washed with 0.2 volume of distilled water. The chloroform layer was removed and evaporated under a stream of nitrogen.

Silica Sep-Pak separation

Hexane (0.2 ml) was added to the dry extract and the solution was applied to the Sep-Pak column using a Pasteur pipette. An additional 0.2 ml of light petroleum (b.p. 20—40°C) was used to rinse the residue left in the test tube and this solution was again passed through the column. The Sep-Pak column was filled with 5 ml of 1.5% diethyl ether in light petroleum. The eluted sample was collected and then evaporated to dryness at 37°C under a nitrogen stream.

Esterification

A modification of a method described by Metcalfe and Schmitz [6] was used for saponification and methylation. A 0.5-ml volume of a 0.5 *M* sodium

hydroxide—methanol solution was added to the dried sample, purged with nitrogen and then heated in a heating block for 15 min at 100°C. Boron trifluoride in methanol (0.5 ml) was added and the mixture was heated for an additional 10 min at 100°C. The reaction mixture was cooled, extracted two times with 1.5 ml of light petroleum, and evaporated to dryness at 37°C in small vial under a stream of nitrogen. The residue was dissolved in 10 μl of *n*-heptane, of which 1 μl was introduced into the injection port of the gas—liquid chromatograph.

TLC separation

To prove the effectiveness of the Sep-Pak separation, the separated fraction from the Sep-Pak column was chromatographed on a TLC plate with a mixture of light petroleum—diethyl ether—glacial acetic acid (80:20:1) as solvent. The TLC plate was sprayed with aqueous sulfuric acid and charred for 10 min at 200°C. For comparison, the conventional TLC separation of the lipid extract was chromatographed and the bands made visible under UV light by spraying the plate with the Rhodamine 6G solution.

The corresponding band of cholesteryl esters on the TLC plate was scraped off and extracted three times with chloroform. The extracted cholesteryl esters were hydrolyzed and the fatty acids methylated and analyzed as described.

GLC determination

A Hewlett-Packard 5830A gas chromatograph, equipped with a hydrogen flame ionization detector, a Hewlett-Packard 18850A GC microprocessor terminal, and a single glass column of 183 cm × 6.4 mm O.D. (2 mm I.D.) packed with 10% SP-2330 on Chromosorb W AW, 100—120 mesh, was used. The column temperature was 200°C, the flow-rate of the nitrogen carrier gas was 20 ml/min.

RESULTS

The identification of cholesteryl esters, after being eluted from the Sep-Pak cartridge, was performed with the TLC system. It showed a very clean thin-layer chromatogram of cholesteryl esters without any additional spot, both with the lipid extraction method of Folch et al. [5] and with our *n*-octane—isopropanol—water procedure (Fig. 1). Both extraction methods were suitable for the Sep-Pak procedure. For the quantitative determination of the fatty acid composition of cholesteryl esters, they were hydrolyzed, methylated and chromatographed by GLC after being eluted from the Sep-Pak cartridge or scraped from a TLC plate. A typical gas—liquid chromatogram of fatty acids of human serum cholesteryl esters is shown in Fig. 2. The linearity of the detector response to different fatty acid methyl esters in the investigated concentration range was established, using pure standards.

The reproducibility of the method was estimated by carrying out six replicates on one serum sample as indicated in Table I. The average analytical coefficient of variation (C.V.), due to the instrument (GLC response) itself, was 1.9%. The coefficient of variation for the within-day assay and the between-day assay was 0.8—4.9% for the major fatty acids ($C_{16:0}$, $C_{16:1}$,

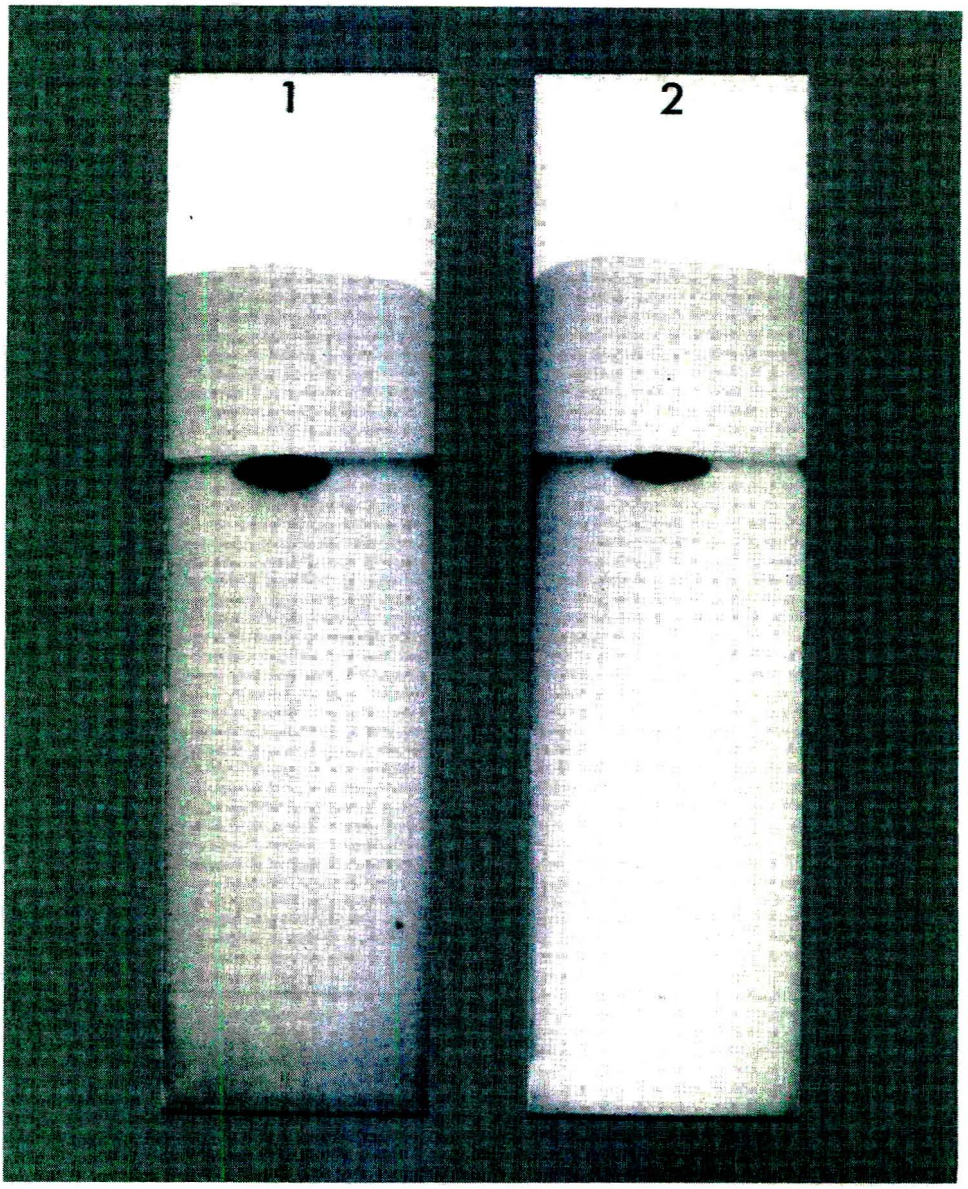

Fig. 1. A clean thin-layer chromatogram of cholesteryl esters after elution from Sep-Pak cartridges: (1) with *n*-octane—isopropanol—water lipid extraction; (2) with chloroform—methanol lipid extraction.

$C_{18:1}$, $C_{18:2}$ and $C_{20:4}$) and 6.7—30.8% for the minor fatty acids (less than 2% of the total, $C_{18:0}$ and $C_{20:0}$). There were no statistically significant differences between the within-day and between-day assay variations. The recovery was determined by assaying a serum sample after the addition of a known amount of cholesteryl ester standards of cholesteryl palmitate, cholesteryl oleate and cholesteryl linoleate. The percent recoveries for the method are shown in

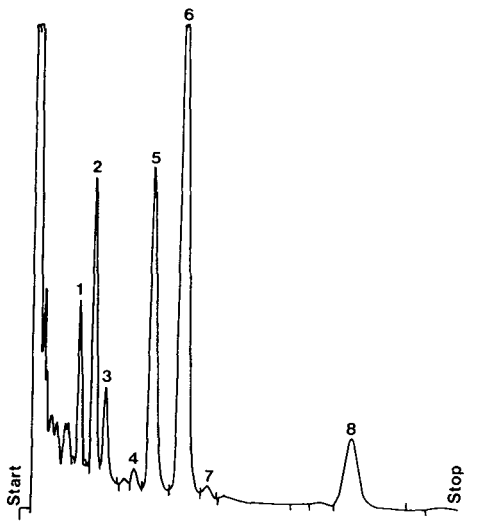

Fig. 2. Gas—liquid chromatogram of fatty acids of human serum cholesteryl esters. Peaks: 1 = $C_{15:0}$, internal standard; 2 = $C_{16:0}$; 3 = $C_{16:1}$; 4 = $C_{18:0}$; 5 = $C_{18:1}$; 6 = $C_{18:2}$; 7 = $C_{20:0}$; 8 = $C_{20:4}$.

TABLE I

PRECISION OF GLC MEASUREMENT OF FATTY ACID COMPOSITION OF CHOLESTERYL ESTERS (n = 6)

Fatty acid	Total fatty acid (%)					
	GLC response (mean ± S.D.)	C.V. (%)	Within-day precision (mean ± S.D.)	C.V. (%)	Day-to-day precision (mean ± S.D.)	C.V. (%)
$C_{16:0}$	11.9 ± 0.2	2.0	11.9 ± 0.2	2.0	12.1 ± 0.3	2.5
$C_{16:1}$	5.0 ± 0.1	2.0	4.9 ± 0.1	2.6	5.2 ± 0.2	3.8
$C_{18:0}$	1.1 ± 0.05	0.5	1.2 ± 0.2	16.6	1.3 ± 0.4	30.8
$C_{18:1}$	22.8 ± 0.1	0.4	22.7 ± 0.6	2.6	22.7 ± 0.9	4.0
$C_{18:2}$	48.5 ± 0.2	0.4	48.4 ± 0.4	0.8	47.9 ± 0.9	1.9
$C_{20:0}$	0.7 ± 0.05	7.1	0.6 ± 0.04	6.7	0.7 ± 0.1	14.3
$C_{20:4}$	10.1 ± 0.1	1.0	10.3 ± 0.3	2.9	10.3 ± 0.5	4.9

TABLE II

RECOVERY OF CHOLESTERYL ESTERS BY THE SEP-PAK CARTRIDGE WITH DIFFERENT LIPID EXTRACTION

	Recovery (%)		
	Chloroform—methanol	Hexane—methanol	n-Octane—isopropanol—water
Cholesteryl palmitate	90.7	88.7	92.8
Cholesteryl oleate	92.3	88.6	94.2
Cholesteryl linoleate	91.0	87.6	92.8

Table II. Both extraction methods of *n*-octane—isopropanol—water and chloroform—methanol showed more than 90% recoveries. The slightly increased recovery with the octane extraction method may be due to the fact that the cholesteryl esters are highly lipophilic.

To check further that our Sep-Pak method measured the true fatty acid composition of cholesteryl esters, we compared our procedure with the conventional TLC method. The result of this study is shown in Table III. There were no significant differences between our Sep-Pak method and the conventional TLC method. The results also showed that the lipid extraction of Folch et al. [5] (chloroform—methanol) can also be used in conjuction with the Sep-Pak column as indicated previously on the qualitative identification. The validity of the Sep-Pak method was further checked on six serum specimens with cholesterol and triglyceride concentrations ranging from 172—296 and 135—282 mg/dl, respectively. As shown in Table IV, the results on the comparison of the Sep-Pak and TLC methods were in good agreement. The value of $C_{18:0}$ minor fatty acid of specimen No. 3 on the Sep-Pak method was slightly higher than the value of the TLC method. This was caused by the unresolved small peak on the gas chromatogram.

TABLE III

FATTY ACID COMPOSITION OF CHOLESTERYL ESTER DETERMINED BY DIFFERENT EXTRACTION METHODS (n = 6)

Values are given as percent total fatty acids (mean ± S.D.).

Fatty acid	Method		
	n-Octane—isopropanol—water (Sep-Pak)	Chloroform—methanol (Sep-Pak)	Chloroform—methanol (TLC)
$C_{16:0}$	11.9 ± 0.4	11.9 ± 0.3	11.3 ± 0.4
$C_{16:1}$	4.9 ± 0.4	5.1 ± 0.1	4.9 ± 0.1
$C_{18:0}$	1.2 ± 0.2	1.1 ± 0.3	0.8 ± 0.1
$C_{18:1}$	22.7 ± 0.6	22.9 ± 0.3	22.6 ± 0.4
$C_{18:2}$	48.4 ± 0.4	48.4 ± 0.4	49.1 ± 0.4
$C_{20:0}$	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.1
$C_{20:4}$	10.3 ± 0.3	10.0 ± 0.5	10.6 ± 0.3

DISCUSSION

A simple procedure has been developed by Peter and Reynolds [7] for the separation of cholesteryl esters from the other lipid components of serum. Cholesteryl esters were extracted into *n*-octane from a mixture of serum and alkaline aqueous isopropanol. Phospholipids, triglycerides and free fatty acids remained in the isopropanol phase. Rockerbie et al. [8] used this method for the analysis of the fatty acid components of cholesteryl esters but Koot-Gronsveld et al. [9] found that the method does not eliminate triglycerides completely from the octane phase and that some isopropanol—fatty acid

TABLE IV

FATTY ACID COMPOSITION OF SERUM CHOLESTERYL ESTER BY TWO DIFFERENT SEPARATION METHODS

Specimen No.	Cholesterol (mg/dl)	Triglycerides (mg/dl)	Fatty acids (%)											
			$C_{16:0}$		$C_{16:1}$		$C_{18:0}$		$C_{18:1}$		$C_{18:2}$		$C_{20:4}$	
			Sep-Pak	TLC	Sep-Pak	TLC	Sep-Pak	TLC	Sep-Pak	TLC	Sep-Pak	TLC	Sep-Pak	TLC
1	251	135	12.2	10.7	3.3	2.7	1.4	1.3	15.0	15.0	55.6	57.9	12.5	12.3
2	271	137	12.3	12.2	9.0	8.5	1.5	1.3	16.0	15.4	50.5	52.0	10.7	10.5
3	172	207	13.3	11.7	4.2	4.5	3.0	0.9	20.7	20.7	52.7	55.5	6.3	6.3
4	213	282	13.2	12.9	5.4	5.2	0.9	0.8	13.0	13.4	54.1	54.3	13.4	13.4
5	296	203	13.0	12.4	4.9	5.2	0.8	0.6	23.1	22.6	49.0	49.6	9.5	9.6
6	266	167	12.9	11.9	4.8	4.6	1.1	1.0	22.0	22.6	49.5	50.0	9.7	9.9
Mean	245	189	12.8	12.1	5.3	5.1	1.5	0.9	18.3	18.3	51.9	53.2	10.4	10.3
S.D.	45	55	0.5	0.8	2.0	1.9	0.8	0.2	4.2	4.2	2.7	3.3	2.5	2.5

esters also contaminate the cholesteryl ester fraction. This was confirmed by Rockerbie et al. [10]. Our method does not present this problem since cholesteryl esters are isolated completely by the Sep-Pak cartridges.

A direct determination of the linoleate to oleate ratio in serum cholesteryl esters has been published by Bernert et al. [11] using reversed-phase high-performance liquid chromatography. The linoleate to oleate ratio tended to have a significantly higher value than that of the GLC method. The reason for this difference has not been clarified. Our Sep-Pak separation of cholesteryl esters may be able to solve this problem by producing the pure fraction of cholesteryl esters.

The silica Sep-Pak cartridge procedure described here offers definite advantages over the traditional TLC separation of cholesteryl esters.

The precision and recovery studies showed that the Sep-Pak method is comparable to the conventional TLC method. There is no statistical significance between these two methods. Although the traditional lipid extraction by chloroform–methanol can be combined with the Sep-Pak method, the *n*-octane lipid extraction offers a slightly better recovery and also the convenience, since the octane layer stays on the top of the mixture. The advantage of speed, convenience and saving of time in sample preparation of the Sep-Pak method provides a rapid and efficient alternative to the TLC method for the separation and analysis of serum cholesteryl esters. The method is simpler, quicker and more readily adaptable than the TLC method.

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CHROMBIO. 1760

GAS CHROMATOGRAPHIC QUANTITATION OF BREATH HYDROGEN AND CARBON MONOXIDE FOR CLINICAL INVESTIGATION IN ADULTS AND IN CHILDREN

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SUMMARY

A simple and suitable method of breath sampling and the gas chromatographic determination of H_2 and CO in breath of children as well as of adults are described. The analytical system allows the measurement of concentrations of H_2 and CO as low as a few ppm.

The proposed method of breath collection (tedlar bag with low resistance valve face mask) appears particularly useful for long storage purposes, because it eliminates the need for additional manipulations and the possible associated sample dilution.

Normalization of H_2 breath amounts to internal standard O_2 to obtain reliable and precise measurements is particularly useful in non-collaborating patients whose observed H_2 peak values increase by 15% after the correction

The overall procedure is fast, inexpensive and accurately recognizes adult or children lactose malabsorbers as well as subjects exposed even to low levels of CO.

INTRODUCTION

Breath analysis represents important information in laboratory diagnosis whenever one needs to assess the concentration of some components of alveolar air of endogenous origin (e.g. H_2 and CO) as well as of exogenous origin (e.g. ambient toxic gases).

H_2 in alveolar air is derived from bacterial activity on unabsorbed carbohydrates reaching the lower gastrointestinal tract; intestinal gas diffuses into the mucosal capillary blood where it is transported to the lungs and excreted in the breath, roughly in proportion to the degree of malabsorption. The use of a breath test for H_2 determination in the expirate after oral carbohydrate loads has therefore been suggested mainly as a diagnostic non-invasive test for (a) lactose malabsorption (alternatively to sequential blood glucose determinations) [1], (b) small bowel bacterial overgrowth (alternatively to duodenal juice cultures) [2], (c) small bowel transit time (alternatively to X-ray procedures [3]. However, the results reported so far do not allow this procedure to be considered among the routine laboratory tests.

CO, besides being an environmental toxic gas to which a subject may be professionally (e.g. foundry and garage workers) or individually (smoking habits) exposed, is also produced during heme catabolism. Since CO is not catabolized in vivo and virtually 100% of CO is excreted via the lungs, the determination of CO excretion rate may be employed in a variety of clinical investigations, such as environmental poisoning and hemolytic anemias, as an alternative to CO blood level determinations. However, the CO breath test too has received little attention as a common laboratory tool [4].

It is evident that the usefulness of breath analysis, besides rapidity, is mainly due to its non-invasivity, because the specimen to be analysed is simply the expired breath. This is particularly advantageous when it is necessary to perform biological monitoring for several hours, because serial blood determinations can be avoided, thus interfering very little with the patient's normal activities.

The analysis of the breath by gas chromatography (GC) with thermoconductivity detection has proved to be the most suitable procedure for the determination of all gases present, even in concentrations as low as a few ppm.

We set up an easy and efficient system of H_2 breath collection and following analysis by means of GC, which turned out to be particularly useful in the study of lactose malabsorption. Our results confirm that H_2 determination in breath may be an alternative (and sometimes more sensitive) to blood glucose determinations. By making appropriate variations in the GC operating conditions (carrier gas, volume of the loop of the sampling valve, etc.), the analytical system described here is capable also of evaluating breath CO.

EXPERIMENTAL

Gas chromatography

The GC system used was constituted by an HP 5840 (Hewlett-Packard, Avondale, PA, U.S.A.) gas chromatograph equipped with a six-port sampling valve with a 1-ml sample loop, thermoconductivity detector and printer-plotter with integrator.

The chromatographic columns for H_2 analysis were made of steel tubing, 1 m × 0.6 mm I.D., filled with 60—80 mesh molecular sieve 5 Å. The columns operated at 60°C in the isothermal mode, the detector was at 100°C. Pure nitrogen was used as the carrier gas, with a 10 ml/min flow-rate. Instrument

sensitivity was set at 1. Before analysis, the columns were equilibrated at 300°C overnight with a nitrogen flow of 10 ml/min, in order to ensure the cleanliness of the tandem columns and to preserve the resolution characteristics of the system.

A 5 cm × 0.5 cm column, packed with magnesium chloride, was inserted in the sampling line to dry the samples.

To detect CO we used the following conditions: steel columns (3 m × 0.6 mm I.D.) filled with 60—80 mesh molecular sieve 5 Å; the columns operated at 100°C in isothermal mode and the detector operated at 80°C. Pure helium was used as carrier gas at a flow-rate of 28 ml/min. The instrument sensitivity was 3. The loop volume of the sampling valve was 3 ml.

Sampling

Expired air samples were obtained by having the subjects exhale through an anesthetic mask equipped with two low-resistance one-way valves or a Ruben valve into a 250 ml tedlar (polyvinylfluoride, Dupont de Némours International S.A., Geneva, Switzerland) vacuum empty bag (see Fig. 1). Stability of the sample on storage was determined by filling the bags with standard gas mixtures. The bags were stored at room temperature and were analysed at intervals throughout a period of 24 h.

Fig. 1. The anesthetic mask equipped with a Ruben valve connected by means of a gas tight cock, to a polyvinylfluoride bag.

During the study we compared three types of breath collection: (1) *after a* deep inhalation followed by a 10-sec breath-holding period, the first volume of exhalation was discarded and the last portion was collected; (2) the subject inhaled deeply, held his breath for 10 sec, and exhaled once completely into the collection bag; (3) several normal tidal volumes of breath were collected. Duration of collection of expired air was not considered crucial, because we did not need to calculate the rate of H_2 excretion, but only determine the change in H_2 concentration in serially collected samples of expired air.

Samples were introduced at atmospheric pressure into the sampling valve of the GC system through a tight connection between valve and bag, by simple compression of the bag so that the loop was filled.

Normalization of the H_2 peak

We used breath O_2 as internal standard to normalize H_2 breath amounts as proposed by Robb and Davidson [5], in order to obtain reliable and precise measurements.

Subjects

In order to test the sensitivity and the specificity of the method, we comparatively studied the H_2 excretion and the rise in blood glucose levels after an oral lactose load in 29 adult subjects without evident gastroenterological disease (age 40—50 years) and 15 children with clinical suspicion of lactose malabsorption (age 7 months to 11 years; mean 3.4 years ± 4 S.D.).

Venous blood samples were obtained in the fasting state and at 20, 40, 60 and 90 min after lactose ingestion. Plasma glucose was determined by the glucose oxidase method. Lactose malabsorption was diagnosed on the basis of an increase of blood glucose of less than 20 mg/dl [6].

Breath samples were taken simultaneously with blood and then were protracted until 240 min (at 30-min intervals) in order to consider the variability of gastric emptying and small bowel transit time.

Increases of H_2 more than 20 ppm were considered to be indicative of lactose malabsorption [8].

Breath CO was evaluated in 30 healthy male hospital workers non-professionally exposed: ten were non-smokers, ten were heavy smokers, the remainder were mild smokers.

RESULTS

Gas chromatographic determination of H_2 and CO

Figs. 2 and 3 show chromatograms of standard gas mixtures of 21 ppm H_2 in N_2 and of 50 ppm CO in N_2, respectively. A clear linearity of the measurements by examining H_2 and CO standard samples at different concentrations was found.

The limit of sensitivity that can be reached using this procedure is 1.0 ppm and 1.5 ppm, respectively, for H_2 and CO analysis.

The intra-assay reproducibility of the method for H_2 measurements was tested by collecting and analysing five breath samples of a patient delivered in rapid succession; the mean values, standard deviation, and coefficient of

variation were 41 ppm, 1.4 ppm and 3.4%, respectively. The inter-assay variability was determined by analysing aliquots of the same sample containing 21 ppm of H_2 on ten occasions over a period of 20 days in the same operating conditions. A mean peak area* of 2.27 mV sec, a standard deviation of 0.03 mV sec and a coefficient of variation of 1.3% were obtained.

The intra- and inter-assay variability for CO measurements showed coefficients of variation of 3.9% and 1.8%, respectively.

The calculation of the peak area was automatically integrated using the external standard method.

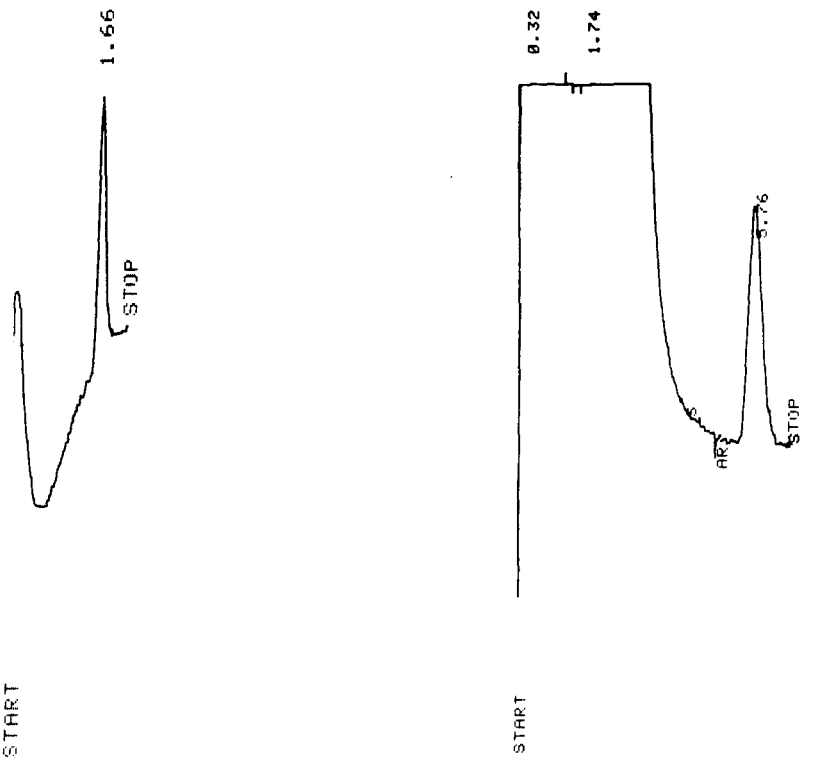

Fig. 2. Chromatogram of a standard gas mixture of 21 ppm H_2 in N_2. Oven temperature 60°C, detector temperature 100°C. Sensitivity 1; attenuation 2^1.

Fig. 3. Chromatogram of a standard gas mixture of 50 ppm CO in N_2. Oven temperature 100°C, detector temperature 80°C. Sensitivity 3; attenuation 2^1.

Sample collection and normalization of the H_2 peak

We observed that types "a" and "b" breath collection gave H_2 concentrations 10% higher than "c" type collection. For this reason, in adult collaborating patients the "a" type of breath collection was routinely utilized, whereas in

*Area units: 1 count = 3.2 μV sec.

non-collaborating children the "c" type of breath collection was utilized with correction of the H_2 concentration to internal standard. The correction to O_2 concentrations in fact increased the observed H_2 peak values by 10—15% only in children, no substantial increase being possible to detect in adult patients. The data reported in the tables are in any case all normalized to O_2 concentration. We stress, however, that the normalization may not be necessary in adults who can exhale alveolar air. It must be noted that the correction to O_2 levels is mainly useful to obtain reproducible results in the same subjects but it does not guarantee the accuracy of the results.

Sample storage

In Table I are reported the analysis of H_2 and CO standard gas mixtures stored in the tedlar bags. The results, expressed as mean percentage original value of five consecutive analyses for each sample at every time, document the good stability of the stored samples.

TABLE I

STABILITY OF H_2 AND CO LEVELS IN STANDARD GAS MIXTURES STORED IN TEDLAR BAGS

Hours	Percentage of original value			
	H_2 21 ppm	H_2 60 ppm	CO 20 ppm	CO 50 ppm
0	100	100	100	100
12	92.2	93.4	100	100
24	86	89.7	99	98
48			96	97
72			96	96

H_2 Excretion in breath after oral lactose load

In Table II we compare the results of the H_2 breath test with those of the blood glucose level increments in the subjects tested.

Evaluation of carbon monoxide in breath

In Table III are reported the results of breath samples of 30 smoker subjects in order to point out the possibility of determining simply and quickly CO in the breath of subjects professionally or individually exposed to toxic gases. These results are in agreement with literature data, obtained using other analytical methods [4, 7].

No normalization of observed CO was performed because the levels of CO in both the working and analytical areas were considered too low to alter the determinations significantly.

TABLE II

COMPARISON OF H_2 BREATH TEST AND GLUCOSE BLOOD LEVEL INCREMENT FOR THE DIAGNOSIS OF LACTOSE MALABSORPTION

Results are expressed as mean ± S.D. and range.

Diagnosis by H_2 breath level increment (ppm H_2)	Diagnosis by glucose blood level increment (mg/dl glucose)			
		Absorbers	Malabsorbers	Borderline
		31 ± 10 (20 — 54)	12.8 ± 6.6 (0 — 23)	(20—25)
Absorbers				
5.4 ± 7.0 (0 — 19)	Children (*n*)	5	—	—
	Adults (*n*)	7	—	—
Malabsorbers				
64 ± 40 (21 — 24)	Children (*n*)	—	10	2
	Adults (*n*)	—	20	4

TABLE III

CO IN BREATH OF SMOKERS AND NON-SMOKERS

Smoking habits	CO in breath
Non-smokers	4.5 ppm
Mild smokers (10—20 cigarettes/day)	18.0 ppm
Heavy smokers (> 20 cigarettes/day)	42.5 ppm

DISCUSSION

Expired breath, one of the most readily available biological materials, has been poorly utilized for clinical investigation until recent times. We experimented with methods for collecting, storing, measuring and quantitating H_2 and CO in expired air for clinical studies of carbohydrate malabsorption and CO production or exposure.

Compared to other methods already published, our procedure shows some advantages. The breath collection in a squeezable bag connected with a low-resistance face mask (especially suitable for children) allows easy, direct introduction of the sample into the GC apparatus by means of a sampling valve (reproducibility of the injected volume). Moreover, it is possible to store the sample in the bag itself, thus eliminating the need to transfer the samples into storage containers, which usually produces a loss of at least 10% of the gases [8]. Storage in tedlar bags has been found useful in all cases when it is not possible to perform the gas analysis immediately after sampling. Furthermore,

our method shares several advantages with the recently reported technique of Christman and Hamilton [9]. In fact, it is possible to examine non-hospitalized patients by means of the collecting bags, the H_2 elution time (approx. 90 sec) is short enough for our purposes, and, finally, operating procedures are just as simple. Our method has the advantage of a linear signal—concentration curve, and the particular GC versatility allows other breath gases to be determined simultaneously, thus permitting the use of O_2 as internal standard in cases where peak normalization is necessary.

Our results show a good agreement between the H_2 breath test and the standard lactose tolerance test (glucose blood level increment). According to clinical symptoms (abdominal malaise, diarrhoea with positive clinitest), to a H_2 production of more than 20 ppm and to a glucose blood level increment of less than 20 mg/dl it has been possible to separate lactose absorbers from lactose malabsorbers.

However, it must be noted that six subjects (four adults and two children) were lactose malabsorbers on the basis of H_2 breath test and clinical response, but they showed a borderline (20—25 mg/dl) glucose blood level increment. The finding of these borderline cases may be explained by the lower reliability of the glucose blood level determination and possibly decreased glucose utilization such as in subclinical diabetes mellitus [10]. No false negative H_2 breath test was found, in agreement with the opinion [11] of the rarity of the absence of colonic bacteria capable of producing H_2 from non-absorbed lactose.

We found a good correlation between CO in the breath and amount of smoked tobacco. Thus the CO breath test may be considered a rapid, efficient and non-invasive method to evaluate exposure to environmental CO also.

As far as normalization of the H_2 peak is concerned, it is known that breath samples are representative of alveolar air only in adults, and in children old enough to cooperate fully [4] in order to give an end-expiratory sample in a single breath. Therefore some authors [5, 8] have proposed the normalization of the observed breath H_2 levels to one of the other respiratory gases, for preschool children. In respect of this suggestion we do not find it necessary to correct for breath H_2 values against CO_2 amounts [10] because (1) we used columns packed with molecular sieve (which traps CO_2 molecules), (2) the alveolar CO_2 concentration is physiologically widely variable, and (3) CO_2 is an end-product of lactose metabolism after oral load. The normalization to respiratory nitrogen [5] proposed in order to avoid variability of the sampling volume was not considered because our GC system is equipped with a gas sampling valve, and we used nitrogen as carrier gas.

We used breath O_2 as internal standard to normalize H_2 breath amounts as proposed by Robb and Davidson [5] in order to obtain reproducible measurements in non-collaborative subjects.

No normalization of the CO peak was applied because all examined subjects were adults.

The method discussed here may be particularly suitable for epidemiological studies of lactose absorption [12] as well as for monitoring subjects exposed to CO even at low levels, in adults and children. Our data contribute to validate the few results produced up to now by this procedure.

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DERIVATIZATION OF CATECHOLAMINES IN AQUEOUS SOLUTION FOR QUANTITATIVE ANALYSIS IN BIOLOGICAL FLUIDS

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SUMMARY

The reaction of methyl chloroformate with catecholamines at the nanogram level in aqueous solution has been estimated. Optimal pH conditions of the aqueous medium were established which provide a quantitative yield of the formate derivative of primary and secondary catecholamines. First the catechol function was blocked by the reaction with methyl chloroformate under mild alkaline conditions (pH 7.2) and subsequently a pH shift was introduced (Δ pH = 2) to improve the reaction of the amine function. The formate derivatives were extracted effectively ($\geq$ 99%) into ethyl acetate and subsequently converted to their O-*tert.*-butyldimethylsilyl, N-formate derivatives. These mixed derivatives appeared to be very suitable for quantitative determination of catecholamines and related compounds in biological fluids by gas chromatography—mass spectrometry. The coefficient of variation estimated in urine samples was 6% (n = 6). The minimal detectable concentration in biological samples was 50 pg ml^{-1} with a signal-to-noise ratio of 5.

INTRODUCTION

The physiologically important catecholamines occur in low concentrations in body fluids. Apart from the difficulty in assessing small amounts, the analysis of catecholamines is hindered by their sensitivity to oxidative degradation and their amphoteric character. These properties necessitate a careful

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sample-handling procedure under very strict conditions. Conventional sample clean-up methods use ion-exchange column chromatography [1] or adsorption to aluminium oxide [2] or boric gel [3]. An essential disadvantage of these methods is the risk of irreversible adsorption [4], which becomes especially serious if very small amounts have to be isolated. Recently, ion-pair extraction of catecholamines [5] and related compounds [6] has been described. This appears to be a very promising method, but the recovery depends on many factors which differ for each individual compound [6]. Brooks and Horning [7] demonstrated the potential utility of acetylation in aqueous media in the isolation of amines from dilute aqueous solutions. This method is based on the fact that amines and phenols easily react with anhydrates [8, 9] and alkyl chloroformates [10—12]. The stable acetyl or carbamate derivatives can be extracted effectively into relatively nonpolar solvents and easily concentrated. Thereafter, compounds containing only phenolic hydroxy groups or amino groups can be analyzed by gas chromatography (GC) or gas chromatography—mass spectrometry (GC—MS) directly from the extract. However, compounds containing additional aliphatic hydroxy groups will in general demand a subsequent derivatization because of their poor chromatographic properties.

In this paper the reaction of methyl chloroformate with the different functional groups of catecholamines in buffered aqueous media is described. The lowest pH values were determined at which the catecholic function is completely blocked by the reaction with methyl chloroformate. Subsequently the pH of the medium can be increased to values necessary for quantitative reaction of the amine function.

In addition, a number of silylation methods were evaluated for their utility in the formation of a singular mixed trialkylsilyl-formate derivative. *tert.*-Butyldimethylsilylation (tBDMS) turned out to be the most versatile silylation method. The tBDMS reagent reacts with the free aliphatic hydroxy group together with a complete substitution of the O-methyl formate group by an O-tBDMS group. The resulting O-tBDMS, N-carbamate derivatives are extremely stable and very useful for the GC—MS determination of catecholamines at physiological concentrations.

MATERIALS AND INSTRUMENTATION

Chemicals

Epinephrine (E), norepinephrine hydrochloride (NE), metaneprine hydrochloride (MN), normetanephrine hydrochloride (NMN), dopamine hydrochloride (DA) and isoprenaline bisulfate (IP) were obtained from Sigma (St. Louis, MO, U.S.A.). 1,2-Dihydroxybenzene was obtained from Merck, (Darmstadt, G.F.R.) and 3,4-dihydroxybenzylamine from Aldrich Europe (Beerse, Belgium). Methyl chloroformate for synthesis (95%) was available from Merck. The silylating reagents hexamethyldisilazane (HDMS), N,O-bis-(trimethylsilyl)trifluoroacetamide + 1% trimethylchlorosilane (TMCS) were supplied by Pierce (Rockford, IL, U.S.A.) and trimethylsilylimidazole (TSIM) by Merck. *tert.*-Butyldimethylchlorosilane (1 mmol)—imidazole (2.5 mmol) in dimethylformamide (1 ml) was obtained from Applied Science Labs. (State

College, PA, U.S.A.). The normal alkanes hexadecane, tetracosane, octacosane and triacontane were obtained from Polyscience (Niles, IL, U.S.A.)

All solvents were of analytical grade.

Buffers

Phosphate buffers used were 1 *M* K_2HPO_4 in distilled water, the pH being adjusted with concentrated phosphoric acid (Merck). The sodium carbonate buffer was a saturated solution of sodium carbonate in distilled water, the pH adjusted with 12 *N* sodium hydroxide to pH 11.

Instrumentation

Gas chromatography. GC analyses were carried out on a Hewlett-Packard 5810 A instrument equipped with a flame ionization detector. The chromatographic column was a fused-silica capillary column 25 m × 0.22 mm I.D., coated with CpSil-5 (Chrompack, Middelburg, The Netherlands). The chromatographic conditions were: injector temperature 250°C, split ratio 30:1, carrier gas (helium) flow-rate 28 cm sec^{-1}. The oven temperature was varied in various experiments.

Gas chromatography—mass spectrometry. GC—MS analyses were performed on a Jeol JMS-D100 instrument with JMA 0231 data system. The chromatographic column was an OV-1701 fused-silica capillary column, 25 m × 0.22 mm I.D. (Chrompack). The oven temperature was 270°C, injection port temperature 300°C, and GC—MS interface temperature 270°C. The capillary column was directly introduced into the ion source of the mass spectrometer. Samples were introduced by a modified solid injector [13]. The MS conditions were: source temperature 250°C, electron energy 70 eV, and emission current 0.3 mA.

METHODS

pH optimization experiments

Reaction with phenolic hydroxy groups. Solutions of 10^{-3} *M* 1,2-dihydroxybenzene in 0.02 *N* hydrochloric acid (0.5 ml), and 1.0 ml of phosphate buffer of a pH ranging from 6.25 to 8.0 were mixed with 25 μl of methyl chloroformate for 10 sec and allowed to react for 5 min. Then the derivatives were extracted into 2 ml of ethyl acetate (containing hexadecane, 100 μg ml^{-1}, as internal standard) by shaking for 5 min; 1 μl of the extracts was analyzed by GC at an oven temperature of 160°C.

Determination of the rate of the reaction of methyl chloroformate with phenolic hydroxy groups was performed in analogous experiments, but in these experiments the pH of the phosphate buffer was kept at 7.5 and extractions were carried out at 1, 5, 10 and 30 min after addition of methyl chloroformate.

Reaction with primary amines. The yield of the reaction of methyl chloroformate with primary catecholamines was determined for dopamine. Volumes ranging from 0.03 to 0.5 ml of 2 × 10^{-3} *M* dopamine in 0.02 *N* hydrochloric acid were made up to 0.5 ml with 0.02 *N* hydrochloric acid. These solutions were mixed with 1.0 ml of phosphate buffer (pH 7.5) and 25 μl of methyl chloroformate. After 5 min the derivatives were extracted into 2 ml of ethyl

acetate, containing tetracosane (100 μg ml^{-1}) as internal standard. The extracts were analyzed by GC at an oven temperature of 240° C.

pH shift. For secondary amines a pH shift was introduced. The influence of the pH of the aqueous medium on the rate of reaction of secondary catecholamines with methyl chloroformate was determined for epinephrine (1 μg) and isoprenaline (1 μg) with norepinephrine (1 μg) as internal standard. To 0.5 ml of this mixture in 0.02 *N* HCl were added 1.0 ml of phosphate buffer (pH 7.5) and 25 μl of methyl chloroformate, and after mixing allowed to react for 5 min. Then a volume of saturated sodium carbonate (0—1.0 ml) of pH 11 and a second volume of 25 μl of methyl chloroformate were added and vigorously mixed. After 10 min the derivatives were extracted into 2 ml of ethyl acetate. After centrifugation, an aliquot (1.5 ml) of the organic phase was evaporated to dryness under a stream of nitrogen in a waterbath at 40° C. The residue was silylated (tBDMS, see below) and analyzed by GC—MS (selected ion detection mode) on the ion at *m/z* 481 for all three compounds.

Determination of the extraction efficiency

Methyl formate derivatives of catecholamines (E, NE and IP, 1 μg of each) were extracted into ethyl acetate at a phase ratio of 1. Then 1 ml of the water phase was transferred to a clean tube and re-extracted with 1 ml of ethyl acetate. An aliquot of both organic layers (0.5 ml) was evaporated to dryness and silylated (tBDMS). The amounts of catecholamines were estimated by GC relative to octacosane as internal standard (5 μg first extract, 250 ng second extract).

Evaluation of silylation conditions

Methyl formate derivatives of catecholamines were treated with various silylating reagents under various conditions.

Identification of the derivatives formed was performed by GC—MS. The GC oven temperature was 240° C and 270° C for TMS and tBDMS derivatives, respectively. The following silylating methods were tested: (1) 100 μl of HMDS + 1% TMCS (pure or 20% in dichloromethane) for 5, 30 and 90 min at 20° C, and for 5, 50 and 240 min at 70° C; (2) 100 μl of BSTFA + 1% TMCS (pure and 10% in acetonitrile) for 5, 30, 90 min and overnight at 20°C, and for 5, 50 and 240 min at 70° C; (3) 100 μl of TSIM (5% in diethyl ether or dichloromethane) for 5, 30 and 90 min at 20° C; (4) 100 μl of tBDMCS—imidazole—dimethylformamide for 90 min at 60, 80 and 110° C, after cooling the reaction mixture to room temperature, 100 μl of hexane were added to dissolve the derivatives.

Calibration curves

Unhydrolyzed urine (0.25 ml) was spiked with epinephrine (range 0—50 ng), norepinephrine (range 0—170 ng) and isoprenaline (internal standard, 51.08 ng in 0.2 ml of 0.02 *N* hydrochloric acid) and processed as given in Fig. 1.

Biological samples

Urine. Catecholamine conjugates in urine were hydrolyzed by boiling acidified urine (HCl, pH $\leqslant$ 1) for 20 min. To an aliquot of 0.5 ml were added 51.08 ng of isoprenaline (internal standard), 25 μl of methyl chloroformate and

2.0 ml of phosphate buffer (pH 7.5). The pH shift was made with 1.0 ml of the carbonate buffer (pH 11). For other conditions see Fig. 1.

Plasma. To 0.5 ml of non-preprocessed plasma were added 1.27 ng of isoprenaline (internal standard). The emulsion, which occasinally occurs after extraction, was broken by stirring with a small glass rod and the sample was centrifuged again.

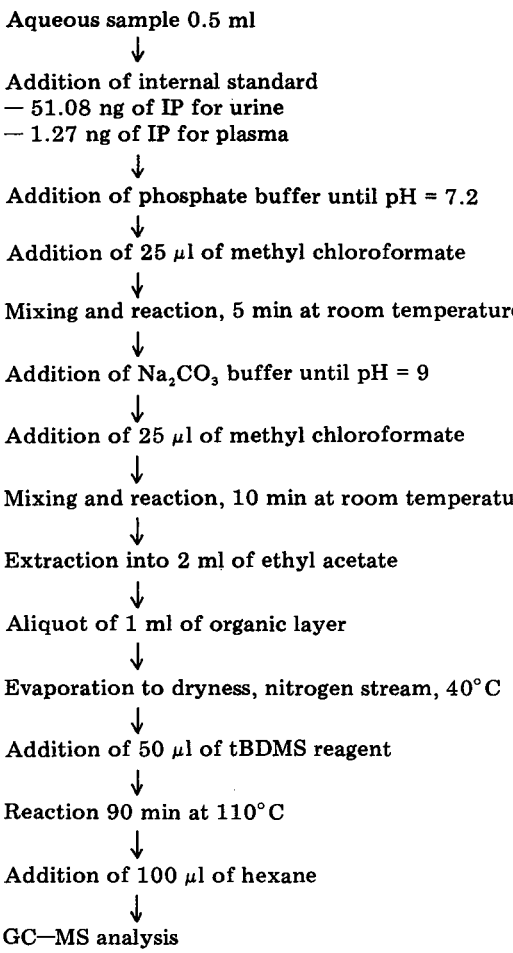

Fig. 1. Flow chart of analytical procedure for determination of catecholamines and metanephrines in biological samples.

RESULTS

pH of aqueous reaction medium

Fig. 2 shows the yield of the reaction of methyl chloroformate with 1,2-dihydroxybenzene as a function of the pH of the aqueous medium. The

maximum yield is reached at pH 7.2. The reaction takes place virtually instantaneously. Maximum yield was obtained for a reaction time of 1 min at pH 7.2. For practical reasons, e.g. reproducibility of sample handling of large series, the reaction conditions have been fixed at a pH of 7.2 and a reaction time of 5 min. In order to estimate simultaneously the reaction with primary amino groups, these conditions were applied to dopamine. No other conditions have been found which resulted in higher yields of the N,O-tricarbamate derivative. A linear relationship between the amount of dopamine and the yield of the derivative was observed over the range studied: $Y = 0.753X - 0.015$; $r = 0.9997$, range 15–150 μg.

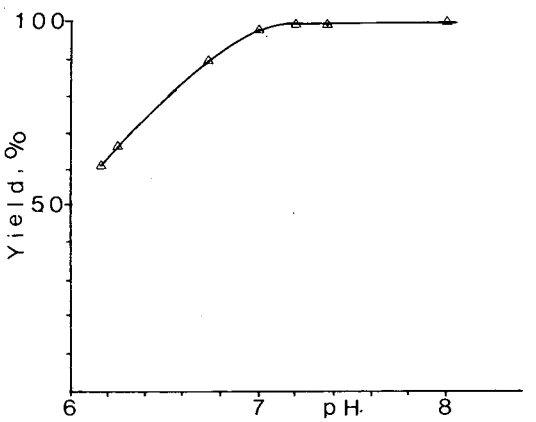

Fig. 2. Yield of the methylformate derivative of 1,2-dihydroxybenzene relative to n-C_{14} as a function of the pH of the aqueous medium. Reaction 30 min at room temperature.

For secondary amines, in particular for isoprenaline, lower yields and less reproducibile results were obtained. Both the yield and the reproducibility were considerably improved by a pH shift of $\Delta pH = 2$ after incubation of the reaction mixture for 5 min at pH 7.2. The effect of the pH shift on the yield of the formate derivative of isoprenaline and epinephrine relative to norepinephrine is shown in Fig. 3.

Isolation of formate derivatives

The extraction recoveries of epinephrine, norepinephrine and isoprenaline were determined for the system ethyl acetate—aqueous medium as 99.65%, 99.50% and 99.83% using a phase ratio of 1. The calculated partition constants are 290, 200 and 605, for E, NE and IP, respectively.

Silylation

In our hands, silylation of formate derivatives of catecholamines with HMDS [11] yielded mixed derivatives of form II and III together with minor amounts of form IV (cf. Fig. 4). Even under milder conditions (e.g. 10% HMDS in ethyl acetate) no single peak of form II could be obtained. Neither with stronger

silylating reagents nor under vigorous conditions has a single derivative been obtained.

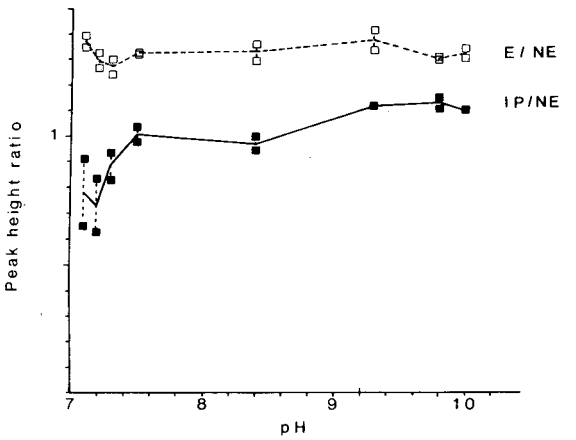

Fig. 3. Influence of the pH shift step on the yield and reproducibility for the reaction of methyl chloroformate with N-alkyl-substituted catecholamines. E = epinephrine, IP = isoprenaline and NE = norepinephrine (internal standard).

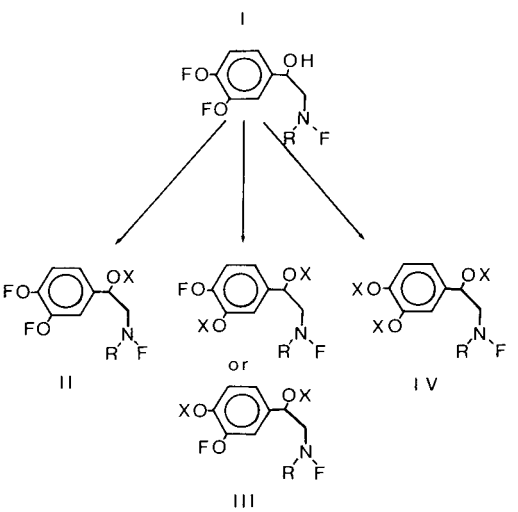

Fig. 4. Trialkylsilyl derivative formation of methyl formate derivatives of catecholamines. F = CH_3O—CO; X = $(alkyl)_3$—Si; R = H, CH_3 or $CH(CH_3)_3$. I = Catecholamine-N,O,O-trismethyl formate derivative. II, III and IV: No, partial and complete exchange of the O-methyl formate groups with silyl groups, respectively.

If the reaction was enforced too strongly, partial N-silylation took place for primary amines (NE, NMN and DA). This has been avoided by using TSIM, a very weak silyl donor for aliphatic amino groups [14]. A 5% mixture of TSIM in dichloromethane or diethyl ether yielded a single peak of form IV, but the yield was not very reproducible. Although the tBDMCS—imidazole reagent is less reactive than most of the TMS reagents [15], it is able to exchange O-

TMS with O-tBDMS [16]. It appears that this ability also includes O-carbamate groups. Substitution of O-carbamate together with silylation of the aliphatic hydroxy group was completed within 90 min at 110°C.

Gas chromatography—mass spectrometry

Fig. 5 shows a mass chromatogram of a synthetic mixture of metanephrines (MN and NMN) and catecholamines (E, NE and IP) as their O-tBDMS, N-carbamate derivatives. Up to 270°C a baseline separation was achieved on a medium polar capillary column (OV-1701). On non-polar capillary columns (SE-30) both the metanephrines (MN and NMN) and the catecholamines (E and NE) eluted together.

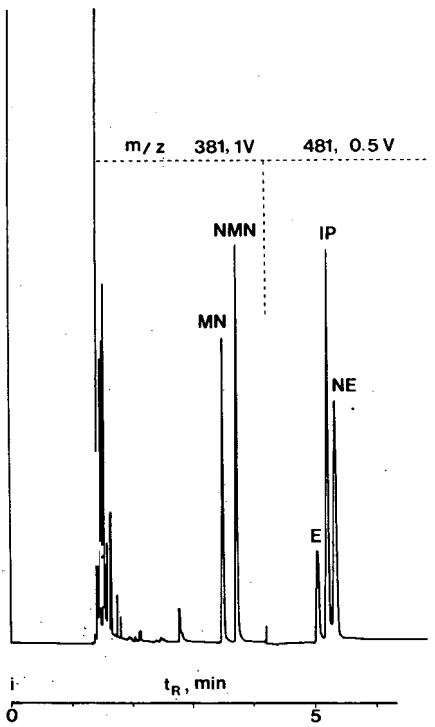

Fig. 5. Mass chromatogram of a synthetic mixture of metanephrines and catecholamines. MN = metanephrine (20 ng), NMN = normetanephrine (40 ng), E = epinephrine (6 ng), IP = isoprenaline (51 ng) and NE = norepinephrine (21 ng).

The retention indices of these compounds are given in Table I. The mass spectra of the compounds of interest are dominated by α-cleavage of the hydroxyethylamino chain, resulting in very intense ions at mass 381 and 481 for metanephrines and catecholamines, respectively. The mass spectral data of the catecholamines are summarized in Table II.

TABLE I

RETENTION INDICES ON OV-1701 OF O-tBDMS, N-CARBAMATE DERIVATIVES OF CATECHOLAMINES

Compound	Retention index
Metanephrine	2696
Normetanephrine	2733
Epinephrine	2908
Isoprenaline	2924
Norepinephrine	2937

TABLE II

MASS SPECTRAL DATA OF O-tBDMS, N-CARBAMATE DERIVATIVES OF CATECHOLAMINES

Compound	Fragment a* % $\Sigma_{70}I$**	Base peak *m/z*	Other peaks *m/z* (relative intensity)
Epinephrine	12.2	481	73 (73), 75 (18), 208 (8), 526 (3)
Norepinephrine	9.1	73	481 (44), 75 (38), 77 (19), 480 (5)
Isoprenaline	15.3	481	73 (93), 75 (22), 77 (20), 554 (2)

*Fragment of α-cleavage of hydroxyethylamino chain.
**% $\Sigma_{70}I$ = Intensity relative to the total ionization in the mass range *m/z* 70 to the molecular ion.

Quantitative results

The linearity of the whole procedure (carbamate formation, extraction, silylation and GC–MS analysis) was determined for physiological ranges of E and NE in urine. These experiments were performed in diluted hydrochloric acid and in spiked unhydrolyzed urine. Apart from the vertical displacement, due to the presence of free endogenous catecholamines in the urine, both calibration curves were identical when expressed in terms of slope, coefficient of regression and coefficient of variation. The calibration curves for the determination of the catecholamines in urine were linear over the range studied. The equations for E and NE were as follows:

E: $Y = 1.31X + 0.01$, $r = 0.9995$, range 3–50 ng
NE: $Y = 1.105X + 0.058$, $r = 0.9993$, range 9—150 ng

where Y is the ratio of the peak heights of the catecholamine and the internal standard and X is the ratio of the amounts (w/w) of the catecholamine and the internal standard.

The precision of the procedure was determined from a set of six identically processed aliquots of a hydrolyzed urine. The coefficients of variation were 5.5% and 1.6% for E and NE, respectively, for a mean sample concentration of 9.7 ng ml^{-1} of E and 74.4 ng ml^{-1} of NE. The analytical recovery was estimated by adding of 7.47 ng of E and 34.84 ng of NE to an aliquot of 0.5 ml of this urine. Recovered were 7.24 ng of E (97%) and 33.1 ng of NE (95%).

The detection limit of the GC–MS was 5 pg with a signal-to-noise ratio of 3.

This sensitivity allowed the determination of catecholamines down to 50 pg ml^{-1}. The determination of catecholamines in normal plasma samples has not yet been evaluated.

Applications

Fig. 6 shows an example of the analysis of metanephrines and catecholamines in a normal urine. An example of an assay in plasma of a patient suffering from pheochromocytoma is given in Fig. 7.

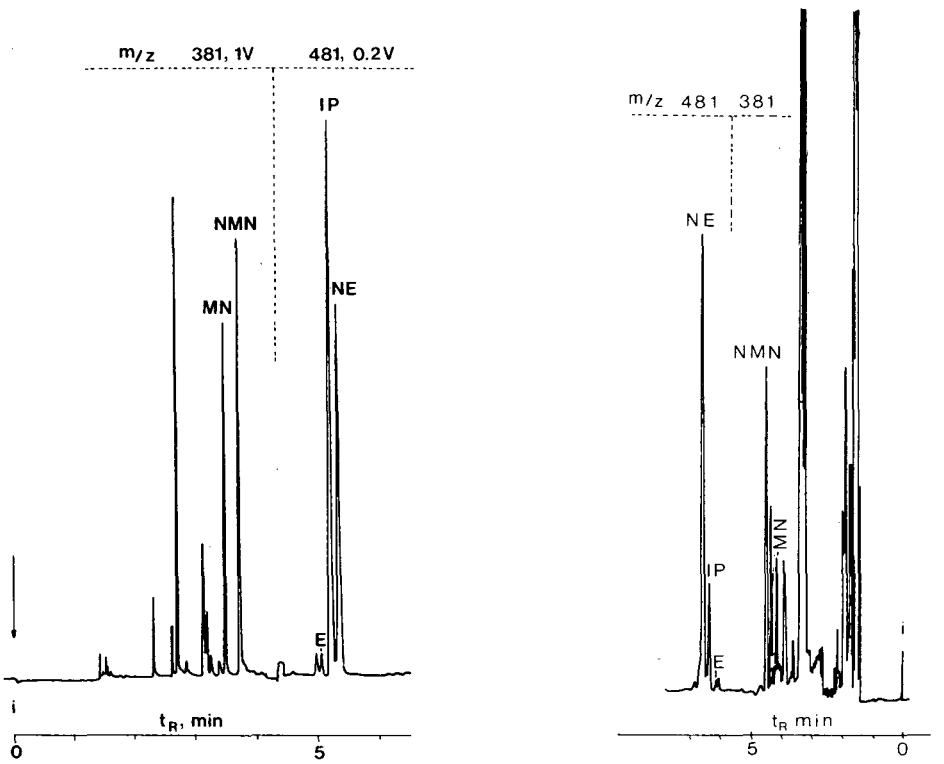

Fig. 6. Analysis of metanephrines and catecholamines in a normal urine. MN = metanephrine (1.15 μmol per 24 h), NMN = normetanephrine (2.25 μmol per 24 h), E = epinephrine (0.03 μmol per 24 h) and NE = norepinephrine (0.80 μmol per 24 h).

Fig. 7. Analysis of metanephrines and catecholamines in plasma of a patient suffering from pheochromocytoma. Amount of internal standard (IP) = 1.27 ng.

DISCUSSION

The kinetics of the reaction of alkyl chloroformates with amines in buffered aqueous media have been described by Ahnfelt and Hartvig [12]. The kinetic model for the reaction

$$AH + ClCOOR \rightarrow ACOOR + H^+ + Cl^- \quad (1)$$

$$H_2O + ClCOOR \rightarrow ROH + CO_2 + H^+ + Cl^- \quad (2)$$

is given by

$$\ln \frac{C_0^{AH}}{C_t^{AH}} = \frac{k_1 \cdot C_t^F \cdot K_a}{k_s \cdot (K_a + a_{H^+})}$$

with $C_t^F = C_0^F\ (1 - e^{-k_s t}) \sim C_0^F$ for $1 >> e^{-k_s t}$ where

C_t^{AH} = concentration of the amine at time t (M)

C_t^F = concentration of chloroformate at time t (M)

k_1 = rate constant for the reaction alkyl chloroformate with the amine ($mol^{-1}\ sec^{-1}$)

k_s = hydrolysis rate constant of the chloroformate (sec^{-1})

K_a = acid dissociation constant of the amine

a_{H^+} = activity of H_3O^+.

Although this equation is not valid for multifunctional compounds, consideration of the variables will be of great help in solving kinetic problems. In the present case, isoprenaline appeared to be less reactive than norepinephrine. This can partially be ascribed to the difference between the K_a of the amine functions of NE (pK_a = 9.78) and IP (pK_a = 10.0). More important for the lower reactivity of isoprenaline is probably the steric hindrance caused by the N-isopropyl group (decrease of k_1). Both negative effects on the yield can effectively be compensated by increasing the pH, so that the right-hand side of eqn. 1 yields a higher numerical value (> 4.6 for > 99% conversion). The observation of lower reactivity of compounds with a bulky N-alkyl substituent is in good agreement with the findings of Gyllenhaal et al. [11] on the carbamate formation of *n*-hexylnorepinephrine. However, increasing the pH is not allowed until the catecholic function has been protected. At elevated pH a considerable amount of the catecholamines would be oxidized before the carbamate formation was completed. The fact that the phenolic hydroxy groups react rapidly with methyl chloroformate at a relatively low pH permits the use of a pH shift.

The formation of tBDMS derivatives has substantial advantages above TMS derivatives: (1) the derivatives are much more stable [15]; (2) the increased mass and the higher intensity of the analyzed ion improves the signal-to-noise ratio in both ways; (3) the substitution of O-carbamate by tBDMS while the N-carbamate group is not affected provides a powerful tool to increase the selectivity. This implies that only amines will retain the carbamate moiety. Consequently, only amines will be specifically detected if a specific alkyl chloroformate has been used. A typical example would be 2,2,2-trichloroethyl chloroformate as reagent and GC with electron-capture detection or GC—MS with electron attachment negative chemical ionization. These mixed derivatives will have comparable properties to the O-TMS, pentafluorobenzylimide derivative of catecholamines as described by Lhuguenot and Maume [17]. However, these imide derivatives can only be formed with primary amines, whereas the derivatives as described here can be formed with both primary and secondary amines.

In conclusion, the derivatization of catecholamines and related compounds (e.g. their synthetic congeners) in aqueous biological samples provides a rapid,

easy and accurate isolation method with a high and reproducible yield. The O-tBDMS, N-carbamate derivative formation provides an excellent method to investigate in a very selective and sensitive way the occurrence of catecholamines in biological fluids. This method therefore represents a meaningful procedure next to the frequently used radioenzymatic [18] and liquid chromatography with electrochemical detection [19] methods.

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STUDIES OF METABOLIC CHANGES IN CELL CULTURES INFECTED WITH FOUR SEROTYPES OF DENGUE FEVER VIRUSES BY FREQUENCY-PULSED ELECTRON-CAPTURE GAS—LIQUID CHROMATOGRAPHY

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SUMMARY

Monkey kidney cell cultures were infected with four serotypes of dengue viruses, and the supernatant fluids of the cell cultures were extracted for amines, alcohols, carboxylic acids,

and hydroxy acids. The derivatized extracts were then analyzed by frequency-pulsed electron-capture gas–liquid chromatography (FPEC–GLC). FPEC–GLC profiles of the hydroxy acids showed peaks that were different for different serotypes and the FPEC–GLC carboxylic acid profiles differed from the control medium. These differences were reproducible when the same lot of medium was used. There were differences in profiles between lots of control media due apparently to different fetal bovine sera used in the growth medium. Therefore, the same lot of medium was necessary to reproduce profiles. The data obtained from the study indicate that FPEC–GLC can be used to detect changes in cellular metabolism caused by viral infection, and that these metabolic changes might be useful for detection of genetic differences in viruses as reflected by detectable changes in the metabolism of the infected cell.

INTRODUCTION

Gas–liquid chromatography has been used to study chemical changes in various chemical components present in body fluids and tissue culture as a results of bacterial and viral infections [1–8], and frequency-pulsed electron-capture gas–liquid chromatography (FPEC–GLC) has been used to detect differences in chemical components in sera and cerebrospinal fluids of persons infected with different types of viral diseases [5, 9].

Diagnosis of viral infections, such as those produced by various serotypes of dengue, through direct serum analysis by FPEC–GLC would be desirable. The major advantage of the new technique would be in the amount of time that would be saved if the virus did not have to be cultured. However, before analyzing more complex human specimens with FPEC–GLC, it seemed important to evaluate the method in controlled conditions such as tissue culture. If metabolic differences detected in infections caused by dengue sero types, which produce epidemics throughout the world [10–12], could b detected in tissue culture, then metabolic differences produced in the host ce may also be detected in disease cases involving humans. In addition these studies may provide clues to metabolic changes that take place in virus infected cells.

MATERIALS AND METHODS*

Tissue culture and virus infection

Rhesus monkey kidney cells, LLC-MK_2, were seeded in 25 cm^2 Falcon tissue culture flasks in aliquots of 2 million cells per 8 ml growth medium per flask. The flasks were incubated at 35°C for three days. The growth medium used was medium-199 with Hanks' salts containing 10% new born calf serum. The pH of the medium was adjusted to 7.4 with sodium bicarbonate. The histories of the virus strains used are described in Table I. All viruses were diluted in virus diluent [20% fetal bovine serum (FBS); pH 7.3]. The viruses were inoculated into replicate cell culture flasks from which growth medium had been removed. After virus adsorption at 35°C for 1 h, each flask was rinsed twice with phosphate buffered saline (PBS) and incubated at 35°C with 5 ml of maintenance medium which contained 2% FBS in medium-199. Flasks

*Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or by the U.S. Department of Health and Human Services.

TABLE I

HISTORY OF DENGUE VIRUS STRAINS USED IN THE STUDY

CDC accession number	Serotype	Virus designation and passage history	Year isolated	Place
CA1816	Dengue 1	Hawaiian prototype One monkey, one mosquito and seven tissue culture passages	1944	Hawaii
CA1817	Dengue 1	H-13806a Four tissue culture passages	1977	Jamaica
CA1818	Dengue 1	H-23333a Four tissue culture passages	1977	Puerto Rico
CA1819	Dengue 1	H-45509a Two tissue culture passages	1980	Mexico
CA1821	Dengue 2	H-14241a Three tissue culture passages	1977	Puerto Rico
CA1822	Dengue 2	H-20919a Two tissue culture passages	1977	Puerto Rico
CA1820	Dengue 2	New Guinea 'C'; prototype Twenty four suckling mouse and six tissue culture passages	1944	New Guinea
CA1823	Dengue 3	H-87; prototype Passages (one monkey and nineteen tissue cultures)	1956	Philippines
CA1824	Dengue 3	PR-6 Thirteen suckling mouse passages	1963	Puerto Rico
CA1825	Dengue 3	H-21326 Two tissue culture passages	1977	Puerto Rico
CA1826	Dengue 4	H-241; prototype Seven suckling mouse and six tissue culture passages	1956	Philippines
CA2039	Dengue 4	H-54101 Two tissue culture passages	1981	Dominica
CA2040	Dengue 4	H-54157 Two tissue culture passages	1981	Saint Barthelemy

inoculated with virus diluent alone and uninfected tissue culture in growth medium served as controls. For the time study, supernatant fluid from each flask was removed daily for seven days and on the tenth day after inoculation, centrifuged at 200 *g* for 10 min, and stored frozen at −70°C until analysis. For other studies, the procedure was the same except that the supernatant fluids were removed on the seventh day after inoculation. All tests except the time study were repeated. Virus titers were determined by means of plaque assay, according to the method of Eckels et al. [13].

Extraction and derivatization procedures

A 2-ml aliquot of each tissue culture supernatant fluid was placed in a 50-ml round-bottomed centrifuge tube with a PTFE-lined screw cap; then heptanoic acid (11.43 nmol in 0.3 ml of distilled water, made basic with sodium hydroxide to obtain solubility), 2-hydroxyisovaleric acid (2.68 μmol

in 0.1 ml of distilled water), and di-*n*-butylamine (1.19 μmol in 0.4 ml of distilled water made acidic to increase solubility) were added to each sample as internal standards. Next, the samples were acidified to about pH 2 with 0.1 ml of 50% (v/v) sulfuric acid, mixed by shaking, and extracted with 20 ml of nanograde chloroform (Mallinckrodt) by shaking them for 5 min on a Burrell Wrist Action Shaker at a setting of 10. The residual aqueous phase was made basic (about pH 10) with 0.3 ml of 8 *N* sodium hydroxide and reextracted with 20 ml of chloroform, as described for the acidic extraction, to obtain the amines. Then, the residual basic aqueous phase was reacidified to about pH 2 with sulfuric acid and extracted with 20 ml of diethyl ether (Fisher reagent grade stabilized with butylated hydroxytoluene) to obtain the hydroxy acids. The acidic chloroform extracts were derivatized with trichloroethanol—heptafluorobutyric anhydride (TCE—HFBA) to form TCE esters of carbxylic acids and HFBA esters of alcohols as described [14, 15]. The basic chloroform extracts containing amines and the acidic diethyl ether extracts containing hydroxy acids were derivatized with HFBA pyridine—ethanol to form amines and esters, respectively, as described [16, 17]. After the TCE and HFBA derivatives of carboxylic acids, alcohols, and amines were prepared, they were dissolved in 0.1 ml of xylene–ethanol (1:1). The HFBA derivatives of hydroxy acids were dissolved in 0.1 ml of ethyl acetate. A 2-μl injection was used for analysis of all derivatives. The techniques for filling and cleaning the syringe have been described [15].

Apparatus

The derivatives were analyzed on a Perkin-Elmer Model 3920 gas chromatograph equipped with dual 10 mCi ^{63}Ni frequency-pulsed electron-capture detectors. Two glass columns (7.3 m × 0.2 cm I.D.) packed with 3% OV-101 on 80–100 mesh Chromosorb W HP (AW-DMCS treated) were used under conditions previously described [14, 15, 17].

A Perkin-Elmer programmable processor (PEP-2) equipped with a Modular Software System (MS-16 revision B) accumulated data from the gas chromatograph, analyzed the data according to a stored method, and prepared a report. An internal standard analysis was performed on the data by using heptanoic acid, di-*n*-butylamine, and 2-hydroxyisovaleric acid, which were added as internal standards [14, 17].

RESULTS

The results of viral replication of one representative test are shown in Table II. While all prototype viruses replicated well in LLC-MK_2 cells as expected, virus titers of many non-prototype virus strains did not reach 4 log. No cytopathic effects and few lysed cells were observed during infection.

The time study indicated that some change in the chemical components in the supernatant fluids in the virus-infected cell cultures was apparent as early as the second day after inoculation, but differentiation between serotypes was best on the seventh day. This was true not only of the cell cultures infected with prototype viruses but also of non-prototype strains despite the fact that the majority of the latter group did not replicate as well as the former group.

TABLE II

REPLICATION OF DENGUE VIRUS STRAINS USED IN FPEC—GLC ANALYSIS

Virus	Inoculum (log PFU/flask)*	Extracellular virus titer (log PFU/ml supernatant fluid) seven days after inoculation
DEN 1 (Hawaii)	3.6	5.9
DEN 1 (H-13806)	3.4	3.5
DEN 1 (H-23333)	3.9	2.8
DEN 1 (H-45509)	3.6	3.2
DEN 2 (New Guinea "C")	3.6	6.8
DEN 2 (H-14241)	3.5	3.4
DEN 2	3.1	2.9
DEN 3	3.3	4.3
DEN 3	3.9	3.7
DEN 3 (H-21326)	3.8	2.7
DEN 4 (H-241)	3.6	4.5
DEN 4 (H-54101)	4.0	5.8
DEN 4 (H-54157)	4.0	6.8

*PFU = plaque forming unit.

Similarly, FPEC -GLC profiles of non-prototype strains were similar to those of prototype virus within a given serotype, with two minor exceptions described below.

As shown in Fig. 1, a dramatic change occurred in the hydroxy acid components in the supernatant fluids of the infected culture (Fig. 1B) as compared with those of the control (Fig. 1A) culture. The major changes in the infected culture (Fig. 1B) were apparent in the removal of the blackened peaks 2, 7, and 8 and to a lesser degree, peaks 1, 2a, and 10. A "U" has been placed over the peaks (Fig. 1B) to indicate utilization or removal by comparison with uninfected control fluid (Fig. 1A). One new (N) peak, labelled 12 was detected in the infected sample.

As shown in Fig. 2 peaks in the profiles of four serotypes of dengue viruses differed both qualitatively and quantitatively from peaks found in the control and in the FPEC—GLC profiles between serogroups. For example, qualitatively peaks 8a, 11a, 12 and 13 were not found in the controls, and dengue serotypes 2 and 3 differed from serotypes 1 and 4 by the production of peaks 8a, 11a, and 13. Serotypes 2 and 3 differed from each other by production of peaks 8a (dengue 2) and 13 (dengue 3). Dengue serotype 1 differed from serotype 4 on the basis of utilization or removal of peak 8 and by the production in serotype 4, above that found in the controls, of peak 9.

The changes in amine profile that occurred during infection are illustrated in Fig. 3. Dengue 1 was the only serotype that produced a different profile from that of the control. The significance of the reduction of peaks 1 and 5 in the dengue 1 profile (Fig. 3A), as compared with the control profile (Fig. 3B), was questionable, since peaks 1 and 5 were absent in another control (Fig. 3C) in which a different lot of growth medium was used. Further, peaks 6 and 7

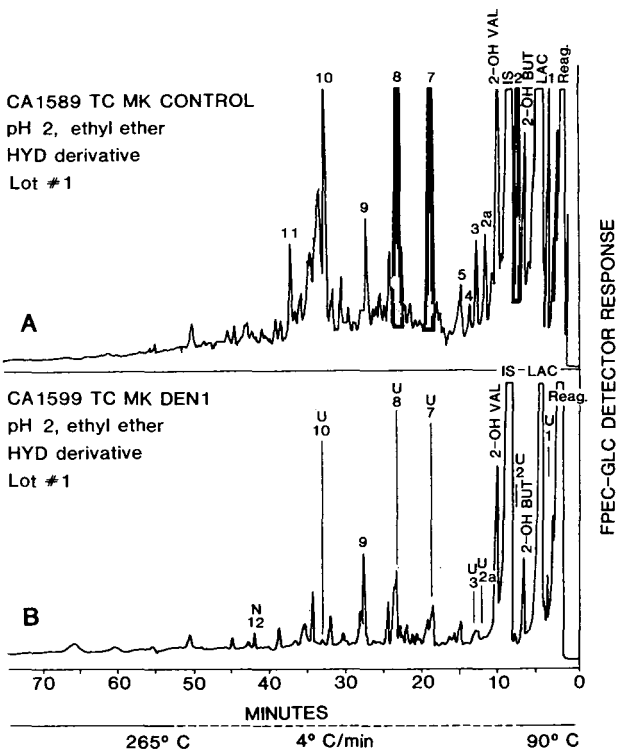

Fig. 1. FPEC—GLC traces of hydroxy acids in the supernatant fluids of (A) control and (B) dengue-infected LLC-MK_2 cell cultures. Column: OV-101. Abbreviations: TC: tissue culture; MK: LLC-MK_2 cells; HYD: heptafluorobutyric anhydride—ethanol derivatized acidic diethyl ether extracts; reag: reagent; LAC: lactic acid; 2-OH BUT: 2-hydroxybutyric acid; IS: internal standard; 2-OH VAL: 2-hydroxy valeric acid. "U" over a peak indicates utilized or removal.

(Fig. 3A) were not reproducible from lot to lot which may indicate that peaks 6 and 7 were intermediates affected by change in the medium.

Fig. 4 shows carboxylic acid profiles of normal and infected cultures. Profile differences among different lots of growth medium were again detected, as demonstrated by the lack of peaks 2 and 4 in lot 2 (compare Fig. 4A and B). The profile of Dengue 1 infected culture (Fig. 4C) is clearly distinguished from that of the control by the appearance of new peaks (3 and 6) and the reduction of peaks (1, C4, and iC5). The concentration of the internal standard (C7) shown in Fig. 4C is slightly lower than that shown for the internal standards in Fig. 4A and B. This difference in concentration as shown by other analyses and by computer evaluation which bases its concentration determination on the relationship of the size of the internal standard to a fixed molar concentration, verified the reduction of peaks 1 and iC5.

Fig. 5 shows that carboxylic acid profiles of all serotypes are different from that of the controls (Fig. 4A and B) because: (1) three peaks (1, 2, and iC5)

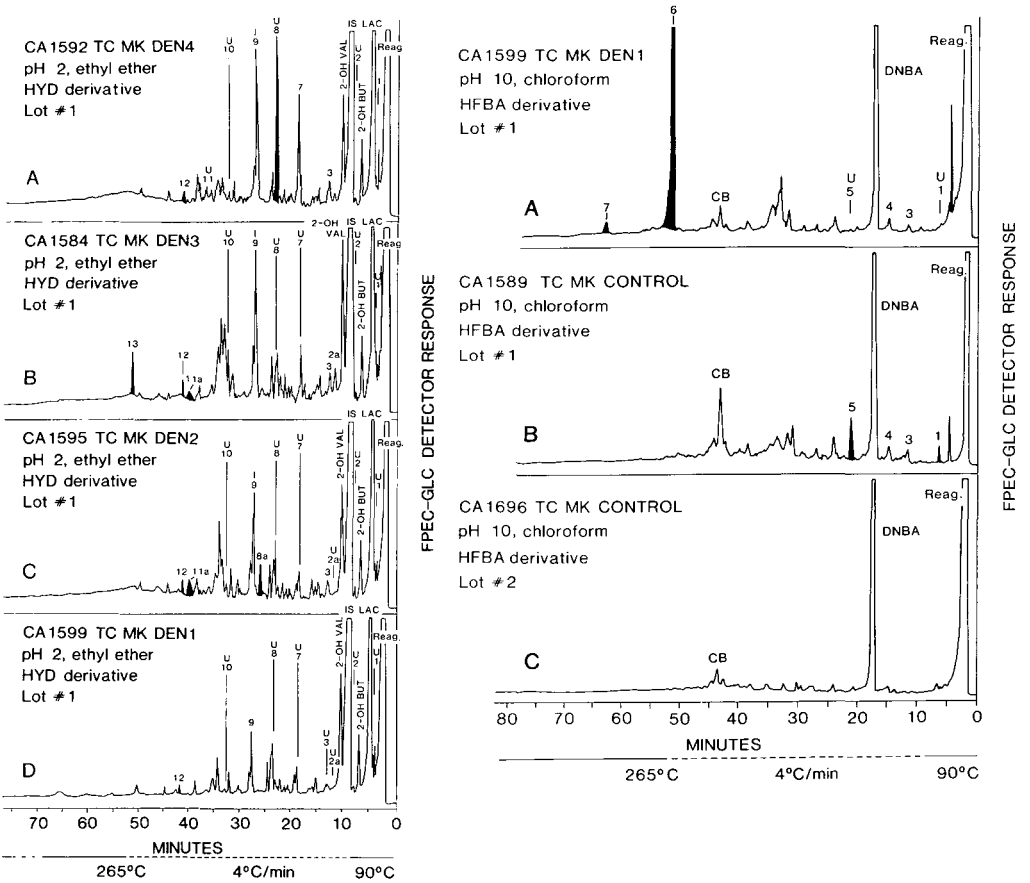

Fig. 2. FPEC—GLC traces of hydroxy acids in the supernatant fluids of LLC-MK_2 cell cultures infected with four dengue serotypes. Column: OV-101. Abbreviations: "I" over a peak indicates that the peak was increased over that found in the control. For other abbreviations, see Fig. 1.

Fig. 3. FPEC—GLC traces of amines in the supernatant fluids of two sets of normal (B, C) and a dengue virus-infected LLC-MK_2 (A) cell cultures. Column: OV-101. Abbreviations: HFBA: Heptafluorobutyric anhydride; DNBA: internal standard, di-*n*-butylamine; CB: column bleed. For other abbreviations, see Fig. 1.

in the control profile were consistently reduced; and (2) a new peak was present (peak 6) that was not detected in the control. Dengue 1 profile for carboxylic acids was further distinguished from the profiles of the other serotypes by a new peak 3 not shared by the others.

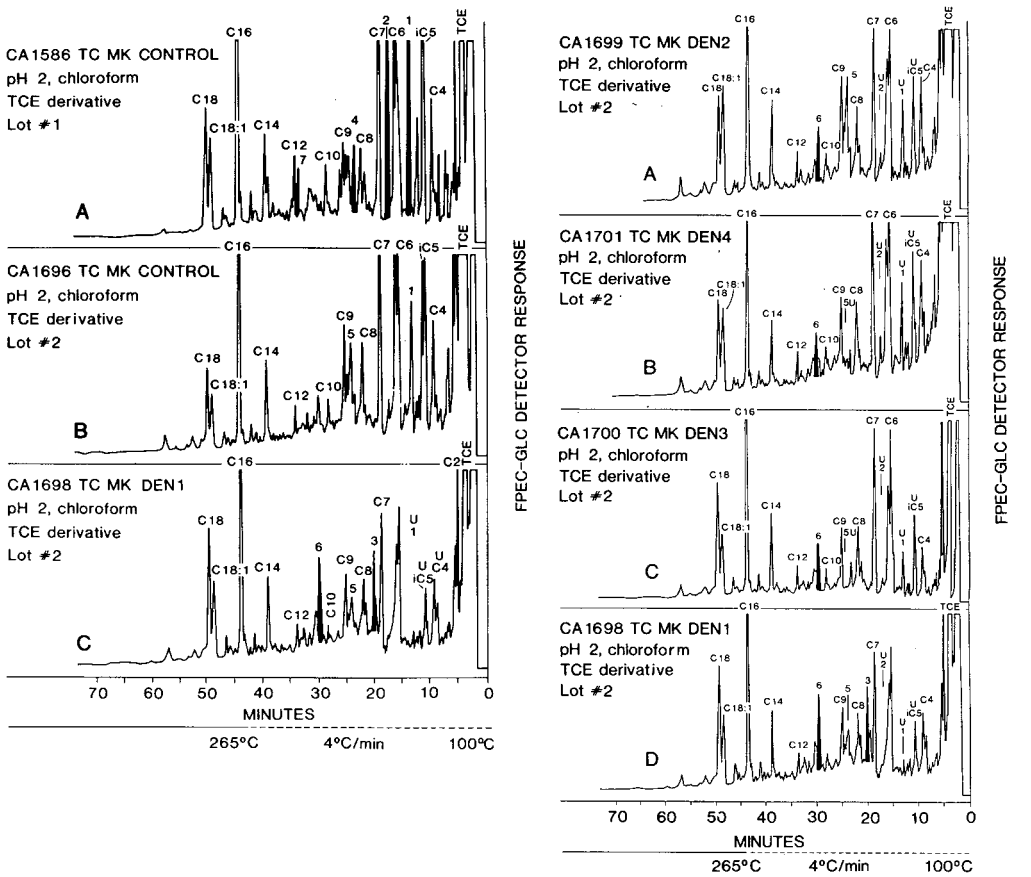

Fig. 4. FPEC—GLC traces of carboxylic acids and alcohols in the supernatant fluids of two sets of normal (A, B) and a dengue virus-infected LLC-MK_2 (C) cell cultures. Abbreviations: TCE: trichloroethanol; C7: internal standard. The letter C followed by a number indicates a saturated straight chain carboxylic acid with the number of carbon atoms indicated by the number. The letter "i" indicates "iso"; and the use of a colon between two numbers indicates unsaturation. For other abbreviations, see Fig. 1.

Fig. 5. FPEC—GLC chromatograms of carboxylic acids and alcohols in the supernatant fluids of LLC-MK_2 cell cultures infected with four dengue serotypes. Column: OV-101. For abbreviations, see Figs. 1 and 4.

DISCUSSION

The results from our studies clearly show that the profiles of metabolites in LLC-MK_2 cell cultures infected with dengue viruses are different from those of uninfected control cultures. Further, the metabolism is altered in a sufficiently different manner to produce FPEC—GLC profiles which show that genetically different viruses can alter the cellular metabolism in a manner that makes distinction by FPEC—GLC possible. Among the compounds studied, hydroxy

acids appear to be the most promising indicators of type differences, since each serotype produced a distinct profile.

FPEC—GLC profiles of dengue strains of a given serotype were homogeneous enough to make classification possible, as long as tests were performed with the same lot of growth medium. Minor variations detected in two lots of media probably were derived from different lots of FBS employed, since all other constituents of the media came from the same lots of ingredients. In a blind coded test only two strains, a dengue 1 (H-13806) and a dengue 3 (PR-6), had different FPEC—GLC profiles from those produced by prototype viruses, dengue 1 (Hawaii) and dengue 3 (H-87), respectively. It is of interest to note that the dengue 3 (PR-6) strain was isolated in Puerto Rico and was demonstrated to be different serologically from the prototype dengue 3 (H-87) which was originally isolated in Southeast Asia [18]. The dengue 1 (H-13806) strain was isolated during the first documented dengue 1 pandemic in the Caribbean.

The main variations between the FPEC—GLC profiles obtained from control cell cultures grown in different lots of growth medium seem to be caused by inherent differences between lots of bovine sera. For this reason, the use of cells grown with serum-free media would appear to be better for FPEC—GLC analysis. An alternative would be the use of predetermined reference serotypes which were cultured and tested by FPEC—GLC with each new lot of medium.

The present study adds support to previous findings [7, 9] that different viral diseases seem to be capable of altering the host cell metabolism or body response in different ways, and possibly offers a way to study changes in cellular metabolism caused by viral infections. If so, these differences may be useful as an aid in the identification of several types of viral diseases including dengue fever. The ultimate dengue diagnostic scheme would involve FPEC—GLC detection of dengue infection through analysis of human body fluids, and preliminary studies indicate further research in this area could be fruitful.

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STUDIES ON STEROIDS

CLXXXVII. DETERMINATION OF SERUM BILE ACIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE LABELING

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SUMMARY

A method for the simultaneous determination of bile acids in serum by high-performance liquid chromatography (HPLC) with fluorescence labeling is described. The bile acid fraction was obtained from a serum specimen by passing it through a BondElut cartridge. Bile acids were derivatized quantitatively into the fluorescent compounds through the hydroxyl group at C-3 by treatment with 1-anthroyl nitrile in the presence of quinuclidine in acetonitrile. These derivatives were separated into the free, glycine- and taurine-conjugate fractions by ion-exchange chromatography on a lipophilic gel, piperidinohydroxypropyl Sephadex LH-20. Subsequent resolution of each fraction into cholate, ursodeoxycholate, chenodeoxycholate, deoxycholate and lithocholate was attained by HPLC on a Cosmosil $5C_{18}$ column using 0.3% potassium phosphate buffer (pH 6.0)—methanol (1:5) and 0.1% potassium phosphate buffer (pH 6.0)—methanol (1:8) as mobile phases. The anthroyl bile acids were monitored by fluorescence detection (excitation wavelength 370 nm; emission wavelength 470 nm), the limit of detection being 20 fmol. The proposed method proved to be applicable to the quantitation of bile acids in serum with satisfactory reliability and sensitivity.

INTRODUCTION

In recent years considerable attention has been directed to the biodynamics of bile acids in patients with hepatobiliary diseases. For this purpose the development of a reliable method for the profile analysis of bile acids in biological fluids is urgently needed. Among various methods high-performance liquid chromatography (HPLC) with fluorescence detection appears to be most

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promising with respect to resolution, sensitivity and versatility. Pre-column labeling with a fluorophore usually involves the carboxyl group of the side-chain [1, 2]. This method, however, requires hydrolysis of the taurine conjugate prior to fluorescence labeling. Recently, novel methods using immobilized 3α-hydroxysteroid dehydrogenase have been developed for the determination of bile acids in serum [3—5]. These procedures, however, have disadvantages in that the resolution of chenodeoxycholate and deoxycholate is unsatisfactory and the sensitivity is still insufficient. In the previous study we developed a new type of fluorescence labeling reagent having a carbonyl nitrile group for use in HPLC of the hydroxyl compounds [6, 7]. The present paper deals with the use of 1-anthroyl nitrile for the derivatization of serum bile acids through the 3α-hydroxyl group, followed by separation and determination by HPLC with fluorescence detection.

EXPERIMENTAL

High-performance liquid chromatography

The apparatus was a Model 638-50 liquid chromatograph (Hitachi, Tokyo, Japan) equipped with a Model 650-10LC fluorescence spectrophotometer (Hitachi) (excitation wavelength 370 nm; emission wavelength 470 nm). A Cosmosil $5C_{18}$ (5 μm) column (15 cm × 4 mm I.D.) (Nakarai Kagaku Co., Kyoto, Japan) was used at ambient temperature.

Materials

Cholic acid, chenodeoxycholic acid, deoxycholic acid and lithocholic acid were purchased from Sigma (St. Louis, MO, U.S.A.) and purified prior to use. Ursodeoxycholic acid was kindly donated by Tokyo Tanabe Co. (Tokyo, Japan). The glycine and taurine conjugates were synthesized by the *p*-nitrophenyl ester method in these laboratories [8, 9]. Sephadex LH-20 was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). All the reagents employed were of analytical grade. Solvents were purified by distillation prior to use. Piperidinohydroxypropyl Sephadex LH-20 (acetate form, PHP-LH-20) [10] (0.6 mequiv./g) and 1-anthroyl nitrile [7] were prepared in the manner described in the previous papers. A BondElut cartridge (Analytichem International, Harbor City, CA, U.S.A.) was washed successively with ethanol (5 ml) and water (5 ml) prior to use. All glassware used was silanized with trimethylchlorosilane.

Preparation of free and conjugated deoxycholate 12-propionates

Deoxycholic acid 12-propionate. To a solution of methyl deoxycholate (2 g) in pyridine (20 ml) was added propionyl chloride (4 ml). The resulting solution was heated at 60°C for 50 min and then poured into ice—water. After extraction with ethyl acetate, the organic layer was washed successively with 5% $NaHCO_3$, water, 3% hydrochloric acid and water, and evaporated down. An oily residue obtained was dissolved in 3% methanolic potassium hydroxide (15 ml) and allowed to stand at room temperature for 4 h. After neutralization with concentrated hydrochloric acid, the solution was concentrated in vacuo, poured into ice-cooled 1% potassium hydroxide and

extracted with diethyl ether. The aqueous layer was acidified with concentrated hydrochloric acid, and the precipitate was collected by filtration and washed with water. Recrystallizaton from acetone gave deoxycholic acid 12-propionate (1.5 g) as colorless needles, m.p. 214—215°C. Anal. calc. for $C_{27}H_{44}O_5$: C, 72.28; H, 9.89. Found C, 72.43; H, 10.01. NMR (C^2HCl_3) δ: 0.72 (3H, s, 18-CH_3), 0.81 (3H, d, J = 6 Hz, 21-CH_3), 0.88 (3H, s, 19-CH_3), 1.17 (3H, t, J = 8 Hz, CH_3CH_2—), 2.35 (2H, q, J = 8 Hz, CH_3CH_2—), 3.62 (1H, m, 3β-H), 5.06 (1H, m, 12β-H).

Glycodeoxycholic acid 12-propionate. To a solution of deoxycholic acid 12-propionate (1 g) in ethyl acetate (40 ml) were added N-hydroxysuccinimide (700 mg) and N,N′-dicyclohexylcarbodiimide (1.5 g), and the solution was stirred at room temperature overnight. The precipitate was removed by filtration and the filtrate was washed with water. After evaporation of the solvent, an oily residue obtained was subjected to column chromatography on silica gel (30 g). Elution with hexane—ethyl acetate and recrystallization of the eluate from hexane—acetone gave deoxycholate N-succinimidyl ester 12-propionate (800 mg) as colorless needles, m.p. 164—165°C. NMR (C^2HCl_3) δ: 0.75 (3H, s, 18-CH_3), 0.85 (3H, d, J = 6 Hz, 21-CH_3), 0.90 (3H, s, 19-CH_3), 1.18 (3H, t, J = 8 Hz, CH_3CH_2—), 2.37 (2H, q, J = 8 Hz, CH_3CH_2—), 2.80 (4H, s, —$COCH_2CH_2CO$—), 3.60 (1H, m, 3β-H), 5.03 (1H, m, 12β-H). To a solution of deoxycholate N-succinimidyl ester 12-propionate (700 mg) in ethyl acetate (15 ml) was added ethyl glycinate · HCl (800 mg) in pyridine (3 ml), and the resulting solution was heated at 60°C for 48 h. The reaction mixture was poured into ice—water and extracted with ethyl acetate. The organic layer was washed with 5% hydrochloric acid and water, and evaporated down. An oily residue obtained was subjected to column chromatography on silica gel (25 g) with hexane—ethyl acetate. Ethyl glycodeoxycholate 12-propionate (200 mg) thus obtained was dissolved in 1.5% methanolic potassium hydroxide (10 ml) and allowed to stand at room temperature for 1 h. The reaction mixture was neutralized with concentrated hydrochloric acid, concentrated in vacuo, added with 3% hydrochloric acid and extracted with ethyl acetate. The organic layer was washed with water and evaporated down. An oily residue was subjected to column chromatography on silica gel (6 g). Elution with chloroform—methanol and recrystallization of the eluate from acetonitrile—methanol gave glycodeoxycholate 12-propionate (100 mg) as a colorless crystalline substance, m.p. 178—180°C. Anal. calc. for $C_{29}H_{47}NO_6$: C, 68.88; H, 9.37; N, 2.77. Found: C, 68.61; H, 9.07; N, 2.87. NMR ($C^2H_3O^2H$) δ: 0.78 (3H, s, 18-CH_3), 0.87 (3H, d, J = 6 Hz, 21-CH_3), 0.94 (3H, s, 19-CH_3), 1.17 (3H, t, J = 8 Hz, CH_3CH_2—), 2.41 (2H, q, J = 8 Hz, CH_3CH_2—), 3.54 (1H, m, 3β-H), 3.83 (2H, s, >NCH_2—), 5.08 (1H, m, 12β-H).

Taurodeoxycholate 12-propionate. To a solution of deoxycholate N-succinimidyl ester 12-propionate (200 mg) in pyridine (5 ml) was added taurine (600 mg) in water (1 ml), and the resulting solution was allowed to stand at room temperature overnight. After evaporation of the solvent, the oily residue obtained was subjected to column chromatography on silica gel (8 g). Elution with chloroform—methanol and recrystallization of the eluate from aceto-

nitrile—methanol gave taurodeoxycholate 12-propionate (70 mg) as a colorless crystalline substance, m.p. 162—165°C. Anal. calc. for $C_{29}H_{49}NO_7S \cdot H_2O$: C, 60.70; H, 8.96; N, 2.44. Found: C, 60.51; H, 9.12; N, 2.51. NMR ($C^2H_3O^2H$) δ: 0.77 (3H, s, 18-CH_3), 0.85 (3H, d, J = 6 Hz, 21-CH_3), 0.93 (3H, s, 19-CH_3), 1.18 (3H, t, J = 8 Hz, CH_3CH_2—), 2.41 (2H, q, J = 8 Hz, CH_3CH_2—), 2.96 (2H, t, J = 7 Hz, >NCH_2CH_2—), 3.52 (1H, m, 3β-H), 3.59 (2H, t, J = 7 Hz, >NCH_2CH_2—), 5.08 (1H, m, 12β-H).

Procedure for determination of serum bile acids

To a serum sample (100 μl) were added free, glycine- and taurine-conjugated deoxycholate 12-propionates (each 250 ng) as internal standards (I.S.), and the mixture was diluted with 0.5 *M* phosphate buffer (pH 7.0) (1 ml) and applied to a BondElut cartridge. After successive washing with water (2 ml) and 1.5% ethanol (1 ml), bile acids were eluted with 90% ethanol (2 ml). A 400-μl aliquot of the effluent was evaporated down, added with 1-anthroyl nitrile (200 μg) in acetonitrile (100 μl) and 0.16% quinuclidine in acetonrile (100 μl), and the mixture was heated at 60°C for 20 min. After addition of methanol (50 μl) for decomposing excess 1-anthroyl nitrile, the mixture was evaporated down under nitrogen. The residue was dissolved in 90% ethanol (1 ml) and applied to a PHP-LH-20 column (100 mg, 18 mm × 6 mm I.D.). Elution was carried out at a flow-rate of 0.2 ml/min. After washing with 90% ethanol (1 ml), free, glycine- and taurine-conjugated bile acids were fractionally separated by stepwise elution with 0.1 *M* acetic acid in 90% ethanol (5 ml), 0.2 *M* formic acid in 90% ethanol (5 ml), and 0.3 *M* acetic acid—potassium acetate (pH 6.3) in 90% ethanol (5 ml). Each fraction was evaporated and the residue obtained was redissolved in methanol (100—200 μl). A 5—10 μl aliquot of the solution was injected into the HPLC system.

When an interfering peak appeared on a chromatogram of the taurine-conjugate fraction, the following prior clean-up was recommended (see Fig. 6): the eluate from a BondElut cartridge was applied to a PHP-LH-20 column (100 mg, 18 mm × 6 mm I.D.) and eluted with 0.3 *M* acetic acid—potassium acetate (pH 6.3) in 90% ethanol (5 ml). The effluent was evaporated down and then applied to a BondElut cartridge in the manner described above for the elimination of inorganic salts.

Recovery test for bile acids

The test samples were prepared by dissolving 200 pmol each of free, glycine- and taurine-conjugated bile acids in human serum (100 μl). After addition of internal standard (each 250 ng), the serum sample was subjected successively to clean-up by a BondElut cartridge, derivatization with 1-anthroyl nitrile, group separation on PHP-LH-20 and determination by HPLC in the manner described above.

RESULTS AND DISCUSSION

Derivatization of bile acids with 1-anthroyl nitrile

In the previous study we developed 1-anthroyl nitrile as a fluorescence

labeling reagent which is effective for a secondary hydroxyl group on the steroid nucleus [7]. Initially, suitable conditions were investigated for the coupling of bile acids through the inherent 3α-hydroxyl group with 1-anthroyl nitrile (Fig. 1). Bile acids were dissolved in various concentrations of triethylamine or quinuclidine in acetonitrile and allowed to stand at 60° C. An aliquot of the resulting solution was applied to HPLC. The yield of the anthroyl derivative was calculated by comparison with the peak area of the standard sample. The reaction rate was significantly influenced by the organic base as illustrated in Fig. 2. In the presence of triethylamine, the yield of the anthroyl derivative was approximately 40% at 1 h. On the other hand, when quinuclidine, a sterically rigid base, was employed, the reaction rate increased along with the reaction time up to 10 min, resulting in the quantitative formation of the anthroyl derivative. In this condition the 7α and 12α axial and 7β equatorial hydroxyl groups underwent no reaction with 1-anthroyl nitrile due to the steric hindrance. Consequently, derivatization occurred selectively at the 3α equatorial hydroxyl group. On the basis of these data, bile acids were treated with 1-anthroyl nitrile in 0.08% quinuclidine in acetonitrile at 60° C for 20 min.

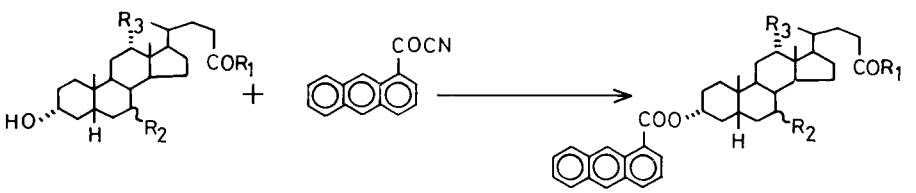

Fig. 1. Transformation of bile acids with 1-anthroyl nitrile into the 3-(1-anthroyl) derivatives. R_1 = OH, $NHCH_2COOH$, $NHCH_2CH_2SO_3H$; R_2 = H, α-OH, β-OH; R_3 = H, α-OH.

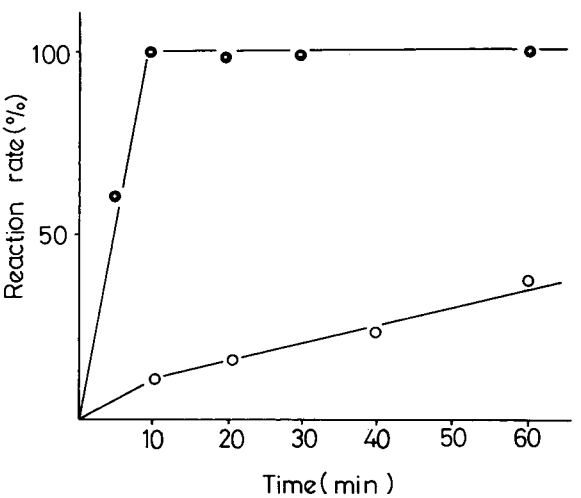

Fig. 2. Time course for derivatization of bile acids with 1-anthroyl nitrile. (•), 0.08% quinuclidine in acetonitrile; (○), 4% triethylamine in acetonitrile.

Separation of bile acid 3-(1-anthroyl) derivatives

Next, effort was directed to the separation of the derivatized bile acids by means of HPLC. Several attempts have been made on the separation of bile acids on ODS columns with various solvent systems [11—15]. In acidic conditions, distinct differences in the k' value are observed among the conjugated forms, but chenodeoxycholate and deoxycholate are not completely resolved. On the other hand, when neutral or weakly alkaline mobile phase is used, the steric interaction between the 12α-hydroxyl group and acidic group of the side-chain takes place, providing efficient separation of the two bile acids [16]. Accordingly, the mobile phase adjusted to pH 6.0—7.8 was employed in the present study.

Various combinations of buffer solution and organic solvent were examined for the suitable mobile phase on Cosmosil $5C_{18}$, which is an ODS column. The use of a potassium phosphate buffer—methanol system appeared to be promising, as it exerts no significant leading or tailing. The chromatographic behaviours of bile acids were investigated in these conditions. The k' values of free bile acids relative to ursodeoxycholic acid were plotted against the pH value using 0.3% potassium phosphate buffer—methanol (1:5) as mobile phase (Fig. 3). Similar chromatographic behaviours were also observed for the glycine and taurine conjugates. It is to be noted that ursodeoxycholate showed a larger k' value than cholate. Although the resolution value of chenodeoxycholic acid and deoxycholic acid decreased along with decreasing pH, the baseline separa-

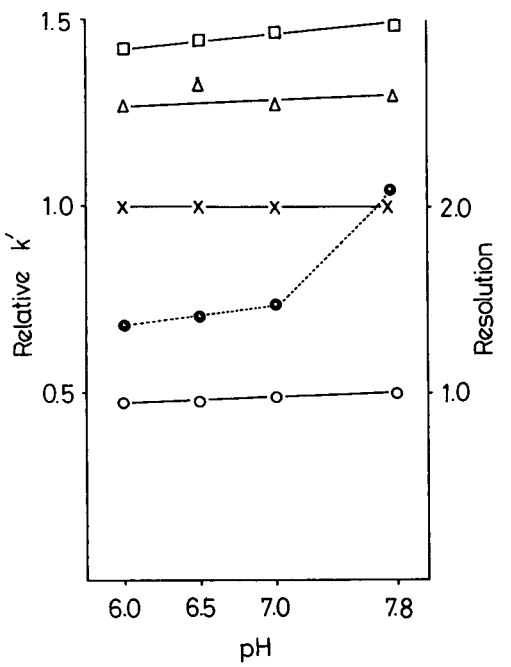

Fig. 3. Effect of pH on the k' values relative to ursodeoxycholate. (○), cholate; (×), ursodeoxycholate; (△), chenodeoxycholate; (□), deoxycholate. The dotted line represents the resolution of chenodeoxycholate and deoxycholate.

tion of these two was achieved even at pH 6.0. The effect of salt concentration in the mobile phase on the retention value was also examined with potassium phosphate buffer (pH 6.0)—methanol (1:5). The k' value was raised with increasing salt concentration up to 0.5%.

On the basis of these data 0.3% potassium phosphate buffer (pH 6.0)—methanol (1:5) and 0.1% potassium phosphate buffer (pH 6.0)—methanol (1:8) were chosen as suitable mobile phases. The k' values of bile acids observed with the two solvent systems are listed in Table I. Typical chromatograms of standard samples are shown in Fig. 4. The anthroyl bile acids were monitored by fluorescence detection (excitation wavelength 370 nm; emission wavelength 470 nm), the limit of detection being 20 fmol. A calibration graph was constructed by plotting the ratio of the peak area of each bile acid to that of free, glycine-, or taurine-conjugated deoxycholic acid 12-propionate against the amount of bile acid.

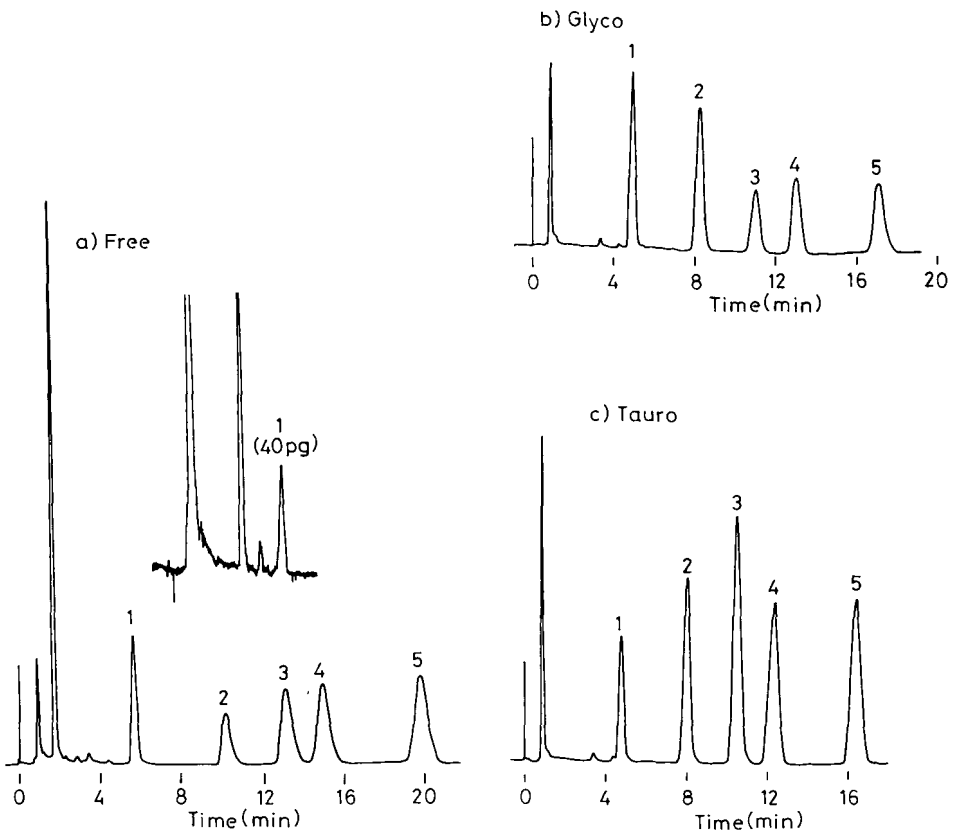

Fig. 4. Separation of 3-(1-anthroyl) derivatives of free and conjugated bile acids. Peaks: 1, cholate; 2, ursodeoxycholate; 3, chenodeoxycholate; 4, deoxycholate; 5, deoxycholate 12-propionate (I.S.).

TABLE I

CAPACITY RATIOS OF 3-(1-ANTHROYL) DERIVATIVES OF FREE AND CONJUGATED BILE ACIDS

Conditions: column, Cosmosil $5C_{18}$; mobile phases, (A) 0.3% potassium phosphate buffer (pH 6.0)—methanol (1:5), (B) 0.1% potassium phosphate buffer (pH 6.0)—methanol (1:8), t_0 = 0.7 min, flow-rate 1.8 ml/min.

Compound	Free		Glycine conjugate (G)		Taurine conjugate (T)	
	A	B	A	B	A	B
Cholate (CA)	7.7	—	7.1	—	6.7	—
Ursodeoxycholate (UDCA)	15.7	—	14.0	—	12.7	—
Chenodeoxycholate (CDCA)	20.5	—	17.5	—	16.8	—
Deoxycholate (DCA)	23.6	5.2	20.7	—	19.9	—
Lithocholate (LCA)	—	15.0	—	14.9	—	14.7
Deoxycholate 12-propionate (I.S.)	30.9	7.7	29.0	—	24.4	—

Clean-up of serum bile acids

The determination of bile acids in blood is markedly influenced by the clean-up procedure employed. For this purpose Amberlite XAD-2 resin has been widely used. This method, however, is not always satisfactory with respect to the recovery rate and reproducibility. In the present study a BondElut cartridge was employed for the clean-up of bile acids in serum. A synthetic mixture of 400 pmol of each of the bile acids was dissolved in 0.5 *M* phosphate buffer (pH 7.0) and applied to the cartridge impregnated with 0.5 *M* phosphate buffer (pH 7.0). After elimination of co-existing substances by washing with water and 1.5% ethanol, bile acids were eluted with 90% ethanol and then determined by HPLC. Bile acids were recovered at a rate of more than 90% in an initial 1.5 ml of the effluent.

It is evident from the data in Table I that each bile acid exhibited an almost identical k' value irrespective of the structure of the side-chain. Therefore, the group separation of bile acids into the three conjugated forms became a prerequisite. A synthetic mixture of anthroyl bile acids was dissolved in 90% ethanol and applied to a column of PHP-LH-20 (acetate form), which is a lipophilic ion-exchange gel. After removal of neutral compounds by elution with 90% ethanol, the group separation was carried out by stepwise elution with 0.1 *M* acetic acid in 90% ethanol, 0.2 *M* formic acid in 90% ethanol and 0.3 *M* acetic acid–potassium acetate (pH 6.3) in 90% ethanol. As shown in Fig. 5, free, glycine- and taurine-conjugated bile acids were completely separated into the three groups.

Determination of bile acids in human serum

A standard procedure for the separation and determination of bile acids in human serum is shown in Fig. 6. As for some serum specimens, especially the pooled human serum, an interfering peak appeared on a chromatogram of the

taurine-conjugate fraction. This problem was readily overcome by ion-exchange chromatography on PHP-LH-20 prior to dervatization with 1-anthroyl nitrile.

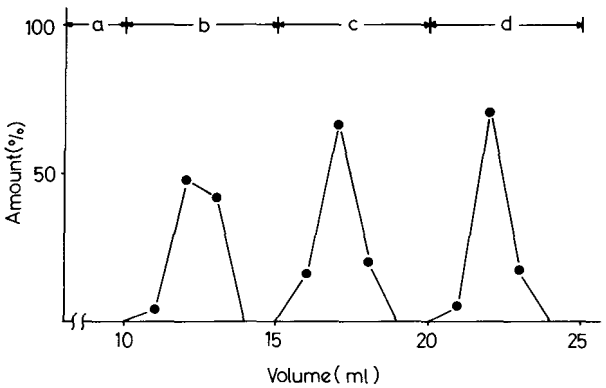

Fig. 5. Group separation of 3-(1-anthroyl) derivatives of bile acids on PHP-LH-20. Eluent: a, 90% ethanol; b, 0.1 *M* acetic acid in 90% ethanol; c, 0.2 *M* formic acid in 90% ethanol; d, 0.3 *M* acetic acid–potassium acetate (pH 6.3) in 90% ethanol.

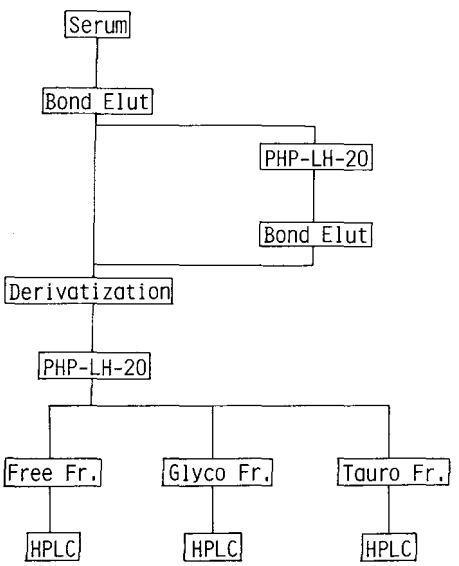

Fig. 6. General scheme for separation and determination of bile acids in human serum.

Applying the standard procedure to human serum, bile acids were determined with satisfactory reproducibility. Known amounts of bile acids were added to human serum and their recovery rates were estimated. As listed in Table II, all bile acids were recovered at a rate of more than 90%. A typical

TABLE II

RECOVERY OF FREE AND CONJUGATED BILE ACIDS ADDED TO NORMAL HUMAN SERUM

Bile acid*	Serum	Added (nmol per 0.1 ml)	Expected (nmol per 0.1 ml)	Found (nmol per 0.1 ml)	Recovery ± S.D.** (%)
CA	<0.005	0.20	0.20	0.196	98.2 ± 4.8
UDCA	0.02	0.20	0.22	0.219	99.6 ± 5.9
CDCA	0.07	0.20	0.27	0.272	100.9 ± 3.6
DCA	0.05	0.20	0.25	0.249	99.4 ± 3.8
LCA	<0.005	0.20	0.20	0.198	99.2 ± 7.5
GCA	<0.005	0.20	0.20	0.196	97.9 ± 5.5
GUDCA	0.01	0.20	0.21	0.208	98.9 ± 4.2
GCDCA	0.10	0.20	0.30	0.300	99.9 ± 4.0
GDCA	0.05	0.20	0.25	0.234	93.7 ± 5.2
GLCA	<0.005	0.20	0.20	0.190	95.1 ± 5.8
TCA	<0.005	0.20	0.20	0.185	92.7 ± 4.6
TUDCA	<0.005	0.20	0.20	0.198	98.9 ± 4.7
TCDCA	<0.005	0.20	0.20	0.192	96.1 ± 5.8
TDCA	<0.005	0.20	0.20	0.180	90.1 ± 4.6
TLCA	<0.005	0.20	0.20	0.181	90.3 ± 4.5

*See Table I for abbreviations.
**$n = 10$.

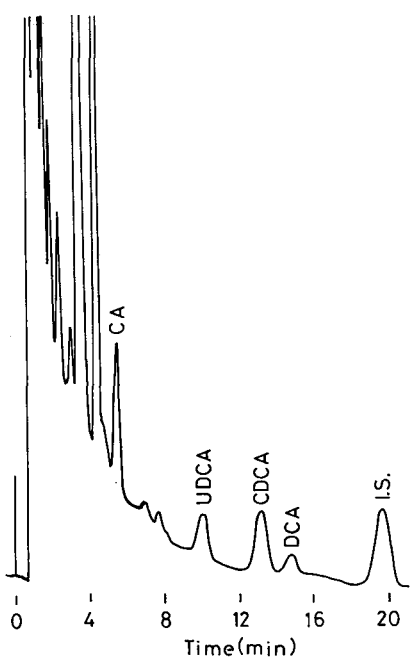

Fig. 7. A chromatogram of free bile acids in serum of a healthy subject. For abbreviations, see Table I.

chromatogram of free bile acids in human serum is illustrated in Fig. 7. The cholic acid peak on the chromatogram represents approximately 500 fmol as an injected amount. The chromatogram with a stable baseline and without leading and tailing is favorable for the determination of bile acids with a quantification limit of 5 pmol per 0.1 ml of serum. Simultaneous determination of bile acids was carried out with serum specimens taken from eight male healthy volunteers. The results obtained are listed in Table III.

It is hoped that the availability of a new method for the simultaneous determination of serum bile acids with satisfactory reliability and sensitivity may provide much more precise knowledge on the metabolic profile of bile acids and may serve as a diagnosis for hepatobiliary diseases.

TABLE III

AMOUNTS OF BILE ACIDS IN SERUM OF HEALTHY SUBJECTS (A—H)

Results are given in nmol/ml.

Bile acid*	A	B	C	D	E	F	G	H
CA	n.d.	n.d.	0.89	0.39	0.36	n.d.**	n.d.	n.d.
UDCA	0.81	0.34	n.d.	0.36	0.30	0.30	0.31	0.16
CDCA	2.09	0.25	1.20	0.28	0.58	0.22	1.50	0.38
DCA	n.d.	0.96	1.12	0.48	0.42	0.34	0.26	0.44
LCA	0.13	n.d.	n.d.	n.d.	0.05	n.d.	n.d.	0.08
GCA	0.84	0.56	0.57	0.86	0.74	0.89	0.24	0.12
GUDCA	0.12	n.d.	0.21	n.d.	0.05	0.05	0.05	n.d.
GCDCA	2.19	1.01	1.90	1.21	3.38	2.87	1.03	1.27
GDCA	n.d.	0.43	1.32	0.15	0.78	0.74	n.d.	0.48
GLCA	0.19	n.d.	0.05	0.05	n.d.	n.d.	n.d.	n.d.
TCA	0.09	n.d.	n.d.	0.06	0.10	0.07	n.d.	n.d.
TUDCA	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
TCDCA	0.20	n.d.	0.06	0.13	0.27	0.26	0.18	0.17
TDCA	0.13	0.11	0.09	n.d.	n.d.	n.d.	0.12	n.d.
TLCA	n.d.	n.d.	0.05	n.d.	n.d.	0.05	n.d.	n.d.
Total	6.79	3.66	7.41	3.92	6.98	5.74	3.64	3.10

*See Table I for abbreviations.
**n.d. = not detectable (<0.05 nmol/ml).

ACKNOWLEDGEMENTS

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CHROMBIO. 1759

RESOLUTION OF VALPROIC ACID FROM DEUTERATED ANALOGUES AND THEIR QUANTITATION IN PLASMA USING CAPILLARY GAS CHROMATOGRAPHY

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SUMMARY

Quantitation of valproic acid and a deuterated analogue in the same plasma sample by capillary gas chromatography without mass spectrometry was illustrated. Specificity was accomplished solely with a 60 m × 0.25 mm fused silica WCOT column coated with OV-351. A hexadeutero and two tetradeutero analogues of valproic acid had resolutions of at least 1.2 from valproic acid. Plasma samples were extracted with carbon tetrachloride following the addition of 2-ethylhexanoic acid as the internal standard. The method is sensitive to at least 0.5 μg/ml and provides the capability of conducting absolute bioavailability and pulsed dosing studies with deuterated drug analogues without a mass spectrometer. The technique was applied to the analysis of plasma samples from dogs simultaneously administered valproic acid and a deuterated analogue.

INTRODUCTION

The merits of stable isotopes in biomedical research has recently been reviewed [1—3]. Major applications in the pharmaceutical area include absolute bioavailability, bioequivalence and pharmacokinetics in chronic drug administration (pulse dosing). The elimination kinetics of a tetradeutero valproic acid isomer was recently studied in chronic epileptic patients on a maintenance dose of valproic acid [4]. Deuterium is the most extensively used heavy stable isotope primarily due to its ready availability, low cost, and high isotopic purity (> 99%). Because of the primary and secondary isotope

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effects associated with many deuterium analogues [3], the use of more costly ^{13}C or ^{15}N analogues has been recommended [1]. However, deuterium may be used provided it is located in a metabolically inert region of the molecule to ensure the absence of significant isotope effects.

The only method currently available to quantitate both the light and heavy isotopes in the same sample is gas chromatography—mass spectrometry (GC—MS). Both conventional and capillary GC systems are used. It has been noted in our laboratory and by Durden and Boulton [5] using a capillary GC—MS system that deuterated and unlabeled analogues are not isographic; deuterated analogues elute a few seconds prior to the unlabeled compounds. In this paper we demonstrate the capability of capillary GC to resolve and quantitate unlabeled from deuterated compounds without MS. The technique is applied to the simultaneous analysis of the anticonvulsant valproic acid and deuterated analogues in dog serum.

EXPERIMENTAL

Chemicals and reagents

Valproic acid (VPA) was obtained from Abbott Laboratories. 4,4′,5,5′-d_4-Valproic acid (I) was synthesized by reducing diallylacetic acid with deuterated hydrazine hydrate (MSD Isotopes, Dorval, Canada) [6]. The 3,3,3′,3′-tetradeutero- (II) and 5,5,5,5′,5′,5′-hexadeuterovalproic acid (III) were purchased from MSD Isotopes. All deuterated valproic acids were free of valproic acid (< 0.15%) as determined by capillary GC. Assigned structures were in agreement with their respective NMR, IR and mass spectra.

Stock aqueous standard solutions of about 1 mg/ml were prepared by dissolving about 50 mg of the acid in 1 ml of 1 *N* sodium hydroxide and adjusting to 50 ml with distilled water. Aqueous standards were stored below 10°C. Plasma standards containing both a deuterated valproic acid and valproic acid in ratios of 0.5 to 2.0 were prepared by appropriate dilution of the stock solutions with fresh bovine plasma to the concentration range of 0.1—50 μg/ml. Internal standard, 2-ethylhexanoic acid (Aldrich, Milwaukee, WI, U.S.A.), was prepared at 150 μg/ml in water.

Carbon tetrachloride was HPLC grade. All other reagents were analytical reagent grade.

Gas chromatography

A fused silica capillary column (60 m × 0.25 mm I.D.) coated with OV-351, film thickness 0.25 μm (J & S Scientific, Crystal Lake, IL, U.S.A.), was used with a Hewlett-Packard Model 5840A gas chromatograph equipped with an autosampler. The injection port containing a 8 cm × 2 mm I.D. fused silica splitless insert was kept at 220°C, the column was operated isothermally at 155°C and the flame ionization detector temperature was 240°C. Hydrogen carrier gas was used at a flow-rate of 41 cm/sec with a split ratio of about 10:1. All three deuterated valproic acids were resolved (resolution > 1.2) from VPA and internal standard, 2-ethylhexanoic acid (Fig. 1).

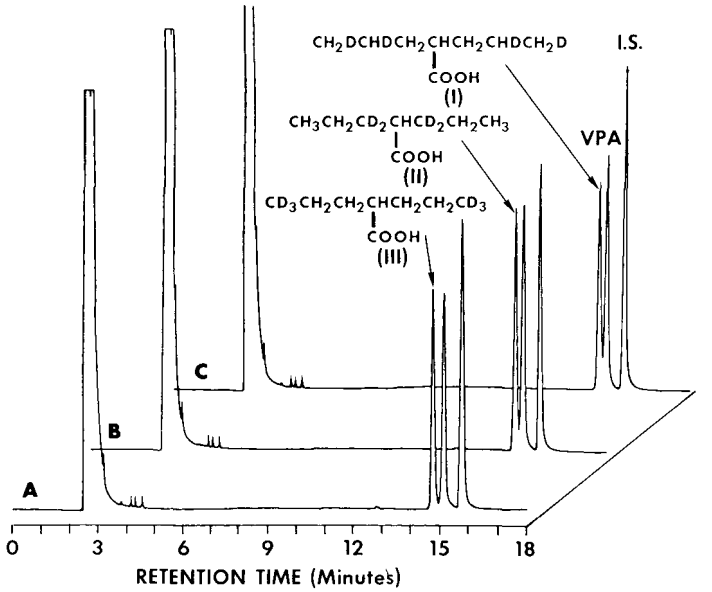

Fig. 1. Chromatograms of plasma extracts of spiked standards. Plasma standards containing: (A) VPA (25.9 μg/ml), III (27.1 μg/ml) and internal standard; (B) VPA (27.1 μg/ml), II (28.0 μg/ml), and internal standard; (C) VPA (23.5 μg/ml), I (24.6 μg/ml), and internal standard.

Assay procedure

A 1-ml plasma sample or standard and 0.2 ml of internal standard solution were added to a 12-ml conical screw cap test tube. A 0.2-ml volume of 22.5% perchloric acid was added and mixed by vortexing. Carbon tetrachloride (0.4 ml) was added followed by vortexing for 10 sec and centrifugation at 1800 *g* for 5 min. The upper aqueous phase was discarded by aspiration leaving a white protein plug above the carbon tetrachloride layer. A Pasteur pipet was used to penetrate the protein layer and cleanly withdraw and transfer the clear carbon tetrachloride to a 0.3-ml microvial. After capping the microvial a 2-μl aliquot was automatically injected into the gas chromatograph. All samples and standards were randomly mixed prior to injection.

Plasma standards of known concentrations of labeled and unlabeled valproic acids were used to construct calibration curves. Three calibration curves were initially constructed for each acid; one curve each for labeled/unlabeled ratios of 2:1, 1:1 and 1:2. Because the calibration curve parameters (slope and intercept) of all three calibration curves were practically identical, in each case the data from the three ratios were combined into a single calibration curve which was used to estimate plasma unknowns. Calibration curve parameters from a plot of peak area ratio (acid/internal standard) versus standard concentrations were obtained by least-squares linear regression analysis. Other than plasma blanks, plasma standard concentrations ranged from about 0.1 μg/ml to 75 μg/ml.

Drug administration to dogs

Fasted beagle dogs weighing 8—10 kg were orally administered 10 ml of a buffered bicarbonate—carbonate (0.025 *M*, pH 9—9.5) solution containing approximately 100 mg each of valproic acid and a deuterated analogue. Actual amounts were adjusted so that equal molar equivalents were administered. Heparinized 5-ml blood samples were obtained prior to dosing and at specified times post-administration. The plasma was isolated into labeled test tubes and held frozen until assayed.

RESULTS AND DISCUSSION

In order to achieve a resolution of at least 1.25 for two components with an α-value of 1.02 a chromatographic column requires a total of at least 65,000 effective theoretical plates [7]. This degree of efficiency, which is necessary to separate an unlabeled component from the same component labeled with deuterium, can be achieved using narrow-bore WCOT capillary GC columns. In this study, two tetradeutero and one hexadeutero analogue of VPA were resolved from unlabeled VPA on a 60 m × 0.25 mm I.D. WCOT fused silica column coated with OV-351 (film thickness 0.25 μm).

The resolutions of I, II and III from VPA at various oven temperatures are shown in Table I. The choice of 155°C was a compromise between resolution and analysis time. The degree of resolution is primarily dependent upon the deuterium content of the molecule. However, the position of the deuterium atoms has a small effect on the chromatographic properties. This is illustrated with the two tetradeutero VPA analogues (I and II) which are positional isomers and have slightly different retention times (Table I).

A small amount of tailing occurs when the underivatized acids are chromatographed (Fig. 1). Tailing factors were kept below 1.3 by occasional cleaning of the fused silica splitless liner. Various split liner insert combinations were examined which contained silylated glass wool or GC packing material coated with 5% FFAP. All split inserts had a detrimental effect on chromatographic efficiency due to increased peak tailing. Using a clean splitless insert, significant tailing never became a problem. If tailing had become

TABLE I

RESOLUTION OF DEUTERATED VPA ANALOGUES FROM VPA AS A FUNCTION OF TEMPERATURE

Hydrogen flow-rate 41.5 cm/sec; resolutions (R_s) are the mean of three determinations; t_R = retention time.

Oven temperature (°C)	I		II		III		VPA
	t_R (min)	R_s	t_R (min)	R_s	t_R (min)	R_s	t_R (min)
145	21.80	1.41	21.75	1.64	21.61	2.25	22.18
150	17.96	1.35	17.92	1.55	17.84	2.17	18.33
155	14.82	1.21	14.79	1.39	14.70	1.96	15.19
160	13.39	1.09	13.36	1.16	13.20	1.74	13.52
Deuterium content (%)	5.40		5.40		7.99		—

TABLE II

CALIBRATION CURVE SLOPES FOR VARIOUS RATIOS OF LABELED/UNLABELED VPA ANALOGUES

Analogue	Ratio (Labelled/unlabelled)	Calibration curve slopes*	
		Deuterated VPA	VPA
I	2:1	0.0312	0.0376
	1:1	0.0310	0.0365
	1:2	0.0305	0.0349
Combined (n=21)		0.0310	0.0358
II	2:1	0.0340	0.0353
	1:1	0.0316	0.0352
	1:2	0.0328	0.0380
Combined (n=21)		0.0331	0.0357
III	2:1	0.0322	0.0361
	1:1	0.0315	0.0345
	1:2	0.0330	0.0360
Combined (n=21)		0.0321	0.0356
None			0.0353

*Individual curves have n=7 points; correlation coefficients were > 0.99 for all curves.

TABLE III

QUANTITATION OF VPA AND *d*-VPA ANALOGUES AT HIGH UNLABELED/LABELED RATIOS

Samples estimated from a calibration curve which had standards at a 1:1 ratio.

Deuterated analogue	Concentration (μg/ml)	Ratio* (VPA/*d*-VPA)	Calculated concentration (μg/ml)	
			d-VPA	VPA***
I	0.97	54:1	0.91 ± 0.10 (11.0)**	53.8 ± 1.8 (3.5)
	30.5	1.7:1	31.4 ± 1.6 (5.1)	51.9 ± 1.9 (3.7)
II	1.02	52:1	0.95 ± 0.09 (9.8)	52.4 ± 2.1 (4.0)
	31.4	1.7:1	31.8 ± 1.4 (4.5)	53.2 ± 2.0 (3.8)
III	0.95	55:1	0.92 ± 0.09 (10.1)	53.1 ± 1.3 (2.4)
	30.4	1.7:1	29.8 ± 1.8 (6.1)	52.5 ± 1.8 (3.4)

*Each ratio determined in quadruplicate.
**Coefficient of variation in parentheses.
***Actual VPA concentration was 52.7 μg/ml.

a problem, esterification would have been required to produce symmetrical peaks. A split ratio of 10:1 provided high sensitivity without significant loss of efficiency.

The extraction process, which was a modification of the method of Dijkhuis and Vervloet [8], was rapid and provided chromatograms free of interfering

peaks (Fig. 1). Calibration curves for the three deuterated acids and VPA were linear to at least 50 μg/ml. The slope parameters for plasma standard combinations of labeled VPA/unlabeled VPA in ratios of 2:1, 1:1 and 1:2 did not significantly differ as shown in Table II. For this reason a single calibration curve from a combination of the three ratio curves was constructed to determine unknown samples. The higher slopes of the VPA calibration curves are primarily due to a slightly lower response of the detector for the deuterated analogues. Because I and II have slightly tailing peaks and are not completely resolved from VPA, a small peak area contribution to the VPA area may also occur. However, analysis of variance indicates no significant difference between the VPA slopes of each analogue suggesting little or no area contribu-

TABLE IV

PRECISION FOR THE DETERMINATION OF VPA AND DEUTERATED ANALOGUES SPIKED IN SERUM

Compound	Serum concentration (μg/ml)			
	Actual	Calculated ± S.D.	*n*	C.V. (%)
4,4′,5,5′-d_4-VPA (I)	0	0		—
	0.36	0.35 ± 0.069	3	19.8
	1.79	1.80 ± 0.133	6	7.4
	3.58	3.64 ± 0.350	3	9.6
	8.94	9.25 ± 0.471	3	5.1
	17.89	17.37 ± 1.224	6	7.0
	26.84	26.65 ± 0.692	3	2.6
	35.78	36.36 ± 2.572	5	7.1
3,3,3′,3′-d_4-VPA (II)	0	0		—
	0.26	0.25 ± 0.045	4	18.1
	1.75	1.76 ± 0.101	7	5.7
	2.62	2.63 ± 0.102	4	3.9
	8.74	8.89 ± 0.429	3	4.8
	17.49	17.75 ± 0.684	7	3.8
	26.23	26.13 ± 1.309	4	5.0
	34.97	35.56 ± 1.740	5	4.9
5,5,5,5′,5′,5′-d_6-VPA (III)	0	0		—
	0.27	0.25 ± 0.052	3	20.9
	1.81	1.75 ± 0.176	5	10.0
	3.62	3.35 ± 0.183	3	5.1
	9.05	8.90 ± 0.400	3	4.5
	18.10	18.07 ± 0.561	6	3.1
	27.15	25.72 ± 0.960	3	3.7
	36.20	35.88 ± 0.492	5	1.4
VPA	0	0		—
	0.34	0.34 ± 0.039	3	11.5
	1.71	1.74 ± 0.099	8	5.7
	3.42	3.36 ± 0.145	4	4.3
	8.54	8.93 ± 0.631	3	7.1
	17.09	17.61 ± 0.850	8	4.8
	25.63	25.48 ± 0.375	5	1.5
	34.17	34.11 ± 0.705	5	2.1

TABLE V

COMPARISON OF DOG PLASMA SAMPLES ASSAYED BY CAPILLARY AND CONVENTIONAL GC

Dog was orally administered 200 mg of valproic acid.

Sample time (min)	VPA concentration* (μg/ml)	
	Capillary GC	Conventional GC
0	0	0
15	69.8	74.6
30	39.1	49.2
45	32.6	34.3
60	25.4	22.5
80	17.1	17.5
100	13.6	13.2
120	11.3	10.9
150	8.8	7.9
180	7.2	7.1
210	6.7	6.4
240	7.8	7.2
300	6.8	6.6
360	4.0	3.6

*Each value is the average of two determinations.

tion by preceding deuterated peaks. All intercepts were not significantly different from zero.

Unlabeled/labeled ratios of 50 and greater are encountered in pulse dose studies in which patients on a maintenance dose of unlabeled drug are administered a single dose of labeled drug [4]. The accuracy and precision of this method is good when applied to plasma samples containing high unlabeled/labeled ratios as shown in Table III. The calibration curves used to estimate the high ratio samples were constructed from standards having unlabeled/labeled ratios of 1:1.

Assay precision is given in Table IV. The deuterated analogues were assayed in the presence of various ratios of VPA. Coefficients of variation were consistently below 10% for concentrations above 1.7 μg/ml. The overall average coefficient of variation (C.V.) for all compounds in the 1.7—37 μg/ml range is 5%. Using the described assay conditions the minimum detectable concentration for all compounds is 0.08 μg/ml. The minimal quantitative concentration is about 0.5 μg/ml. The coefficient of variation at this concentration averages about 15% for the three analogues.

The capillary GC method was compared to a conventional GC method [9] by using both methods to assay plasma samples from a dog orally administered 200 mg of valproic acid. Two assays were conducted with each method on separate days and the average results (Table V) are in good agreement.

Dog samples

The samples from each dog were randomized and assayed under blinded conditions. The plasma concentrations of unlabeled and labeled VPA were similar for all three deuterated analogues suggesting no pronounced isotope

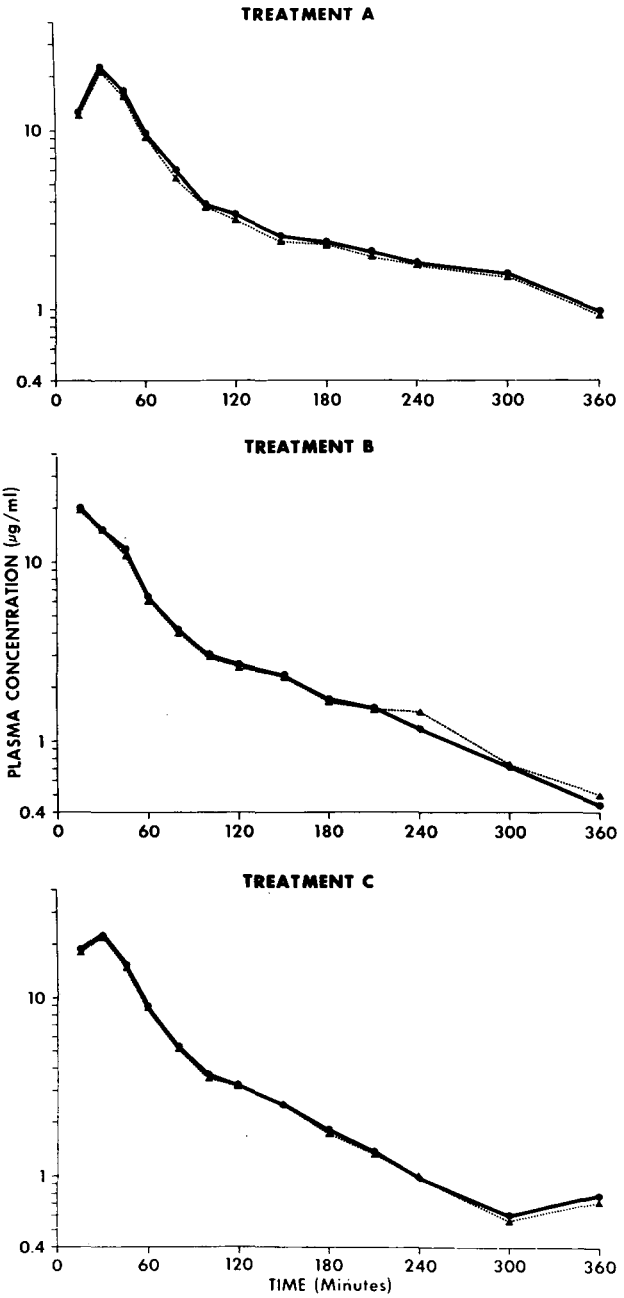

Fig. 2. Plasma concentration vs. time profiles of three dogs simultaneously administered VPA and a deuterated VPA analogue. Treatment A, 104.0 mg of II (●——●) plus 99.7 mg VPA (▲ · · · ▲) to Dog No. 2; Treatment B, 102.8 mg of I (●——●) plus 99.6 mg VPA (▲ · · · ▲) to Dog No. 3; Treatment C, 104.3 mg of III (●——●) plus 102.0 mg VPA (▲ · · · ▲) to Dog No. 6.

effect. The plasma concentration–time curves (Fig. 2) of the deuterated analogues are almost identical to those of VPA even to the last sample time (6 h). This was somewhat surprising since VPA is extensively metabolized [10].

The major advantage of this technique is the elimination of a costly mass spectrometer for certain stable isotope applications. Also, the use of conventional GC detectors (e.g., flame ionization detector, alkali flame ionization detector and electron-capture detector) are less susceptible than a mass spectrometer to sample contamination and maintenance problems. The disadvantages of this technique are that it is probably limited to deuterated analogues and that the chromatography is 2—3 times longer than GC—MS. However, deuterated analogues are satisfactory for bioavailability or pulse dosing studies provided they are labeled, if possible, in metabolically inert positions. The increased analysis time is not a major problem with modern instrumentation, which have automatic samplers and stable detection systems. Because of the good stability of this system, up to 60 VPA samples were assayed daily on a routine basis.

Resolution (R_s = 1.4) has currently been achieved in our laboratory with an experimental drug having a deuterium content of only 3%. Achieving resolution at even lower deuterium contents will further increase the flexibility of the technique.

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CHROMBIO. 1739

SENSITIVE GAS CHROMATOGRAPHIC QUANTITATION OF ZOMEPIRAC IN PLASMA USING AN ELECTRON-CAPTURE DETECTOR

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(First received May 28th, 1982; revised manuscript received March 30th, 1983)

SUMMARY

A highly sensitive, specific and precise gas chromatographic method for the determination of the non-narcotic analgesic agent, zomepirac, in plasma is described. The pentafluorobenzyl ester derivative of zomepirac has been prepared. This enables the detection of zomepirac down to picogram levels using electron-capture detection. The lowest concentration of zomepirac which can be measured accurately and precisely (coefficient of variation <15%) is 5 ng/ml in a 2-ml plasma sample or 25 ng/ml in a 0.1-ml plasma sample. Two previously reported high-performance liquid chromatographic (HPLC) assays have detection limits of 50 ng/ml for 1-ml samples and 10 ng/ml for 2-ml samples, respectively. The present method is very useful when small sample size or interference is causing problems with the HPLC assay. This assay has been employed successfully in analyzing plasma samples from humans and monkeys as well as samples from rat milk.

INTRODUCTION

Zomepirac sodium [sodium 5-(4-chlorobenzoyl)-1,4-dimethyl-1H-pyrrole-2-acetate dihydrate] is a new non-narcotic analgesic drug [1—3]. The pharmacokinetics and the disposition of zomepirac in man have been reported [4—6].

Two high-performance liquid chromatographic (HPLC) assays for the determination of zomepirac in plasma have been published [7, 8]. These HPLC methods offer sufficient sensitivity (detection limits [7, 8]: 10 ng/ml for 2-ml samples and 50 ng/ml for 1-ml samples) for analyzing clinical plasma samples. However, when analyzing plasma samples from different animal species or from patients with concomitant medication, small sample size and interference may cause problems with the HPLC methods. The availability of another highly specific and sensitive assay would be very helpful in these situations.

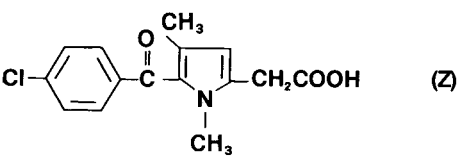
This paper describes a gas chromatographic (GC) asay for the determination

of zomepirac in plasma. The assay can readily be adapted for micro-samples. This paper also describes the pentafluorobenzylation of zomepirac by extractive alkylation. Extractive alkylation affords a method of isolating polar compounds with simultaneous derivatization. The derivatization step is carried out by adding the organic phase containing the derivatizing agent (pentafluorobenzyl bromide) to the aqueous phase containing the compound. The compound is derivatized and extracted into the organic phase in a single step.

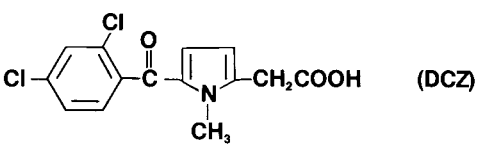
EXPERIMENTAL

Reagents

Hydrochloric acid, potassium carbonate, toluene and isoamyl alcohol were analytical grade (Mallinckrodt, St. Louis, MO, U.S.A.). Ethyl acetate was nanograde (Mallinckrodt). Heptane was glass-distilled grade (Pollard and Company, Wilmington, DE, U.S.A.). Diethyl ether was reagent grade (anhydrous diethyl ether, Mallinckrodt). Pentafluorobenzyl bromide (PFBB) was from Pierce (Rockford, IL, U.S.A.).



(Z)



(DCZ)

A dichloro analogue of zomepirac (DCZ) was used as the internal standard. Zomepirac (Z) was obtained as the sodium salt dihydrate. DCZ was obtained as the free acid (McNeil Pharmaceutical, Spring House, PA, U.S.A.).

Plasma standard solutions

Plasma standards (volume: 10.0 ml) with zomepirac free acid concentrations ranging from 5 to 500 ng/ml were prepared as follows: 0.5 ml of an aqueous solution of zomepirac sodium, containing the appropriate amount of the zomepirac free acid, was added to 9.5 ml of drug-free plasma to give a total volume of 10.0 ml.

The internal standard solution was prepared by first dissolving 3.0 mg of DCZ in 1.0 ml of methanol. This was followed by two successive dilutions (1:100 and 1:1000) with diethyl ether to arrive at a final concentration of 30 ng/ml.

Glass equipment

Disposable screw-top bottles (volume: 28.3 ml) with polyethylene-lined caps and 15-ml centrifuge tubes with PTFE-lined screw caps were used for extraction and derivatization, respectively. Prior to use, all glassware was soaked in

chromic acid for 1 h, rinsed thoroughly with distilled water and treated for 3 h at 270°C. The PTFE-lined screw caps were soaked in *n*-heptane for 1 h and dried at 60°C.

Extraction and derivatization procedure

To each sample of plasma (0.1—2.0 ml), containing zomepirac as standard or unknown in a 28.3-ml disposable screw-top bottle, were added 1.0 ml of 1 *N* hydrochloric acid and 10.0 ml of diethyl ether containing 300 ng of internal standard. The capped bottle was then shaken for 15 min on a table-top shaker (Eberbach) at 120 oscillations per minute and centrifuged at 681 *g* for 10 min. An 8.0-ml aliquot of the supernatant diethyl ether layer was transferred to another 28.3-ml bottle containing 9.0 ml of 0.1 *N* sodium hydroxide. The mixture was shaken for 15 min and centrifufed for 10 min. The supernatant ether layer was aspirated and discarded. Two ml of 1 *N* hydrochloric acid and 10.0 ml of 1.5% isoamyl alcohol in heptane were added to the aqueous layer. The mixture was shaken for 15 min and centrifuged for 10 min. An 8.0-ml aliquot of the supernatant organic layer was transferred to a 15-ml centrifuge tube and evaporated to dryness under a stream of dry nitrogen at room temperature. To the dried plasma extract, 1.0 ml of 1 *M* K_2CO_3 and 1.0 ml of ethyl acetate solution containing 0.5% v/v PFBB were added. The centrifuge tube was capped and heated in an oven at 40°C overnight. After cooling to room temperature, 0.5 ml of supernatant organic phase was pipetted into another centrifuge tube and evaporated to dryness under nitrogen. The residue was reconstituted with 500 μl (50 μl for 0.1-ml plasma samples) of toluene and mixed on a Vortex mixer for 5 sec. A 3-μl aliquot of the resulting solution was then injected directly into the gas chromatograph.

Mass spectrometry

For the positive identification of the pentafluorobenzyl (PFB) ester derivatives of zomepirac (PFB-Z) and the internal standard (PFB-DCZ), a Finnigan 3300 quadrupole mass spectrometer was employed in conjunction with the manufacturer's Model 9500 gas chromatograph and a Finnigan Model 6100 data system. The GC—mass spectrometric (MS) system was operated in the chemical ionization (CI) mode using methane as the reagent gas. A 61.0 × 0.2 cm I.D. silanized glass column packed with 3% OV-17 on Gas-Chrom Q (60—80 mesh) was used with a methane flow-rate of 20 ml/min. During analysis the interface and the transfer line were maintained at 250°C. The column temperature was 235°C and the injection port temperature was at 280°C. The CI source was operated without external heating. The source pressure was maintained at 1 torr. The electron energy was 100 eV and electron beam emission was adjusted to 0.5 mA.

Gas chromatography

A Perkin-Elmer Model 900 gas chromatograph equipped with a ^{63}Ni electron-capture detector (ECD) was used. The column was a 122.0 × 0.4 cm I.D. silanized glass column packed with 3% OV-17 on Gas-Chrom Q (60—80 mesh). Prior to use, the column was conditioned at 280°C overnight with an argon—methane (95:5, v/v) carrier at a flow-rate of 30 ml/min.

The chromatographic conditions for the analysis were: column oven 230°C, injection port 260°C, detector 295°C. The carrier gas (argon—methane, 95:5, v/v) flow-rate was 70 ml/min. The retention times for the PFB ester derivatives of zomepirac (PFB-Z) and of the internal standard (PFB-DCZ) were 6.4 min and 9.0 min, respectively (Fig. 1).

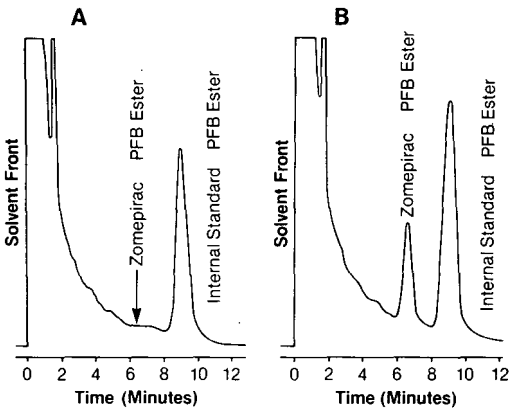


Fig. 1. Gas chromatograms from (A) predose monkey plasma sample (samples without internal standard showed no peak at around 9.0 min) and (B) monkey plasma sample 12 h after oral administration of 10 mg/kg of zomepirac sodium showing 53 ng/ml of zomepirac and 100 ng/ml of internal standard.

Quantitation

Standard curves for zomepirac in plasma were prepared by analyzing standard plasma solutions according to the procedure described above. Ratios of the peak areas (zomepirac/internal standard) were plotted against concentrations of zomepirac. Linear regression analysis was performed on the data.

RESULTS AND DISCUSSION

Reaction conditions

Pentafluorobenzylation of organic acids and phenols has previously been investigated [9, 10]. Derivatization occurs when the acid or phenol is heated with PFBB in strongly basic acetone or alcohol for some hours. More recently, an extractive alkylation technique has successfully been employed [11, 12] for derivatization. In extractive alkylation, organic acids in a basic aqueous medium are extracted by means of a positively charged counter ion (e.g. tetrabutylammonium ion) into an organic phase containing PFBB. In a poorly solvating organic phase, the anion of the organic acid becomes a highly reactive nucleophile in the displacement of bromide ion from the derivatizing reagent. However, if the partition ratio between the organic and aqueous phases is very low, as in the case of short chain fatty acids, the extraction of the organic acids into the organic phase will be inefficient and the reaction will be very slow and incomplete [13]. Since the partition coefficient of an organic acid is a function of the organic phase (e.g., lipophilicity or polarity properties) and of the pH of

the aqueous phase, reaction conditions for an individual organic acid can be optimized by manipulation of these two parameters.

With zomepirac (Z) and the internal standard (DCZ), pentafluorobenzylation in the presence of tetrabutylammonium ion was rapid and complete when using ethyl acetate as the organic phase and 1 *M* K_2CO_3 as the aqueous phase. At pH values greater than 12, however, an additional derivative of zomepirac was obtained. The exact structure of the second derivative has not been identified. Preliminary results from MS (CI) indicated that this derivative contained two PFB groups. It was also found that in the absence of the counterion, the derivatization was completed for both Z and DCZ within 4 h at 40°C. By leaving out the counter ion, the derivatization reaction became selective. This resulted in much cleaner chromatograms. Because of the relatively long (4 h) reaction time, the reaction mixture was kept overnight in the oven.

Reaction mixtures were subjected to GC—MS analysis. Only one derivative was found for both zomepirac (Z) and the internal standard (DCZ). The chemical ionization spectrum for the PFB derivative of zomepirac (PFB-Z) is shown in Fig. 2. Major *m/e* peaks observed were: 472 $(M+1)^+$, 500 $(M+29)^+$, 512 $(M+41)^+$, 139 $(ClC_6H_4CO)^+$, 436 $(M+1—HCl)^+$, 246 $(M+1—PFB\ formate)^+$ and 360 $(M+1—chlorobenzene)^+$. These ions are consistent with the addition of one PFB group to the carboxy group in zomepirac (MW = 471).

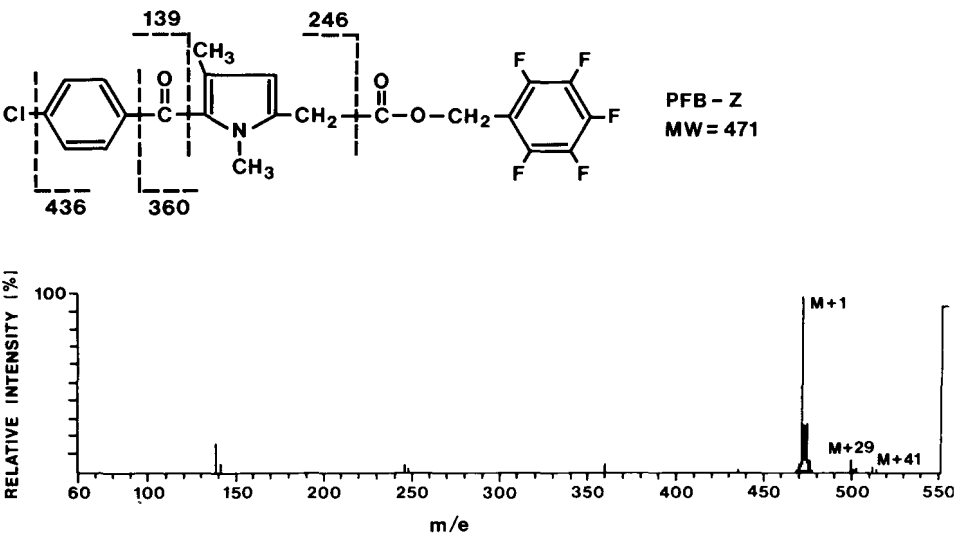


Fig. 2. Chemical ionization mass spectrum and proposed fragmentation pattern of the pentafluorobenzyl derivative of zomepirac (PFB-Z).

Sensitivity

The PFB ester derivative of zomepirac (PFB-Z) is highly electron-capture sensitive. Ten pg of this derivative when injected into the gas chromatograph under the stated conditions gave a peak with a signal-to-noise ratio of eight.

The detection limit of zomepirac that has been determined accurately and precisely (C.V. < 15%) in a 2-ml plasma sample was 5 ng/ml, which is more

than adequate for all clinical samples. Furthermore, the detection limit in a 0.1-ml plasma sample was found to be 25 ng/ml, which is still adequate for most purposes. The reasons behind the low detection limit for smaller samples is quite obvious. Since only 0.3% of the derivatives in each sample was injected into the gas chromatograph in the present procedure, the percentage of sample utilized can be readily increased by using a smaller amount of toluene for reconstitution and/or by injecting a larger amount of the toluene solution. The background noise or interference in the chromatogram was found to be directly proportional to plasma sample size.

Interference

No interference peaks due to endogenous materials have ever been observed using the present procedure (Fig. 1). This procedure, to date, has been employed successfully to analyze over 500 zomepirac plasma and milk samples. The major circulating metabolite of Z (glucuronide conjugate) will not interfere in this procedure because it is eliminated in the extraction step.

Stability

Freshly prepared plasma standard solutions were compared with plasma standard solutions at the same drug concentrations which had been kept frozen at −10° C for two months. The variations in the peak area ratios at each drug level between 5 ng/ml and 500 ng/ml were insignificant ($p < 0.05$).

Toluene solutions of derivatized plasma extract containing PFB-Z and PFB-DCZ were examined by repeated injections of aliquots into the gas chromatograph. It was found that both PFB ester derivatives were stable in the presence of plasma extract for at least two days at room temperature. The stability of PFB compounds has previously been demonstrated [9—14].

Response curve

The linearity of the ECD response was demonstrated by injecting samples of different concentrations of the PFB ester derivatives of both Z and DCZ into the gas chromatograph. Peak area values were plotted against the absolute amounts of the derivatives injected to obtain response curves. The response curve for the zomepirac derivative was linear between 0.01 ng and 5.00 ng (as zomepirac) and the response for the internal standard derivative was linear between 0.05 ng and 5.00 ng. The detector response for both derivatives was found to be non-linear above the 5.00-ng level.

Recovery

GC response curves which correlate peak area with the amount of PFB-Z and PFB-DCZ per sample offer the possibility of determining total yields after extraction and derivatization for zomepirac (Z) and internal standard (DCZ) in this procedure. For 300 ng of Z and 300 ng of DCZ seeded in 2 ml of plasma, the total recoveries after extraction and derivatization were 76% (C.V. = 4.2%) for Z and 70% (C.V. = 4.8%) for DCZ (eight determinations).

Standard curve

Standard curves have been prepared by analyzing 0.1—2.0 ml of the plasma

standard solutions. Excellent correlation was observed between the peak area ratios and the zomepirac plasma concentrations. Linear regression analysis gave correlation coefficients of greater than 0.99 in all cases.

The reproducibility of the assay was very good as is shown in Table I. For six independent determinations at each concentration over the course of two weeks, the coefficients of variation were less than 5% in the concentration range of 50—500 ng/ml and were less than 15% in the concentration range of 5–50 ng/ml.

TABLE I

MEAN PEAK AREA RATIO OF PFB-Z TO PFB-DCZ, STANDARD DEVIATION AND COEFFICIENT OF VARIATION OF WORKING STANDARD CURVES PREPARED BY ANALYZING SEEDED PLASMA SAMPLES OVER THE COURSE OF TWO WEEKS (300 ng OF DCZ PER SAMPLE)

Zomepirac plasma concn. (ng/ml)	No. of determinations	Mean peak area ratio	Standard deviation	Coefficient of variation
0	6	0	0	0
5	6	0.026	0.004	0.15
50	6	0.252	0.009	0.04
200	6	1.121	0.029	0.03
350	6	2.023	0.063	0.03
500	6	2.952	0.057	0.02

For 2-ml plasma samples, the standard curve was non-linear above 500 ng/ml. Therefore, samples with concentrations above 500 ng/ml should be diluted with blank plasma before analysis.

Applications

The present method has been employed successfully in analyzing human and monkey plasma samples as well as rat milk samples. The long reaction time for derivatization does not affect sample throughput. An average of 28 plasma samples can be analyzed routinely during an 8-h working day. For each set of samples, extractions were performed in the afternoon with the derivatization step performed overnight, and injections were done the following morning with analysis of results completed before noon.

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ELECTRON-CAPTURE DETERMINATION OF METOCLOPRAMIDE IN BIOLOGICAL FLUIDS USING FUSED SILICA CAPILLARY COLUMNS

APPLICATION TO PLACENTAL TRANSPORT STUDIES IN SHEEP AND HUMANS

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SUMMARY

An electron-capture gas—liquid chromatographic assay for metoclopramide using cross-linked fused silica capillary columns which provides improved selectivity and sensitivity is reported. A 25 m × 0.31 mm fused silica capillary column was used for all analyses. Linearity was observed in the range of 4—40 ng of metoclopramide base per 0.25—0.5 ml of plasma. This represents from ca. 0.9—9.0 pg at the detector employing a split ratio of 30:1 and an injection volume of 2 μl. Applicability of the method is demonstrated by the analysis of human and sheep plasma (maternal, fetal and neonatal) from metoclopramide placental transfer studies.

INTRODUCTION

Metoclopramide (MCP), 4-amino-5-chloro-2-methoxy-N-(2-diethyl-amino-ethyl)benzamide (I), is a procainamide analogue used clinically in the treatment of nausea and vomiting and to promote gastric emptying [1—7].

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A number of analytical methods have been reported for the measurement of MCP in biological fluids including colorimetry [8], thin-layer chromatographic photodensitometry [9, 10] and high-performance liquid chromatography (HPLC) [11—14]. Most, however, lack specificity [8—10] and require the extraction of large plasma volumes (2—5 ml) [8—14] in order to achieve adequate sensitivity in the low nanogram range for thorough pharmacokinetic studies. Several electron-capture gas—liquid chromatographic (GLC—ECD) methods have also been developed. These do provide good sensitivity following the extraction of small plasma volumes (<1.0 ml) and have been used for pharmacokinetic studies in small animals such as rats [15] and in man [16, 17]. While sensitive, these packed column GLC—ECD methods, like HPLC [13], demonstrate considerable potential for interference from endogenous substances when used at higher sensitivities as well as from other drugs in clinical studies.

The aim of the present paper is to describe a GLC—ECD procedure whereby better resolution and high sensitivity have been achieved using recently introduced nonreactive cross-linked fused silica capillary columns.

EXPERIMENTAL

Materials

Metoclopramide (MCP), 4-amino-5-chloro-2-methoxy-N-(2-diethyl-aminoethyl)benzamide monohydrochloride monohydrate (MCP·HCl·H_2O), (lot Nos. A105 and 9207) and MCP·HCl·H_2O injectable, 5 mg/ml (Reglan® Injectable, 2 ml ampule, lot No. 80246, for sheep study) were supplied by A.H. Robins, Montreal, Canada. MCP·HCl·H_2O injectable 5 mg/ml (Maxeran® Injectable, 2 ml ampule; Nordic Pharmaceuticals, Montreal, Canada, for human study) was supplied by Pharmacy, The Vancouver General Hospital, Vancouver, Canada. Maprotiline, N-methyl-9-10-ethanoanthracene-9(10H)propanamine (lot No. A11663096472-0) was supplied by Ciba Pharmaceuticals, Mississauga, Canada. Heptafluorobutyric anhydride (HFBA) sequanal grade and triethylamine (TEA) were purchased from Pierce, Rockford IL, U.S.A. Benzene and toluene (distilled in glass) were purchased from Caledon Laboratories, Georgetown, Canada. Solutions of 1 *N* and 5 *N* sodium hydroxide, 1 *N* hydrochloric acid and 4% ammonium hydroxide were prepared from ACS reagent-grade chemicals (American Scientific and Chemical, Seattle, WA, U.S.A.). Deionized distilled water was used in the preparation of all reagents, stock solutions and throughout analysis.

Instrumentation and chromatographic conditions

A Model 5840A Hewlett-Packard gas chromatograph equipped with a ^{63}Ni electron-capture detector (ECD) and a Model 18835B capillary inlet system was used for all analyses. A Model 18850A Hewlett-Packard integrator system was used for peak area integration and quantitation.

A 25 m × 0.31 mm I.D. cross-linked SE-54 fused silica capillary column (5% phenylmethylsilicone, Ultra No. 2, film thickness 0.15 μm, siloxane-deactivated; Hewlett-Packard, Avondale, PA, U.S.A.) was used for all plasma analyses.

The split injection mode employing a silanized, unpacked Jennings split liner (Hewlett-Packard) was used, with a 2-μl sample being injected. A fused silica insert (78 mm × 2 mm I.D.) may be used interchangeably with the Jennings split liner provided that it is packed with a tight silanized glass wool plug extending 1 cm from the base to 3 cm from the top. This is illustrated by the close agreement of the following respective calibration curve parameters obtained by injecting the same calibration curve samples using first the unpacked Jennings liner ($y = 0.0241x + 0.0086$; $r = 0.9996$) and immediately followed using a packed fused silica insert ($y = 0.0243x + 0.004$; $r = 0.9997$). A more complete evaluation of these two split liner configurations and their use in plasma MCP analysis has been made [18].

The operating conditions for routine analysis were: injection temperature, 220°C; column temperature, 235°C; detector temperature (ECD), 350°C; carrier gas (hydrogen, ultra high purity) flow-rate 1.0 ml/min ($\bar{\mu}$ = 63 cm/sec); split vent flow-rate, 30 ml/min (split ratio 30:1); inlet pressure 0.65 bar; septum purge flow-rate, 1.5 ml/min; make-up gas (argon—methane, 95:5) flow-rate, 60 ml/min.

Stock solutions

Metoclopramide hydrochloride (0.04 μg/ml, equivalent to base) and the internal standard, maprotilene hydrochloride (II) (0.4 μg/ml, equivalent to base), were prepared by dissolving these compounds in water. The solutions were stored at 4°C, following preparation, for up to three weeks.

Extraction and derivative formation

To 0.25—0.5 ml of plasma obtained from MCP treated parturient human patients or pregnant sheep were added 0.1 ml of maprotiline hydrochloride and 0.5 ml of 1 *N* sodium hydroxide in a 15-ml PTFE-lined screw-capped disposable culture tube. The final volume was adjusted to 2.1 ml (pH ≈ 14) with water. Benzene (6 ml) was added and the aqueous phase was extracted by shaking for 20 min on a rotary shaker (Labquake Tube Shaker, Model 415-110; Lab Industries, Berkeley, CA, U.S.A.). After centrifugation at 2300 *g* for 10 min, 5 ml of the organic phase were removed and back-extracted for 20 min with 2 ml of 1 *N* hydrochloric acid. The samples were centrifuged for 5 min and the organic layer aspirated and discarded. The remaining aqueous layer was washed with two 4-ml aliquots of benzene, alkalinized by adding 0.5 ml of 5 *N* sodium hydroxide (pH ≈ 14) and then re-extracted for 20 min with 6 ml of benzene. Following centrifugation at 2300 *g* for 5 min, 5 ml of the organic layer were removed and dried under a gentle stream of nitrogen in a 40°C water bath. The residue was reconstituted to a volume of 200 μl with 150 μl of toluene and 50 μl of 0.05 *M* TEA in toluene. A 20-μl volume of HFBA was added, the sample vortexed and placed in an oven at 55°C for 60 min. After cooling to room temperature, the excess derivatizing agent was removed by hydrolysis with 0.5 ml of water (vortex for 10 sec) and neutralizing with 0.5 ml of 4% ammonium hydroxide (vortex for 10 sec). Following centrifugation at 750 *g* for 1 min the derivatized organic layer was immediately transferred to a clean, dry 15-ml culture tube. Aliquots of 2 μl were used for GLC—ECD analysis.

Preparation of the calibration curve

A 0.5-ml sample of blank human or sheep plasma was spiked with serial amounts (4, 8, 16, 24, 32, or 40 ng) of the prepared metoclopramide hydrochloride stock solution and then 0.1 ml of the maprotiline hydrochloride solution (0.4 μg/ml) and 0.5 ml of 0.5 *N* sodium hydroxide were added. The aqueous phase was adjusted to a total volume of 2.1 ml (pH ≈ 14) with water and the samples extracted and derivatized as described above. Quantitative estimation of MCP in plasma was accomplished by plotting the area ratios of the heptafluorobutyryl (HFB) derivatives of MCP and maprotiline against the range of indicated MCP concentrations.

Human experiments

The placental transfer of MCP from mother to fetus was examined in normal healthy women in labour who required MCP as a pre-anaesthetic medication prior to undergoing elective Caesarian section.

MCP was administered by intravenous (i.v.) infusion over 1—2 min at the rate of 0.15 mg/kg. All patients received a standardized general anaesthetic regimen of thiopental, halothane, *d*-tubocurare and succinylcholine. One-point blood samples were drawn at delivery from a maternal vein (MV) and from a double-clamped section of the umbilical cord [umbilical vein (UV), umbilical artery (UA)]. The blood samples were immediately centrifuged, the plasma removed and stored at —20°C until analysis.

Sheep experiments

A preliminary experiment on the placental transfer of MCP was carried out on a pregnant ewe with vascular catheters chronically implanted in the mother and fetus [19]. A 10-mg i.v. dose of MCP was administered over a 3-min period via the implanted maternal jugular venous catheter. Blood samples for MCP determination were simultaneously withdrawn from implanted maternal femoral arterial and fetal venous (lateral tarsal vein) catheters at —5, 1, 5, 15, 30, 60, 90, 120, 150, and 180 min. Normally the fetal femoral arterial site is used for sampling but this catheter was not patent for this preliminary study. The blood samples were immediately centrifuged, the plasma removed and stored at —20°C until analyzed.

RESULTS

Application of the developed method to sheep plasma analysis is shown in Fig. 1. No interfering peaks from endogenous plasma components were observed in the plasma extracts (Fig. 1a). Peaks with retention times of 3.20 and 4.57 min were the HFB derivatives of MCP and maprotiline, respectively (Fig. 1b). Essentially identical chromatograms were obtained with blank and MCP-spiked human plasma.

The data for a representative calibration curve used in the quantitation of MCP in maternal and fetal sheep plasma are presented in Table I. Linearity was observed over the concentration range studied (4—40 ng/ml) with the line of best fit through the data points being described by $y = 0.0254x - 0.0023$ with a correlation coefficient of $r = 0.9993$. The precision of the assay for this

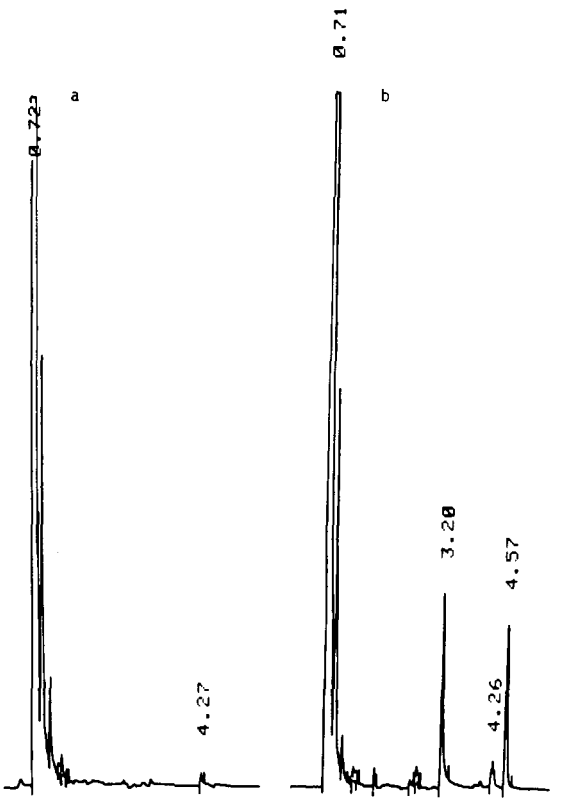

Fig. 1. Representative chromatograms obtained from blank (a) and MCP-spiked (b) plasma extracts on the 25-m SE-54 fused silica column. The spiked sample contained MCP, 139.58 pg/μl (40.20 ng/ml) and maprotiline 138.89 pg/μl (2-μl injection; split ratio, 30:1).

TABLE I

CALIBRATION CURVE DATA FOR SHEEP PLASMA

n = 2, duplicate injections.

MCP (ng/ml)	A.R.* (± S.D.)	C.V. (%)
4.02	0.1055 ± 0.0114	10.85
8.04	0.1893 ± 0.0159	8.41
16.08	0.4263 ± 0.0119	2.80
24.12	0.5939 ± 0.0235	3.95
32.16	0.8094 ± 0.0178	2.20
40.20	1.0256 ± 0.0127	1.24

*Area ratio, drug/internal standard ± 1 S.D.

calibration curve is included in Table I. In general, a range of coefficients of variation (C.V.) from 2—10% was observed for all calibration curves (human or sheep plasma) with an average of 5—6%. This is illustrated by a more extensive study of C.V. values in Table II for two extreme calibration curve MCP concentrations.

TABLE II

REPRODUCIBILITY STUDY

A fused silica insert packed with a "tight" silanized glass wool plug was used in this study.

Sample*	No. of samples**	A.R.***	C.V. (%)
8.02	6	0.3416 ± 0.0159	4.65
40.20	5	1.2152 ± 0.0815	6.71

*MCP·HCl·H_2O concentration (equivalent to MCP base), ng/ml.
**Triplicate injections.
***Mean area ratio (HFB-MCP/HFB-maprotiline) ± 1 S.D.

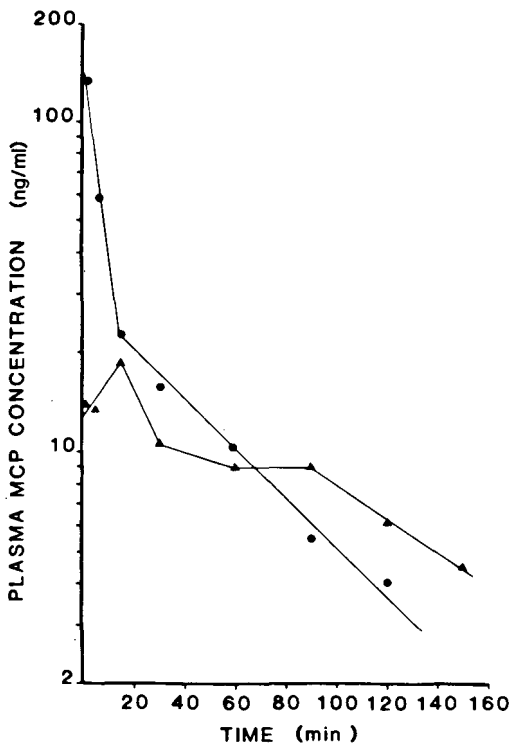

Fig. 2. A semi-logarithmic plot of the plasma profile of MCP in a pregnant ewe following a 10-mg i.v. dose (●——●) maternal plasma levels, (▲——▲) fetal plasma levels. The terminal elimination half-lives in the ewe and fetus were calculated to be 40 and 54 min, respectively.

Semilogarithmic plots of the determined MCP concentrations in maternal and fetal sheep plasma against time following administration of the 10-mg i.v. dose to the ewe are shown in Fig. 2. Maternal plasma MCP elimination was observed to follow a biexponental decay described by the following equation: $C_p = Ae^{-\alpha t} + Be^{-\beta t}$ where C_p is the plasma concentration at time t. A and B are the intercepts of the α and β phases, respectively. The parameters α and β represent the distribution and terminal elimination rate constants, respectively. The half-life of the α phase ($t_{1/2\alpha}$) was calculated to be 6 min, indicating rapid

TABLE III

HUMAN PLASMA ANALYSIS DATA

Patient code	Maternal		MCP i.v. dose (mg/kg)	MV sample*, elapsed time (min)	Umbilical samples*, elapsed time (min)	Plasma MCP concentration** ng/ml)		
	age (years)	wt. (kg)				MV	UV	UA
2S	28	68	0.15	36	29	61.16±0.22	18.82±0.13	18.28***
9S	23	80	0.15	21	30	63.01±3.12	38.75±3.27	32.78***

*Time elapsed between administration of MCP and sampling and delivery, respectively.
**Mean of two separate determinations ± 1 S.D.
***Single determination.

distribution of MCP following an i.v. injection. A maternal elimination half-life ($t_{½\beta}$) of 40 min was calculated for the terminal phase.

Transfer of MCP to the fetus was observed to be rapid with measurable concentrations at 1 min post-injection to the ewe. Plasma concentrations peaked in the fetus at 20 min and were observed to exceed maternal concentrations at 90 min. An elimination half-life ($t_{½}$) of 54 min was calculated for MCP in the fetus.

Data from preliminary studies of the placental transfer of MCP in human subjects are shown in Table III. In both instances, the MV plasma concentrations were greater than those measured in either UV or UA samples.

DISCUSSION

The high efficiency associated with glass open tubular (capillary) columns has been applied for many years to resolve complex mixtures. Analysis of environmental pollutants in air and water and use in the petroleum, food, flavor and fragrance industries is widespread. More recently this technology has been applied to the analysis of biological samples for metabolic profiling [20—22] of certain disease states, the measurement of human urine and plasma drug concentrations [23—25] and the determination of drug pharmacokinetics in man [23, 25]. The recent introduction of inert fused silica columns [20] facilitates reliable and reproducible analysis of a variety of underivatized (acidic, basic, neutral, polar) drugs [26]. Previously such procedures for underivatized substances using more reactive glass columns were often only marginally successful unless the surface was carefully deactivated.

Before a placental transfer study of MCP in hospitalized human patients undergoing multiple therapy during general or spinal anaesthesia could be undertaken, an analytical method with high separation efficiency was required, due to potential interference from structurally related compounds (e.g., local anaesthetics). In addition, a planned study of this drug's placental transfer in sheep required an assay with improved sensitivity such that the analysis of small plasma volumes (0.25—0.5 ml) was possible after serial blood sampling (1.0 ml) of the catheterized fetus.

A packed column GLC—ECD assay method developed for use in our laboratory [16] demonstrated the potential for interference from other drugs as well as from endogenous plasma components during trace drug analysis. On this basis then, the development of a capillary column GLC—ECD method was

undertaken that would provide both improved sensitivity and selectivity.

Initial tests conducted with glass capillary columns (OV-225, Silar 10-C) provided generally unsatisfactory results, with broad severely-tailing MCP peaks being obtained, possibly due to incomplete surface deactivation [20, 22].

Two Carbowax deactivated methylsilicone fluid fused silica columns were tested following experimentation with glass columns. Both provided good resolution of MCP from plasma components as well as a marked improvement in peak shape. These columns are, however, subject to phase stripping with aromatic solvents. Hexane and isooctane are recommended solvents for capillary column GLC—ECD analysis, however they provided extremely variable chromatographic results in this study. Both lack sufficient solvent strength to adequately extract and solubilize MCP and their use did not permit quantitation in the desired 4—40 ng/ml MCP concentration range.

Polysiloxane-deactivated fused silica columns with cross-linked phases (methylsilicone, phenylmethylsilicone) exhibit a high degree of inertness, excellent thermal stability (maximum temperature 325—350°C) and very low solvent extractability [20, 26]. This technological advance virtually eliminates phase stripping thus allowing the use of aromatic solvents such as benzene and toluene which possess good extractability and solubility characteristics towards MCP. The use of a cross-linked SE-54 fused silica column coupled with toluene as the derivatizing and injection solvent permitted quantitation in the range of 4—40 ng/ml MCP.

Resolution of MCP and maprotiline is excellent as illustrated in Fig. 1b; no interference from endogenous plasma (sheep) components was observed (Fig. 1a). Analysis times are short and repeat injections of plasma extracts every 5—6 min are possible without interference from late eluting plasma components. An identical chromatographic pattern was obtained with blank and clinical patient plasma; again no interference from plasma components or from the drugs used during general anaesthesia (viz., thiopental, halothane) was observed. Diazepam, a commonly prescribed therapeutic agent, and a congener, prazepam, have also been shown not to interfere. The antiarrhythmic procainamide, a close structural analogue of MCP, also shows no interference eluting well before MCP.

A series of samples containing 20 ng of MCP and 40 ng of maprotiline were incubated in the presence of the catalyst TEA, for periods ranging from 0—180 min to determine the optimum time required for reaction of both drugs with HFBA. No significant differences were observed in area ratios of MCP to internal standard between 20—180 min; a 60-min reaction time was subsequently chosen to ensure complete derivatization.

Derivatized samples have been found to be stable for at least four days when stored at −4°C with repeat injections showing no significant decline in area ratios over this time period.

The developed assay method has been found to show good linearity over the 4—40 ng/ml concentration range studied with replicate calibration curves having correlation coefficients of at least $r = 0.989$. This represents ca. 0.9—9 pg at the detector employing a 30:1 split ratio (2 μl injection) and demonstrates the sensitivity of the method.

Within-run precision (repeatability) of a representative calibration curve is shown in Table I with coefficients of variation ranging from 1.24—10.85%. Between-run precision studies (reproducibility) have shown the method to be reliable and reproducible with average coefficients of variation between 5 and 6% (Table II).

The plasma concentration data in Table III illustrate the applicability of the method to the analysis of human plasma samples in the clinical setting. The low sensitivity limit of the method (ca. 4 ng/ml) has also been found to be suitable for a preliminary pharmacokinetic assessment of plasma MCP concentration vs. time profiles in chronically catheterized maternal and fetal sheep (Fig. 2).

In summary, the developed capillary GLC—ECD method:

(1) provides a short analysis time; a desired property for a routine analytical method;

(2) is reliable, reproducible and offers improved sensitivity over the previous packed column GLC—ECD method in use in our laboratory [16];

(3) has been applied to patient samples in the clinical setting without interference from the other drugs used during general anaesthesia in this study;

(4) has been used to demonstrate placental transfer of MCP both in humans and in sheep, which has not been reported to date [5];

(5) has shown pharmacokinetic applicability to the study of MCP placental transfer in sheep.

More extensive evaluations of the pharmacokinetics of MCP placental transfer in human patients as well as in pregnant and non-pregnant sheep are currently underway in our laboratory using the method described in this paper.

ACKNOWLEDGEMENTS

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DETERMINATION OF THE ANTIALLERGENIC AGENT, *TRANS*-3-[6-(METHYLTHIO)-4-OXO-4H-QUINAZOLIN-3-YL]-2-PROPENOIC ACID, IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A rapid and selective high-performance liquid chromatographic (HPLC) assay was developed for the determination of the antiallergenic compound, *trans*-3-[6-(methylthio)-4-oxo-4H-quinazolin-3-yl]-2-propenoic acid, [I], in plasma. The assay involves acetonitrile protein precipitation followed by the analysis of an aliquot of the protein-free fraction by reversed-phase HPLC with fluorescence detection (excitation at 245 nm, with emission greater than 418 nm). The overall recovery of [I] from plasma was 103 ± 10%. The sensitivity limit of the assay was 0.125 μg/ml of plasma. The analogous compound, *trans*-3-[6-[(1-methylethyl)thio]-4-oxo-4H-quinazolin-3-yl]-2-propenoic acid, [II], is used as the internal standard. The assay was used to monitor the plasma concentration—time fall-off profile of [I] in the dog and in man. The stability of [I] was demonstrated in dog plasma on long-term storage for up to 180 days at —17°C and —70°C.

INTRODUCTION

The compound, *trans*-3-[6-(methylthio)4-oxo-4H-quinazolin-3-yl]-2-propenoic acid, [I] (Fig. 1), synthesized by LeMahieu [1], is under investigation as an active mediator release inhibitor for use in the treatment of allergic bronchial asthma [2].

Studies on the biotransformation of [I] in the rat indicated that the compound was converted by S-oxidation to the sulfoxide, *trans*-3-[6-(methylsulfinyl)-4-oxo-4H-quinazolin-3-yl]-2-propenoic acid, [I-A], and by hydrogenation of the side chain to the dihydro compound, 6-(methylthio)-4-oxo-4H-quinazoline-3-propenoic acid, [I-B] (Fig. 1) [3].

A sensitive and selective high-performance liquid chromatographic (HPLC)

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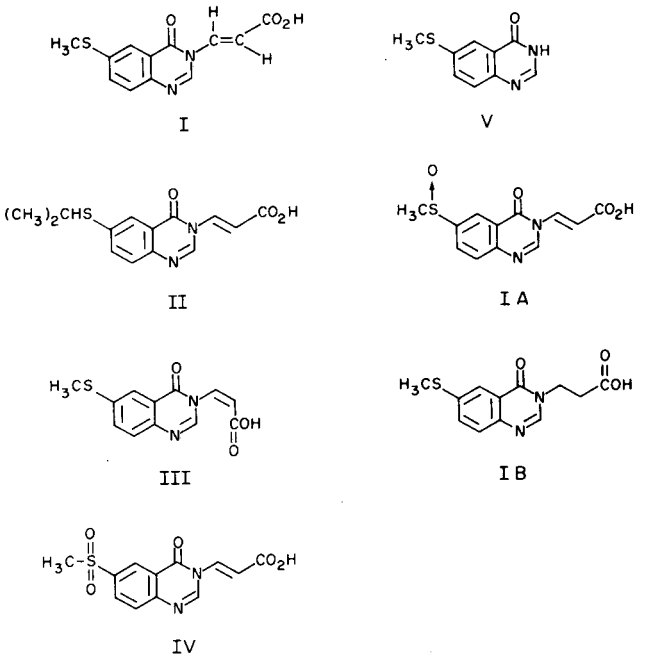

Fig. 1. Chemical structures for the compounds referred to in the text.

assay with automated injection was developed to monitor the biopharmaceutic and pharmacokinetic profile of the drug in the dog. The parent compound [I] and its methylethyl analogue, *trans*-3-[6-[(1-methylethyl)thio]-4-oxo-4H-quinazolin-3-yl]-2-propenoic acid, [II] (Fig. 1) used as the internal standard are quantitated in the protein-free fraction after precipitation of the plasma proteins with acetonitrile. An aliquot of the protein-free fraction is diluted with the HPLC mobile phase, introduced via a WISP auto-injector and analyzed by reversed-phase HPLC using fluorescence detection with excitation at 245 nm and emission greater than 418 nm.

The assay was used to monitor the plasma concentration—time fall-off profile of [I] in the dog and in man. The stability of [I] was demonstrated in dog plasma on long-term storage for up to 180 days at —17°C and —70°C.

EXPERIMENTAL

Column

A prepacked 30 cm × 3.9 mm I.D. column containing μBondapak C_{18}, particle size 10 μm (Waters Assoc., Milford, MA, U.S.A.) generating 16,680 plates per meter was used.

Instrumental parameters

The HPLC system consisted of a Waters Model 6000 A reciprocating piston pump, a Waters Intelligent Sample Processor (WISP™) Model 710B, and a Schoeffel Model FS-970 LC fluorometer operated at 245 nm for excitation and emission at wavelengths greater than 418 nm (Kratos, Schoeffel Instru-

ments, Westwood, NJ, U.S.A.). The isocratic mobile phase used was a mixture of acetonitrile—methanol—0.001 *M* ascorbic acid (pH 3.25) (36:28:36, v/v) at a pressure of ca. 7 MPa and constant flow-rate of 1 ml/min. The fluorescence detector sensitivity was 1.0 μA full scale and the chart speed on the 10-mV recorder, Model 7132A (Hewlett-Packard, Palo Alto, CA, U.S.A.) was 1.27 cm/min. The WISP auto-injector was programmed to run for 12 min (10 min run time + 2 min purge and rinse) per sample using methanol as the rinse solvent. Under these conditions 25 ng of [I] and 40 ng of [II] injected gave nearly full-scale pen response. The retention times of [I] and [II] were 4.42 min and 6.26 min (Table I) with corresponding capacity factors (k') of 0.55 and 1.19, respectively. The minimum detectable amount of [I] was 1.25 ng injected equivalent to 0.125 μg/ml of plasma.

TABLE I

RETENTION TIMES AND CAPACITY FACTORS (k') OF COMPOUNDS REFERRED TO IN THE TEXT AND IN FIG. 1

Compound	Retention time (min)	k'
[I]	4.42	0.55
[I-A]	3.06	0.07
[I-B]	3.98	0.39
[II]	6.26	1.19
[III]	3.47	0.21
[IV]	3.20	0.10
[V]	3.98	0.40

Reagents

All reagents were of analytical-reagent grade (> 99% purity). They include acetonitrile, methanol (Burdick & Jackson Labs., Muskegon, MI, U.S.A.); 0.001 *M* ascorbic acid, pH 3.25 (0.176 g/l), USP-FCC grade (Roche Chemical Division, Hoffmann-La Roche, Nutley, NJ, U.S.A.); concentrated ammonium hydroxide (29.3% ammonia), Baker analyzed reagent grade (J.T. Baker, Phillipsburg, PA, U.S.A.).

Analytical standards

Compound [I], *trans*-3-[6-(methylthio)-4-oxo-4H-quinazolin-3-yl]-2-propenoic acid ($C_{12}H_{10}N_2O_3S$, MW 262.3, m.p. 271—272°C); compound [I-A], *trans*-3-[6-(methylsulfinyl)-4-oxo-4H-quinazolin-3-yl]-2-propenoic acid ($C_{12}H_{10}N_2O_4S$, MW 278.3, m.p. 276—277°C); compound [I-B], 6-(methylthio)-4-oxo-4H-quinazoline-3-propenoic acid ($C_{12}H_{12}N_2O_3S$, MW 264.3, m.p. 192—193°C); compound [II], *trans*-3-[6-[(1-methylethyl)thio]-4-oxo-4H-quinazolin-3-yl]-2-propenoic acid ($C_{14}H_{14}N_2O_3S$, MW 290.4, m.p. 238—239°C); compound [III], *cis*-3-[6-(methylthio)-4-oxo-4H-quinazolin-3yl]-2-propenoic acid ($C_{12}H_{10}N_2O_3S$, MW 262.3, m.p. 233—234°C); compound [IV], *trans*-3-[6-(methylsulfonyl)-4-oxo-4H-quinazolin-3-yl]-2-propenoic acid ($C_{12}H_{10}N_2O_5S$, MW 294.3, m.p. 284—290°C) and compound [V], 6-(methylthio)-4-(3H)-quinazolinone ($C_9H_8N_2OS$, MW 192.3, m.p. 203—204.5°C). All compounds

were of pharmaceutical grade purity (> 99%), with the exception of compounds [I-A] and [III] which were approximately 98% pure.

Preparation of analytical standards

Prepare stock solutions of compounds [I] and [II] as described below:

(A) 1 mg of [I] per ml in methanol—conc. ammonium hydroxide (99:1). Dissolve 10 mg of [I] in 10 ml of methanol—conc. ammonium hydroxide (99:1).

(B) 100 μg of [I] per ml in methanol—conc. ammonium hydroxide (99:1). A 1-ml aliquot of Solution A diluted to 10 ml with methanol—ammonium hydroxide (99:1).

(C) 1 mg of [II] per ml in methanol—conc. ammonium hydroxide (99:1). Dissolve 10 mg of [II] in 10 ml of methanol—ammonium hydroxide (99:1).

Preparation of mixed standard solutions 1—7: aliquots of Solution A, B and C are diluted to 10 ml in methanol as follows:

Solution	Aliquots (μl) of standard			Final concn. (ng per 100 μl of solution)	
	A	B	C	[I]	[II]
1	—	25	80	25	800
2	—	50	80	50	800
3	—	100	80	100	800
4	20	—	80	200	800
5	50	—	80	500	800
6	100	—	80	1000	800
7	—	—	80	0	800

Aliquots (100 μl) of solutions 1, 2, 3, 4, 5 or 6 are added to separate 200-μl specimens of control plasma and processed along with the samples to establish a recovered standard calibration curve for the direct quantitation of unknowns.

Aliquots (50 μl) of the above solutions are added to a 0.5-ml aliquot of the protein-free fraction of control plasma and diluted to 1.5 ml with HPLC mobile phase as the external standard calibration curve to establish the linearity and performance of the HPLC system.

Analytical procedure

The flow diagram of the precipitation procedure is shown in Fig. 2. Into a glass-stoppered 15-ml centrifuge tube, transfer a 100-μl aliquot of solution 7 (equivalent to 800 ng of [II], the internal standard), 700 μl of acetonitrile, and mix for a few seconds on a Vortex super-mixer (Lab-Line Instruments, Melrose Park, IL, U.S.A.). Add 200 μl of unknown plasma (aliquots of less than 200 μl taken of those unknowns with expected concentrations above the highest calibration point are diluted to 200 μl with control plasma) and mix again for 10 sec at the highest speed setting of the Vortex mixer. Centrifuge the samples in a refrigerated centrifuge (Model PR-J, Rotor No. 253, Damon/IEC, Needham, MA, U.S.A.) at 5°C for 10 min at 2100 rpm (1100 *g*). Transfer 0.5 ml of the supernatant protein-free fraction into a standard 4-ml glass vial

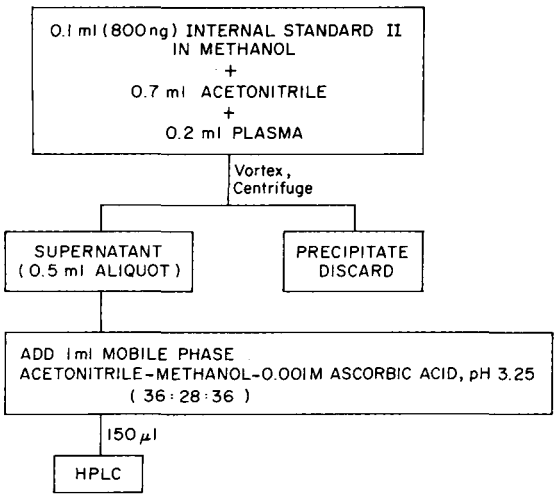

Fig. 2. Flow diagram of the precipitation procedure of Compounds [I] and [II] from plasma.

(Waters part No. 73001). Add 1 ml of the mobile phase, acetonitrile—methanol—0.001 *M* ascorbic acid, pH 3.25 (36:28:36), and seal with an H-style vial cap (Waters part no. 72711) fitted with a PTFE septum (Waters part No. 73005). Program the auto-injector (WISP 710B) to inject 75—150 µl out of a total volume of 1.5 ml for HPLC analysis.

Along with the samples, process seven 200-µl specimens of control plasma, one to be used as a control blank to which 100 µl of methanol are added and six to be used for the preparation of the recovered standards to which 100 µl of solutions 1, 2, 3, 4, 5 or 6, equivalent to 25, 50, 100, 200, 500 ng and 1 µg of [I] and 800 ng of [II] per 200 µl of plasma (representing 0.125, 0.25, 0.5, 1, 2.5 or 5 µg of [I] and 4 µg of [II] per ml of plasma), respectively, are added. These standards are used to establish the recovery curve for the direct quantitation of the unknowns.

In order to verify the linearity and performance of the HPLC assay, a matrix external standard calibration curve is prepared by adding six 200-µl specimens of control plasma to separate mixtures of 100 µl methanol and 700 µl acetonitrile. To 0.5-ml aliquots of the resultant protein-free fractions, 50 µl of solutions 1, 2, 3, 4, 5 or 6 are added along with 0.95 ml of mobile phase. Aliquots (75 or 150/1500 µl) are programmed on the WISP for automated injection. Typical chromatograms are shown in Fig. 3.

Calculations and assay validation

The concentration of [I] in the unknowns was determined by interpolation from a least squares regression equation (power equation: $Y = mX^b$) of the calibration data (processed by a Hewlett-Packard Model 3354B Laboratory Data System) of the recovered standards processed along with the unknowns using peak height ratios (peak height of compound [I] to peak height of internal standard [II]) versus concentration of [I] per ml of plasma. A typical calibration curve as defined by the equation $Y = 0.330X^{0.946}$ is linear from

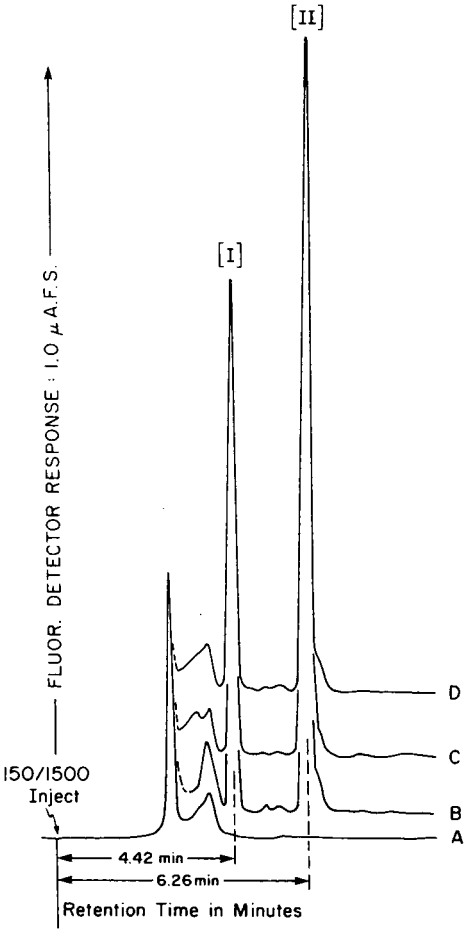

Fig. 3. Chromatograms of Compound [I] and Compound [II], the internal standard. (A) Control (0 h) dog plasma supernatant, (B) 1.5-h dog plasma supernatant following a single 8 mg/kg i.v. dose of [I], (C) authentic standards recovered from control dog plasma, and (D) authentic standards added to the supernatant of control dog plasma (matrix external standards).

0.125—5 μg of [I] per ml of plasma. The correlation coefficient (r) is equal to 0.9997 and the average deviation from the line is 2.71%. Intra- and inter-assay validation data over the linear concentration range (0.125—5.0 μg/ml of plasma) are summarized in Table II. The mean intra- and inter-assay coefficients of variation are 1.7% and 4.0%, respectively.

RESULTS AND DISCUSSION

A rapid, sensitive and selective HPLC assay was developed for the determination of compound [I] from plasma using a WISP auto-injector and fluorescence detection for quantitation. This method enabled the rapid and accurate quantitation of compound [I] with high sample throughput required

TABLE II

STATISTICAL VALIDATION OF THE HPLC ASSAY FOR COMPOUND [I]

Amount added (μg/ml)	Amount found (μg/ml)	*n*	Coefficient of variation (%)
Intra-assay variability			
0.125	0.131 ± 0.023	3	1.8
0.250	0.243 ± 0.004	3	1.8
0.500	0.480 ± 0.010	3	2.0
1.00	1.00 ± 0.02	3	1.8
2.50	2.49 ± 0.05	4	1.8
5.00	5.11 ± 0.04	3	0.8
		Average	1.7
Inter-assay variability			
0.125	0.130 ± 0.007	14	5.5
0.250	0.246 ± 0.009	13	3.8
0.500	0.481 ± 0.015	13	3.0
1.00	1.00 ± 0.07	13	6.5
2.50	2.54 ± 0.10	14	4.1
5.00	5.05 ± 0.20	12	4.0
		Average	4.0

for pharmacokinetic and biopharmaceutic studies. The corrected excitation and emission spectra of compound [I] occur at 245 and 425 nm, respectively. The Schoeffel Model FS-970 fluorescence detector with excitation at 245 nm and emission greater than 418 nm allowed for quantitation of [I] and [II] in the nanogram range.

Reversed-phase HPLC analysis is the method of choice, since it is amenable to an acetonitrile protein precipitation step followed by direct injection of the supernatant after dilution with mobile phase.

Compound [II] was chosen as the internal standard in the assay, because of its similar precipitation and chromatographic behavior to compound [I]. Compound [II] has not been identified as a metabolite of [I].

Percent recovery and sensitivity limits

The overall recovery of [I] was 103 ± 10% (S.D.) over the concentration range of 0.125–5 μg per ml of plasma. The ⩾ 100% recovery was attributed to a matrix enhancement effect, which necessitated that the external standard samples be added to the protein-free fraction of control plasma [4, 5]. The sensitivity limit of the assay was 0.125 μg of [I] per ml of plasma.

Chromatographic behavior of [I] and [II]

Compounds [I] and [II] exhibited UV absorbances sufficiently intense for detection at 254 nm. However, the use of ascorbic acid as a stabilizer/buffer in the mobile phase created a significantly high background UV absorbance, making UV detection impossible. Also, the fact that the UV chromatogram

of the control plasma samples contained significant interfering matrix peaks eluting in the region of compound [I], made fluorometric detection a necessity.

The HPLC system is flushed initially with methanol—water (50:50) to remove deposits from the column accumulated from previous use. The mobile phase [acetonitrile—methanol—0.001 *M* ascorbic acid, pH 3.25 (36:28:36)] is allowed to recycle through the system overnight, or, in some instances, for 48 h or more at 0.5 ml/min until equilibrium is attained when the desired separation and resolution are obtained. Non-equilibration may result in interference from small artifact peaks eluting close to [I] and/or [II], or a change in k' values. Several μBondapak C_{18} columns used during the course of this project showed variation in equilibration time from column to column with concomitant variation in retention time of ± 0.5 min for [I] and ± 1 min for [II].

It was also noted that retention times of [I] and [II] changed significantly when aliquots of mixed standard solutions 1—7 in methanol were injected without the presence of biological matrix. Preparing [I] and [II] in methanol—conc. ammonium hydroxide (99:1) for stock solutions A—C and subsequent preparation of mixed working standard solution 1—7 also in methanol—conc. ammonium hydroxide (99:1) instead of in methanol alone alleviates this discrepancy. However, due to the volatility of the ammonia from the methanol—ammonia solution (which would change its concentration), the working mixed standard solutions 1—7 were prepared in 100% methanol and added to the biological matrix. No adverse effect was observed in quantitation of [I] or [II]. Also, no significant change in chromatography was observed when sample volumes between 10 and 150 μl were injected.

Selectivity of the assay

The biotransformation [3] and the instability of [I] due to isomerization under specific conditions necessitate the use of chromatographic procedures which ensure the stability and selectivity of the assay. Ascorbic acid buffer was chosen in place of the more commonly used phosphate buffer because of its antioxidant property. The resultant chromatography improved the peak shape of compounds [I] and [II] and their resolution. A fresh solution of 0.001 *M* ascorbic acid is prepared every month.

Under the reported procedure, the biotransformed products [I-A], [I-B] and [III] (*cis*-isomer, Table I) were > 90% resolved from each other and from [I] and [II] (Fig. 4). Compound [IV] eluted very soon after [I-A] such that both compounds were not fully resolved from the solvent front. Compound [V] eluted at the same retention time as [I-B], and was not fully resolved from [I] on certain μBondapak C_{18} columns. In this case further equilibration and/or a new C_{18} column was required.

None of these compounds, however, would interfere with the quantitation of [I] (Fig. 4).

Application of the HPLC method

Biopharmaceutic and pharmacokinetic studies in the dog. The HPLC method was applied to a pilot study of single 2 mg/kg doses of [I] administered intra-

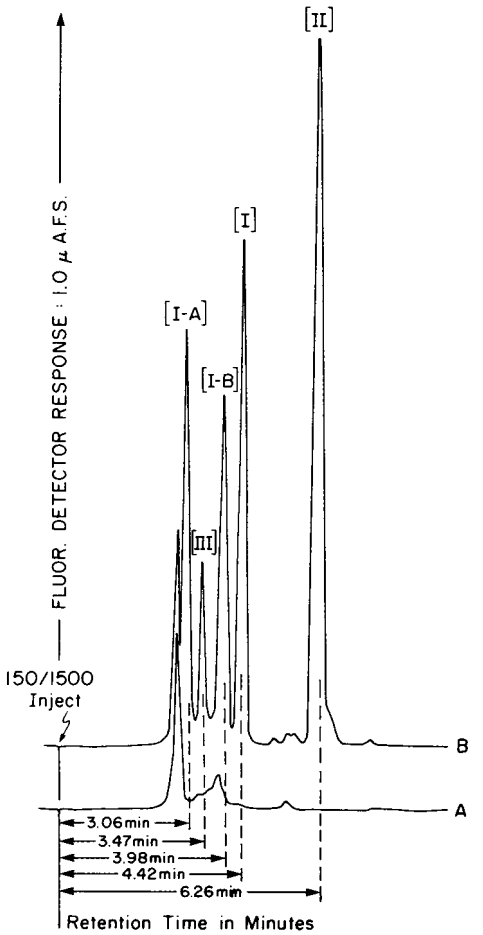

Fig. 4. Chromatogram of Compounds [I], [I-A], [I-B], [II] and [III]. (A) Control dog plasma supernatant and (B) authentic standards recovered from control dog plasma.

venously (i.v.) and orally as a solution and capsule (handpacked) in the dog. Following this dosing regimen plasma concentrations were measurable up to 4 h.

Studies in man. Plasma concentrations of [I] were determined in four normal volunteers following the oral administration of a single 350-mg dose of the drug in suspension. Blood samples were collected at appropriate time points in heparinized tubes and centrifuged; the plasma was separated and stored frozen at −70°C until analysis. The mean data plotted in Fig. 5 suggest that peak concentrations of about 7 μg/ml occur at about 2 h and that the plasma concentrations decline, being measurable at 9 h, but not 12 h post dosing.

Stability of [I] in plasma

Stability of [I] in dog and human plasma at ambient temperature. Compound [I] added to fresh control dog plasma was analyzed by HPLC after

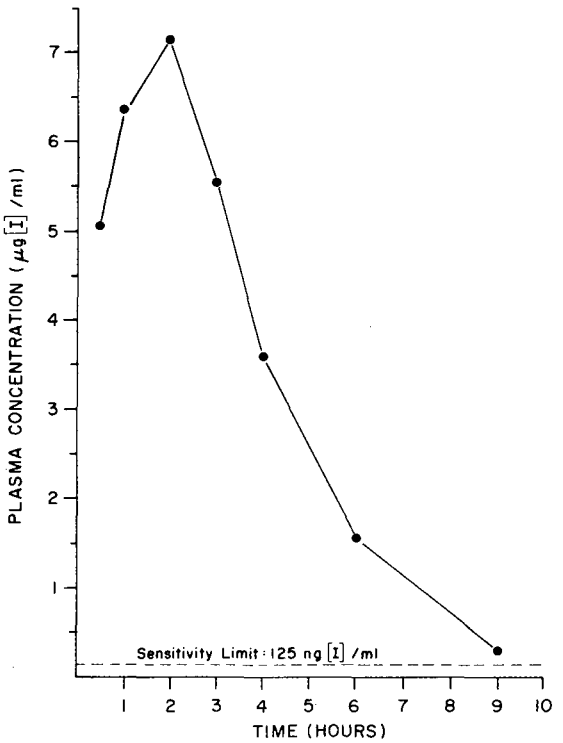

Fig. 5. Mean plasma concentrations of [I] following the oral administration of a single 350-mg dose to four subjects.

remaining at room temperature (23°C) from 0—48 h, and found to be stable for up to 6 h. The recovery dropped to 93 and 86% at 24 and 48 h, respectively, indicating apparent instability. Similar results were obtained for compound [I] in fresh human plasma. Under these conditions, Compound [I] is stable under the sample preparation techniques described with no additional precautions required.

Stability of [I] in dog plasma on storage at −17°C and −70°C up to 180 days. The stability of [I] in dog plasma was evaluated by HPLC analysis at three concentrations following storage at −17°C and −70°C for a period of 7, 30, 61, 90 and 180 days. The plasma samples were prepared as follows: transfer 12.5 μl of solution B (see Experimental section) into a 10-ml glass stoppered amberized volumetric flask, dilute to 10 ml by adding fresh control dog plasma slowly, mix well by sonication to yield a plasma stock solution 1 whose concentration is 0.125 μg/ml (representing the sensitivity limit of the assay). Transfer 10 μl of solution A into a 10-ml glass stoppered amberized volumetric flask, dilute to volume with plasma to yield plasma solution 2 whose concentration is 1 μg/ml (representing a mid-point value on the calibration curve). Transfer 50 μl of solution A into a 10-ml glass stoppered amberized volumetric flask, dilute to volume with plasma to yield plasma solution 3 whose concentration is 5 μg/ml (representing the upper limit on the calibration curve).

Storage. Transfer 0.8-ml aliquots of plasma solutions 1, 2, and 3 (0.125, 1, and 5 μg/ml, respectively) into ten 1-dram vials for each concentration (30 vials). Five vials at each concentration (total of 15) were stored at −17°C with the remaining five vials at each concentration (total of 15) stored at −70°C.

The stability-indicating plasma samples from days 0, 7, 30, 61, 90 and 180 stored at −17°C and −70°C were analyzed in triplicate as unknowns along with the external standards and recovered calibration standards at similar concentrations added to fresh control plasma. The data are tabulated in Table III and were analyzed by least-squares regression. The data indicated that [I] was stable throughout the storage interval at −17°C and −70°C, although the recovery of samples stored at the higher temperature was consistently a few percentage points lower though not statistically significant. The coefficients of correlation of all samples as compared to day 0 were ⩾ 0.999, indicating overall stability.

TABLE III

STABILITY OF [I] IN DOG PLASMA STORED AT −17°C AND −70°C FOR UP TO 180 DAYS

n = 3 for all samples except those denoted with asterisk.

Day	Mean concn. found ± S.D. (% S.D.) (0.125 μg/ml added)	Mean concn. found ± S.D. (% S.D.) (1.00 μg/ml added)	Mean concn. found ± S.D. (% S.D.) (5.00 μg/ml added)
Stability of [I] stored at −17°C			
0	0.124 ± 0.004 (3.3)	1.01 ± 0.02 (1.6)	5.24 ± 0.08 (1.5)
7	0.123 ± 0.000 (0.0)	0.85 ± 0.01 (0.6)	4.58 ± 0.03 (0.6)
30	0.138 ± 0.004 (3.0)	0.96 ± 0.03 (3.0)	4.80 ± 0.05 (1.1)
61	0.132 ± 0.000 (0.0)	0.86 ± 0.01 (1.4)*	4.48 ± 0.03 (0.6)
90	0.130 ± 0.000 (0.0)	0.89 ± 0.01 (1.1)	4.48 ± 0.09 (2.0)
180	0.114 ± 0.000 (0.0)	0.85 ± 0.05 (5.8)	4.55 ± 0.07 (1.5)
Stability of [I] stored at −70°C			
0	0.124 ± 0.004 (3.3)	1.01 ± 0.02 (1.6)	5.24 ± 0.08 (1.5)
7	0.123 ± 0.000 (0.0)	0.91 ± 0.02 (2.0)	4.81 ± 0.19 (3.9)*
30	0.143 ± 0.000 (0.0)	1.00 ± 0.01 (0.9)	5.03 ± 0.04 (0.8)
61	0.132 ± 0.000 (0.0)*	0.89 ± 0.02 (2.0)	4.71 ± 0.23 (4.9)
90	0.130 ± 0.000 (0.0)	0.91 ± 0.02 (1.6)	4.77 ± 0.04 (0.9)
180	0.114 ± 0.000 (0.0)	0.89 ± 0.02 (2.1)	4.75 ± 0.09 (1.9)

*n = 2 for these samples.

The plasma samples collected from dogs in the biopharmaceutic and pharmacokinetic studies and from man in the single-dose pharmacokinetic study were stored at −70°C prior to analysis.

ACKNOWLEDGEMENTS

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CHROMBIO. 1731

SIMULTANEOUS DETERMINATION OF DIPROPHYLLINE, PROXYPHYLLINE AND THEOPHYLLINE IN SERUM BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A selective and reliable high-performance liquid chromatographic assay for the simultaneous determination of diprophylline, proxyphylline and theophylline is described. The method involves a single extraction procedure followed by separation on an ODS reversed-phase column using a ternary solvent system. The assay is sufficiently rapid and sensitive to be applied for pharmacokinetic studies as well as for routine monitoring of patient's serum after therapeutic doses of the combined preparation. The practicability and utility of the proposed method is demonstrated in a pharmacokinetic study on four healthy volunteers.

INTRODUCTION

The methylxanthine derivatives diprophylline [7-(2,3-dihydroxypropyl)-theophylline] and proxyphylline [7-(2-hydroxypropyl)-theophylline] have therapeutic properties similar to those of theophylline and can be used for the treatment of obstructive lung diseases. There is some evidence that a combined preparation of these agents with theophylline (Neo-Biphylline^{R}) may exhibit less frequent adverse side-effects than an equivalent dose of theophylline alone [1, 2].

Although guidelines for the monitoring of these xanthines during therapy have not yet been developed, it can be expected that serum concentration measurements will be required in the same situations as proposed for theophylline [3]. Several methods exist to measure theophylline in biological fluids, the most popular being spectrophotometric assay [4], enzyme immunoassay [5], gas chromatography [6] and high-performance liquid chromatography (HPLC) [7—17]. In situations, however, where a combination of

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different xanthines is used, immunological methods are not suitable and, therefore, only chromatographic methods can be applied to measure these drugs simultaneously.

In the present study we describe a sensitive and selective HPLC method, using an isocratic reversed-phase system, to determine diprophylline, proxyphylline and theophylline in serum or plasma. By using this method, the pharmacokinetics of these three methylxanthines have been studied in four healthy volunteers after an oral administration of this drug combination.

MATERIALS AND METHODS

Ragents

All reagents were analytical grade. Proxyphylline and diprophylline were obtained from G. Streuli (Uznach, Switzerland). Theobromine was from Siegfried (Zofingen, Switzerland) and 8-chlorotheophylline from Aldrich (Milwaukee, WI, U.S.A.). All other reagents were purchased from E. Merck (Darmstadt, G.F.R.).

Chromatographic conditions

Analyses were performed on an HPLC system consisting of a Constametric II pump (Milton Roy, Philadelphia, PA, U.S.A.) and a Tracor Model 970 variable-wavelength absorbance detector (Tracor, Austin, TX, U.S.A.) set at 274 nm and 0.04 a.u.f.s. A 25 cm × 4.6 mm I.D. Ultrasphere 5-μm ODS reversed-phase column (Beckman, Berkeley, CA, U.S.A.) was used together with a 5 cm × 3.2 mm I.D. precolumn dry-filled with 30—38 μm Co:Pell ODS (Whatman, Clifton, NJ, U.S.A.). The system was operated at 40°C at a flow-rate of 1.5 ml/min developing a pressure of about 170 bar. The mobile phase consisted of 0.01 *M* sodium acetate buffer (pH 5.2)–acetonitrile–methanol (91:6:3, v/v), and was degassed and filtered before use.

Sample preparation

Serum (0.5 ml) or plasma from patients and calibration standards were transferred to a 12-ml glass-tube followed by 0.2 ml of 0.1 *M* phosphate buffer, pH 7.0. Proteins were precipitated by addition of 3 ml of 2-propanol, containing 1.5 μg of 8-chlorotheophylline as internal standard. The mixture was intensively stirred for 5 sec at 40,000 rpm using a high-speed dental micromotor (Bien-Air, Bienne, Switzerland) equiped with a Teflon mixing head. After centrifugation for 2 min at 3500 *g* the supernatant was transferred to a conical glass-tube and evaporated at 60°C under a stream of nitrogen. The residue was dissolved in 50 μl of methanol and 10 μl were injected using a WISP autosampler (Waters Assoc., Milford, MA, U.S.A.). All analyses were performed in duplicate.

Preparation of standard curves

For the standard curves stock solutions of theophylline, proxyphylline and diprophylline were made in water–ethanol (80:20, v/v). These solutions were further diluted with drug-free human serum to give final concentrations of 0.25, 1, 2, 4, 8, and 12 μg/ml. Peak height ratios between drug and internal

standard were plotted against drug concentrations and analysed with a linear regression method yielding straight lines for all three components.

Recovery and reproducibility

In order to estimate the analytical recovery, serum samples were spiked with 10 μg/ml theophylline, proxyphylline, diprophylline and 8-chlorotheophylline, and extracted as before. Peak height ratios of these samples were then compared with samples to which the same amounts of the three components were added after the preparation procedure just before injection. Within- and between-day reproducibility and accuracy were determined by analysing ten samples containing 1.5 and 10 μg/ml of the three components, on the same day and on ten different days.

Stability of serum samples

In order to assess the stability of theophylline, proxyphylline and diprophylline, fresh serum samples were stored at 4°C or 21°C, respectively, and assayed after 0, 1, 2, and 7 days.

Drug administration and sample collection

Four healthy volunteers (two males and two females) aged 23—32 years and weighing 51—82 kg received an oral dose of 300 mg of proxyphylline, 300 mg of diprophylline and 200 mg of theophylline dissolved in 40 ml of water. Blood samples were collected immediately before and at 5, 10, 20, 30, 45 min, and 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 18, and 24 h after administration. After centrifugation plasma was stored frozen at −20°C until assayed.

RESULTS AND DISCUSSION

A number of solvents at various pH values has been evaluated for the HPLC analysis to give an optimal resolution of the xanthine analogues in the shortest time possible. With the ternary solvent system finally used and, due to the high resolution power of the 5-μm ODS column, the desired separation could be achieved within 16 min (Fig. 1). The pH of the solvent had to be carefully controlled as the retention time for diprophylline was very sensitive to small changes of pH. The retention times of the various compounds are listed in Table I. The interference of the caffeine metabolite 1,7-dimethylxanthine (paraxanthine) could be minimized, whereas in most of the previous published studies using a reversed-phase separation system, paraxanthine either could not be separated from theophylline or was not investigated at all [7—14]. However, some authors have reported a good separation of paraxanthine, using either a straight-phase HPLC system [16] or reversed-phase ion-pair gradient elution [17]. In order to reduce the viscosity of the mobile phase and, as a consequence, to reduce the back-pressure, the column temperature was maintained at 40°C during the HPLC analysis. Because we used only a single extraction step, a precolumn was found essential to prolong the life of the analytical column. The precolumn was replaced after 60 injections.

The analytical recoveries for theophylline, proxyphylline and diprophylline were between 94.7% and 96.0%, and for the internal standard, 8-chloro-

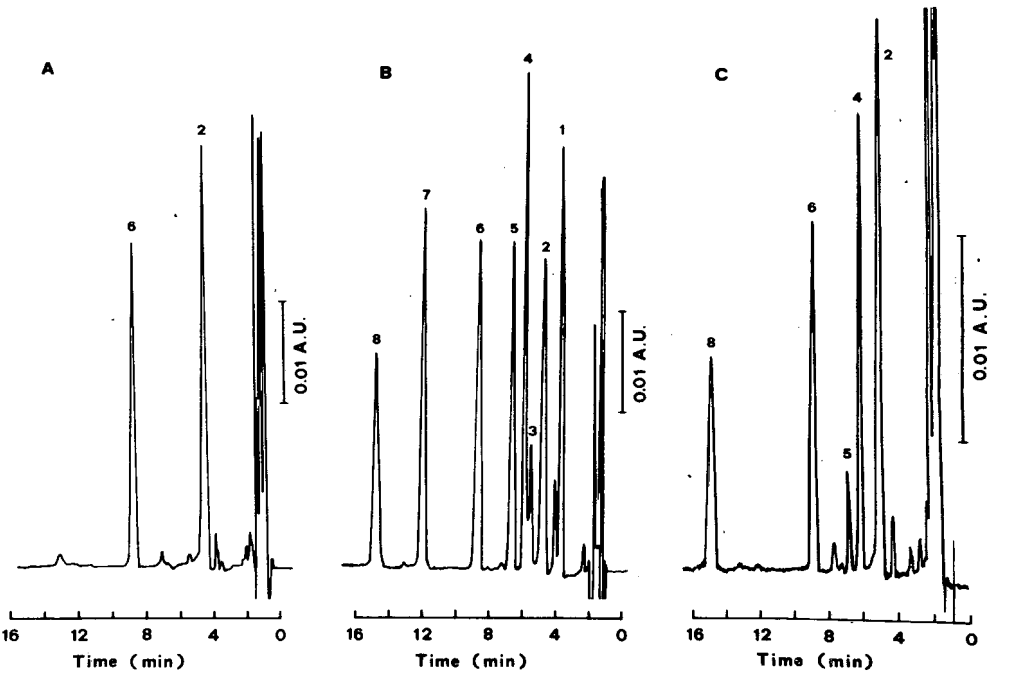

Fig. 1. Chromatograms of (A) control serum, (B) serum spiked with a mixture of xanthines, and (C) serum obtained after a single dose of 300 mg of diprophylline, 300 mg of proxyphylline and 200 mg of theophylline. 1 = Theobromine, 2 = unknown serum constituent, 3 = paraxanthine, 4 = theophylline, 5 = diprophylline, 6 = internal standard (8-chlorotheophylline), 7 = caffeine, 8 = proxyphylline.

TABLE I

RETENTION TIMES OF THE INVESTIGATED XANTHINE DERIVATIVES

Conditions of separation were as described in the text.

Xanthine derivatives	Retention time (min)
Theobromine	3.8
Paraxanthine	5.7
Theophylline	5.95
Diprophylline	6.7
8-Chlorotheophylline	8.85
Caffeine	12.1
Proxyphylline	15.1

theophylline, this recovery was 90.1%. Standard curves for all three xanthines are displayed in Fig. 2. They are strictly linear with coefficients of correlation better than 0.999.

Within-day and between-day reproducibility and accuracy for serum samples containing 1.5 and 10 μg of diprophylline, proxyphylline and theophylline per ml was excellent as summarized in Table II. Based on a signal-to-noise ratio of

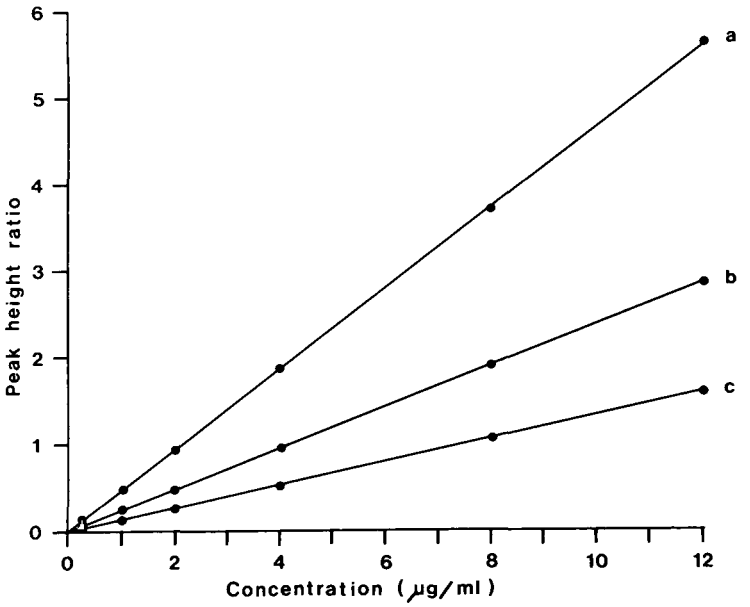

Fig. 2. Standard curves of theophylline (a), diprophylline (b) and proxyphylline (c) using 8-chlorotheophylline as internal standard.

TABLE II

PRECISION AND ACCURACY OF THE HPLC METHOD FOR THE DETERMINATION OF THEOPHYLLINE, DIPROPHYLLINE AND PROXYPHYLLINE IN PATIENT'S SERUM

Compound	Serum conc. (μg/ml)	Within-day (n = 10)			Day-to-day (n = 10)		
		Mean conc. (μg/ml)	C.V.* (%)	M.E.** (%)	Mean conc. (μg/ml)	C.V.* (%)	M.E.** (%)
Diproxyphylline	1.5	1.56	1.28	4.0	1.50	2.67	0
	10	10.1	0.89	1.0	9.96	1.6	0.4
Proxyphylline	1.5	1.53	1.31	2.0	1.48	2.03	1.3
	10	10.1	1.88	1.0	9.89	1.11	1.1
Theophylline	1.5	1.56	0.64	4.0	1.50	1.33	0
	10	9.97	0.90	0.3	9.95	1.11	0.5

*C.V. = coefficient of variation.
**M.E. = mean error.

3:1, the detection limits were 0.2 μg/ml for diprophylline, 0.25 μg/ml for proxyphylline and 0.1 μg/ml for theophylline.

The stability of all three investigated xanthines in serum at 4°C or room temperature was good. For up to seven days no significant reduction of the serum concentration could be observed. This is of practical importance, as

often the turn-around time for patient samples might be extended, especially over weekends.

The utility of the proposed HPLC method could be demonstrated by a pharmacokinetic study in four healthy volunteers. A dose of 300 mg of diprophylline, 300 mg of proxyphylline and 200 mg of theophylline was given orally to each of the participants. In Fig. 3 an example of the concentration—time course of one volunteer is displayed. After a very rapid absorption of the oral solution, proxyphylline and theophylline serum concentrations could be followed for at least 25 h, and the more-rapidly eliminated diprophylline for about 8 h. The serum concentrations were fitted to a one-compartment open model for oral dosage and the corresponding pharmacokinetic parameters were calculated on a Hewlett-Packard HP 85 desktop computer using the G-PHARM pharmacokinetic program developed by Gomeni and Gomeni [18]. The pharmacokinetic data for all three xanthines are listed in Table III. There is no evidence that the pharmacokinetics are influenced by administering these three xanthines together, as the calculated parameters were similar to those published before for single administration of each of these drugs [19—21]. The following antibiotics and other drugs sometimes used concomitantly with xanthines in patients with chronic bronchitis did not interfere with the HPLC method: carbenicillin, cefoperazone, cephacetril, penicillin G, diphenylhydantoin, phenobarbital and heparin. Only cefoxitin had a retention time similar to that of the internal standard.

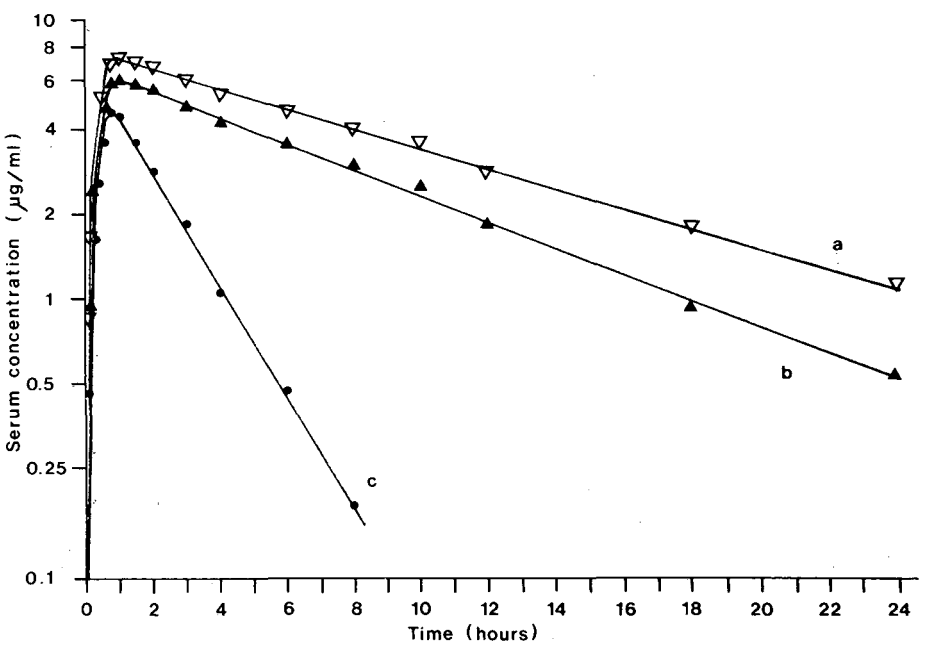

Fig. 3. Serum concentration—time curves after oral administration of 200 mg of theophylline (a), 300 mg of proxyphylline (b) and 300 mg of diprophylline (c).

TABLE III

PHARMACOKINETIC PARAMETERS* CALCULATED FROM SERUM CONCENTRATIONS AFTER ORAL ADMINISTRATION OF 300 mg OF DIPROPHYLLINE, 300 mg OF PROXYPHYLLINE AND 200 mg OF THEOPHYLLINE TO FOUR HEALTHY VOLUNTEERS

Values are expressed as mean ± S.D.

	Compound		
	Diprophylline	Proxyphylline	Theophylline
k_a (h^{-1})	2.47 ± 0.56	2.48 ± 1.30	2.14 ± 1.22
$t_{½a}$ (h)	0.29 ± 0.07	0.33 ± 0.16	0.39 ± 0.16
C_{max} (mg l^{-1})	4.55 ± 0.42	6.87 ± 1.53	5.97 ± 1.27
T_{max} (h)	1.25 ± 0.61	1.12 ± 0.55	1.50 ± 0.58
β (h^{-1})	0.348 ± 0.057	0.080 ± 0.003	0.105 ± 0.007
$t_{½\beta}$ (h)	2.03 ± 0.30	8.69 ± 0.28	6.64 ± 0.46
Cl** (l h^{-1} kg^{-1})	0.295 ± 0.044	0.051 ± 0.003	0.048 ± 0.019
V_β** (l kg^{-1})	0.862 ± 0.079	0.637 ± 0.041	0.464 ± 0.014

*k_a = absorption rate constant, $t_{½a}$ = absorption half-life, C_{max} = maximum serum concentration, t_{max} = time to reach C_{max}, β = elimination rate constant, $t_{½\beta}$ = elimination half-life, Cl = total body clearance, V_β = apparent volume of distribution.
**Cl and V_β are calculated assuming total absorption of the drugs [19, 22, 23].

CONCLUSION

With the highly selective HPLC method proposed in this study it is possible to determine diprophylline, proxyphylline and theophylline simultaneously in patient serum after therapeutic doses. The assay is fast, simple and reliable and is, therefore, very suitable for the routine laboratory. In addition, due to its sensitivity, this method can also be used for pharmacokinetic studies.

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CHROMBIO. 1733

AUTOMATED LIQUID CHROMATOGRAPHIC ANALYSIS OF THE ANTI-TUMORIGENIC DRUGS ETOPOSIDE (VP 16-213) AND TENIPOSIDE (VM 26)

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SUMMARY

A method is described for the fully automated analysis of large numbers of 1—2 ml serum and plasma or urine samples containing the anti-tumorigenic drugs etoposide and teniposide and their aglycone. The blood samples are hydrolysed by a proteolytic enzyme, subtilisin A, prior to preconcentration on a small precolumn. The hydrolysis step serves both to release the strongly protein-bound drugs and to prevent clogging of the chromatographic system. On-line preconcentration is carried out with precolumns packed with PRP_1, a microparticulate divinylbenzene—styrene copolymeric sorbent. Chromatography takes place, after column switching, in a C_{18}/methanol—water system. After a post-column clean-up step using continuous extraction with dichloroethane in an autoanalyzer system, native fluorescence of these analytes is used for detection of the drugs. Recovery of etoposide and teniposide from spiked serum and plasma samples was 100%.

Calibration curves of etoposide and teniposide typically show correlation coefficients of 0.9994 over a two-to-three order linear range. The detection limit of etoposide is approx. 8 ng per sample. Repeatability was found to be excellent. Unattended overnight routine analysis is possible without any problems. This method, considering optimal sample throughput, reliability and selectivity, competes favourably with existing techniques for the analysis of etoposide and teniposide.

INTRODUCTION

Recently several liquid chromatographic procedures for the determination of the antineoplastic agents etoposide (VP 16-213) [1] and teniposide (VM 26) have been described [2—5]. In these procedures off-line preconcentration by

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liquid—liquid extraction and evaporation to dryness is carried out, followed by reversed-phase chromatography in a C_{18}/methanol—water system and detection either by UV absorption at 254 and 280 nm or by native fluorescence. Recently, electrochemical detection has also been described [6].

A major drawback of the procedures reported in the literature is the time-consuming and cumbersome off-line sample preparation. Further, special care has to be taken to ensure that quantitative extraction of the strongly protein-bound drugs [1] takes place and that the structure of the drugs remains unimpaired.

In this paper we describe the use of mild protein hydrolysis by a proteolytic enzyme as a sample preparation step to release the drugs and to allow repeated injection of large (0.2—2 ml) amounts of blood samples directly into the chromatographic system (cf. ref. 7). The hydrolysed serum and plasma samples are preconcentrated on a short precolumn and, after column switching, analysed in a C_{18}/methanol—water system followed by continuous post-column liquid—liquid extraction as a clean-up step, and fluorescence detection.

EXPERIMENTAL

Liquid chromatography

Automated preconcentration and high-performance liquid chromatographic (HPLC) analysis were carried out with a Kontron (Zürich, Switzerland) liquid chromatograph, consisting of two Model 410 pumps, an MSI 660 autosampler, a Model 200 programmer, a prototype of the MCS 670 column switching apparatus and a Perkin-Elmer (Norwalk, CT, U.S.A.) 3000 fluorescence detector set at λ_{ex} = 230 nm and λ_{em} = 328 nm. Signals were recorded on a W + W 900 (Kontron) recorder. Quantitation of the signals was carried out manually. The apparatus used is schematically shown in Figs. 1 and 2.

The post-column extraction system consisted of a Technicon (Tarrytown, NY, U.S.A.) AutoAnalyzer pump Model II, equipped with Acidflex tubing in order to pump the extraction solvent dichloroethane. The flow-rate of the reagent stream was 0.6 ml/min, the flow-rate through the fluorescence detector

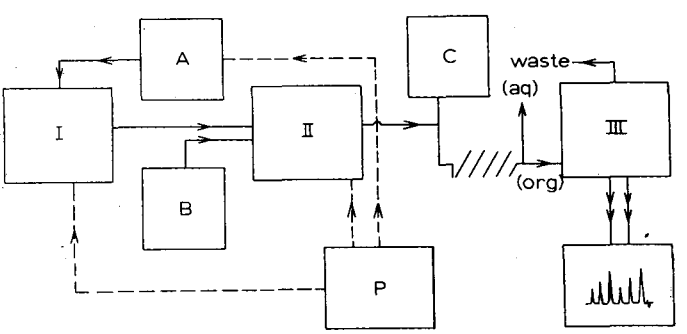

Fig. 1. Scheme of the apparatus for automated analysis of etoposide and teniposide. I, Autosampler; II, column switching apparatus; III, fluorescence detector; A and B, HPLC pumps; C, autoanalyzer pump; P, programmer.

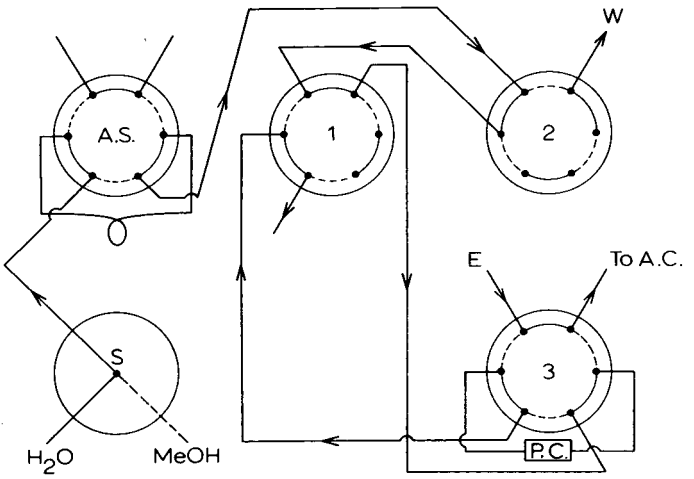

Fig. 2. Switching valve configuration. A.S., autosampler injector equipped with 1.6-ml loop. S, low-pressure selector valve. 1, 2, 3, high-pressure switching valves. A.C., analytical column. E, eluent, P, precolumn. W, waste. The indicated configuration (solid line) applies for the preconcentration step.

0.3 ml/min. Extraction took place in 2 mm I.D. standard Technicon glass reactors with ten or twenty coils. The phase separator was a modified Technicon one with PTFE insert [8].

Chromatography was carried out with a 125 × 4.0 mm I.D. LiChroCart column, prepacked with 10-μm LiChrosorb RP-18 (Merck, Darmstadt, G.F.R.) or with a 100 × 4.1 mm I.D. stainless-steel column, home packed with 5-μm Nucleosil C_{18} (Macherey & Nagel, Düren, G.F.R.). Methanol—water mixtures with or without 1% (v/v) glacial acetic acid were used as mobile phase. The mobile phase flow-rate was 1 ml/min.

Preconcentration was carried out on home-made 2 × 4.6 mm I.D. or 10 × 2 mm I.D. precolumns which were slurry-packed by micro-spatula or by syringe [9] with Nucleosil C_{18} or PRP_1 (Hamilton, Bonaduz, Switzerland), a micro-particulate (10-μm) divinylbenzene—styrene copolymer.

Chemicals

Etoposide, teniposide and aglycone were received as a gift from J.M.S. van Maanen (Dutch Cancer Institute, Amsterdam, The Netherlands). The structures of these compounds are given in Fig. 3. Aglycone was used as internal standard for etoposide.

Subtilisin A was purchased from Novo Industry A/S (Bagsvaerd, Denmark), and proteinase K from Merck. All solvents were purchased from Baker (Deventer, The Netherlands) and were of analytical grade. All aqueous solutions were prepared with demineralized water, treated in a Milli Q (Millipore, Bedford, MD, U.S.A.) ultrafiltration system. Eluents and urine samples were filtered through an all-glass filtration apparatus with 0.2-μm filters (Millipore) and degassed under vacuum before use. Frozen sterilized calf serum was

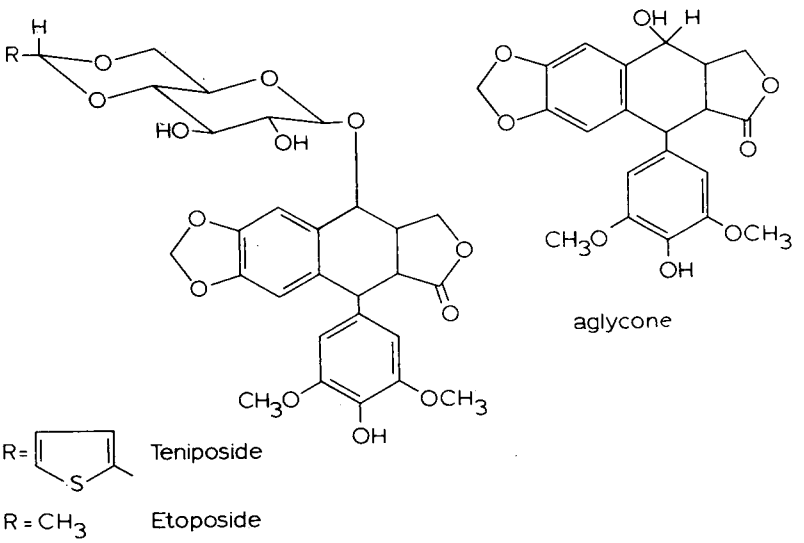

Fig. 3. Structures of etoposide, teniposide and aglycone.

obtained from Flow Laboratories (Irvine, Great Britain). Frozen human plasma was received as a gift from the blood transfusion service of the Onze Lieve Vrouwe Gasthuis (Amsterdam, The Netherlands).

PROCEDURE

Enzymatic hydrolysis

Aliquots of water, plasma or serum samples spiked with etoposide and/or teniposide were incubated with equal amounts of 1 mg/ml aqueous subtilisin A solution for 15 min at 50°C. Incubation with proteinase K took place for 10 min at ambient temperature.

Column switching

Automated preconcentration, precolumn clean-up and precolumn switching were carried out according to the procedure described in Table I. The individual steps of the procedure were designed to effect optimal performance regarding analysis time, sample throughput, recovery of analyte, sample clean-up, absence of cross-contamination and absence of clogging problems.

Hydrolysed blood and/or filtered urine samples were put in the tray of the autosampler and subsequently transferred to the precolumn using water as the carrier stream at a flow-rate of 1 ml/min (see Fig. 1). The precolumn was then backflushed with 4 ml of water at a flow-rate of 2 ml/min to remove large protein fragments, or other, hydrophilic, matrix compounds and to effect a partial sample clean-up. The precolumn was then switched on-line with the analytical column and backflushed for 30 sec using a methanol—water mixture. Sample analysis took 5—10 min. Meanwhile, the precolumn was backflushed with 6 ml of methanol and a flow-rate of 2 ml/min to remove strongly retained

TABLE I

PROGRAM FOR THE AUTOMATED ANALYSIS OF ETOPOSIDE, TENIPOSIDE AND AGLYCONE USING A PRECOLUMN

Time (min)	Event	
0	start FILE 12	
0	start FILE 11	
0	start autosampler	Filling sample loop
2.7	start FILE 10 (% B 100, flow 1 ml/min, AUX 2 DUR 4)	Preconcentration on PC* with water
4.7	AUX 1 DUR 7.5 (flow 2 ml/min)	Backflush washstep with water
6.7	AUX 3 DUR 0.5	Elution of sample from PC
	AUX 5 DUR 0.01	Changing from water to methanol (to waste)
	flow 10 ml/min	
7.2	AUX 2 DUR 3	Backflush washstep of PC
	flow 2 ml/min	with methanol**
10.2	AUX 5 DUR 0.01	Changing from methanol to water (to waste)
	flow 10 ml/min	
10.7	AUX 2 DUR 1	Backflush PC with water
	flow 2 ml/min	to remove methanol
11.7	flow 0	
11.8	END	End/start again at t_0 depending on number of samples programmed in file 12

*PC = precolumn.

**Extra wash step to remove plasma components from the precolumn that would interfere with a subsequent etoposide analysis at high sensitivity.

compounds and then reconditioned with 2 ml of water at a flow-rate of 1 ml/min. The total program for the Model 200 microprocessor is given in Table I. The switching valves configuration is depicted in Fig. 2.

RESULTS AND DISCUSSION

Selection of packing materials

Preconcentration of etoposide from aqueous solution on a short (2 × 4.6 mm I.D.) precolumn packed with C_{18} chemically bonded silica could not be carried out with 100% recovery as breakthrough occurred even after 2 ml, due to insufficient retention. Since it is necessary to backflush the precolumn with a few milliliters of water after sampling 1—2 ml of serum or plasma (cf. ref. 7), a breakthrough volume of 2 ml is not sufficient for the analysis of patient samples. Therefore, PRP_1 was used instead of the C_{18} material as this sorbent displays 10—20 times higher retention towards aromatic compounds than C_{18} packing materials [10]. With short (2 × 4.6 mm I.D.) PRP_1 precolumns, breakthrough of etoposide was not observed even after flushing with 20 ml of water. As chromatography of etoposide was not very efficient on PRP_1, C_{18} analytical columns were used in combination with PRP_1 precolumns.

Enzymatic hydrolysis

The necessity of enzymatic hydrolysis prior to preconcentration of blood samples in order to prevent clogging of the present chromatographic system was demonstrated as follows. Already after one injection of 0.5 ml of preconcentrated plasma (1:1 diluted with water) the pressure drop over the analytical column increased by 6 MPa. With subtilisin A-treated samples no increase of pressure was observed even after several hours of continous operation and passage of 30-ml hydrolysed blood samples. Upon injection of 0.5 ml of non-hydrolysed (1:1 diluted) plasma the recovery of etoposide was only 50%, while 100% recovery was obtained after protein hydrolysis with subtilisin A or proteinase K. Incubation of etoposide with subtilisin A at 55°C for 2 h was found not to affect the structure of the drug. The advantage of proteinase K over subtilisin A is that it can be used at ambient temperature. A disadvantage is its high price. For the present application, subtilisin A and proteinase K were found to be equally effective in protein hydrolysis. Further experiments were all carried out with subtilisin A.

Detection

Unfortunately, clean-up of the preconcentrated samples via washing with 6 ml of water was not sufficiently effective to remove all the compounds which interfere with the detection of the drugs by means of UV or fluorescence monitoring. Therefore a post-column extraction step with dichloroethane was introduced which was found to provide extremely low fluorescence background signals with hydrolysed blood samples and reasonably low background signals with urine samples. Due to the higher background with urine samples the detection limit was somewhat higher (30 ng) than with hydrolysed (1:1 diluted) blood samples (8 ng) of the same volume (1.6 ml). The concentration range of etoposide in human plasma during the first 24 h after an intravenous therapeutic dose of 100 mg/m^2 typically is 20—0.5 μg/ml [3, 5].

Quantitation

Internal standardization. Both teniposide and aglycone can be used as internal standards in the analysis of etoposide. Fig. 4 shows chromatograms obtained after duplicate injection of plasma samples spiked with etoposide and teniposide. Separation of aglycone and etoposide requires a lower methanol content in the eluent (45%) than that of teniposide and etoposide (55% methanol). Since the use of less methanol causes a lower noise level in the extraction detector, the use of aglycone as internal standard for etoposide is to be preferred. Calibration curves for subtilisin A-treated plasma and serum samples spiked with etoposide using either teniposide or aglycone as internal standard were found to be linear over the concentration range 0.01—25 μg/ml. The correlation coefficient typically was 0.9994 (n = 10). The detection limit of etoposide, in plasma, is approx. 8 ng (see Fig. 5). This figure is of the same order of magnitude as with HPLC and electrochemical detection [6] and better than with HPLC and fluorescence detection using liquid—liquid extraction as sample pretreatment step (150 ng/ml) [3].

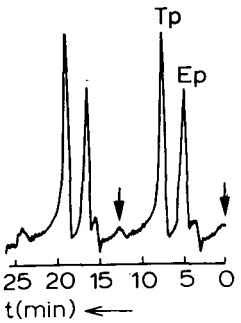

Fig. 4. Duplicate injection (see Table I) of 1.6 ml of a mixture of etoposide (Ep) and teniposide (Tp). Amounts injected: teniposide, 1.09 μg; etoposide, 503 ng. Chromatographic conditions: column, LiChroCart C_{18}; fluorescence detection after post-column extraction; mobile phase, methanol—water (55:45, v/v); flow-rate, 1 ml/min. Further conditions, see text.

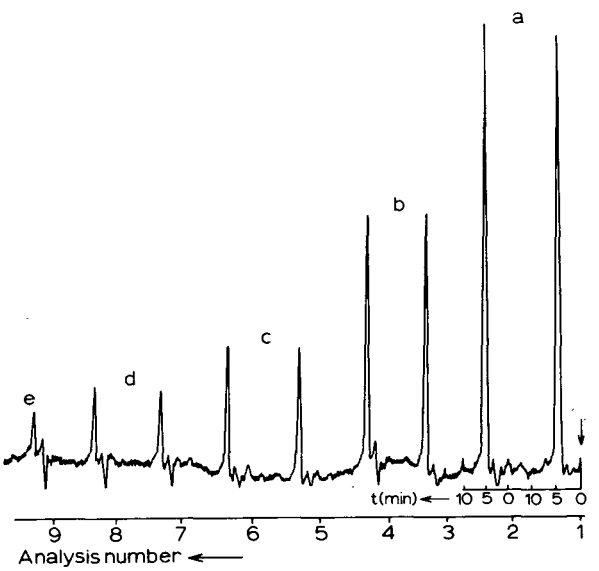

Fig. 5. Automated determination of the calibration curve and detection limit of etoposide in 0.5 ml of hydrolysed plasma samples. Amount of etoposide (in ng): (a) 257, (b) 158, (c) 82, (d) 43, (e) 23. Procedure: see text. Chromatographic conditions: column, 5-μm Nucleosil C_{18}; mobile phase, methanol—water—glacial acetic acid (46:64:1), pH 3.3; flow-rate, 1 ml/min. Further conditions, see Fig. 4.

External standardization. Plotting of peak area versus concentration of etoposide in hydrolysed plasma samples and aqueous solutions yielded identical calibration curves. In other words, the recovery of the drug from the hydrolysed blood samples is quantitative. This excellent result is caused by the efficient action of subtilisin A.

Plotting of peak heights instead of areas resulted in calibration curves with steeper slopes for the aqueous samples than for the blood samples (see Fig. 6). This is caused by a 85% increase in peak width upon preconcentration from

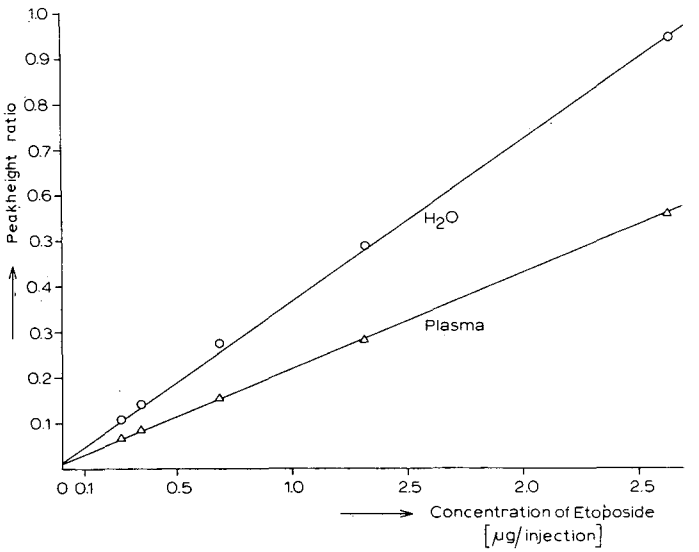

Fig. 6. Calibration curves of etoposide in water (○) and hydrolysed plasma (△). Ordinate: ratio of peak height of etoposide/aglycone. Abscissa: concentration of etoposide (µg/ml). Sample size: 1.6 ml.

plasma as compared to preconcentration from purely aqueous samples. The additional peak broadening is due to the fact that the surface of the sorbent in the precolumn is largely occupied by matrix constituents, which causes spreading of the analyte zone. With aged repeatedly frozen and thawed serum samples, which are known to contain smaller protein fragments [7], additional band broadening was much less than with fresh plasma samples.

Routine analysis

The present method has been used routinely without problems for several months. The repeatability was ± 2.2% (mean relative S.D.) (n = 10). Due to fluctuations in the response of the fluorescence detector, daily calibration was necessary. Using the program given in Table I, unattended operation was possible even with relatively large injection volumes, without increase of pressure or baseline shifts. As the precolumns are easily replaced, newly packed precolumns were installed every day, in order to prevent problems due to loss of performance of the stationary phase material. In all probability, however, precolumn lifetime is much longer than a single day.

One of the interesting aspects of the use of (inexpensive) precolumns is the possibility of sample collection and storage. Etoposide-spiked, hydrolysed plasma samples were preconcentrated and the precolumns stored for 40 h at −20° C. After analysis, the recovery was still found to be 100%.

CONCLUSIONS

A selective and sensitive method for the automated liquid chromatographic analysis of total etoposide and teniposide in biological samples has been devel-

oped. The method can be carried out without cumbersome sample preparation steps even with repeated injection of relatively large (1—2 ml) serum and plasma samples. Preconcentration is carried out on short (2 × 4.6 mm I.D.) PRP_1 precolumns and is followed by a backflush wash step with water and subsequent separation in a C_{18}/methanol—water system. Selective detection takes place after post-column extraction to 1,2-dichloroethane using the native fluorescence of the drugs. Continuous, unattended routine analysis of enzymatically hydrolysed blood samples is possible. The detection limits are comparable to those described in the literature, but the selectivity of the present method is much better.

The principle of on-line sample clean-up by a post-column extraction system presented here may well be more widely applicable and should be further investigated.

ACKNOWLEDGEMENTS

J.M.S. van Maanen (Dutch Cancer Institute, Amsterdam, The Netherlands) is thanked for the gift of etoposide, teniposide and aglycone. Kontron Ltd. (Zürich, Switzerland) is thanked for the loan of the column switching unit and other apparatus to carry out this work.

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DETERMINATION OF ETRETINATE AND ITS MAIN METABOLITE IN HUMAN PLASMA USING NORMAL-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

An analytical method for the determination of two aromatic retinoids in human plasma (etretinate and its main metabolite) is described, using normal-phase high-performance liquid chromatography. The method is highly sensitive (4 ng/ml) and selective, and allows good separation of isomerization products. Both compounds are well extracted (95%) from plasma in the practically important concentration range (10—1000 ng/ml). After chromatographing the compounds together with an internal standard, they are quantified by spectrophotometry.

INTRODUCTION

Vitamin A analogues have been subjected to considerable investigational effort, which has been intensified in recent years and is still increasing [1—4]. One of the prerequisites for all experimental work are analytical methods for the determination of certain compounds selectively, quantitatively and in very low concentrations in different kinds of biological samples. Many different methods have been developed for the separation of natural vitamin A-related compounds, most of them using liquid chromatography [5—13]. Radioimmunoassays [14] and bioassays [15] for vitamin A have been described.

For one of the best known retinoids, the aromatic compound etretinate, several analytical procedures have been developed, based both on normal-phase as well as on reversed-phase high-performance liquid chromatography (HPLC) [16—18]. However, these methods need to be improved to meet the special requirements encountered in routine analysis during clinical and pharmacokinetic studies. The limit of detection is usually too high, which is a handicap

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when investigating prolonged elimination phases in which the concentrations of both parent compound as well as main metabolites in plasma are low. Good assay reproducibility is a special requirement for all clinical and pharmacokinetic studies.

Closely related compounds, such as *cis—trans* isomers, must be separated. This is a general need in the field of vitamin A analogues, but especially in the case of etretinate, where such isomers are present as biotransformation products. It is the aim of the present report to describe and discuss an analytical method which is, compared to other recently published procedures [16—18], highly sensitive and selective. The use of adsorption chromatography has the advantage of avoiding solvent programming as described in the publications aldready mentioned. In addition, the life time of normal-phase columns, in our hands, was found to be superior to that of reversed-phase columns. The method allows routine determination of both parent drug (etretinate) and its main metabolite (Ro 10-1670), the analogous carboxylic acid. In addition, it provides estimates of various isomerization products of both compounds which, from the point of view of quality control, is important. The method has been used mainly in several clinical pharmacokinetic studies, but also with slightly modified extraction procedures to determine plasma levels in several animal species.

EXPERIMENTAL

Materials

The reagents were of analytical grade unless otherwise stated: *n*-hexane (E. Merck, Darmstadt, G.F.R.), tetrahydrofuran, ethyl acetate, glacial acetic acid, methyl acetate (Merck, synthetic grade); diethyl ether (ad narcosin, Siegfried AG, Zofingen, Switzerland), isopropanol; saturated aqueous sodium sulphate solution at room temperature; nitrogen gas 99.99% (Carba AG, Basle, Switzerland).

Solutions

Mobile phase for HPLC. The mobile phase (*n*-hexane—tetrahydrofuran—glacial acetic acid 200:3:1.2, v/v) was prepared by mixing the three solvents after degassing. The mobile phase was recycled to achieve constant composition.

Standard solutions. Stock standard solutions of all three substances (etretinate, its main metabolite the analogous carboxylic acid Ro 10-1670, and retinoic acid) were prepared separately every two months by dissolivng 10 mg of the solid material in 100 ml of isopropanol to give a concentration of 100 ng/ml.

Working standard solutions containing both etretinate and Ro 10-1670 in two concentrations (10 ng/μl and 1 ng/μl) were freshly prepared every week by diluting 10 ml of each corresponding stock solution to 100 ml with isopropanol, giving a concentration of 10 ng/μl. From this solution 10 ml were then diluted to 100 ml.

Internal standard (retinoic acid) working standard solution was separately prepared by diluting 0.5 ml of the stock standard to 100 ml with isopropanol.

Note: Because of extreme sensitivity of this class of compounds to isomerization under the influence of light, all laboratory work was performed under shortwave light protection and substances were handled in amber or aluminium foil-wrapped glassware.

Plasma standards. Plasma standards were freshly prepared for each assay run, either to establish linearity of the method or for the daily calibration curve. Samples were spiked by pipetting the final volumes of working standard solutions into 0.5 ml of plasma.

Extraction procedure

A 0.5-ml volume of pooled human plasma, delivered by a blood bank, was pipetted into a 30-ml round-bottomed centrifuge tube using an Oxford pipettor. The 100 μl of internal standard working solution were added by an SMI-pettor, and vortexed for 5 sec; 0.5 ml of methyl acetate was added with an Oxford pipettor, and vortexed again for 5 sec. Then 0.5 ml of saturated sodium sulphate solution was added with an Oxford pipettor followed by 10 ml of ethyl acetate and the mixture extracted for 20 min on a Heidolph extractor at 20 rpm. After centrifuging for 10 min at 2000—3000 *g*, the organic phase was transferred into a 30-ml tapered centrifuge tube and evaporated to dryness at 40° C under nitrogen. The glass wall was rinsed with 2—3 ml of ether and again evaporated to dryness. The residue was stable for at least one week in a stoppered tube at 5° C.

High-performance liquid chromatography

Equipment. This comprised a Pye-Unicam LC-UV spectrophotometer (Pye-Unicam, Cambridge, Great Britain) set at 360 nm, a 250 × 3.2 mm column packed with LiChrosorb Si 60, 7 μm particle size (Merck), giving a theoretical efficiency of 10,000—15,000 plates at a flow-rate of 1 ml/min and ambient temperature, a Milton Roy 5000 pump (Milton Roy Company, St. Petersburg, PA, U.S.A.), a Rheodyne 7120 loop injector and a W+W 1100 recorder (Kontron, Zürich, Switzerland).

Procedure. The dry extract was dissolved in 200 μl of mobile phase, vortexed for 15 sec, and 100 μl were injected into the HPLC system.

Retention times were 2 min 50 sec, 4 min, and 6 min 50 sec, for etretinate, retinoic acid, and the main metabolite Ro 10-1670, respectively.

Calibration and calculation

Linearity of the complete assay procedure was established in the range 4—1000 ng/ml of plasma by analyzing spiked human plasma samples covering this range. A calibration curve, consisting of four different concentrations within the expected range of the plasma samples to be analyzed, was generated for each assay run by least-squares regression of the peak height ratios (spiked drug/internal standard) against the concentration of the drug. The unknown concentrations of plasma samples were determined from the calculated peak height ratio by interpolation from this calibration curve.

RESULTS AND DISCUSSION

Selectivity and choice of the internal standard

In developing an analytical method for the separation of closely related retinoids and in the search for an internal standard possessing similar chromatographic properties, several compounds of the vitamin A type were considered as potential candidates. The two main vitamin A components in human plasma, the palmityl ester and the free alcohol (retinol), can easily be separated in any appropriate chromatographic system. Many different human plasma samples from patients and healthy volunteers were screened for possible endogenous compounds which could interfere with the retinoids to be analyzed (Fig. 1, I). Most of these blank samples were completely free of such substances. An additional difficulty with the retinoids is the possibility of rapid isomerization under the influence of UV irradiation. The chromatographic system, therefore, must be able to separate these potential photoisomerization products [19]. Spiked plasma samples were exposed to light before starting the analytical procedure. The chromatograms obtained, compared with those from light-protected samples, showed several additional small peaks grouped around each main substance peak (Fig. 1, II). No effort was made to obtain baseline separation, because quantification of one of the isomers was not a major goal in the development of this method. However, there was one peak with a slightly shorter retention time than that of Ro 10-1670, the main metabolite, which appeared only in the chromatograms from patients undergoing chronic etretinate therapy (Fig. 1, III). As the influence of light could be excluded, formation of metabolite was the most probable explanation. Various synthetic isomers were added to these plasma extracts, and the 13-*cis* isomer of Ro 10-1670 co-chromatographed with this unknown component in two different mobile phases.

The ideal internal standard should have a polarity intermediate between that of etretinate and its main metabolite and should not, of course, be either a metabolite or an isomerization/decomposition product of the parent drug. Retinoic acid fulfils these requirements, as shown in earlier investigations [13].

Although retinoic acid appears regularly as a biotransformation product of vitamin A in plasma, its concentration is negligible compared with the internal standard-spiked concentration. Representative chromatograms are shown in Fig. 2. The average endogenous concentrations of retinoic acid are 1 ng/ml [20]; this is to be compared with an internal standard concentration, used here, of 50 ng/ml.

Recovery

In order to determine the extraction efficiency of both parent drug and its main metabolite, human plasma samples were spiked with ^{14}C-labelled material according to the spiking procedure described (see Experimental). Plasma samples at concentrations of 10, 100 and 500 ng/ml were prepared, extracted, and an aliquot of the final organic solution was counted in a liquid scintillation counter (W+W, Kontron) (Table I). The percentage of recovered radioactivity was about 97% for both compounds at all concentrations.

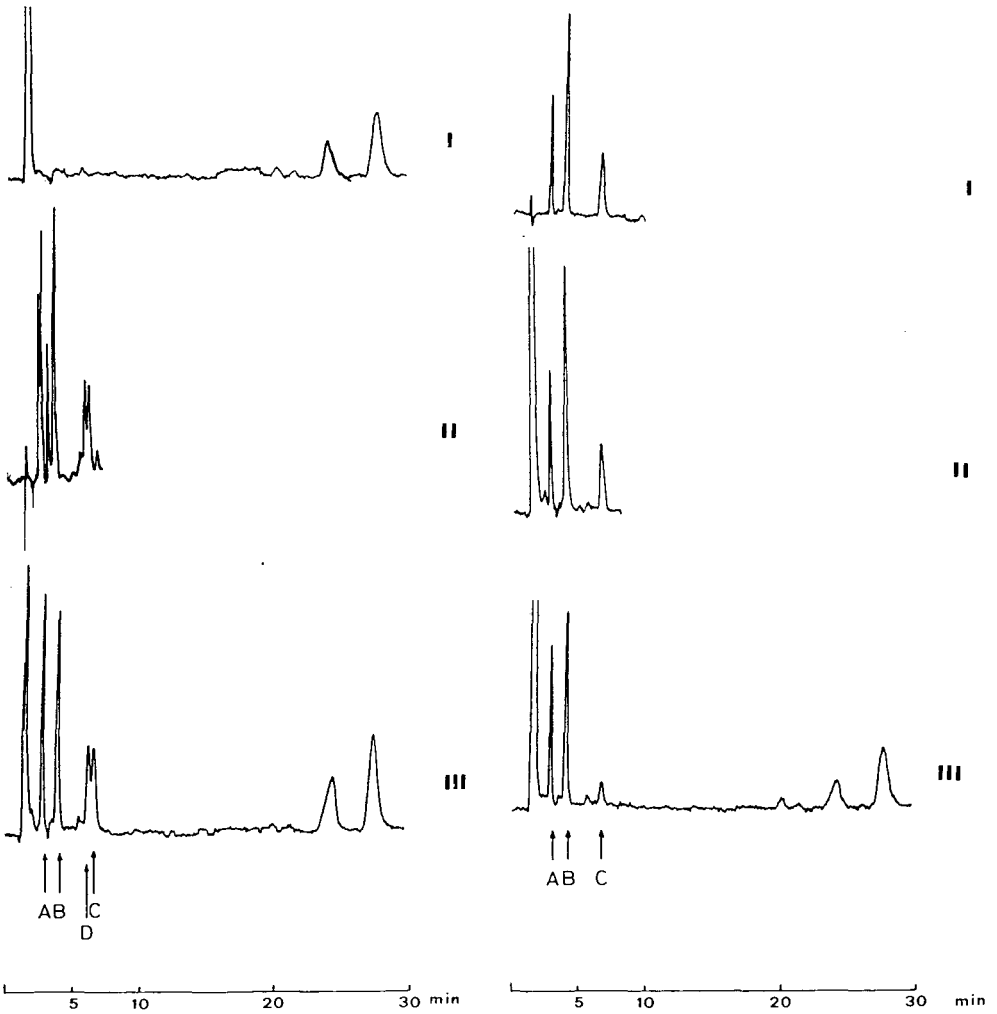

Fig. 1. (I) Chromatogram of a human blank plasma. (II) Chromatogram of a spiked human plasma sample, which was exposed to short-wavelength fluorescent light for 10 min. (III) Chromatogram of plasma from a patient undergoing chronic dosing. The 13-*cis* isomer (D) of Ro 10-1670 is shown.

Fig. 2. (I) Chromatogram of a mixture of etretinate (A), retinoic acid (B) and Ro 10-1670 (C) in mobile phase. (II) Chromatogram of human blank plasma, spiked with all three compounds (see Fig. 1), at a concentration of 25 ng/ml for A and C and 50 ng/ml for B. (III) Chromatogram of a volunteer's plasma 2.5 h after a single dose of 25 mg of etretinate.

Limit of detection

A signal-to-noise ratio of 3:1 corresponded to a concentration of 4 ng/ml etretinate and main metabolite.

TABLE I

EXTRACTION YIELD OF [^{14}C]ETRETINATE AND ^{14}C-LABELLED Ro 10-1670 FROM PLASMA

Spiked conc.* (ng/ml)	Etretinate			Ro 10-1670 (main metabolite)		
	Recovery (%)	C.V.** (%)	No. of replicates	Recovery (%)	C.V. (%)	No. of replicates
10	93.1	2.4	3	96.4	2.0	3
100	96.6	1.0	3	95.5	0.5	3
500	96.5	0.3	3	96.2	0.6	3

*Solvent concentration in spiked plasma samples was <1%.
**C.V. = coefficient of variation.

Linearity

Calibration samples were prepared from plasma samples taken from volunteers taking part in pharmacokinetic studies or from patients undergoing etretinate therapy. A linear correlation between peak height ratios and concentration over the range 4—2000 ng/ml was found. Coefficients of correlation calculated from individual time-independent calibration curves showed acceptable variation. Since 90% of the analyzed samples showed concentrations below 500 ng/ml, standard curves were routinely set up from 10 to 500 ng/ml.

Stability

Spiked plasma samples were stored under varying conditions over different periods of time and analyzed for etretinate. No degradation was found after eight weeks of storage at −20°C, neither did repeated thawing and freezing give rise to degradation. For the same storage period, about 20% loss was observed at +4°C; part of the degradation was due to hydrolysis to Ro 10-1670.

In order to obtain information about possible enzymatic degradation in both whole blood and plasma, biological samples were spiked with etretinate or Ro 10-1670 and incubated at 37°C for 1 h and 16 h, respectively. No Ro 10-1670 could be detected, either in blood after 1 h or in plasma after 16 h. The concentrations of etretinate remained constant for at least 8 h.

Accuracy and precision

In Table II reproducibility data are summarized. Spiked plasma samples covering a concentration range of 10—1000 ng/ml were analyzed in duplicate on five different days over a period of four weeks. The intra-assay reproducibility was calculated from the variation of duplicates, whereas the inter-assay reproducibility was established from the variation of the means of duplicate values. Inter-assay precision for etretinate and its main metabolite Ro 10-1670, calculated for the whole concentration range, was less than 4% and 3%, respectively. Inter-assay accuracy was 95%.

TABLE II

REPRODUCIBILITY DATA

Etretinate						Ro 10-1670 (main metabolite)					
Conc. added (ng/ml)	Found (%)	Found — expected (%)	No. of replicates	C.V. (%)		Conc. added (ng/ml)	Found (%)	Found — expected (%)	No. of replicates	C.V. (%)	
				Inter-assay	Intra-assay					Inter-assay	Intra-assay
10	94	—6	5	5.8	3.1	10	110	+10	5	6.4	14.4
100	95	—5	5	3.8	1.0	100	100	±0	5	1.5	1.3
500	94	—6	5	4.2	2.5	500	94	—6	5	3.5	1.6
1000	95	—5	5	3.2	1.7	1000	96	—4	5	2.5	1.9

Application of the method to biological samples

The method was developed primarily to analyze human plasma samples, either from patients on chronic etretinate therapy in whom plasma concentrations have to be controlled for safety reasons from time to time, or from volunteers who were administered etretinate in order to establish the pharmacokinetics of the drug. The method has also been used, with certain minor modifications, to determine etretinate concentrations in animal plasma and different tissues. The main problems hampering the method under these circumstances were endogenous compounds extracted from the tissues which interfere, chromatographically, with the compounds to be determined.

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CHROMBIO. 1748

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF 4′-(9-ACRIDINYLAMINO)METHANESULFON-*m*-ANISIDIDE IN PLASMA

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SUMMARY

A rapid and selective high-performance liquid chromatographic method for the measurement of 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (AMSA), a new anticancer drug, has been developed. The method employed an analogue of AMSA, 4′-(3-methyl-9-acridinylamino)methanesulphonanilide as internal standard. Plasma samples were acidified, washed with hexane, readjusted to pH 9.0 and extracted with diethyl ether. The evaporated extract was chromatographed on a Radial-Pak C_{18} column using acetonitrile—water containing 0.01 mol/l triethylamine phosphate as mobile phase. Detection was by UV absorbance at 254 nm. Chromatography time for each sample was 5.5 min. Using 0.5 ml of plasma, AMSA concentrations as low as 50 nmol/l could be measured with acceptable accuracy and precision. Patients' samples remained stable when stored at —20°C for up to one month. Plasma AMSA concentrations were followed for 24 h after 200 mg/m² infusions in two patients with acute myeloid leukemia. This method appears eminently suitable for investigation of the pharmacokinetics of AMSA in patients and laboratory animals.

INTRODUCTION

4′-(9-Acridinylamino)methanesulfon-*m*-anisidide (AMSA) (I) is an acridine derivative (Fig. 1) currently undergoing clinical evaluation as a chemotherapeutic agent for the treatment of human tumors. The drug has demonstrated significant activity against leukemias and lymphomas [1—5], with moderate activity against metastatic breast cancer [6—8] and malignant melanoma [9]. Although there is some information on the distribution of radiolabelled AMSA in rodent [10] and man [11], there is a lack of good pharmacokinetic studies in man using a specific analytical technique. A better knowledge of the kinetics of AMSA may provide a basis for its more rational use with regard to increased

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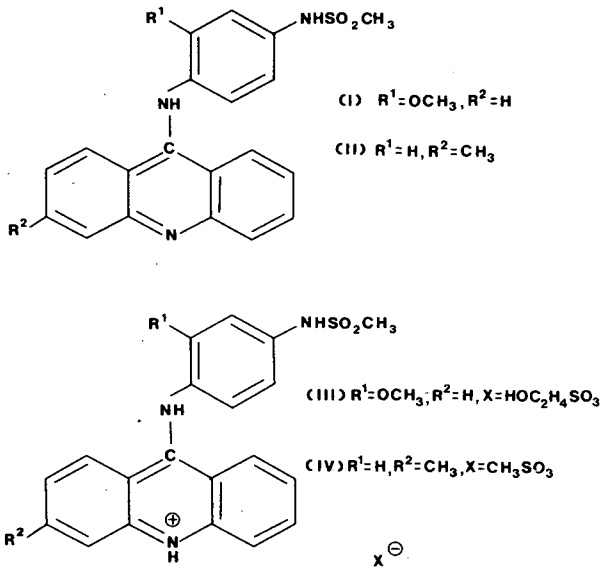

Fig. 1. Structures of (I) AMSA, (II) internal standard, and (III) and (IV) the respective salts of these compounds.

efficacy or avoidance of toxicity in particular patients such as those with hepatic or renal dysfunction who may be at risk.

Determination of AMSA in plasma has been reported using a non-specific fluorescence method [12] and gas chromatography (GC) [13]. This latter method did not use an internal standard and reported recoveries were only 85% on average. To our knowledge, with the exception of an abstract from Malspeis et al. [14], no high-performance liquid chromatographic (HPLC) method for AMSA has been published in full. We wish to report a selective and relatively rapid HPLC method using 4′-(3-methyl-9-acridinylamino)-methanesulfonanilide (II) as internal standard. This method can determine AMSA concentrations as low as 50 nmol/l in 0.5 ml of plasma with acceptable accuracy and precision.

EXPERIMENTAL

Materials

The 2-hydroxyethanesulfonic acid salt of AMSA (III, Fig. 1) and the methanesulfonic acid salt of 4′-(3-methyl-9-acridinylamino)methanesulfonanilide (IV, Fig. 1) were kindly supplied by Dr. B. Baguley and associates, Cancer Chemotherapy Research Laboratory, University of Auckland School of Medicine Auckland, New Zealand. Both salts were used without further purification.

The solvents used were UV-grade acetonitrile, methanol (both from Waters Assoc., Milford, MA, U.S.A.), anhydrous diethyl ether and hexane (both from J.T. Baker, Phillipsburg, NJ, U.S.A.). Sodium tetraborate, hydrochloric acid, triethylamine (all from BDH Chemicals, Poole, Great Britain) and phosphoric acid (J.T. Baker) were all Analar grade. All aqueous solutions were prepared

using Millipore Milli-Q water. All glassware was routinely washed in chromic acid solution.

Standard solutions

Stock solutions of AMSA (10.0 mmol/l) and the internal standard, 4'-(3-methyl-9-acridinylamino)methanesulfonanilide (1.0 mmol/l) were prepared in methanol. The internal standard stock solution was further diluted 1:50 with methanol to 20 μmol/l for use in the assay. The stock AMSA solution was further diluted 1:500 with fresh blood bank plasma to give a plasma concentration of 20 μmol/l which was further serially diluted to yield plasma calibration concentrations of 10, 8, 6, 4, 2, 1, 0.8, 0.6, 0.4, 0.2, 0.1 μmol/l for use as the standard curve. In addition, pure AMSA was weighed out and dissolved in four large plasma pools to give concentrations of 10.0, 0.5, 1.0 and 0.5 μmol/l for the determination of the accuracy and precision of the assay. These plasma pools were aliquoted into 1-ml volumes, stored at −20°C and used in each subsequent assay as a quality control check and to determine the inter-assay precision. All standard solutions were stored at −20°C when not in use.

Mobile phase

The HPLC mobile phase was prepared by adding 10 ml of stock triethylamine phosphate (TEAP) solution to 990 ml of acetonitrile—water (40:60) to give a final concentration of 0.01 mol/l TEAP and pH 3.80. The stock solution of TEAP (1.0 mol/l) was made up by adding 13.9 ml triethylamine to 60 ml of water, adjusting to pH 3.0 with phosphoric acid and adjusting the final volume to 100 ml with water. All HPLC solvents were filtered through a 0.45-μm filter (Type HA) (Millipore, Bedford, MA, U.S.A.) and thoroughly degassed before use.

Apparatus

The chromatographic separations were performed with a Waters Assoc. Model 6000A pump, U6K injector and a radial compression system (RCM-100) containing a 10 × 0.8 cm Radial -Pak C_{18} column (10 μm particle size). The mobile phase flow-rate was 7 ml/min with detection by a Model 440 UV detector at 254 nm. Peak areas were determined using a Perkin-Elmer Sigma 10 chromatography data station.

Collection of samples

Blood samples (5 ml) from patients were collected in heparinised Venoject tubes by venipuncture of a peripheral arm vein or from a surgically implanted arterial catheter. Blank samples were collected for each patient prior to commencement of the AMSA infusion. Immediately after collection the samples were centrifuged at 1720 *g* at 10°C for 15 min, the plasma removed and stored in capped glass tubes at −20°C until analysis.

Assay procedure

The internal standard (100 μl, 2 nmol) was added to 12-ml screw-cap glass culture tubes and the methanol evaporated off under nitrogen at 35°C. Plasma

(0.5 ml) was added to each tube and adjusted to pH 3.0—4.0 by addition of 120 μl of hydrochloric acid (0.5 mol/l). After gentle vortexing, 5 ml of hexane was added, the tube capped and shaken for 20 min followed by centrifugation at 1720 *g* for 10 min. The upper hexane layer was discarded. The remaining plasma was adjusted to pH 9.0 with 0.5 ml saturated sodium tetraborate solution and re-extracted for 15 min with 6 ml diethyl ether. After centrifugation at 1720 *g* for 15 min the ether layer was transferred to a tapered glass tube and evaporated at 35°C under a gentle flow of nitrogen. The residue was reconstituted in 100 μl of methanol and 20—40 μl injected into the liquid chromatograph.

Calculations

Peak area ratios of AMSA to internal standard were plotted against corresponding AMSA concentrations in the calibration plasma samples and the least-squares unweighted regression line was calculated. Quantitation of AMSA in unknown samples was then achieved by calculating peak area ratio for unknown, and using the calibration curve to compute a concentration.

RESULTS

The UV spectra of AMSA and internal standard in the HPLC mobile phase are shown in Fig. 2. Both compounds exhibited absorption maxima at 270 ± 2 nm. However, detection at 254 nm, which resulted in a 20% loss of sensitivity, was used in the assay as this was the fixed wavelength of the available detector.

Using the chromatographic conditions described, baseline separation of AMSA and internal standard was achieved (Fig. 3A), with retention times of

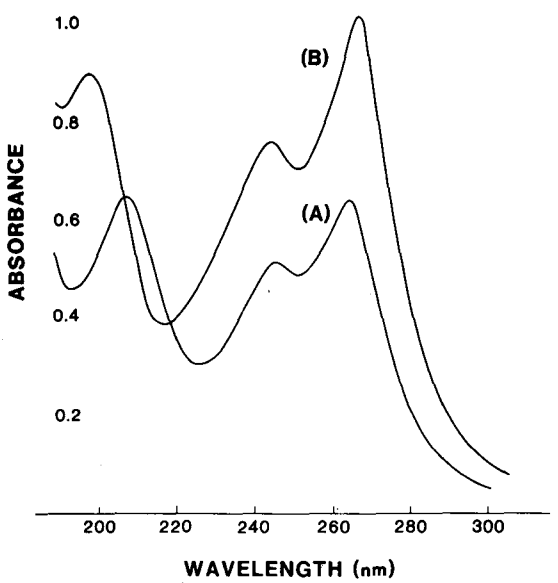

Fig. 2. UV absorption spectra of (A) AMSA and (B) internal standard in the HPLC mobile phase.

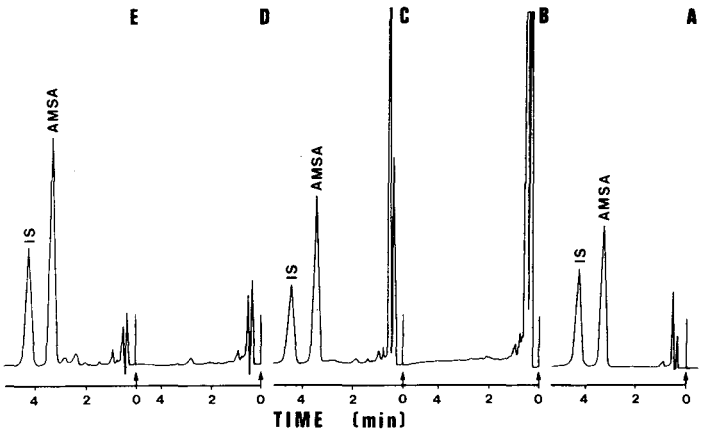

Fig. 3. Chromatograms of (A) blood bank plasma spiked with AMSA (5 μmol/l) and internal standard (IS) (4 μmol/l), (B) and (D) pre-infusion samples from the two patients, (C) and (E) correspondingly post-infusion AMSA samples from the two patients.

3.4 and 4.3 min, respectively. The total run time for each sample was 5.5 min. No interfering peaks were observed in the areas where AMSA and internal standard elute either in normals, in blood bank plasma or in patients' samples (Fig. 3B and D). However, the sample clean-up procedure of Malspeis et al. [14] was used to remove an endogenous plasma compound with retention time 9.8 min which was detected in blank plasma after a single extraction at pH 9.0. The use of ethyl acetate instead of diethyl ether also extracted this compound plus greater quantities of other more polar endogenous compounds. A number of other anticancer drugs including adriamycin, chlorambucil, cytosine arabinoside, 5-fluorouracil, lomustine, melphalan, methotrexate, prednisolone, 6-thioguanine, vincristine and vinblastine did not interfere with the AMSA or internal standard peaks under these chromatographic conditions. The reported in vitro thiolytic cleavage products of AMSA, 4-amino-3-methoxymethanesulfonanilide, 9-aminoacridine, 9(10H)-acridone [15] had retention times of 0.60, 1.47 and 2.16 min, and also did not interfere. 9-Aminoacridine has previously been identified as a metabolite in the plasma and urine of patients receiving AMSA [16]. In the present study, a small peak with a retention time identical to that for 9-aminoacridine was observed in the chromatograms of post-infusion plasma samples obtained from patient 2. However, the small size of the peak did not permit accurate measurement or precise identification.

The relationship between the peak area ratio of AMSA to internal standard and AMSA concentration was linear from 0 to 20 μmol/l. The standard curve was set up over the range 0.1 to 10.0 μmol/l and was represented by the equation $y = 0.216x - 0.002$ ($r = 0.9998$, $p < 0.001$), where y is the peak area ratio of AMSA/internal standard and x is the concentration of AMSA. Using 0.5 ml of plasma, the lower limit of the assay was 50 nmol/l. This could be further reduced to approximately 20 nmol/l by increasing the volume of plasma used and by decreasing the amount of internal standard. Estimates of the accuracy and the intra-assay precision of the method are given in Table I. Each plasma

TABLE I

INTRA-ASSAY PRECISION AND RECOVERY OF AMSA ADDED TO PLASMA

Amount added (μmol/l)	Mean conc. measured (μmol/l)	*n*	Standard deviation	Coefficient of variation (%)	Recovery (%)
10.0	11.49	8	0.26	2.29	115
5.0	5.25	8	0.09	1.78	105
1.0	1.11	8	0.03	2.32	111
0.5	0.52	8	0.01	2.66	104

TABLE II

INTER-ASSAY PRECISION OF AMSA MEASUREMENT IN PLASMA

Plasma pool	No. of consecutive assays	Mean concn. measured	Standard deviation	Coefficient of variation (%)
1 (10 μmol/l)	9	11.75	0.38	3.23
2 (5 μmol/l)	9	5.39	0.13	2.34
3 (1 μmol/l)	9	1.08	0.04	3.96

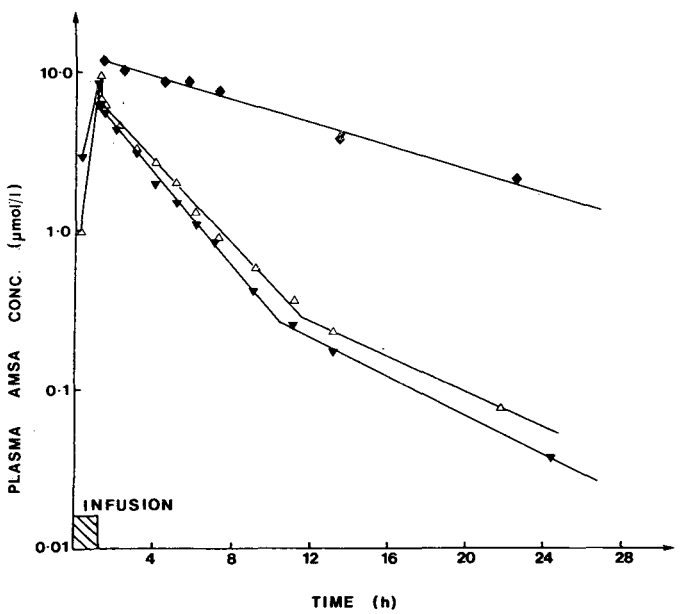

Fig. 4. Plasma concentrations of AMSA as a function of time in two patients after a 1-h infusion of 200 mg/m² AMSA. (♦) patient 1, (▼, △) two infusions 24 h apart in patient 2. Each point is the mean of duplicate determinations.

pool with added AMSA was analysed eight times in one run. Mean recoveries ranged from 104—115% with mean coefficients of variation from 1.78—2.66%. Inclusion of aliquots of three of these pools in eight subsequent assays gave acceptable estimates of inter-assay precision with coefficients of variation ranging from 2.34—3.96% (Table II).

Application of this method for measuring plasma AMSA concentrations in two patients receiving 200 mg/m^2 AMSA infusions (a single infusion in one patient and a double infusion 24 h apart in the other) as part of their treatment for acute myeloid leukemia is illustrated in Fig. 4. Each point was the mean of duplicate determinations. Although both patients had apparently normal hepatic and renal function, very different elimination profiles were observed. One patient exhibited a slow mono-exponential decline with a elimination half-life of 8.4 h, whereas in the other, AMSA elimination appeared to follow a bi-exponential curve with initial and terminal half-life values of 2.06, 2.32 and 4.9, 5.4 h respectively for the first and second infusion. Further information on the stability of AMSA in patients' plasma samples stored at −20°C was obtained by repeated assaying of four samples over a one-month period. These results are shown in Table III. No significant alterations in concentration were observed over this period.

TABLE III

STABILITY OF AMSA IN PATIENTS' SAMPLES STORED AT −20°C

Each measured value is the mean of duplicate extractions.

Time after sampling (weeks)	Concn. of AMSA measured (μmol/l)			
	1	2	3	4
1	6.24	5.64	2.01	1.54
2	6.25	5.82	1.93	1.51
4	6.25	5.75	1.99	1.52
Mean	6.25	5.74	1.98	1.52
Standard deviation	0.01	0.09	0.04	0.01
Coefficient of variation (%)	0.09	1.58	2.10	1.00

Haemolysis of the blood sample during collection led to an apparent reduction in plasma AMSA concentration. Further in vitro studies with grossly haemolysed blood indicated significant reductions in the absolute amounts of AMSA and internal standard as measured by the HPLC method. Careful collection of blood samples overcame the problem of haemolysis.

DISCUSSION

A relatively rapid and selective HPLC method for AMSA has been developed which allows the measurement of AMSA in plasma as low as 50 nmol/l in 0.5 ml of plasma with acceptable accuracy and precision. In addition, patients' samples do not deteriorate when stored at −20°C for up to one month.

Significant reductions in AMSA levels were observed in plasma obtained from haemolysed blood samples. The reason for this is not known. However, in vitro studies [17] have shown that a reaction may occur between AMSA and plasma proteins in human blood. Nucleophilic displacement of the anilino substituent by protein thiol groups results in covalent adducts. This displace-

ment reaction was found to occur at a greater rate in the presence of red blood cells. The effect of haemolysis on AMSA levels has not been previously reported, but would probably also result in lower AMSA concentrations by the other published methods for determination of AMSA [12—14].

The HPLC method described appears eminently suitable for further investigation of the pharmacokinetics of AMSA in patients and in laboratory animals such as the rabbit, with the one restraint that sample collection must be accomplished without causing haemolysis.

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CHROMBIO. 1755

SIMULTANEOUS ANALYSIS OF 1-β-D-ARABINOFURANOSYLCYTOSINE, 1-β-D-ARABINOFURANOSYLURACIL AND SODIUM SALICYLATE IN BIOLOGICAL SAMPLES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A reversed-phase high-performance liquid chromatographic column switching system is described for the rapid and complete separation of 1-β-D-arabinofuranosylcytosine (Ara-C), 1-β-D-arabinofuranosyluracil (Ara-U) and sodium salicylate using an internal standard of sodium cefmetazole. The system is highly selective and separates these compounds from interfering compounds commonly in biological matrices. The system was tested by following the pharmacokinetics of Ara-C after rectal administration in the presence of sodium salicylate which is an aid to drug absorption. The chromatographic system is also suitable for monitoring levels of Ara-C and its metabolite Ara-U after intravenous administration of Ara-C.

INTRODUCTION

1-β-D-Arabinofuranosylcytosine (Ara-C) is administered by intravenous or subcutaneous routes for the treatment of acute myelocytic leukemia and acute lymphocytic leukemia [1—4]. After intravenous injection, plasma Ara-C exhibits biphasic pharmacokinetics. There is an initial rapid metabolism

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of Ara-C by deamination to 1-β-D-arabinofuranosyluracil (Ara-U) followed by a slow elimination phase ($t_{½}$ = 2—2.5 h) [5, 6]. The initial deamination of Ara-C by pyrimidine neucleoside deaminase (cytidine aminohydrolase) occurs rapidly with a half-life of ca. 11 min, and is a saturable process [7, 14]. Thus to determine the pharmacokinetics and bioavailability of Ara-C, it is necessary to monitor both Ara-C and Ara-U levels in the blood.

We have chosen to administer Ara-C via a novel route, the rectum, using adjuvant enhanced absorption technology that has been developed in our laboratories. The adjuvant of choice for this work was sodium salicylate, and the vehicle of administration was a rectal suppository. Previous analytical methods have suffered from at least one of the following disadvantages: relative insensitivity, complex preparation, lack of specificity or long elution times. The methods have included biological assay using tissue cultures [8, 9], radiolabelling of Ara-C [7, 8], paper chromatography [7, 10] or high-performance liquid chromatography (HPLC) [6, 11, 12].

EXPERIMENTAL

Reagents

Cytidine, cytosine, uridine, Ara-U, formic acid (95—97%) and sodium salicylate (99+%) were obtained from Sigma (St. Louis, MO, U.S.A.). Witepsol S55 was obtained from Kay-Fries (Montvale, NJ, U.S.A.). Sodium cefmetazole was obtained from Merck Sharp and Dohme (West Point, PA, U.S.A.). Ammonium hydroxide was obtained from Fischer (Pittsburgh, PA, U.S.A.). Ara-C was obtained from Upjohn (Kalamazoo, MI, U.S.A.). Tetrahydrouridine (THU) was obtained from Calbiochem-Beliring (La Jolla, CA, U.S.A.).

HPLC equipment

The plan and key for the system are illustrated in Fig. 1. Pumps 1 and 2 were Waters Model M45; pump 3 was an Eldex Model A-60-5, and pump 4 was a Fluid Metering lab pump. All three valves (Rheodyne Model 7001) were activated via Rheodyne Model 7000 controllers, and all events were controlled by a Waters system controller. The UV detector was a dual-channel Waters

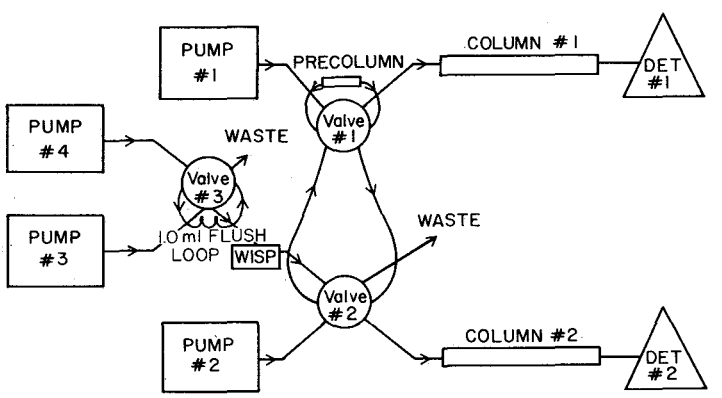

Fig. 1. Plan and key of the HPLC system used.

Model 440 absorbance detector set at 254 nm. The data were recorded on a Waters data module. Samples (20 μl) were injected using a Waters WISP 710B. The pre-packed columns were obtained from Ranin and contained a 5-μm particle size reversed-phase C_{18} material (Ranin OD-MP RP-18). The precolumn was a Ranin OD-MP RP-18 Spheri 5 of 30 mm × 4.6 mm. Column 1 consisted of two Ranin OD-MP RP-18 Spheri 5, 100 mm × 4.6 mm, and column 2 consisted of one Ranin OD-MP RP-18 Spheri 5, 100 mm × 4.6 mm. Pump 4 was activated by a Waters system controller.

Sample preparation

Biological samples. Blood samples (300 μl) were collected in serum collecting tubes containing 5 μg of THU to eliminate deamination of Ara-C [5, 6]. The tubes were centrifuged for 10 min at 900 *g* and 100 μl of serum were removed. An internal standard, sodium cefmetazole, was added to the serum (25 μl containing 0—5 μg of sodium cefmetazole). The serum was then deproteinated using 200 μl of acetonitrile and centrifuged for 5 min at 900 *g*. The supernatant was collected and evaporated to dryness under a nitrogen gas stream. The sample was reconstituted with 100 μl of water then analyzed by HPLC.

Standard samples. Standard aqueous solutions containing Ara-C, Ara-U and sodium salicylate were prepared daily. Aliquots of the standard solutions were added to blank pooled blood samples containing THU. The spiked standard blood samples were prepared for HPLC analysis in identical fashion to the biological samples.

HPLC conditions

The conditions are summarized in Table I for the solvents, flow-rates and pressures, and Table II for the events sequence. The key in each table is to be used in conjunction with Fig. 1. The starting conditions are valves 1, 2 and 3 in a clockwise position causing pump 1 to bypass valve 1 and elute from column 1; pump 2 to bypass valve 2 and elute from column 2; pump 3 to bypass valve 3 through valves 2 and 1 and then to precolumn and to waste; pump 4 was off but connected to bypass valve 3 to waste.

TABLE I

SOLVENT SYSTEMS, FLOW-RATES AND PRESSURES REFERRED TO IN FIG. 1 AND TEXT

Pump No.*	Flow-rate (ml/min)	Pressure (bars)	Solvent system and code
1	2	140	0.017 *M* ammonium hydroxide—formic acid buffer adjusted to pH 3.0 with formic acid (I)
2	2	70	0.0068 *M* ammonium hydroxide—0.01 *M* formic acid—20% acetonitrile (II)
3	2		0.017 *M* ammonium hydroxide—formic acid buffer adjusted to pH 3.0 with formic acid (I)
4	2		acetonitrile (III)

*See Fig. 1.

TABLE II

EVENT SEQUENCE CONTROLLED THROUGH SYSTEMS CONTROLLER (SEE FIG. 1)

Event	Time (min)	Process*	Effect
Inject	0.0	WISP injection	Sample through valves 1, 2 and precolumn to waste
Pulse 1	0.3	Valve 1 CCW	Precolumn in for system 1 (column 1 and solvent 1)
Pulse 3	0.4	Valve 2 CCW	System 2 flow through bypass port of valve 1
Pulse 2	1.0	Valve 1 CW	Precolumn out of system 1 and into system 2
Pulse 4	3.0	Valve 2 CW	Precolumn out of system 2; solvent II through precolumn from pump 3 to waste
Pulse 5	3.1	Valve 3 CCW	Flush loop through precolumn to waste
Pulse 6	4.1	Valve 3 CW	Flush loop out of precolumn loop
on 7	4.2	power on pump 4	Reload flush loop with solvent III
off 7	4.7	power off pump 4	Reload complete

*CW = clockwise, CCW = counterclockwise.

The column switching was designed so that the fraction containing Ara-C and Ara-U was transfered from the precolumn to column 1, and the fraction containing sodium salicylate and sodium cefmetazole was transfered from the precolumn to column 2. It was also necessary to alter solvent systems to elute sodium salicylate and sodium cefmetazole. Initially, a 20-μl sample was injected and eluted onto the precolumn with solvent I; the rapidly eluting components that interfere with the assay were eluted to waste. When this was complete, the precolumn outflow was switched to column 1. The fraction containing Ara-C and Ara-U was then eluted onto column 1 and resolved using solvent system I. After transfer from the precolumn was completed, the precolumn outlet was switched to column 2. Column 1 was then eluted with solvent system I using pump 1. The eluent was monitored by UV spectroscopy at a wavelength of 254 nm (see Figs. 2A, 3A, 3B and 4). The precolumn after switching was eluted with solvent system II to elute sodium salicylate and sodium cefmetazole onto column 2. When the transfer was completed, the precolumn was switched to waste and flushed with solvent system III (from pump 4) to remove any of the sample compounds remaining on the precolumn. Column 2 was eluted with solvent system II using pump 2. The eluent was monitored by UV spectroscopy at 254 nm (see Figs. 2B, 3C and D). When flushing with solvent III was completed, the precolumn was reconditioned by eluting with solvent system I in preparation for the next injection.

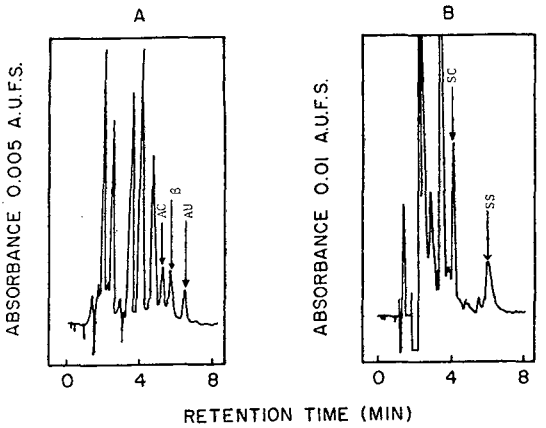

Fig. 2. High-performance liquid chromatograms of pooled serum samples spiked with (A) Ara-C (AC) 2 μg/ml and Ara-U (AU) 0.5 μg/ml for column 1; and (B) sodium salicylate (SS) 20 μg/ml and sodium cefmetazole (SC) 5 μg/ml for column 2.

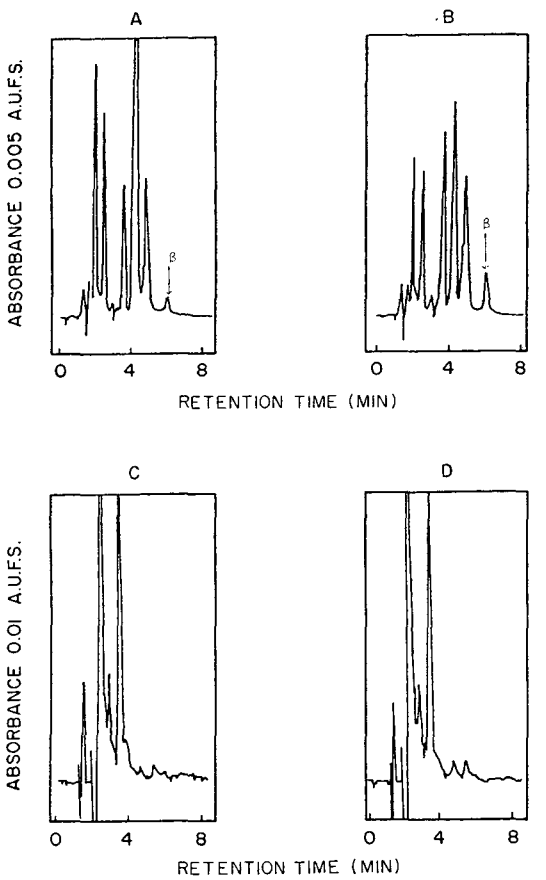

Fig. 3. High-performance liquid chromatograms of blank serum samples from two dogs (A) and (C) for column 1, (B) and (D) for column 2.

RESULTS AND DISCUSSION

Chromatographic separation

Fig. 2A (for column 1) and 2B (for column 2) show typical chromatograms of pooled, spiked serum samples containing Ara-C (2 μg/ml). Ara-U (0.5 μg/ml), sodium salicylate (20 μg/ml) and sodium cefmetazole (5 μg/ml). Peak β (Fig. 2A), which elutes between the Ara-C and Ara-U peaks, varies in height from dog to dog (see Fig. 3A and B). A similar variation in peak heights of inter-animal HPLC traces for an Ara-C/Ara-U system has been reported by Linssen et al. [6]. In their study, the variable peak had the same elution properties as Ara-U. Fig. 3A—D shows blank serum samples from two dogs. Fig. 3A and B are chromatograms of column 1 and Fig. 3C and D are chromatograms of column 2. The blank serum chromatogram in Fig. 3A—D shows no interfering substances with the same eluting properties as Ara-C, Ara-U, sodium salicylate and sodium cefmetazole. In the development of the assay, the pH of the Ara-C/Ara-U column solvent system is critical. At pH 3.0 ± 0.1, good resolution was obtained between Ara-C and peak β. Fig. 4 shows the chromatogram from column 1 of a mixture containing cytosine (I), cytidine (II), uridine (III), Ara-C (IV) and Ara-U (V) which have caused interference with Ara-C and Ara-U peaks on other chromatographic systems [6, 7, 11].

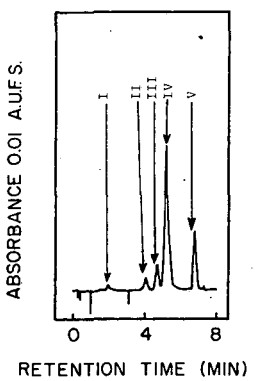

Fig. 4. High-performance liquid chromatogram from column 1 of serum samples spiked with (I) cytosine (0.3 μg/ml), (II) cytidine (0.3 μg/ml), (III) uridine (0.3 μg/ml), (IV) Ara-C (5 μg/ml) and (V) Ara-U (1 μg/ml).

Calibration curves and accuracy

Spiked standard samples were analyzed before each batch of biological samples to check for any changes in chromatographic characteristics. None were detected in the study. As an internal standard was used, peak height ratios (PHR), with respect to the internal standard, were used for Ara-C, Ara-U and sodium salicylate. A plot of Ara-C PHR vs. concentration in the range 0.125—8.000 μg/ml serum (n = 3 at each of the eight points on the curve) gives a linear correlation coefficient of 0.9951 and passes through the origin. The relative coefficient of variation at 0.125 μg/ml was 15% and at 8.000 μg/ml was 0.3%. The percent recovery was 98 ± 1% at 4 μg/ml (n = 3) with respect to aqueous solutions.

A plot of Ara-U PHR vs. concentration in the range 0.0625—2.0000 μg/ml serum (n = 3 at each of the seven points on the curve) gives a linear correlation coefficient of 0.9972 and passes through the origin. The relative coefficient of variation at 0.0625 μg/ml was 2.1% and at 2.0000 μg/ml was 0.6%. The percent recovery was 108 ± 0.2% at 1.0 μg/ml (n = 3) with respect to aqueous solutions.

For sodium salicylate, PHR vs. concentration in the range 1.25—80 μg/ml serum (n = 3 at each of the eight points on the curve) gives a linear correlation coefficient of 0.9940 and passes through the origin. The relative coefficient of variation at 1.25 μg/ml was 17% and at 80.0 μg/ml was 0.02%. The percent recovery was 113 ± 0.4% at 40 μg/ml (n = 3) with respect to aqueous solutions.

Biological data

Figs. 5 and 6 show the serum levels of Ara-C/Ara-U and sodium salicylate, respectively, after the administration of a suppository containing Witepsol S55 (696 mg), Ara-C (32 mg), water (262 μl) and sodium salicylate (322 mg) to beagle dogs (n = 6). Ara-C is rapidly absorbed to a maximum serum level after 20—30 min. Ara-U levels increase slowly in the serum to a plateau value which is maintained from 30—120 min. Sodium salicylate reaches its peak serum level after 90 min. The prolonged serum levels of Ara-C via the rectal route may provide a viable alternative to slow infusion therapy currently in use. The pharmacokinetic implications of this study will be reported more extensively at a later date [13].

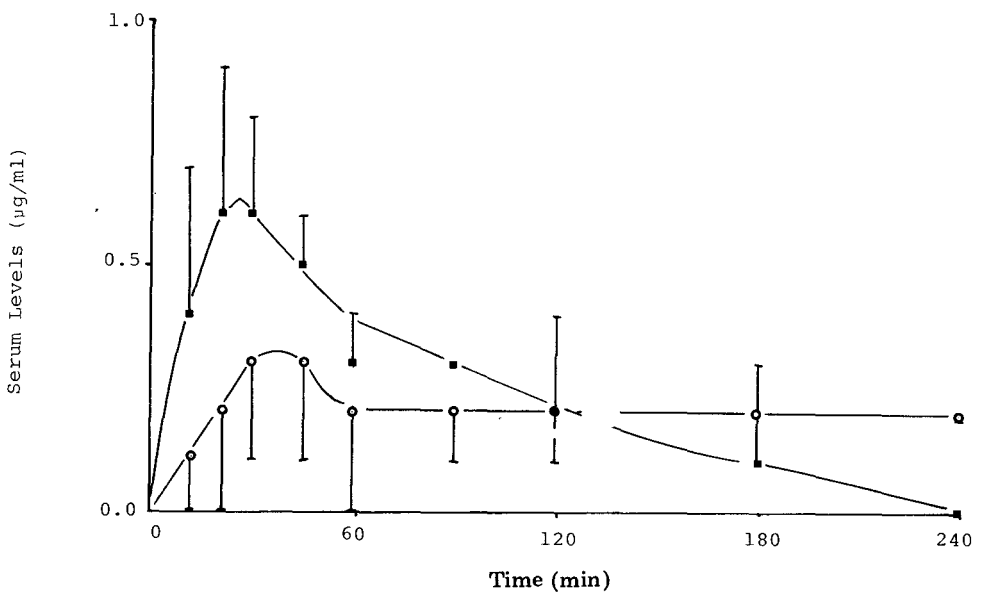

Fig. 5. Serum levels of Ara-C (▪) and Ara-U (○) after rectal administration of a suppository containing Ara-C and sodium salicylate.

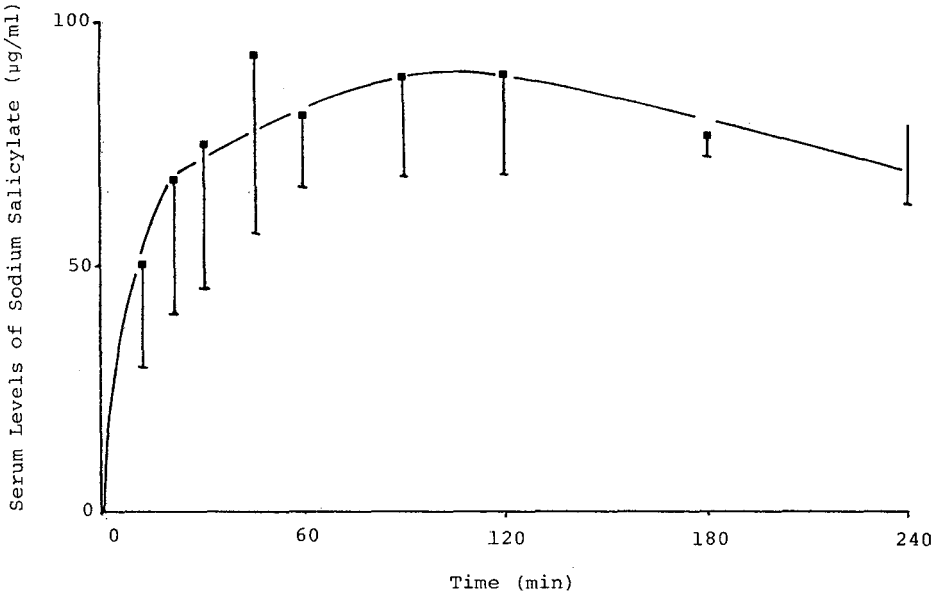

Fig. 6. Serum levels of sodium salicylate after rectal administration of a suppository containing Ara-C and sodium salicylate.

CONCLUSIONS

A method has been described for the analysis of Ara-C, Ara-U and sodium salicylate with the following advantages over other published methods: decreased analysis time due to a rapid elution of compound enabling analysis of a large number of samples per day (ca. 77 samples per 9-h day); selectivity with respect to interferences; small sample size; minimal sample preparation; simultaneous analysis of Ara-C, Ara-U and sodium salicylate; and ready applicability to Ara-C and Ara-U monitoring for intravenous therapy currently in use.

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DETERMINATION OF AMIKACIN IN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ULTRAVIOLET DETECTION

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SUMMARY

A procedure for the determination of amikacin in serum is described. The aminoglycoside is extracted from serum by using a disposable cation-exchange column. The eluate of this column is derivatized with 1-fluoro-2,4-dinitrobenzene and subsequently analysed by reversed-phase high-performance liquid chromatography with ultraviolet detection at 365 nm. The absolute recovery of amikacin by this procedure is 72%. Kanamycin is used as the internal standard. The sensitivitiy is 1 mg/l for amikacin with samples of 200 μl. Precision, expressed as the coefficient of variation, is about 3% in the therapeutic concentration range. The 2,4-dinitrophenyl derivative of amikacin is synthesized on a preparative scale by a new method and its structure is demonstrated to be the fully derivatized amikacin. The analysis of serum samples obtained in an in vivo experiment correlates well with the results from a microbiological assay.

INTRODUCTION

Previously it was shown that 2,4-dinitrophenylation of aminoglycosides produces derivatives with good chromatographic properties in reversed-phase high-performance liquid chromatographic (HPLC) systems and with sufficiently high absorbance at 365 nm to allow therapeutic drug monitoring [1—3]. Recently, a 2,4-dinitrophenylation method was described for amikacin [4]. The present paper reports also a method for quantitating amikacin in serum by means of precolumn derivatization with 1-fluoro-2,4-dinitrobenzene (FDNB), but with a simpler sample treatment and with the use of an internal standard. Furthermore, we succeeded in obtaining values for the absolute recovery of the determination.

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EXPERIMENTAL

Instrumentation

The chromatographic equipment was described previously [1]. Chromatography was performed on columns (30 cm × 3.9 mm I.D.) packed with LiChrosorb RP-18, particle size 10 μm (Merck, Darmstadt, G.F.R.). High-resolution proton nuclear magnetic resonance (H-NMR) spectra were recorded on a Bruker WP200 (Bruker, Rheinstetten, G.F.R.) at the Laboratory for Organic Chemistry, University of Utrecht, under supervision of Dr. de Bie. The National Institute of Public Health (RIV), Bilthoven, The Netherlands, did the microbiological assays, using an agar-well diffusion technique [5].

Materials

Unless mentioned otherwise, demineralized water was used. Ethanol, sodium hydroxide, sodium bromide and sodium sulphate decahydrate were of European Pharmacopoeia quality. Dimethyl sulphoxide (DMSO) was of British Pharmacopoeia quality. Acetic acid, acetone, FDNB, methanol and ethyl acetate were of analytical grade. Acetonitrile "zur Synthese" and tetrabutylammonium hydroxide (TBAH) 40% in water "zur Synthese" were from Merck. CM-Sephadex C-25 cation exchanger was from Pharmacia (Uppsala, Sweden) and kanamycin sulphate from Sigma (St. Louis, MO, U.S.A.). Ampoules (2 ml) of Amukin® containing the equivalent of 500 mg of amikacin as the sulphate salt, and amikacin base were obtained by courtesy of Pfizer (Rotterdam, The Netherlands). Weights of amikacin indicate weights of the chemical substance amikacin base, not microbiological potencies, unless otherwise indicated.

Pooled human serum was obtained from ambulatory patients, and was frozen and stored at −18°C within three days of collection.

Stoppered polypropylene centrifuge tubes of 1.5 ml capacity disposable glass pasteur pipettes of 5.4 mm I.D., glasswool and ampoules of 0.5 and 5 ml capacity were also used.

Procedures

Procedure A: HPLC determination of amikacin in serum. Remove most of the stem of a pasteur pipette and plug the remainder of the stem with glass wool. Incubate CM-Sephadex C-25 in a 0.2 mol/l sodium sulphate solution in water at room temperature for at least 24 h. Fill the pasteur pipette with sufficient of the Sephadex slurry to obtain a column height of 1.5 cm. Pipette 200 μl of the serum sample into a centrifuge tube and add 20 μl of a solution containing 250 mg/l kanamycin sulphate in water (internal standard). Vortex and dispense the contents of the centrifuge tube on top of the column. Elute the column with 2 ml of a solution containing 0.001 mol/l hydrochloric acid and 0.2 mol/l sodium sulphate (initial eluent). Discard the eluate. Elute the column with 250 μl of 0.05 mol/l sodium hydroxide. Discard the eluate (dead volume of the column). Elute the column with 1 ml of 0.05 mol/l sodium hydroxide and collect this eluate in a 5-ml ampoule. Add 2.5 ml of a solution of FDNB in methanol (30 g/l). The formation of a precipitate is observed, which redissolves upon mixing. Heat-seal the ampoule and place in boiling

water for 5 min. After cooling, break the seal and inject 150 µl into the chromatograph.

Prepare the mobile phase by mixing 470 ml of acetonitrile with 530 ml of water (both filtered through a 0.2-µm filter) and 1 ml of acetic acid, and deaerate ultrasonically. Pump the mobile phase at 2.5 ml/min and monitor the eluent at 365 nm.

Procedure B: synthesis of the 2,4-dinitrophenyl derivative of amikacin. Amukin (2 ml) was mixed with a sufficient quantity of sodium hydroxide solution (10 mol/l) to obtain a pH of 10. Then, 20 ml of DMSO were added. Alternately, FDNB and TBAH were added, guided by HPLC analysis of the reaction mixture after each addition. The final reaction mixture was diluted with 100 ml of a mixture of ethanol and water (25:75, v/v). The resulting precipitate was purified by dissolving in the smallest possible volume of a mixture of 630 ml of acetone and 370 ml of glass-distilled water.

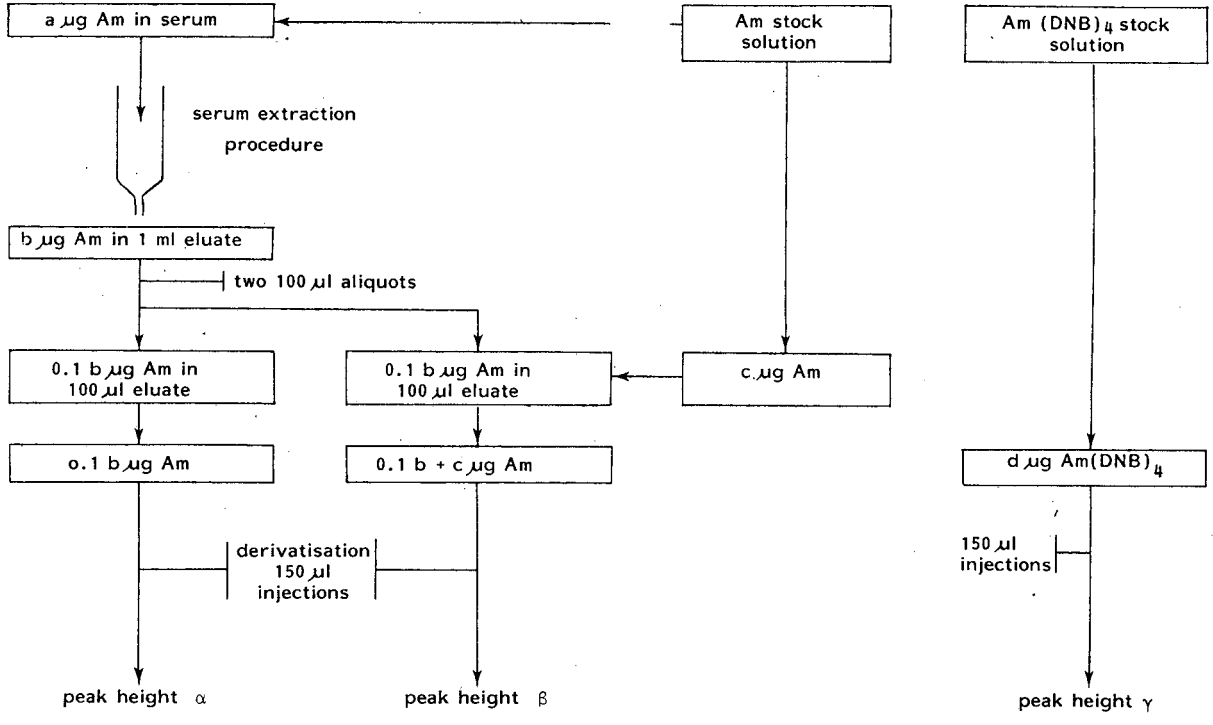
Aliquots of this solution were injected into the chromatograph, eluted with the same acetone–water mixture, and the appropriate fractions were collected. The combined fractions of repeated injections were diluted with glass-distilled water and extracted with ethyl acetate. The combined ethyl acetate extracts were evaporated and the residue was placed in a hygrostat, above a mixture of sodium bromide and water (2:1, w/w) until constant weight was obtained.

Procedure C: recovery measurements. Amikacin base was investigated for content of the chemical substance amikacin by non-aqueous titration [3]. Absence of related compounds was established by thin-layer chromatography [6].

Serum was spiked with an aqueous stock solution containing amikacin base and kanamycin sulphate, to a serum concentration of 16 mg/l of both antibiotics. Samples of 200 µl of this spiked serum were processed by means of a cation-exchange column as described under procedure A. The 1-ml eluate fraction which contained the aminoglycosides was collected and weighed in order to calculate the exact volume of the eluate. Two 100-µl portions were taken and to one aliquot 5 µl of the stock solution of amikacin and kanamycin were added (standard addition technique). After the addition of 250 µl of FDNB (30 g/l in methanol) both mixtures were derivatized and chromatographed. Also, samples of a solution of the purified amikacin derivative in the mobile phase were chromatographed. Serum extraction recoveries of amikacin and kanamycin, the derivatization yield of amikacin and the overall absolute recovery of amikacin were then calculated as shown in Fig. 1.

Procedure D: in vivo experiment and bioassay comparison study. A 69-kg healthy volunteer received 1 ampoule of Amukin by intramuscular injection. Blood samples were collected at regular time intervals and left in polypropylene tubes for some hours. The clot of erythrocytes was removed and the remaining serum samples were analysed by HPLC and by microbiological assay.

For both analytical techniques identical spiked serum samples were used for calibration.



$$\text{Recovery of the serum extraction procedure} = \frac{b}{a} \cdot 100\% = \frac{c\,\alpha 1000}{a\,(\beta - \alpha)}\%$$

$$\text{Yield of derivatisation reaction} = \frac{(\beta - \alpha)\, d\, M_r(Am)}{\gamma\, c\, M_r(Am(DNB)_4)} \cdot 100\%$$

Fig. 1. Scheme of the measurement and calculation of the serum extraction recovery and derivatization yield of amikacin. Am = amikacin, $Am(DNB)_4$ = amikacin derivative, M_r = moleculer weight.

RESULTS

Representative chromatograms are shown in Fig. 2. Identical chromatograms were obtained with serum samples from patients.

Linearity and precision

A calibration line was constructed. The results are summarized in Table I. Precision is reported in Table II.

Characterization of the derivative of amikacin

(1) Part of the high-resolution H-NMR spectrum of the derivative is shown in Fig. 3. In the H-NMR spectrum of the 2,4-dinitrophenyl group, doublets are expected for $H_{(3)}$ and $H_{(6)}$ because of the coupling between $H_{(3)}$ and $H_{(5)}$, and $H_{(6)}$ and $H_{(5)}$, respectively. $H_{(5)}$ is coupled with both $H_{(3)}$ and $H_{(6)}$, giving rise to quadruplets.

The H-NMR spectrum of the amikacin derivative shows four doublets of $H_{(6)}$ in the range 6.9—7.6 ppm and also four doublets of $H_{(3)}$ in the range 8.5—8.9 ppm. The four quadruplets of $H_{(5)}$ in the range 8.1—8.3 ppm are not completely resolved. Four 2,4-dinitrophenyl groups are therefore present,

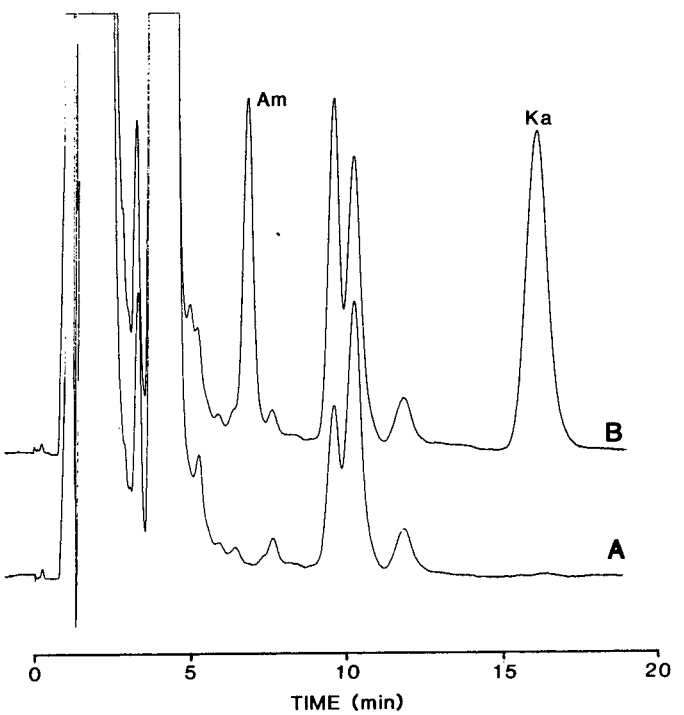


Fig. 2. HPLC of serum samples. Detector setting: 0.02 a.u.f.s. Chromatogram A is obtained with blank serum without internal standard. Chromatogram B is obtained with serum spiked with amikacin (16 mg/l) and with internal standard. Am = amikacin derivative, Ka = kanamycin derivative (internal standard).

TABLE I

STANDARD CURVE

Duplicate determinations were made on every concentration tested.

Amikacin concentration (mg/l)	PHR*	PHR/concentration
1	0.062	0.062
2	0.117	0.059
4	0.240	0.060
8	0.495	0.062
16	1.01	0.063
32	2.04	0.064
64	4.30	0.067

*Mean value of peak height ratio of derivatized amikacin to derivatized kanamycin (internal standard).

indicating that all four primary amino groups are derivatized. This was also concluded by Wong et al. [4] from other H-NMR data.

(2) A solution of the prepared derivative in the mobile phase was investigated for chromatographic purity by HPLC. Besides the major peak of the amikacin derivative, one minor peak was observed. The area of the peak of the amikacin derivative was 98.7% of the total peak area.

(3) The nitrogen content of the purified derivative was found to be 13.16% (S.D. = 0.04%, n = 2). The calculated nitrogen content for $C_{46}H_{51}N_{13}O_{29}$ is 14.57%. The purity of the prepared derivative was taken as

$$\frac{13.16}{14.57} \cdot 98.7\% = 89\%$$

TABLE II

WITHIN-RUN PRECISION

Amikacin concentration (mg/l)	Precision*	
	C.V. (%)	n
1	5.3	5
2	3.4	6
16	3.1	6
32	3.2	6
64	1.5	6

*C.V. = coefficient of variation of measured peak height ratios; n = number of determinations.

Recovery measurements

The results, corrected for the content of amikacin base and for the purity of the amikacin derivative, are given in Table III.

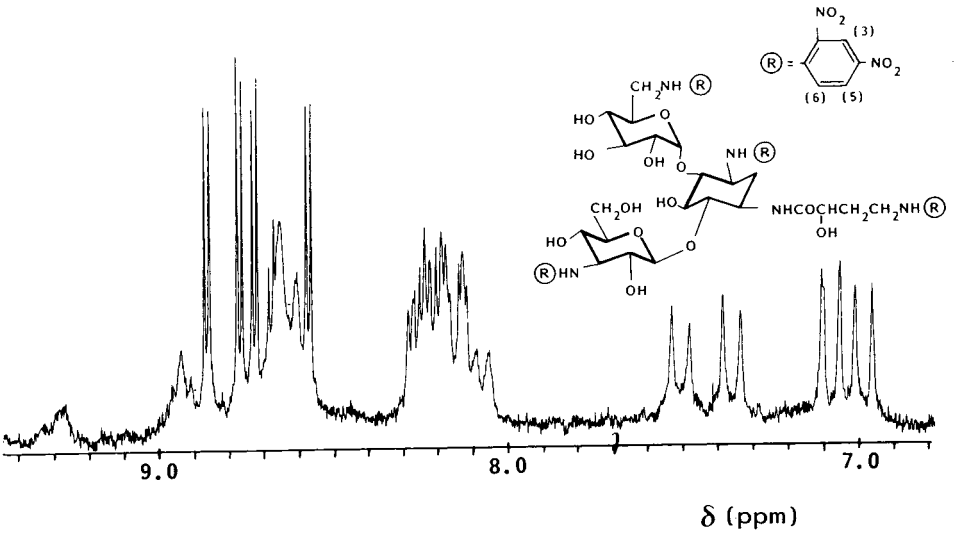


Fig. 3. Part of the 200 mHz H-NMR spectrum of the 2,4-dinitrophenyl derivative of amikacin. Chemical shift (δ) relative to tetramethylsilane. Conditions: saturated solution in DMSO-d_6 at 33°C. Assignment of peaks: see text. Inset: structure of the 2,4-dinitrophenyl derivative of amikacin.

TABLE III

RESULTS OF ABSOLUTE RECOVERY MEASUREMENTS

	Recovery (%)	n*	C.V. (%)
Serum extraction recovery of amikacin	95	7	6
Serum extraction recovery of kanamycin	92	8	9
Derivatization yield of amikacin	77	8	10
Overall recovery of amikacin	72	7	9

*n = number of determinations.

In vivo experiment and bioassay comparison study

The results are summarized in Table IV.

DISCUSSION

Serum extraction

Determinations of gentamicin, sisomicin and tobramycin in serum, by 2,4-dinitrophenylation were performed using a protein precipitation step with acetonitrile [1-3]. For amikacin, this failed because of the relatively high polarity of the 2,4-dinitrophenyl derivative of amikacin, making its separation from the peaks near the solvent front impossible.

The serum extraction procedure presented in this paper is a modification of

TABLE IV

RESULTS OF IN VIVO EXPERIMENT AND BIOASSAY COMPARISON STUDY

Serum level determinations by two methods on samples obtained after a single intramuscular dose of amikacin from one volunteer.
Linear regression analysis (HPLC values = X, microbiological values = Y): Y intercept = 0.02, standard deviation = 0.84; slope = 0.92, standard deviation = 0.05; r = 0.993.

Time after injection (h)	Amikacin concentration (mg/l by potency) found by	
	HPLC*	Microbiological assay**
0.5	16.2	14.3
1	24.2	23.7
1.5	24.8	22.1
2	21.2	18.5
3	13.8	13.7
5	6.1	6.0
8	2.4	1.8

*Standards and samples analysed once.
**Standards and samples analysed in duplicate.

the technique described by Anhalt and Brown [7]. In their procedure 0.2 mol/l sodium sulphate is used instead of 0.001 mol/l hydrochloric acid in 0.2 mol/l sodium sulphate as the initial eluent.

Eluting the cation-exchange column with neutral solutions sometimes yields low and irreproducible recoveries, due to the alkaline reaction of some serum samples. The amino groups of the aminoglycosides are deprotonated in alkaline solution, the cation exchanger will not retain these deprotonated aminoglycosides. Our findings were confirmed by Anhalt [8].

Derivatization

Tsuji et al. [9] and Helboe and Kryger [10] reported 2,4-dinitrophenylation of aminoglycosides in a water–methanol mixture in the presence of a borate buffer pH 9.0 with reaction temperatures and reaction times of 100°C and 45 min, and 60°C and 60 min, respectively. These authors reported no derivatization yields for their methods. We measured the derivatization yields for amikacin obtained by these two methods and found 86% and 90%, respectively. We also observed that derivatization, in sodium hydroxide solutions of concentrations ranging from 0.01 mol/l to 0.06 mol/l at 100°C for 5 min with the same reagent concentration, resulted in essentially the same derivatization yield, i.e. 92%. When this technique was applied to the eluate obtained after the serum extraction, somewhat lower derivatization yields were obtained, i.e. 77% (see Table III).

The only difference between the conditions in which these two results were obtained is the presence of a low concentration of residual sodium sulphate in the derivatization mixture obtained from the cation-exchange column. The negative influence of sodium sulphate upon the yield of the derivatization

reaction was confirmed by experiments in which increasing amounts of sodium sulphate were added to the derivatization mixture.

Chromatography

The 2,4-dinitrophenyl derivatives of amikacin and kanamycin show good chromatographic properties on RP-18 columns with mobile phases composed of water and acetone, or water and acetonitrile. However, only the acetonitrile–water mixture was useful as the mobile phase in the analysis of serum samples. Kanamycin is composed of three components: kanamycin A, the major component, and kanamycin B and C, the two minor congeners. As equimolar amounts of kanamycin and amikacin give rise to roughly equal peak areas, it may be concluded that the observed peak of kanamycin is the peak of the derivative of the major component of kanamycin, i.e. kanamycin A.

Preparative synthesis of the amikacin derivative

Bunnett and Hermann [11] showed that the reaction between FDNB and amino groups is very fast in DMSO. In this solvent amikacin base, FDNB and the derivative of amikacin are soluble. Amikacin sulphate is not soluble, so the salt was converted into the free base before the addition of DMSO.

We did not succeed in obtaining a "pure" derivative, because of its hygroscopic properties and our inability to find a suitable solvent for the recrystallization of the derivative. However, a derivative of known purity instead of a pure derivative also allowed measurements of the recoveries of the derivatization and determination. A chromatographic purification of the raw derivative was carried out using solvents without nitrogen in their molecular structure. So, the nitrogen content of the obtained purified derivative indicated its content of amikacin (2,4-dinitrophenyl)$_4$, the remainder being water.

CONCLUSIONS

With the proposed method, amikacin can be determined in 200-μl serum samples with sufficient accuracy, precision and sensitivity to make therapeutic drug monitoring possible.

The method described by Wong et al. [4] uses only 25 μl of serum, but in their approach a concentration step and several sample clean-up steps are required. A second difference with this method is the use of an internal standard, which is an important factor in the reliability of the assay [2].

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SIMULTANEOUS ANALYSIS OF 5′-DEOXY-5-FLUOROURIDINE AND 5-FLUOROURACIL IN PLASMA BY ANALYTICAL ISOTACHOPHORESIS

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SUMMARY

Analytical isotachophoresis was used for the determination of 5-fluorouracil and 5′-deoxy-5-fluorouridine in plasma. The inclusion of spacers in the system greatly improved the separation and quantitation. The method can be employed for simultaneous measurements of different fluorinated pyrimidines used in clinical practice.

INTRODUCTION

5′-Deoxy-5-fluorouridine (5′-dFUR) is a recently synthesized fluoropyrimidine nucleoside (Roche 21-9738) reported in preliminary investigations to have an antineoplastic activity in vivo against several rat and murine tumor lines superior to that of 5-fluorouracil (5-FU), 2′-deoxy-5-fluorouridine and ftorafur [N_1-(2′-furanidyl-5-fluorouracil] [1, 2]. The results observed with 5′-dFUR show a dramatic reduction in host toxicity. Further, an excellent activity has been observed after per oral administration [3].

The mechanism through which 5′-dFUR induces its antineoplastic activity is not known. In vitro studies have not revealed evidence for the formation of an active novel metabolite in sensitive Ehrlich ascites tumor cells [4]. In investigations of its cellular metabolism it has been found that 5′-dFUR is converted to 5-FU by the enzyme uridine phosphorylase. There is a greater activity of this enzyme in tumor tissues than in normal tissues. No doubt 5′-dFUR is an interesting new fluoropyrimidine. The present method was worked out as a tool for pharmacokinetic studies of this new 5-FU derivative.

We have recently reported that 5-FU can be analysed in body fluids by isotachophoresis [5, 6]. The principle of isotachophoresis has been known for

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many years, but the technique has not been used much until recently [7].

The tachophor used was equipped with an UV absorbance detector which gives an immediate response to an absorbing zone, and because of the high resolution of the detector the quantitative evaluation of the UV signal is easy. By using non-UV-absorbing compounds as discrete spacers, the identification and quantitation of fluorinated pyrimidines in biological samples can be improved.

In this paper we describe a specific and sensitive assay for 5-FU and 5′-dFUR in plasma by analytical isotachophoresis.

MATERIAL AND METHODS

Chemicals

5-FU and 5′-dFUR were supplied by Roche AB, Stockholm, Sweden. HPMC (hydroxypropylmethylcellulose), Methocel 90HG, 15,000 CPS, was obtained from Dow Chemical Company, Midland, MI, U.S.A. The resin Ag 1-X8 (formate), 200–400 mesh, was purchased from Bio-Rad Labs., Richmond, CA, U.S.A. All other chemicals used were of analytical grade and commercially available.

Instrumentation

The isotachophoretic separations were performed in an LKB 2127 Tachophor (LKB Instruments, Stockholm, Sweden). The UV signal (254 nm) was recorded at a chart speed of 6—10 cm min^{-1}. The separations were carried out in a 23-cm standard capillary.

Pretreatment of plasma samples

A 1-ml volume of plasma was diluted to 5 ml with picric acid [5]. The precipitate formed was removed by centrifugation at 3000 *g* for 5 min. The supernatant was brought to pH 11 by the addition of 0.1 mol/l potassium hydroxide solution. The Ag 1-X8 resin was equilibrated with 5 volumes of 6 mol/l formic acid for 1 h. Pasteur pipettes (150 × 5 mm) were used as columns. The resin was washed with 30 column volumes of glass-distilled water. The flow-rate was approximately 1 ml/min. The deproteinized plasma sample was loaded onto the column, then 20 column volumes of glass distilled water were passed through the column and discarded. 5-FU and 5′-dFUR were eluted with 5 column volumes (3.5 ml) of 0.1 mol/l formic acid and evaporated to dryness at 60° C under a gentle stream of nitrogen (approx. 60 min). Ten columns can be handled simultaneously. The evaporated sample was dissolved in 20 μl of water and 1–4 μl were then injected into the Tachophor. A second injection with spacer solution, 1–3 μl, was then performed.

Quantitation

In isotachophoresis the zone length is directly proportional to the amount of a compound in equilibrium with the leading electrolyte [8].

In this paper the zone widths at half the peak height was determined when more than 100 pmol were injected. In the range 10–100 pmol of a compound

the peak heights of the ions were measured. Zones representing 5-FU and 5'-dFUR were measured and quantitated by comparison with standard curves [5].

Reproducibility and linearity

Calibration curves were constructed by adding increasing amounts of 5-FU and 5'-dFUR to pooled plasma from healthy blood donors. The addition of 5-FU and 5'-dFUR was followed by immediate deproteinization. Day-to-day variations were tested by repeat runs of pooled plasma samples. The within-day variation was determined in the same way. Further, plasma from cancer patients treated by intravenous bolus injections or slow intravenous infusions was used for analysis. For these samples 5'-dFUR 0.1—20 nmol were added to 1 ml of plasma.

RESULTS

Extraction procedure

5-FU and 5'-dFUR cannot be quantitatively separated by isotachophoresis when whole plasma is injected into the Tachophor. Deproteinization with picric acid and ion-exchange chromatography were therefore used as purification steps. The recovery of known amounts of 5-[^{3}H]FU and 5'-dFUR subjected to the extraction procedure was 88 ± 0.8% and 78 ± 1.2%, respectively, as calculated from ten separate experiments.

Determination of optimal isotachophoretic conditions

In aqueous solution 5-FU and 5'-dFUR is well separated with 5 mmol/l leading electrolyte at a pH interval of 7.0—8.6. However, in order to obtain a perfect separation of 5-FU and 5'-dFUR in plasma the pH of the leading electrolyte is critical. Two different leading electrolyte systems were finally selected. In samples where other compounds such as allopurinol, hypoxanthine and oxypurinol are to be quantitated together with fluorinated pyrimidines a pH of 8.4 was selected (Table I). In samples where only 5-FU and 5'-dFUR are to be quantitated a pH of 7.6 is convenient. The terminator finally selected was 40 mmol/l of recrystallized glycine with barium hydroxide added to pH 9.4.

TABLE I

ELECTROLYTE SYSTEM FOR THE SEPARATION OF 5-FLUOROURACIL AND 5'-DEOXY-5-FLUOROURIDINE

	Leading electrolyte	Terminating electrolyte
Anion	Cl^-	Glycine*
Concentration	0.005 *M*	0.04 *M*
Counterion	Tris	Ba^{2+}
pH	8.4	9.4
Additive	0.25% HPMC	None

*Glycine recrystallized.

During the investigation different lengths of capillary were used. For routine purposes a capillary 23 cm long (I.D. 0.5 mm) was satisfactory. The analysis time was 20—23 min at 18°C. To reduce the separation time, the experiments were started at an elevated current (95 μA) which was reduced to 45 μA prior to detection. As spacers, 4-morpholineethanesulfonic acid (MES), 4-morpholinepropanesulfonic acid (HEPES), and tris(hydroxymethyl)methylaminopropanesulfonic acid (TAPS) were used (Table II).

TABLE II

SPACER COMPOUNDS FOR SEPARATION OF 5-FLUOROURACIL AND 5'-DEOXY-5-FLUOROURIDINE

The leading electrolyte, 0.005 *M* HCl in 0.25% HPMC, was titrated with Tris to pH 8.4. The terminating electrolyte, 0.04 *M* glycine, was titrated with saturated $Ba(OH)_2$ to pH 9.4.

	pK_a
4-Morpholineethanesulfonic acid (MES)	6.5
4-Morpholinepropanesulfonic acid (MOPS)	7.20
N-2-(Hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES)	7.5
4,2-(Hydroxyethyl)1-piperazineethanesulfonic acid (HEPES)	7.55
Tris(Hydroxyethyl)methylaminopropanesulfonic acid (TAPS)	8.40

Calibration and quantitation

Fig. 1 shows an isotachopherogram of a normal plasma sample where known amounts of 5-FU and 5'-dFUR have been added to the plasma pretreated as described above. To identify 5'-dFUR in this experiment the sample was rerun together with pure 5'-dFUR (Fig. 2) using the non-UV-absorbing spacers. The resolution of the separation was greatly improved. In Fig. 3 a plasma sample from a patient receiving 5-FU combined with allopurinol is demonstrated. It was empirically found that the peak height was proportional to the 5-FU and 5'-dFUR concentration in the range 10—80 pmol. This had earlier been demonstrated for hypoxanthine [9].

Reproducibility

The within-day coefficient of variation was calculated from the results of ten separate runs at two different concentrations of 5-FU and 5'-dFUR, 1.0 and 20.0 μmol/l. The coefficients of variation for 5-FU and 5'-dFUR were 7% and 8%, respectively, for the low concentration and 4% and 5%, respectively, for the high concentration.

Application to biological samples

Blood samples were taken from patients with gastrointestinal cancer during 5-FU therapy. The concentration of 5-FU after intravenous bolus injection of 15 mg kg^{-1} body weight reached its highest level 5 min after injection and varied between 120 and 260 μmol/l (four patients), which is in good agreement with the concentrations reported after the same dose schedule analysed by gas chromatography—mass spectrometry [10].

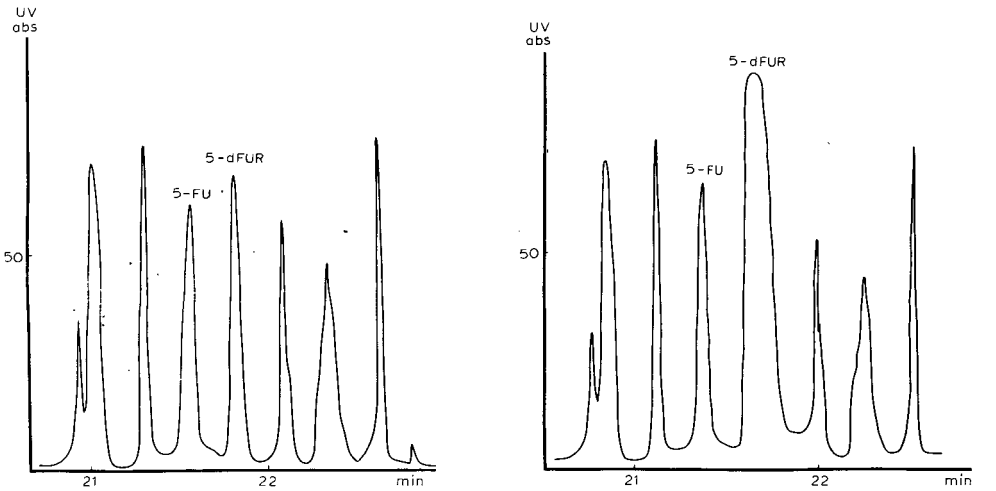

Fig. 1. Isotachopherogram of plasma sample; 2 μl were injected into the Tachophor (corresponding to 0.1 ml of plasma). The leading electrolyte was 0.005 *M* HCl in 0.25% HPMC titrated with Tris to pH 8.4. The terminating electrolyte, 0.04 *M* glycine, was titrated with saturated $Ba(OH)_2$ to pH 9.4. Capillary 43 cm long, current 45 μA. The wavelength of 254 nm was recorded and the temperature was 18°C. A 2-μl volume of spacer solution (see Table II) was also injected.

Fig. 2. Isotachopherogram of the same plasma sample as in Fig. 1 together with injection of 1 nmol of pure 5'-dFUR. The working conditions were the same as in Fig. 1.

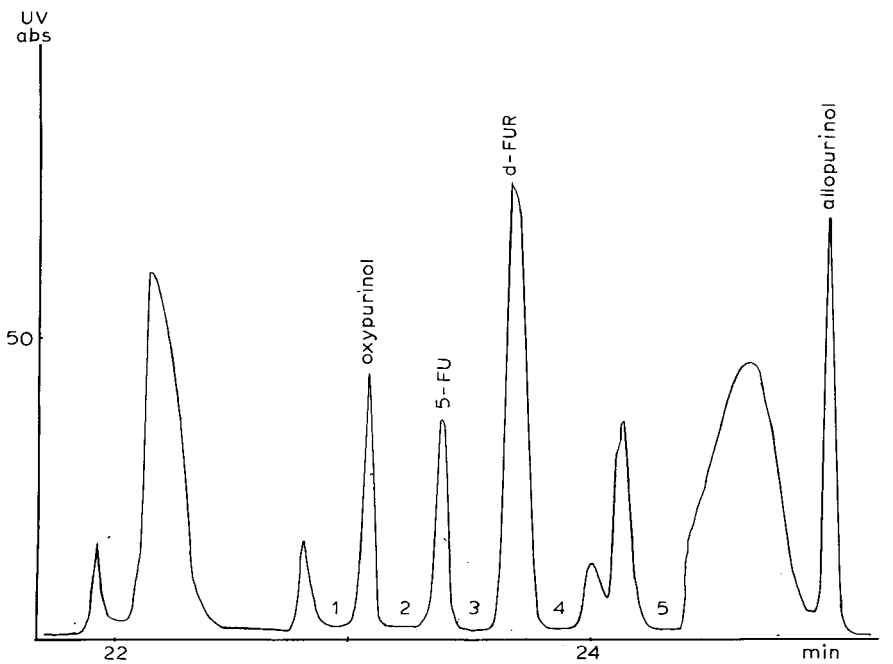

Fig. 3. Isotachopherogram of plasma sample from a patient treated with a continuous infusion of 5-FU, 15 mg kg^{-1} 24 h^{-1} and allopurinol 100 mg × 3 per os. A 2-μl volume was injected (corresponding to 0.1 ml of plasma) together with 3 μl of spacer solution (see Table II).

DISCUSSION

The results of the present study indicate that analytical isotachophoresis is a sensitive and specific method which can be employed for simultaneous measurements of different fluorinated pyrimidines and oxipurines. As previously reported, prepurification of biological samples is necessary to obtain an optimal separation. We have earlier reported that deproteinization with picric acid followed by ion-exchange chromatography is a simple and accurate procedure for this purpose [5].

In isotachophoresis the resolved zones are forced to run in immediate contact with each other. This can lead to practical difficulties as reported in the original method description [5]. As early as 1965 Vestermark [11] drew attention to this problem and suggested addition of intermediate-mobility compounds to the sample solution.

Briefly, spacing can be obtained by using a continuous gradient or by using discrete spacers [12]. In principle, a discrete spacer is a single compound chosen to have a mobility intermediate to those of two sample compounds of interest. In this method we have used non-UV-absorbing compounds and found that this technique is extremely useful in order to improve identification and quantitation of 5-FU and 5′-dFUR, especially in samples with low (< 100 pmol) concentrations, or in samples where other purines or pyrimidines are to be quantitated. By using spacers, the pH in the leading electrolyte will not be as critical as reported in our original method to obtain optimal separation. Thus, as demonstrated in Fig. 3, the spacer MOPS and TES "framed" the 5-FU zone while the spacer HEPES moved directly behind the 5′-dFUR zone. It was further found that in patients given allopurinol, the parent drug and its metabolite oxipurinol were eluted in the same fraction as the fluorinated pyrimidines. It thus seems likely that the isotachophoretic technique described should be readily adapted for simultaneous analysis of not only fluorinated pyrimidines. In fact, Oerlemans et al. [13] recently reported that isotachophoresis offers a simple and rapid way to determine urinary purines and pyrimidines. These authors further concluded that the high reproducibility (day-to-day) variation and repeatability), and short analysis time justify the conclusion that isotachophoresis offers interesting possibilities for the analysis of UV-absorbing compounds both for experimental and diagnostic purposes.

Until now, to our knowledge, only one method for the determination of 5′-dFUR has been reported. Using high-performance liquid chromatography Armstrong and Diasio [4] reported that using a 50-μm C_8 reversed-phase column with a 5-μm reversed-phase precolumn, 5-FU and 5′-dFUR could be separated in plasma. The retention time for 5′-dFUR was 46 min.

This present study demonstrates that isotachophoresis offers possibilities for the determination of 5′-dFUR and other fluorinated pyrimidines in plasma. Since 5′-dFUR represents an interesting new fluoropyrimidine with clinical potential the described method could be used for further studies of the physiological disposition of this drug.

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Note

Rapid method for quantitative analysis of N,N-dibutylnitrosamine, N-butyl-N-(4-hydroxybutyl)nitrosamine and N-butyl-N-(3-carboxypropyl)-nitrosamine in rat urine by gas chromatography—thermal energy analysis

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In 1964 Druckrey et al. [1] reported that among 63 N-nitroso compounds tested, only N,N-dibutylnitrosamine (DBNA) and its ω-hydroxylated derivative, N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN), were carcinogenic to the rat urinary bladder. Since then, the metabolism of these compounds has been elucidated and their urinary metabolites have been identified [2]. One of the DBNA metabolic pathways leads to the formation of BBN which is further oxidized to the corresponding acidic derivative N-butyl-N-(3-carboxypropyl)-nitrosamine (BCPN). BCPN has been shown to be the proximate metabolite of both DBNA and BBN responsible for the induction of urinary bladder tumors in rats [2].

DBNA has been observed as a pollutant in tobacco smoke, corrosion inhibitors, food and more recently in several rubber products including baby bottle nipples, toys, and possibly new motor cars [3—8]. Because of the widespread distribution of DBNA in the environment, a specific, sensitive analytical method for evaluating human exposure to this N-nitroso compound is very much needed. This paper describes the development of a gas chromatographic (GC) method for the quantitative analysis of DBNA, BBN and BCPN extracted from urine of rats given DBNA. BBN was measured as its trimethylsilyl ether (BBN-TMS) and BCPN as its trimethylsilyl ester (BCPN-TMS). The high sensitivity required was achieved by using a gas chromatograph coupled with a thermal energy analyzer (GC—TEA).

The application of the method to human urine samples will be discussed elsewhere.

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EXPERIMENTAL

Reagents

N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA), trimethylchlorosilane (TMCS) and pyridine were obtained from Pierce Chemical Co., Rockford, IL, U.S.A.; Clin Elut™ 1020 extraction columns were produced by Analytichem International, Harbor City, CA, U.S.A.; β-glucuronidase/arylsulfatase was obtained from Boehringer (Mannheim, G.F.R.).

Standards

DBNA was purchased from Eastman Kodak Co., Rochester, NY, U.S.A.; BBN and BCPN were kindly donated by Dr. Masashi Okada of the Tokyo Biomedical Research Institute, Japan.

Urine extraction

Male CD-COBS rats (body weight 180 ± 10 g) obtained from Charles River, Italy, were fasted overnight before treatment and housed individually in metabolic cages. DBNA was administered orally at the dose of 5 mg/kg. Urine samples were collected for 24 h on 500 mg of ammonium sulfamate, added to inhibit unwanted nitrosation. Each 24-h urine sample was divided into two and the volume of each half was made up to 20 ml with water if less than that, and the pH was adjusted to 5. Half the samples were incubated with β-glucoronidase/arylsulfatase (50 μl/sample) at 37°C overnight. All samples were extracted twice with 10 ml of ethyl acetate. The organic phases were combined (18 ml) and added to Clin Elut columns to which 20 ml of 0.1 *N* hydrochloric acid had been added and absorbed. After 2--5 min a second 18 ml of ethyl acetate was added to the columns; the column eluate was collected and the volume was reduced to 0.2 ml; 1—4 μl were analyzed by GC—TEA for DBNA content.

The samples were then evaporated to dryness. The dry residue was dissolved in 50 μl of ethyl acetate and 50 μl of reagent mixture containing pyridine—BSTFA—TMCS (50:45:5); 1—4 μl were analyzed by GC—TEA for BBN and BCPN content. In order to confirm the identity of the gas chromatographic peaks, GC—mass spectrometry (MS) analyses were carried out.

Gas chromatography and gas chromatography—mass spectrometry

A DANI 3800 gas chromatograph equipped with a TEA 543 detector (Thermal Energy Analyzer, Thermo Electron) was used. The glass column (2 m × 2 mm I.D.) was packed with 3% OV-1 on Gas-Chrom Q, 100—120 mesh. DBNA was analyzed at 140°C. For analysis of BBN and BCPN the oven temperature was kept at 140°C for 2 min, then programmed from 140 to 170°C at a rate of 15°C/min. The carrier gas (helium) flow-rate was 30 ml/min. The GC—TEA interface temperature was 250°C and the pyrolyzer temperature was 500°C.

GC—MS analysis was performed on an LKB 2091-051 gas chromatograph—mass spectrometer equipped with an LKB 2130 computer system for data acquisition and calculation and used in the electron impact mode. GC—MS operating conditions were as follows: glass WCOT open tubular column 25 m × 0.3 mm I.D. coated with OV-1, film thickness 0.15 μm; column head

pressure, 2 bars. DBNA analysis was performed at 120°C; for the analysis of BBN and BCPN the temperature was kept at 120°C for 2 min then programmed from 120 to 200°C at a rate of 4°C/min; ion source temperature, 250°C; electron energy, 70 eV; trap current, 50 μA; accelerating voltage, 3.5 kV; resolution 600; scan speed 3.

RESULTS AND DISCUSSION

In the GC—TEA condition described, DBNA had a retention time of 2 min 30 sec; BBN-TMS and BCPN-TMS had retention times of 5 and 6 min, respectively. A linear response was observed for all three compounds for injected amounts ranging from 0.5 to 4 ng, the correlation coefficient r being 0.999, 0.9993 and 0.9992 for DBNA, BBN, and BCPN, respectively.

Extraction and clean-up efficiency was evaluated by adding known amounts (25—200 ng) of standard DBNA, BBN, and BCPN to urine samples (20 ml) from untreated rats. Recovery values for DBNA, BBN and BCPN were 70 ± 2%, 67 ± 4% and 72.5 ± 2% (mean ± S.D.), respectively. The lowest detectable amount of the three compounds was 10 ng excreted in 24 h.

Fig. 1 shows a typical GC—TEA chromatogram of a blank urine sample (Fig. 1a), a urine sample spiked with 50 ng of DBNA (Fig. 1b) and a urine sample from DBNA-treated animals (Fig. 1c).

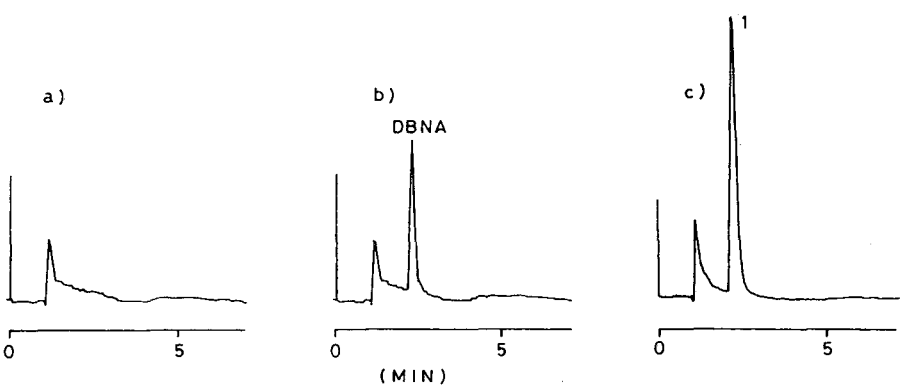

Fig. 1. GC—TEA chromatograms of a blank urine sample (a), a urine sample spiked with 50 ng of DBNA (b), and a urine sample from DBNA-treated animals (c). Peak 1 had the same retention time as standard DBNA.

Fig. 2 shows a typical GC—TEA chromatogram of a blank sample of urine after derivatization with BSTFA—TMCS (Fig. 2a) and a urine sample spiked with BBN and BCPN (50 ng each) and analyzed after derivatization with BSTFA—TMCS (Fig. 2b). Fig. 3 shows a typical GC—TEA chromatogram of a urine sample from DBNA-treated animals analyzed for BBN and BCPN content before (Fig. 3a) and after (Fig. 3b) hydrolysis with β-glucuronidase/aryl-sulfatase. Samples were derivatized with BSTFA—TMCS before analysis.

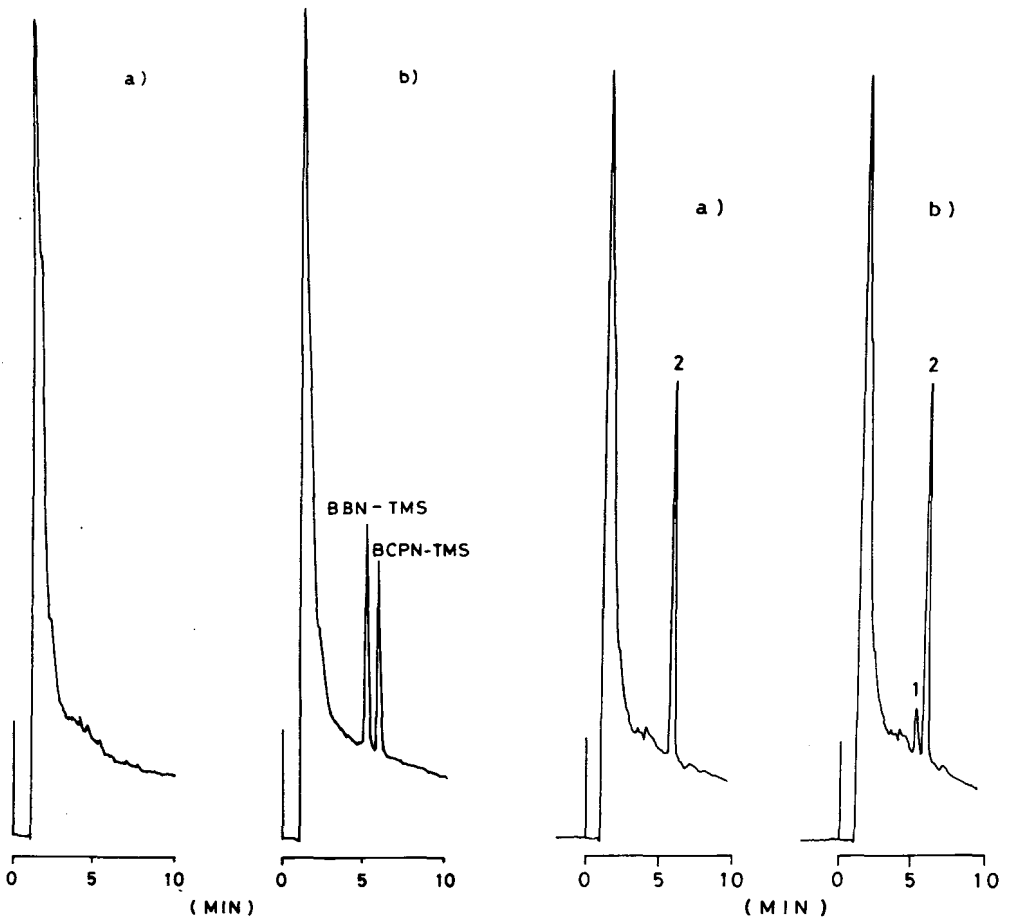

Fig. 2. GC—TEA chromatograms of a blank urine sample after derivatization with BSTFA—TMCS (a) and a urine sample spiked with BBN and BCPN (50 ng each) and analyzed after derivatization with BSTFA—TMCS (b).

Fig. 3 GC—TEA chromatograms of a urine sample from DBNA-treated animals analyzed for BBN and BCPN content before (a) and after (b) hydrolysis with β-glucuronidase/arylsulfatase. Samples were derivatized with BSTFA—TMCS. Peaks 1 and 2 had the same retention time as standard BBN-TMS and BCPN-TMS, respectively.

Urinary levels of DBNA, BBN and BCPN in rats given DBNA at the oral dose of 5 mg/kg are reported in Table I. About 0.3% of the administered DBNA is excreted unchanged in the 24-h urine; BBN is present only in the conjugated form and amounts to 0.05% of the administered DBNA. BCPN is about 21% of the administered DBNA and is present as free acid. Quantitatively, these results differ slightly from those published in an earlier paper [9], whose purpose, however, was to identify DBNA urinary metabolites rather than to quantify them.

TABLE I

DBNA, BBN AND BCPN IN URINE OF RATS GIVEN DBNA 5 mg/kg PER OS

Compound	μg per 24 h (mean ± S.E.)	
	Free	Glucuronic acid/aryl-sulfate conjugated
DBNA	2.7 ± 0.75	—
BBN	n.d.*	0.46 ± 0.13
BCPN	191.1 ± 21	n.d.

*n.d. = not detectable (< 10 ng per 24 h).

The identity of the GC—TEA peaks of DBNA, BBN and BCPN extracted from urine of animals treated with DBNA was verified by GC—MS. Because of the small amount of BBN excreted in the urine, several samples had to be pooled to give enough material for a mass spectrum. The three mass spectra were exactly the same as those obtained by analyzing authentic standards.

The DBNA fragmentation pattern was in agreement with the data reported in the literature [10]. In the mass spectra of BBN-TMS and BCPN-TMS, the molcular ions at *m/z* 246 and 260, as well as in the mass spectra of most silylated compounds, are never present, but the $M^+ - CH_3$ peaks (*m/z* 231 and 245, respectively) can be used for determination of molecular weight [11]. The fragment assignments for BBN-TMS and BCPN-TMS mass spectra are reported in Table II.

TABLE II

MASS SPECTRAL DATA OF BBN AND BCPN AS THEIR TMS DERIVATIVES

BBN		BCPN		Fragment
m/z	%	*m/z*	%	
246	0	260	0	$[M]^+$
231	6	245	6	$[M - CH_3]^+$
229	11			$[M - OH]^+$
216	5	230	34	$[M - NO]^+$
172	7	186	7	$[M - (C_3H_7+HNO)]^+$
158	12	172	21	$[M - (NO+CH_3+C_3H_7)]^+$
		159	18	$[M - (C_3H_7+HNO+HCN)]^+$
126	100	140	29	$[M - (NO+TMS\text{-}OH)]^+$
		112	47	$[M - (COOTMS+HNO)]^+$
		98	31	$[M - (NO+TMS\text{-}OH+C_3H_6)]^+$
84	47	84	62	$CH_3\text{-}CH_2\text{-}CH_2\text{-}CH{=}\overset{+}{N}{=}CH_2$ or $CH_3\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}\overset{+}{N}{\equiv}CH$

Because of its high sensitivity and specificity, the method described appears to be useful for assessing DBNA contamination by measuring the amount of the nitrosamine and its oxidized metabolites BBN and BCPN in urine. The amount of urinary BCPN is the better indicator of the degree of contamination

since this metabolite is excreted in higher concentrations than BBN or DBNA itself; moreover, it is the proximate metabolite responsible for the induction of urinary bladder tumors in rats.

The method will be used to check the urine of human populations likely to be exposed to DBNA, e.g. rubber industry workers, or bottle-fed babies.

ACKNOWLEDGEMENTS

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CHROMBIO. 1735

Note

Rapid separation of plasma steroids by reversed-phase high-performance liquid chromatography with timed collection of fractions

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(Received February 9th, 1983)

The separation of steroid mixtures using high-performance liquid chromatography (HPLC) has been the subject of numerous studies [1—5]. Often, however, such publications involve gradient analyses, and when the necessary equipment is unavailable the operator wishing to separate steroids of widely differing polarity under isocratic conditions is frequently confronted with lengthy separation procedures, impractical to apply to routine analyses in the clinical chemical laboratory.

We have therefore developed conditions permitting the separation and isolation of the steroids cortisol, androstenedione, testosterone, 17α-hydroxyprogesterone and progesterone from plasma samples in less than 15 min by isocratic reversed-phase HPLC. The incorporation of a partial purification of plasma extracts prior to HPLC eliminates the need of frequent regeneration of the column by removing late-eluting UV-absorbing components.

Measurement of these steroids is most useful for the diagnosis of congenital hyperplasia due to 21-hydroxylase deficiency and offers an index for the adequacy of treatment of such patients.

EXPERIMENTAL

Materials and methods

[^{3}H]Cortisol (56.0 Ci/mmole), [^{3}H]androstenedione (90.0 Ci/mmole), [^{3}H]testosterone (50.4 Ci/mmole), 17α-[^{3}H]hydroxyprogesterone (50.0

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Ci/mmole) and [^{3}H]progesterone (101.0 Ci/mmole) were obtained from The Radiochemical Centre, Amersham, Great Britain. Portions of each steroid were diluted in 0.2% (v/v) aqueous ethylene glycol so that a 100-μl aliquot contained approximately 5000 cpm. Non-radioactive steroids were obtained from Steraloids, Wilton, NH, U.S.A. Radioactivity was measured in a Packard Tri-carb liquid scintillation counter (Packard Instrument, Downers Grove, IL, U.S.A.) using Aqua Luma^R scintillation liquid (Lumac Systems, Titusville, FL, U.S.A.). Counting efficiency was 48—50%.

Liquid chromatography was performed using a Model 6000A solvent delivery pump, a U6K injector, a Model 480 LC spectrophotometer and a Model 730 data module, all from Waters Assoc., Milford, MA, U.S.A. A Hypersil 5 ODS column (150 × 4.6 mm), purchased from Chrompack, Middelburg, The Netherlands, was used for the analytical separations. A guard column, packed with pellicular C_{18} material was used to protect the analytical column. The solvent composition was methanol—tetrahydrofuran—water (3:2:5, v/v) at a flow-rate of 1 ml/min, resulting in a column pressure of 14 MPa.

Retention times were determined using authentic standards (10 ng each) and were reproducible to within 0.2% over a 4-h period.

Plasma samples were taken from pooled plasma, obtained from healthy adults (male and female blood donors).

Sep-Pak^R C_{18} cartridges were obtained from Waters Assoc. and were attached to Eppendorf combitips 12.5 ml (Eppendorf, Hamburg, G.F.R.) to facilitate sample application and elution.

Extraction procedure

Following incubation with tritiated steroids (approx. 5000 cpm in 100 μl of 0.2% aqueous ethylene glycol) for 30 min at 37°C, 2-ml plasma samples were extracted with diethyl ether (16 ml). After evaporation of the extracts the residues were taken up in 5 ml of 0.9% (w/v) aqueous sodium chloride and were kept at 37°C for 15 min to dissolve the steroids. Upon cooling to room temperature the suspensions were applied to Sep-Pak C_{18} cartridges which had been washed previously with methanol (10 ml), water (10 ml), and 0.9% aqueous sodium chloride (10 ml). The cartridges were washed with sodium chloride solution (5 ml) and water (10 ml) and then eluted with methanol—tetrahydrofuran—water (3:2:5, v/v; 7.5 ml). The eluates from the cartridges were concentrated to about 4 ml under a stream of purified air at 37°C and were then extracted with diethyl ether (2 × 10 ml). The combined ether extracts were evaporated and dissolved in 50 μl of methanol—tetrahydrofuran—water (3:2:5, v/v) for subsequent liquid chromatography.

Fraction collection

The Model 730 data module was used to actuate a three-port solenoid switching valve 24 VDC (Pharmacia, Uppsala, Sweden), thus allowing the timed collection of fractions of the eluate from the LC column into an LKB 2070 Ultrorack fraction collector. The circuit diagram of the interface (home-made) required to operate the valve and fraction collector is shown in Fig. 1. A brief explanation of its principle of operation follows.

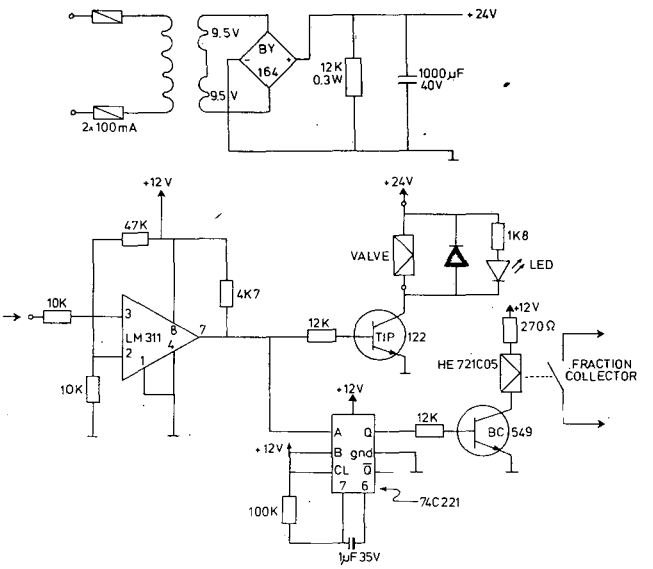

Fig. 1. Circuit diagram of interface and connections between data module, fraction collector (pins 10 and 13 of external input) and valve. K = kΩ, 4K7 = 4700 Ω, 1K8 = 1800 Ω.

The 12 V d.c. output on the data module is used to feed the interface. The timed event output (normally + 5 V d.c.) drops to ground potential upon activation, thus triggering the voltage comparator LM 331 which in turn triggers transistor TIP 122 and the valve is actuated. When the timed event output reverts to + 5 V d.c. the valve returns to its normal position, diverting the eluate from the LC column to a waste container. Through IC SN74C221 triggering transistor BC 549 the voltage comparator activates the Reed relay which causes the fraction collector to step. A light-emitting diode is connected in parallel to the valve's coil and provides visual inspection of the switching of the valve.

RESULTS AND DISCUSSION

Binary mixtures of methanol, acetonitrile or tetrahydrofuran and water were unsuccessful in resolving the steroids of interest under isocratic conditions within 15 min analysis time. Therefore ternary mixtures were used to determine optimal LC conditions since such mixtures have been shown [6—8] to possess powerful selective properties. Although a radially compressed C_{18} column (100 × 8 mm, 5 µm particle size; Waters Assoc.) in combination with acetonitrile—tetrahydrofuran—water (4:1:5, v/v) was satisfactory with respect to resolution and analysis time, the Hypersil 5 ODS column in combination with methanol—tetrahydrofuran—water (3:2:5, v/v) was selected because, due to a smaller internal diameter and lower flow-rate, peak volumes were smaller (1 ml vs. 3—4 ml). This will undoubtedly be advantageous in off-line quantitation, e.g. radioimmunoassay.

The extent to which cross-contamination of fractionated steroids might occur was determined in several trial experiments. Following measurement

of retention times of authentic standards (non-radioactive, 10 ng of each) and correcting for the delay time from detector to collector, the data module was programmed to collect fractions from 10—30 sec before, until 40—60 sec after, the corrected retention times thus obtained. Mixtures of standards, charged with separate ^{3}H-labelled steroids (approx. 5000 cpm) were then chromatographed and the percentage distribution of radioactivity was determined across collected fractions from each analysis. If necessary, the time interval for collecting individual fractions was then adjusted until cross-contamination was less than 5% (Fig. 2).

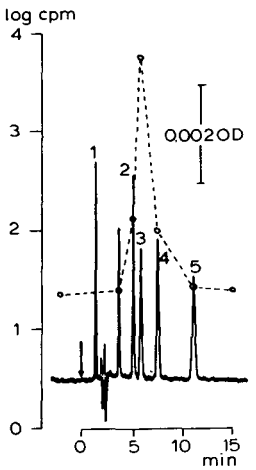

Fig. 2. HPLC separation of a mixture of steroids charged with [^{3}H]testosterone on a Hypersil 5 ODS column (150 × 4.6 mm) using methanol—tetrahydrofuran—water (3:2:5, v/v) as the mobile phase at 1 ml/min. Absorbance measured at 240 nm. Injected volume 45 μl (10 ng of each component). (o- - - -o), amount of radioactivity contained in each fraction collected (note logarithmic scale). Peaks: 1 = cortisol, 2 = androstenedione, 3 = testosterone, 4 = 17α-hydroxyprogesterone, 5 = progesterone.

Extraction of plasma steroids was originally conducted as described by Cannell et al. [9]. In short, this procedure involves loading Sep-Pak C_{18} cartridges with plasma samples diluted (1:10) with acetate buffer and eluting the adsorbed steroids with methanol. Although this method gave satisfactory results on 1-ml plasma samples we found the HPLC column to be rapidly contaminated, necessitating frequent regeneration. We therefore modified the method of Cannell et al. by loading Sep-Pak cartridges with ether extracts instead of whole plasma and eluting the adsorbed steroids with methanol—tetrahydrofuran—water (3:2:5) in place of methanol. In this way injection of methanol (1.5 ml), following elution of the progesterone peak, adequately cleaned the column between separations. Since recoveries of steroids were lower, due to the incorporation of two solvent extraction steps, 2-ml plasma samples were used (Fig. 3).

Recoveries of the individual steroids through the extraction procedure ranged from 65% (cortisol) to 95% (testosterone), as determined from radioactivity measurements. With the exception of androstenedione, recoveries

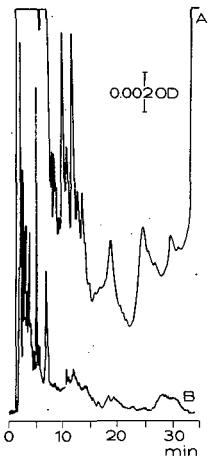

Fig. 3. Liquid chromatogram of Sep-Pak eluates of plasma samples. (A) 1 ml of whole plasma, eluted with methanol. (B) Ether extract of 2 ml of plasma eluted with methanol—tetrahydrofuran—water (3:2:5, v/v). Conditions as in Fig. 2.

in the HPLC fractionation procedure were between 75% and 80%. The low recovery of androstenedione (56%) was the price paid for keeping cross-contamination <5%. Therefore the overall recovery of androstenedione averaged 45% while the other steroids showed recoveries of 55—65%.

CONCLUSIONS

The HPLC fractionation of plasma extracts purified on Sep-Pak C_{18} cartridges as described in this paper provides a rapid and simple means of isolating the steroids cortisol, androstenedione, testosterone, 17α-hydroxyprogesterone and progesterone. Yields are more than adequate for subsequent quantitation by radioimmunoassay. The extraction and purification procedures can easily be applied to large sets of plasma samples and the HPLC fractionation procedure allows rapid, consecutive separations suitable for automation.

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Note

Extraction and quantitation of cortisol by use of high-performance liquid affinity chromatography

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Quantitative determination of cortisol in urine by radioimmunological techniques is inexact and non-specific, probably due to co-determination of other corticosteroids or polar metabolites of cortisol. As a reference method mass spectrometry [1] has been suggested. However, this method is expensive and time-consuming due to the need for derivatization. Low-pressure affinity chromatography has been shown [2] to be useful to enrich cortisol from serum samples prior to high-performance liquid chromatographic (HPLC) quantitation.

A new and faster technique, called high-performance liquid affinity chromatography (HPLAC), has recently been introduced and shown to be useful for enzyme, protein and carbohydrate purification [3, 4]. The technique is based on covalent linkage of a ligand to a solid silica matrix. The ligand, i.e. an antigen or an antibody, has high affinity to the compound of interest and can be absorbed and reversibly eluted with a high ion strength buffer or a compound with high affinity to the substance. In this paper the technique has been used to purify and quantitate cortisol in one step from plasma and urine samples. The extract is directly analysed by HPLC.

EXPERIMENTAL

Materials

Porous silica gel Si 60 (LiChrosorb Si 60, 40 μm) and LiChrosorb RP-18 (5 μm) were obtained from E. Merck, Darmstadt, G.F.R. γ-Glycidoxypropyltrimethoxysilane (Silane Z-6040) was purchased from Dow Chem. (Midland, MI, U.S.A.). Cortisol-3-carboxymethyloxime—bovine serum albumin (BSA)

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was synthesized according to the description of Erlanger et al. [5]. All solvents and other chemicals, obtained from commercial sources, were of analytical grade or HPLC grade and were used without further purification.

Equipment

A Waters Model U6K injector (Waters Assoc. Milford, MA, U.S.A.), a Model 6000 pump and a Model 440 UV detector was used in the chromatographic separations. The LiChrosorb RP-18 column (25 cm × 5 mm I.D.) was eluted and equilibrated with methanol—water (60:40). The flow-rate was set to 1.0 ml/min and the absorbance was monitored at 254 nm. ^{3}H-Radioactivity of collected fractions was measured in Insta-gel (10 ml) on a Packard liquid scintillation spectrometer (Packard, Downers Grove, IL, U.S.A.).

Modification of silica Si 60

Silica Si 60 was coupled to γ-glycidoxypropyltrimethoxysilane according to the method of Glad et al. [4] with minor modifications. A 10-g amount of Si 60 was refluxed with 15 ml of γ-glycidoxypropyltrimethoxysilane for 4 h. A small amount of triethylamine (250 μl) was added. The substituted Si 60 was filtered off and washed with 100 ml of acetone and 50 ml of diethyl ether. Then 2.5 g of the substituted Si 60 were mixed with 3 ml of water, acidified to pH 3 with 0.1 *M* sulphuric acid and heated to 90° C for 1 h. After filtering and washing (10 ml of water, 10 ml of acetone, 10 ml of diethyl ether) the diol compound was oxidized with 25 ml of sodium metaperiodate (0.06 *M* aqueous solution) at ambient temperature for 0.5 h. The substituted Si 60 was then ready for antibody coupling after washing successively with water, acetone and diethyl ether.

Coupling to anti-cortisol antibodies

An antiserum against cortisol-3-carboxymethyloxime—BSA (anticortisol) was raised in rabbits. The antibody solution was fractionated on an ion-exchange DEAE Sepharose Cl 6B column (Pharmacia, Uppsala, Sweden) and eluted with 0.05 *M* Tris, pH 7.4. The immunoglobulin (Ig) fraction was collected. Reamining proteins were eluted with 0.1 *M* NaCl in Tris, pH 7.4, followed by 0.2 *M* NaCl in Tris, pH 7.4. The collected Ig fractions were poured into a dialysis tube and concentrated by means of crystalline PEG 2000. The enriched fraction was dialysed against water. The recovery was 95%, estimated from the absorbance of the fractions at 280 nm. A 4.5-ml volume of anti-cortisol antibody solution dissolved in 10 ml of 0.1 *M* sodium hydrogen carbonate solution was allowed to react with 2.0 g of the modified silica. The mixture was left at 4° C for 22 h with gently stirring. The absorbance at 280 nm of a small portion of the solution was checked intermittently to monitor the coupling efficiency. After coupling, remaining aldehyde functions on the silica matrix were reduced with 80 mg of sodium borohydride, which was suspended in 500 μl of water and added slowly to the solution. The solution was left overnight at room temperature. The material was then filtered off on a G 3 glass filter and washed with 100 ml of 0.1 *M* sodium hydrogen carbonate buffer and 200 ml of distilled water. The yield of the modified silica gel was enough for packing three 5 cm × 5 mm I.D. stainless-steel columns. The columns were packed

with the upward slurry packing technique [6] in water—methanol (30:70) at 14 MPa. The material was stored under water in a refrigerator when not in use.

RESULTS AND DISCUSSION

Test of binding efficiency

The loop of the U6K injector (Waters Assoc.) was replaced by an antibody packed column. A LiChrosorb RP-18 (particle size 5 μm) column was equilibrated with methanol—water (60:40). Ten microlitres of a 10 ng/μl cortisol standard in 0.1 *M* Na_2HPO_4, pH 7.5, buffer were incubated with 10 μl of a [^{3}H]cortisol solution (2.21 μCi/ml) and injected slowly into the antibody column. The antibody column was washed with 1 ml of 0.1 *M* NaH_2PO_4 buffer and 1 ml of water by means of a 2-ml syringe. The cortisol was then eluted with methanol–water (60:40) from the antibody column through the RP-18 column. Fractions of 1.0 ml were collected and counted in a liquid scintillator after the addition of 10 ml of Insta-gel. The data on this and similar experiments show that the ^{3}H-radioactivity was quantitatively (95%) recovered if less than 2 μg of cortisol standard (maximum binding) was injected. Repetitive ion-binding tests after one month's use of the column showed a decrease in binding capacity (20%) but the recovery was unchanged.

Analysis of standard samples

In Fig. 1a is shown a chromatogram of a standard preparation (20 μl of a 1 ng/μl solution). The loop of the injector was replaced by the silica–anti-cortisol column and the knob on the injector was placed in the load position. The injected volume of cortisol solution was 20 μl. After washing the immunosorbent with 1 ml of phosphate buffer and 1 ml of water, the flow direction was changed and the methanol—water (60:40) mixture was introduced to the immunosorbent. A 2-ml volume of this mobile phase was enough to wash out the bound cortisol. The injector was immediately set to the load position again and washed with 2 ml of water. A new injection could be started after washing the antibody column. As seen in the chromatogram (Fig. 1a) cortisol is eluted through the RP-18 column and separated from impurities. Volumes larger than 20 μl could be injected but a tendency to get broad peaks was observed when the volume was 100 μl or more.

Analysis of cortisol in serum and urine samples

A 2-ml volume of serum (or 5 ml of urine) was extracted with 5 ml of dichloromethane. The dichloromethane phase was evaporated to dryness with nitrogen and redissolved in 50 μl of methanol. A 25-μl aliquot was injected onto the antibody column. The column was washed with 2 ml of water. The absorbed fraction was then introduced to the RP-18 column by the procedure given above. The resulting chromatogram (Fig. 1b) shows a cortisol peak which was well separated from minor amounts of impurities. An attempt to analyse cortisol in serum without extraction was also done but after a few injections the RP-18 column became contaminated with impurities probably due to the difficulty in completely washing the antibody column. An external standard

preparation was used to quantitate cortisol because it was difficult to find suitable internal reference compounds which bind to the antibody column.

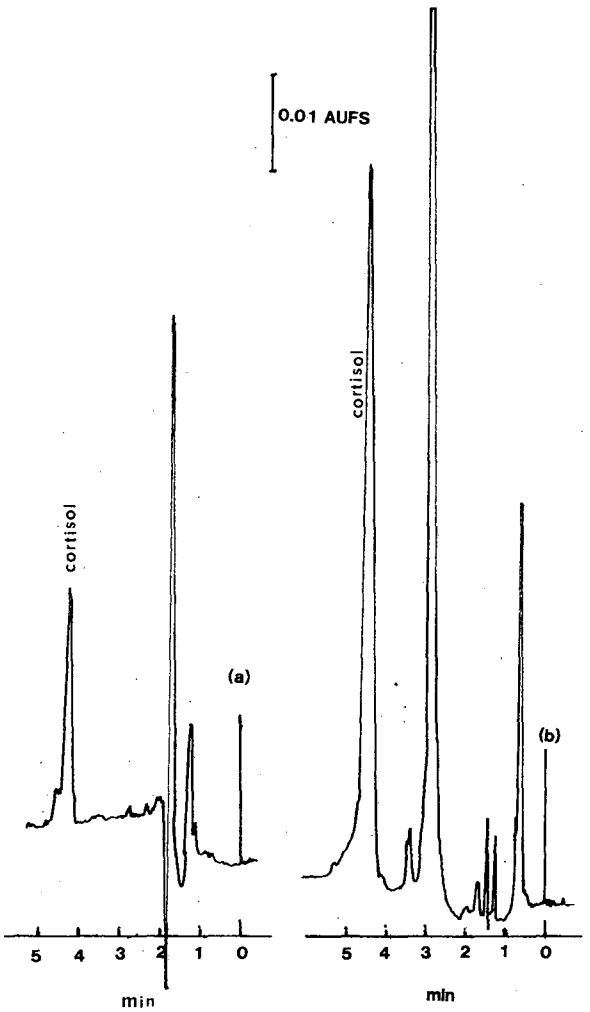

Fig. 1. HPLC chromatogram of (a) a standard preparation (20 ng) and (b) a serum extract. For conditions, see text.

CONCLUSIONS

Enrichment and quantitation of cortisol in one step by use of one affinity chromatography column and one reversed-phase column offer many advantages. One advantage is that a simple non-gradient methanol–water elution system can be used; another is that rather few impurity peaks interfere in the analysis which in turn results in a short analysis time. The anticortisol

column can be used several times and is stable for at least six months if stored in a neutral phosphate buffer (with sodium azide) in a refrigerator. The reported technique can probably find many applications in clinical chemistry laboratories where radioimmunological assays are non-specific.

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CHROMBIO. 1749

Note

Determination of S-sulfocysteine in urine by high-performance liquid chromatography

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S-Sulfocysteine (cysteine-S-sulfonic acid) was first obtained by Clarke [1] as a reaction product from cystine and sulfite and was later identified [2] as an intermediate in the conversion of inorganic sulfate to organic sulfur compounds by moulds. It was furthermore reported that sulfocysteine was present in the urine of rats after injection of cystine [3], and remarkably high concentrations of sulfocysteine have been found in the urine of the blotched Kenya genet [4]. This compound has also been demonstrated in plasma and urine from animals exposed to sulfur dioxide or sulfite in the diet [5, 6], and high concentrations of sulfocysteine are present in the urine of patients suffering from sulfite oxidase deficiency [7].

When present in high concentrations in biological material, sulfocysteine may be determined with a conventional amino acid analyzer [7, 8]. A thin-layer chromatographic screening technique has also been reported for the detection of elevated concentrations of sulfocysteine caused by sulfite oxidase deficiency [9]. Furthermore, a gas chromatographic method for the determination of sulfocysteine after enzymatic hydrolysis of sulfonated proteins has been reported [10]. Unfortunately, neither of these methods is sufficiently sensitive and specific for the determination of the fairly low concentrations of sulfocysteine present in the urine of normal human beings. Sulfocysteine is a thiosulfate ester and we recently developed a liquid chromatographic method for the determination of urinary thiosulfate using a mercury-based electrochemical detector [11]. However, this mode of detection was not applicable to urinary sulfocysteine. Liquid chromatography with fluorometric detection was then considered as an attractive alternative, provided that a fluorescent derivative of sulfocysteine could be prepared. Pre-column derivatization with

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o-phthalaldehyde in the presence of mercaptoethanol [12], succesfully used for the determination of amino acids in physiological fluids, appeared unsuitable to the determination of sulfocysteine, as the latter would react with mercaptoethanol to give cysteine [13], which yields an *o*-phthalaldehyde derivative with low fluorescence [12]. On the other hand, the Dns derivatives of amino acids [14] show very strong fluorescence and the preparation of Dns-sulfocysteine would not be expected to cause problems.

We have now developed a method for the determination of urinary sulfocysteine based on a preliminary separation of sulfocysteine from interfering compounds by ion-exchange chromatography followed by its conversion to the Dns derivative. The latter is then determined by high-performance liquid chromatography with fluorometric detection.

EXPERIMENTAL

Materials

Sulfocysteine synthesized as described previously [15] was chromatographically pure when examined by our liquid chromatographic procedure. On the other hand, we found that a commercially available product (Pierce, Rockford, IL, U.S.A.) contained an unidentified impurity which reacted with Dns chloride. The latter was a product from Fluka (Buchs, Switzerland). Ion-exchange resins AG 50W-X8 (H^+, 100—200 mesh) and AG 3-X4A (Cl^-, 200—400 mesh) were obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.) and used as received.

Procedure

Human urine was collected for 24 h with thymol—isopropanol as a preservative. A 1-ml aliquot was applied to a 3.2 × 1.0 cm column of AG 50W-X8 and the column was washed with 6 ml of water. The combined effluent and washings were neutralized with 1 *M* sodium hydroxide solution and applied to a 3.2 × 1.0 cm column of AG 3-X4A. The column was washed with 8 ml of water and the effluent and washings were discarded. The sulfocysteine was then eluted with 8 ml of 1 *M* sodium chloride solution. A 1.5-ml aliquot of the eluate was then transferred to a 10-ml test tube with a PTFE-lined screw-cap and 0.5 ml of 0.12 *M* lithium carbonate solution, adjusted to pH 9.5 with hydrochloric acid, and 1 ml of a solution of Dns chloride (1.5 g/l) in acetonitrile, were added. After vortex-mixing, the reaction mixture was left in the darkness overnight at room temperature and was then taken to dryness at 60°C using a Vortex Evaporator (Buchler Instruments Inc., Fort Lee, NJ, U.S.A.). The residue was then suspended in 0.5 ml of the mobile phase used for chromatography as described below, by vortex-mixing for two 3-min intervals separated by a 3-min sonification period. After centrifugation, 100 μl of the clear supernatant were taken for chromatography.

Our liquid chromatographic system consisted of a Constametric III pump (Laboratory Data Control, Riviera Beach, FL, U.S.A.), a Rheodyne (Berkeley, CA, U.S.A.) Model 7125 sample injector with a 100-μl sample loop, an Apex Silica column, 5 μm, 250 × 4.5 mm (Jones Chromatography, Llanbradach, Great Britain) and a Fluoromonitor III fluorometric detector (Laboratory

Data Control) with a strip chart recorder. The detector was operated with a mercury lamp and a 360-nm excitation filter and a 418—700-nm emission filter. The mobile phase was toluene—pyridine—acetic acid—ethanol (8:0.2:2:1) and was delivered to the column at a flow-rate of 1 ml/min at room temperature. Peak heights were measured for quantitative determination of sulfocysteine and a standard curve was prepared from sulfocysteine solutions of known concentrations.

RESULTS AND DISCUSSION

Preliminary experiments showed that sulfocysteine was easily converted to its Dns derivative using a procedure slightly modified from that of Tapuhi et al. [14]. As the fluorescence of Dns-amino acids is considerably lowered in polar solvents [16] we decided to use normal-phase liquid chromatography on a silica column for the separation of Dns-sulfocysteine from other Dns derivatives of urinary compounds. The mobile phase system benzene—pyridine—acetic acid—methanol described by Bayer et al. [17] was taken as a starting point for further development of the method. However, we replaced benzene with the less toxic toluene and obtained a better separation of sulfocysteine with a higher concentration of acetic acid than in the original phase system. Furthermore, we observed that the retention time of sulfocysteine varied from day to day when methanol was a component of the system. When ethanol was substituted for methanol the retention time became more reproducible, presumably due to a decreased esterification rate of ethanol with acetic acid. When liquid chromatography was attempted on urine samples after Dns derivatization, no Dns-sulfocysteine could be demonstrated due to the presence of interfering compounds.

Chromatography on a cation-exchange resin eliminated neutral and basic amino acids [2], but liquid chromatography after Dns derivatization of the pretreated sample still gave unsatisfactory results. A subsequent anion-exchange chromatographic step [18] removed remaining interfering compounds. A typical chromatogram of human urine after this clean-up procedure is shown in Fig. 1.

The identity of the peak attributed to Dns-sulfocysteine was verified by subjecting the material present in this peak to two-dimensional thin-layer chromatography on silica gel G (E. Merck, Darmstadt, G.F.R.) using the solvent system [19] methyl acetate—isopropanol—ammonia (9:7:4) in the first dimension and methyl ethyl ketone—propionic acid—water (15:5:6) in the second. Only one fluorescent component which migrated as authentic Dns-sulfocysteine was found in this peak.

The standard curve was linear at least up to concentrations of sulfocysteine corresponding to 15 μmol/l of urine. The recovery of authentic sulfocysteine added to urine samples of known sulfocysteine concentration to increase the latter by 5 μmol/l was 91 ± 11% (mean ± S.D., n = 5) and the detection limit was estimated at 0.1 μmol/l. The intra-assay precision evaluated from seven replicate analyses of a urine sample was 2.9% (coefficient of variation) and the inter-assay precision obtained from analysis of a urine sample on seven different days was 8.1%. The excretion of sulfocysteine in ten healthy males

and ten healthy females on a free diet was 11.8 ± 5.5 μmol per 24 h and 8.0 ± 2.7 μmol per 24 h (mean ± S.D.), respectively. As there was no significant difference between the sexes the results were combined giving an overall mean of 9.9 ± 4.7 μmol per 24 h (range 3.9—25.1 μmol per 24 h).

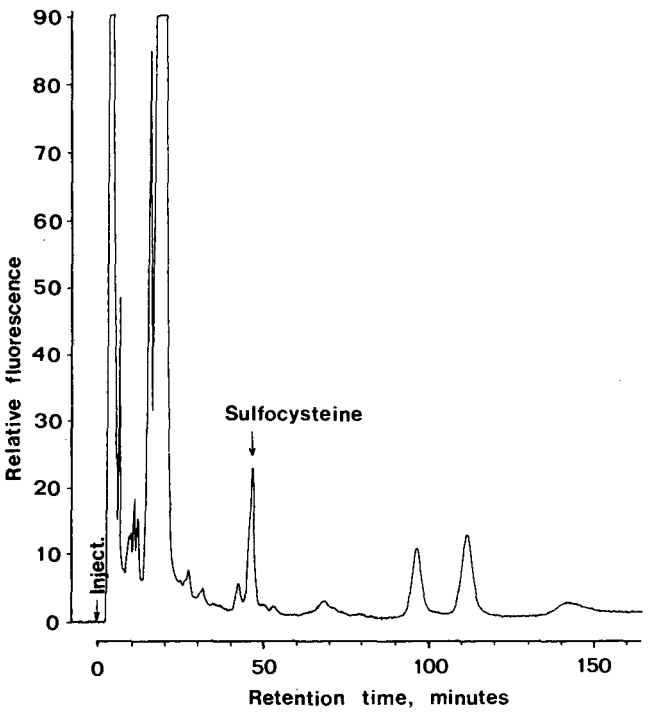

Fig. 1. Chromatogram of human urine.

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CHROMBIO. 1747

Note

Determination of orotate in ruminant milk by high-performance liquid chromatography

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Orotate is an intermediate in pyrimidine biosynthesis and therefore a component of all cells. The concentration of orotate in the tissues, including bovine tissues, is low [1]. Significant concentrations of orotate were found in milk of animals in all three main families of the suborder Ruminantia [2].

Until now, orotate has been determined either by microbiological assays [3], chemical colour reactions [4, 5], or enzymatically [6]. However, differences between the results of several authors are mainly due to assay procedure and to a lesser extent to real differences [3]. Also, because these methods are either expensive or time-consuming, we decided to develop a rapid high-performance liquid chromatographic (HPLC) method to screen large numbers of bovine milk samples for orotate, to study the effects of diet on orotate concentration in milk of ruminants. Orotate was shown to induce fatty liver in rats, and cow's milk is the major source of orotate in the human diet. Knowledge of the influence of the cow's diet on orotate excretion in milk may provide a means for regulating orotate in milk if evidence suggests that orotate in milk poses a problem [7].

EXPERIMENTAL

Reagents

Orotic acid monohydrate (6-carboxy-2,4-dihydropyrimidine) was obtained from Sigma (St. Louis, MO, U.S.A.).

Water was filtered through the Milli-Q Purification System (Millipore, Bedford, MA, U.S.A.). All other reagents were of analytical reagent grade. Mobile phase was degassed and filtered using Pyrex filter holders with 0.5-μm pore diameter filters from Millipore.

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Apparatus

The HPLC system used consisted of a Waters M6000A liquid chromatographic pump (Waters Assoc., Etten-Leur, The Netherlands), a Waters M441 UV/VIS discrete absorbance detector, a Waters automatic sample injector (WISP 710B) and a Spectra Physics SP4100 computing integrator (Spectra Physics, San Jose, CA, U.S.A.).

The column was a reversed-phase μBondapak C_{18} (10 μm; 30 cm × 3.9 mm I.D.) from Waters Assoc., combined with a guard column (2.0 cm × 4.5 mm I.D.) packed with 40-μm C_{18}/Corasil (Waters Assoc.).

Procedure

Samples of milk were obtained from Friesian dairy cows during the morning milking. Milk was deproteinized with trichloroacetic acid (TCA) according to ref. 1, with some modifications: 1 ml of milk was diluted with 4.0 ml of chilled water and 0.2 ml of 50% (w/v) TCA was added; after at least 4 h standing at 4°C, the sample was mixed thoroughly and centrifuged (15 min, 4000 *g*). After filtering the samples, orotate was determined by HPLC.

The chromatographic procedure was as follows. The mobile phase consisted of 0.05 *M* phosphate, pH 7.0. Flow-rate was 1.5 ml/min. A 30-μl aliquot of the filtered supernatant was injected into the column. Column effluent was monitored at 280 nm and at 0.2 absorbance units full scale. Peak areas were calculated into concentrations (mg/l) by the integrator.

RESULTS AND DISCUSSION

The standard calibration curve for orotate in 2% (w/v) TCA was linear in the concentration range 0—202 mg/l. The regression line was (triplicate measurements): $Y = 1053.7X - 64.6$ in which Y = peak area and X = orotate concentration. The standard deviation of the slope was 4.6 and the correlation coefficient (R) was 0.9999.

The chromatographic pattern of orotate standard (Fig. 1A) and of orotate in the filtered supernatant of milk (Fig. 1B) are shown in Fig. 1.

The accuracy of the determination of orotate by HPLC was measured with addition of standards: instead of 4.0 ml of water, 3.0 ml of water and 1.0 ml of orotate standard at four different levels were added to milk in duplicate. After deproteinization with TCA, the orotate concentration was determined and the recovery was calculated (Table I). The average recovery was 100.13 ± 1.50% (± standard deviation, S.D.).

To measure the precision of the determination of orotate, we deproteinized 18 milk samples in triplicate and the concentration in each filtered supernatant was measured in duplicate. The average coefficient of variation (C.V.) of the concentration of orotate due to variations in the chromatographic system (pump, injector, detector, integrator) was 0.55 ± 0.43% (± S.D., $n = 54$). The average coefficient of variation of the total determination (triplicate measurements) was 1.29 ± 0.81% (± S.D., $n = 18$). The concentration of these milk samples ranged from 9.5 to 119.1 mg/l.

The day-to-day variation of the determination of orotate was 0.92% at 62.6 mg/l and 0.57% at 125.5 mg/l orotate in milk ($n = 10$).

TABLE I

RECOVERY OF OROTATE ADDED TO COW'S MILK

Orotate conc. in milk (mg/l)	Orotate added (mg/l)	Measured conc. (mg/l)	Recovery (%)
64.9	60	124.7	99.8
64.9	60	128.6	103.0
64.9	120	183.2	99.1
64.9	120	188.0	101.7
55.3	30	85.5	100.2
55.3	30	84.8	99.4
55.3	90	143.1	98.5
55.3	90	144.3	99.3

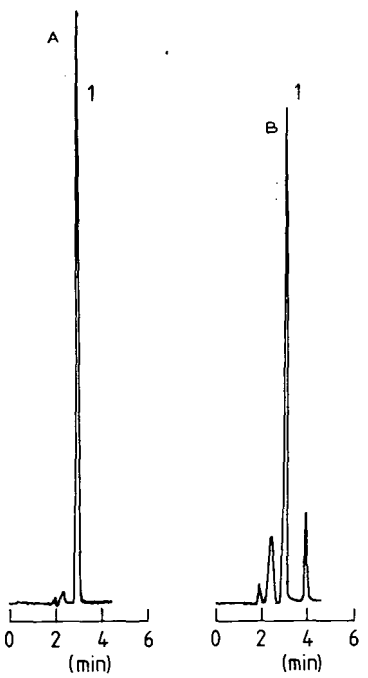

Fig. 1. Chromatographic profiles of (A) 67.25 mg/l standard orotate (1), and (B) orotate (1) in the filtered supernatant of milk (58.7 mg/l).

We did not find any interfering peaks in the 2250 milk samples analysed up to now.

The coefficients of variation of the determination of orotate by chemical analysis and by bioassay with *Lactobacillus jugurt* were 6.9% and 12.8%, respectively [3]. Therefore, the determination of orotate in milk by HPLC is rapid, sensitive and precise. The automated HPLC system is very useful in determining orotate in large numbers of milk samples (more than 1000).

Preliminary results show that the mean concentration of orotate in the milk of 2250 cows is 53.8 mg/l. The distribution was asymmetrical with skewness to the right. The average standard deviation of the distribution was 21.1 mg/l. These results agree with the results obtained by Jesse et al. [8].

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CHROMBIO. 1750

Note

Rapid determination of purine enzyme activity in intact and lysed cells using high-performance liquid chromatography with and without radiolabelled substrates

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(First received February 4th, 1983; revised manuscript received March 21st, 1983)

Rylance et al. [1] recently published methods enabling rapid screening for inherited enzyme defects of purine metabolism associated with uric acid overproduction, using high-performance liquid chromatography (HPLC). The advantage of such methods is their speed as well as the cost reduction and the avoidance of the use of radiolabelled substrates. The drawback is that they may only be used to measure enzyme activity directly in lysed cell preparations employing a specific substrate for each individual enzyme.

This paper describes an adaption of the method of Rylance et al. [1] to a rapid fully automated HPLC system which can be used with an on-line radiodetector for intact cell studies. The use of radiolabelled substrates is essential for the latter in order to evaluate the metabolic fate of a particular substrate. The method described for the simultaneous monitoring of UV absorbance and radioactivity is more rapid than those recently published [2].

METHODS

Studies in intact and lysed cells

The methods used for the enzyme assays in cell lysates, as well as metabolic studies in intact cells, have been described in previous publications [3, 4]. The enzyme assays investigated by HPLC included adenosine deaminase (ADA), purine nucleoside phosphorylase (PNP), adenine (APRT) and hypoxanthine—guanine phosphoribosyltransferase (HGPRT) and phosphoribosyl-pyrophosphate synthetase (PP-rib-PS).

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All incubations using lysed cells were carried out for 15 min at 37°C, initially using radiolabelled substrates (8-^{14}C-labelled adenosine, inosine, adenine and hypoxanthine and/or guanine respectively). The assays were terminated either by the addition of 40% trichloroacetic acid (TCA) followed by extraction to neutrality with water-saturated diethyl ether (APRT, HGPRT), or heating at 100°C for 2 min, (ADA, PNP) [3, 4].

Extracts were run in duplicate on the HPLC system described below, using a dual-channel detector capable of simultaneous monitoring of absorbance at 254 nm and 280 nm. Duplicate extracts were then re-run on the high-performance liquid chromatograph, using a radiodetector in place of the 280-nm UV monitor, allowing simultaneous comparison of the distribution of the label in substrate and product(s) with the 254-nm UV trace.

For the intact cell studies, $1 \cdot 10^6$ cells were incubated for 2 h at 37°C with 25 μM [8-^{14}C]deoxyadenosine (New England Nuclear) in RPMI (Flow Laboratories) supplemented with 10% heat-inactivated foetal calf serum (FCS). The final volume of the incubation mixture was 100 μl (specific activity 37 kBq/ml).

Cells and medium were separated by centrifugation (400 g, 5 min at 4°C) and the pellet washed once (400 g, 5 min at 4°C) with 200 μl of ice-cold 0.85% sodium chloride.

Medium and pellet were mixed with cold TCA (25 μl of 40% TCA and 100 μl of 8% TCA, respectively) and centrifuged for 1 min (12,000 g, Beckman Microfuge). Supernatants were extracted to neutrality with water-saturated diethyl ether and analysed by HPLC as for the red cell lysates.

Chromatography

The HPLC system used was a Waters Assoc. (Cheshire, Great Britain) trimodule fully-automated system, consisting of a WISP 710B automatic injector, a twin pump Model 6000A solvent delivery system, a Model 440 dual-channel UV detector (254 nm, 280 nm), linked to a System Controller and Data Module integration and printout system. The radiodetector was a Precision Radioactivity Monitor with a heterogeneous flow cell of 200 μl capacity supplied by Reeve Analytical (Glasgow, Great Britain).

A Z-module radial compression system, containing a 10-μm reversed-phase radial-Pak C_{18} cartridge (100 × 8 mm I.D.), was used for the lysed cell separations. Buffer A contained KH_2PO_4 2.7 g/l (20 mM) pH 4.45; Buffer B consisted of methanol—water (60:40). A linear gradient (gradient 6) was used for all separations.

A gradient increasing to 40% B in 17 min was used for the ADA assay, with a 3-min equilibrium delay between injections. For the PNP assay, gradient 6 to 30% B in 10 min was used. For APRT and HGPRT, the same gradient to 32% B and 24% B, in 9 min and 8 min, respectively, was used. The flow-rate was 3 ml/min in all instances.

For the intact cell studies, the 280-nm channel recorder was disconnected and a Reeve Analytical radioactivity monitor was attached to the Data Module of the trimodular system (pen 1) for continuous monitoring of radioactivity, in parallel with the UV absorbance at 254 nm using pen 2. The radiodetector contained a flow cell (200 μl void volume) packed with solid scintillant (99/

3811 GSI glass scintillant powder, Grade W, 63—80 μm) obtained from Koch-Light (Colnbrook, Great Britain).

For detection of radioactivity in nucleosides and bases in the medium, the above reversed-phase system was employed. For the nucleotides in the cell extracts, a Partisil 10 SAX cartridge (10 μm, 100 × 8 mm I.D.) was used. This anion-exchange system employed a phosphate gradient: Buffer A contained KH_2PO_4 0.68 g/l (5 m*M*) pH 2.65; Buffer B 25 g/l KH_2PO_4 plus 25 g/l KCl, pH 3.85. The flow-rate was 2 ml/min using a linear gradient (gradient 6) increasing to 100% B in 20 min.

The phosphate used was Aristar grade from BDH Chemicals (Poole, Great Britain), the potassium chloride Analar grade also from BDH. The methanol used was a special HPLC grade from Rathburn Chemicals (Walkerburn, Great Britain).

RESULTS

Cell lysates

A typical chromatogram for each of the four different enzyme assays obtained by HPLC is given in Fig. 1a—d. Fig. 1e shows the radiodetector trace

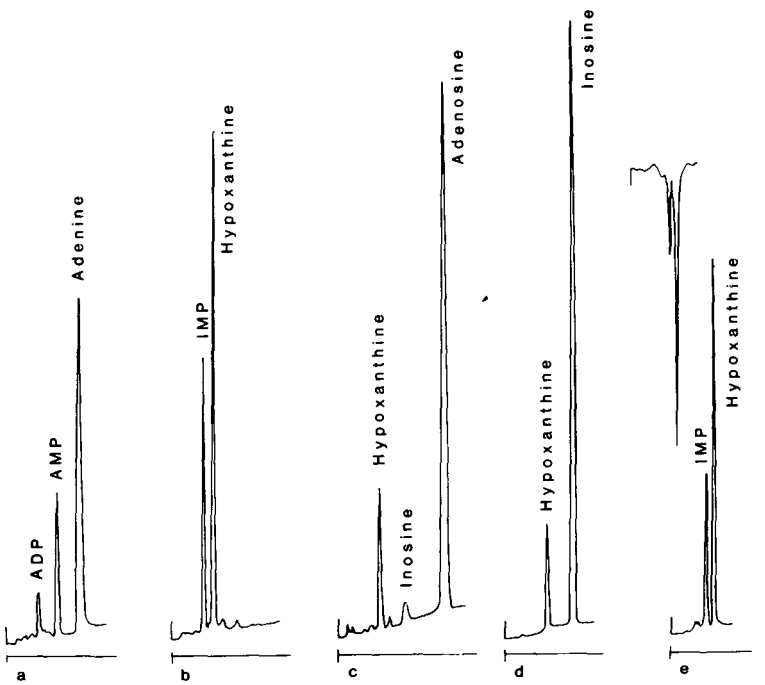

Fig. 1. Representative HPLC trace at 254 nm obtained for four different enzyme assays using haemolysate of healthy controls. (a) 10-μl injection of extract from a typical APRT assay; (b) 10-μl injection of extract from an HGPRT assay; (c) 50-μl injection of assay extract from an ADA assay; (d) 5-μl injection of assay extract from a PNP assay; (e) 7-μl injection of assay for HGPRT as shown in (b). The HPLC trace at 245 nm (lower) printout is compared (in e) with the radiodetector (upper) printout in place of the 280-nm channel printout. The traces were recorded at 0.1—0.5 absorbance units full scale (a.u.f.s.) and a chart speed of 0.2 cm/min. Other conditions are given in detail in the Methods section.

(upper) from an HGPRT assay compared with the direct HPLC assay (lower) demonstrating that these enzymes can be assayed rapidly in lysed cells using labelled or unlabelled substrate. Furthermore, they confirm the reproducibility of the method. Quadruplicate analyses agreed within 2%. Day-to-day variability was also within this range but varied in the long term with the age of the column which was controlled by external standard quantification.

Difficulties were encountered in the use of HPLC alone for the PP-rib-PS assay because the first step utilises ATP which is converted to AMP in the process [5]. Since AMP is also a product of the second step, the enzyme activity could thus not be determined directly by monitoring the UV absorption by HPLC of substrate and product. The assay could be applied to HPLC by using [8-^{14}C]adenine in the second step and using the radiodetector as in the intact cell studies to evaluate the percentage conversion of substrate.

Intact cells

Fig. 2 shows the metabolism of deoxyadenosine (dAR) using a normal lymphocyte cell line [6] established in long term culture using Epstein Barr

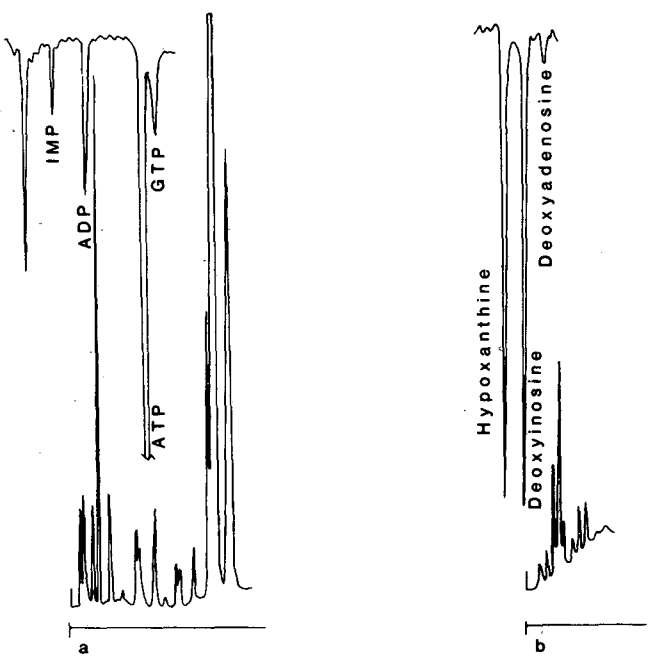

Fig. 2. HPLC trace showing the metabolism of deoxyadenosine in an Epstein Barr virus genome positive B cell line (Wil) established from normal lymphoblasts [6]. (a) Trace obtained from the cell extracts by simultaneous monitoring of UV absorbance and radioactivity, using the anion-exchange system described in the Methods section, showing the incorporation of radioactivity into the different nucleotide pools: IMP, ADP, ATP, GTP. The peak eluting at the front of the chromatogram corresponds to radioactivity in the medium not removed by centrifugation. 0.05 a.u.f.s., chart speed 0.1 cm/min. (b) The HPLC trace of the medium, after incubation, using the reversed-phase system described in methods. The UV trace at 254 nm is recorded in the (lower) printout at 0.05 a.u.f.s., chart speed 0.15 cm/min. The radiodetector trace is recorded in the (upper) printout, in place of the normal HPLC trace from the 280-nm UV detector.

(EB) virus. Fig. 2b shows a typical radiolabel trace (upper) obtained following a 25-μl injection of medium, showing that distribution of radiolabel is predominantly in deoxyinosine (dHR) and hypoxanthine, due to the rapid degradation of dAR by ADA and PNP. Within the cell (Fig. 2a), further metabolism of hypoxanthine to IMP by HGPRT has resulted in counts being predominantly in the peaks corresponding to ATP and ADP. Some of the substrate has also been metabolised to [8-^{14}C]GTP as indicated in the upper trace obtained with the injection of 75 μl of cell extract (Fig. 2b).

DISCUSSION

The use of a fully automated system for the type of studies described here is advantageous for several reasons. First, the speed with which a large number of samples can be processed; both the APRT and HGPRT assays can be run at a rate of six per hour, the PNP and ADA assays at approximately three per hour. Secondly, the 48-sample capacity of the fully automated system allows overnight analysis. Furthermore, the results obtained by HPLC agree closely in all instances with the values obtained previously where reaction products were separated first by high-voltage electrophoresis on thin-layer plates, visualised under UV, scraped directly into scintillation vials and subsequently counted. The saving in cost as well as time is also obvious — one step instead of four.

The ability to couple the radiodetector to the high-performance liquid chromatograph has proved invaluable for the intact cell studies. Particularly for the nucleotides, where it was not always easy in the past to separate all the different nucleotides using high-voltage electrophoresis. The use of the Z-module enables rapid change from the reversed-phase system to the anion-exchange system, and is invaluable for the intact cell studies.

We have investigated the application of a fifth enzyme assay, PP-rib-PS to HPLC analysis. However, in our hands this did not prove satisfactory because the assay is a two-step process using ATP in the first step to generate PP-rib-P. However, using [8-^{14}C]adenine in the second step together with the radiodetector attachment, as for the intact cell studies, the synthetase assay could be processed by HPLC.

These studies confirm that not only can much of the tedium and cost of routine enzyme assays be overcome by adaption to HPLC, but the system can also be used as a valuable tool for research purposes.

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CHROMBIO. 1746

Note

Measurement of phencyclidine and two hydroxylated metabolites by selected ion monitoring

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Phencyclidine (PCP) is a commonly abused street drug, especially among adolescents — often the same adolescents with unplanned pregnancies. However, the extent of PCP use during pregnancy is unstudied and thus its pharmacology and effect on the mother, fetus, and neonate are largely unknown. Nevertheless, PCP has well defined effects in children [1] and adults [2] and a recent case report suggested that it may be teratogenic [3].

In order to undertake a study of the use of PCP during pregnancy and its pharmacology during the peripartum period, a sensitive method for the analysis of PCP and its hydroxylated metabolites in samples from asymptomatic mothers and neonates was needed. Previous assays for PCP have been developed based on thin-layer chromatography [4], gas—liquid chromatography (GLC) with flame ionization [5] or nitrogen—phosphorus detectors [2, 6, 7], GLC with capillary columns [8], gas chromatography—mass spectrometry (GC—MS) [9, 10], and radioimmunoassay [11, 12]. Most of these assays have been either nonspecific, insensitive, require too large a sample size, are not applicable to the metabolites, or have extraction procedures that are too time consuming.

We would like to report an easy, rapid and sensitive technique using selected ion monitoring GC—MS for the analysis of PCP in plasma and PCP and its hydroxylated metabolites in urine.

MATERIALS AND METHODS

Reference compounds

Reference crystals of phencyclidine hydrochloride (PCP), 4-phenyl-4-

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piperidinocyclohexanol (PPC), 1-(1-phenylcyclohexyl)-4-hydroxypiperidine (PCHP), and the deuterated internal standards, [phenyl-$^2H_5$]-1-(1-phenylcyclohexyl)piperidine and [phenyl-$^2H_5$]-1-(1-phenylcyclohexyl)-4-hydroxypiperidine were all provided by Research Triangle Institute (Research Triangle Park, NC, U.S.A.), through the Research Technology Branch of the National Institutes of Drug Abuse. Stock solutions of all compounds were prepared with methanol to yield concentrations equivalent to 1 μg/ml free base.

Apparatus

A Hewlett-Packard 5995A quadrupole table-top mass spectrometer equipped with a direct probe inlet was used to obtain 70-eV electron impact mass spectra of the reference compounds. For selected ion monitoring the gas chromatograph was interfaced to the mass spectrometer with a glass jet separator. The chromatograph was fitted with a 1.3 m × 2 mm I.D. AW DMCS treated glass coil packed with 2% OV-17 coated on 80—100 mesh Supelcoport (Applied Sciences Labs., Bellefonte, PA, U.S.A.). The instrument conditions for PCP analyses were: carrier gas flow-rate 20 ml/min; injection port temperature 200°C; and oven temperature 200°C, the analyzer and ion source temperatures were set at 180°C and 150°C, respectively, to avoid thermal degradation of PCP occurring with temperatures above 200°C [9]. Ion intensities at *m/z* 200 and 205 were monitored for PCP and its deuterated internal standard, respectively, with a window width of 0.10 a.m.u. Total run time was 2.1 min. Ions 91 and 96 in addition to 200 and 205 could also be included for additional confirmation of PCP.

Changes in the conditions for analysis of PPC and PCHP were: injection port temperature 250°C; oven temperature 224°C; analyzer and ion source temperature both 276°C. Ions selected for monitoring were *m/z* 96, *m/z* 200 and *m/z* 288 for the trimethylsilyl derivatives of the internal standard, PPC and PCHP, respectively, with a run time of 2.2 min. For both procedures the optics of the mass spectrometer were optimized by autotuning at *m/z* 502 and the GC column was treated daily with Silyl-8 [13] (Pierce, Rockford, IL, U.S.A.).

Procedure

Urine samples were obtained from asymptomatic pregnant patients attending the obstetrical clinics of Cleveland Metropolitan General Hospital. Those with positive drug histories for PCP use were followed throughout pregnancy by urine analysis at each clinic visit. At delivery both maternal and cord blood samples were drawn. In addition, six 6-h urine collections were attempted and maternal and neonatal blood samples were drawn at approximately 24, 48 and 72 h post partum. Blood samples were separated by centrifugation and the plasma and urine were stored frozen until analyzed by GC—MS.

Prior to extraction, urine samples (0.2—2 ml) were mixed with 1 ml 0.1 *M* sodium acetate buffer (pH 5) and 0.1 ml β-glucuronidase preparation (Sigma, St. Louis, MO, U.S.A.) per 1 ml urine and incubated for 19 h at 37°C in order to deconjugate the hydroxylated metabolites. Following the addition of 25 ng of both deuterated internal standards, plasma and urine samples and spiked blank urine or plasma standards were made basic with 0.5 ml of 2 *M* sodium carbonate solution saturated with sodium chloride. After extraction with 5 ml

diethyl ether, the samples were centrifuged and following flash freezing with methanol and carbon dioxide, the organic layer was transferred to a 5-ml reactivial (Pierce). The ether was carefully evaporated under nitrogen at room temperature and the extract reconstituted with 30 μl of benzene. A 2-μl aliquot of the final solution was injected into the GC—MS system for PCP analysis. Standard curves were prepared and the samples quantitated using the Hewlett-Packard software for automatic quantitation of selected ion monitoring data by area normalization on the *m/z* 205 peak for deuterated PCP. Standard curves ranged from 1.56 to 200 ng/ml.

In order to measure PPC and PCHP, the urine samples from the final step (above) were evaporated to dryness. They could be allowed to sit overnight at this point. Trimethylsilyl derivatives were formed by adding 10 μl pyridine and 100 μl bis(trimethylsilyl)trifluoroacetamide (Pierce) under dry nitrogen, and without heat to the reactivials containing the sample residue. These were sealed immediately. If desired, plasma samples could also be evaporated and silylated using only 5 μl pyridine and 50 μl BSTFA. Samples were then heated at 80°C in a reactivial heating block (Pierce) for 1 h and allowed to return to room temperature. Aliquots (2 μl) were then injected into the GC—MS system with conditions as described above for the metabolites. Samples were quantitated by normalization on the *m/z* 96 internal standard peak. Standard curves ranged from 1.56—200 ng/ml.

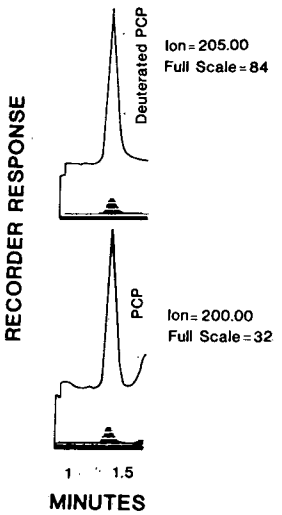
RESULTS

The mass spectra of PCP, the trimethylsilyl derivatives of PPC and PCHP were identical to those published previously using electron impact ionization [9]. The spectra for the two silylated deuterated internal standards, PCP and PCHP, were comparable with the base peaks being *m/z* 205 and 96, respectively.

The selected ion chromatogram of a urine extract containing 12.5 ng/ml PCP and 25 ng/ml internal standard is shown in Fig. 1. The retention times for PCP and deuterated PCP are approximately 1.44 and 1.42 min, respectively. Full scale refers to the amplification required to plot all chromatograms the same size (the higher the number the less required). Fig. 2 is the same urine extract following silylation and reinjection at the higher temperatures. The sample contained 12.5 ng/ml of both PPC and PCHP and 25 ng of the deuterated internal standard. Retention times were approximately 1.40, 1.33 and 1.41 min for the internal standard, PPC and PCHP, respectively. Problematic interference from other metabolites was not noted.

Calibration curves were linear to 500 ng/ml for both PCP and the two metabolites. The least-squares linear regression line which describes a typical PCP curve is $y = 3.251x - 0.674$. Typical curves for PPC and PCHP, respectively, are $y = 8.756x - 3.37$ and $y = 1.821x - 1.542$. Using these curves, samples containing less than 200 pg/ml of PCP, less than 2 ng/ml PPC and less than 1 ng/ml PCHP in urine could be quantitated. In plasma, this method is sensitive to 6.25 ng/ml; sensitivity can be increased with an acid back-extraction step.

The precision of the method was determined by repeat analysis of spiked urine samples containing low (6.25 ng/ml) and high (50 ng/ml) concentrations



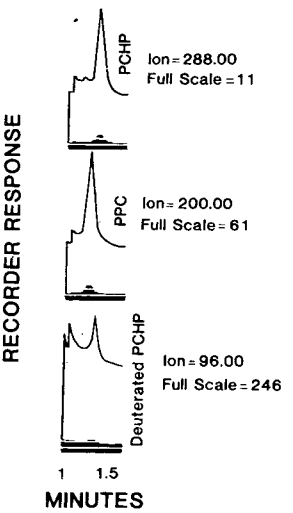


Fig. 1. Selected ion chromatogram of a urine extract.

Fig. 2. Selected ion chromatogram of a urine extract following silylation.

TABLE I

DRUG LEVELS IN SPOT URINE COLLECTIONS FROM ASYMPTOMATIC PREGNANT PATIENTS

Patient	Concentration ng/ml (ng/mg creatinine)				Metabolite/total product ratio
	PCP	PPC	PCHP	Total products	
(1) RS*	20.0 (81.0)	9.4 (37.6)	6.6 (26.7)	36.0	0.44
(2) PB	57.4 (67.2)	35.6 (41.6)	10.9 (12.7)	103.9	0.45
(3) MM	512.0 (300.7)	56.0 (32.9)	15.0 (8.8)	583.0	0.14
(4) NB	138.6 (76.6)	28.6 (15.8)	10.2 (5.6)	177.4	0.28
(5) JB*	99.1 (311.7)	14.7 (46.3)	9.7 (30.4)	123.5	0.20
(6) LC	25.4 (11.1)	9.1 (4.0)	—**	34.6	0.26

*Admitted ingestion 24 h earlier.
**Below detection limits.

of PCP, PPC and PCHP in urine. For PCP the relative standard deviation (coefficient of variation) was 4.01 and 3.81 for ten high and low samples, respectively. Repeatability of high and low concentrations for PPC resulted in a 7.19% and 9.46% relative standard deviation and with 7.59% and 9.11% values for PCHP. The precision for PCP in plasma was 6.60% for the 50-ng sample. Day-to-day repeatability was determined using 50 ng/ml frozen urine samples. The values were 2.94% for PCP, 7.69% for PPC and 7.66% for PCHP.

Using the method described, PCP, PPC and PCHP were quantitated in urine from asymptomatic pregnant patients during routine prenatal screening. The values for six of these patients are shown in Table I. Furthermore, PCP and both metabolites have been quantitated in maternal and neonatal urine post partum.

DISCUSSION

This method is very sensitive, quantitative, easy to perform, and it utilizes relatively inexpensive MS equipment increasingly common in hospital chemistry and other laboratories. In addition, the automatic quantitation feature of the HP 5995A system greatly decreases analysis time; one technician can extract, analyze and quantitate 30 samples and standards in one day. Finally, the ability to easily quantitate PPC and PCHP in urine is of more than academic interest because recent studies have suggested that these compounds are pharmacologically active [14].

This method can be used in conjunction with a routine screening procedure such as the Emit® Phencyclidine screening test (Syva, Palo Alto, CA, U.S.A.). However, the reported sensitivity of this method is only 75 ng/ml. We found that urine values higher than 10—15 units above the "blank" often warranted further analysis by GC—MS.

As reported previously [15], we also noted that diphenhydramine can interfere with the analysis of PCP. It has significant ion intensities at *m/z* 200 and a similar retention time. However, the presence of PCP and not diphenhydramine can be verified by also monitoring the ratio of *m/z* 91/200 and quantitating the PCP metabolites.

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Note

Sensitive gas—liquid chromatographic method for chloramphenicol in animal tissues using electron-capture detection

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Chloramphenicol (CP) is considered to be an effective and almost essential antibiotic for the treatment of enteric and respiratory diseases of animals, where resistance has developed to the commonly used antibacterial drugs [1—3]. In the many countries where CP is used there is however, concern regarding the effects on consumers of CP residues in meat from treated animals. Varying degrees of bone marrow depression have occurred, relatively frequently, in humans undergoing therapy with this drug, and there is no data to indicate the minimal amount of drug or residue which can produce this situation [4, 5]. In view of these circumstances the F.A.O./W.H.O. Expert Committee on Antibiotics have recommended a zero tolerance for CP in meat products.

To accomplish this goal, the drug assay used to ascertain safe drug withdrawal periods and for monitoring residues in meat, should be capable of detecting chloramphenicol in the low ng/g range in tissue. A search of the relevant literature has failed to reveal a practical residue assay for animal tissue with this level of sensitivity.

The tissue residue studies reported to date involve the use of this drug in swine, cattle, and poultry [1, 6—10]; however, the assay procedures were not very sensitive (1 μg/g to 0.1 μg/g). In some studies the assay procedures were microbiological methods which lacked specificity. A gas—liquid chromatographic (GLC) method has been reported by Jacobson et al. [11] which appears to meet the required sensitivity level. It is too time consuming however, for routine residue assay procedures and has not been adopted by public health agencies.

Several methods of detection of CP in serum, urine and milk using GLC

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[12—16] or high-performance liquid chromatography (HPLC) [17, 18] were found. No practical sensitive assay procedures were reported for use in edible animal tissues.

The GLC methodology described in this paper was developed to provide a practicable tissue assay test with a lower limit of detection of $\leqslant$ 5 ng of CP per g of tissue. The test is not excessively time consuming, uses standard analytical techniques and is suitable for drug depletion studies and residue monitoring programs.

EXPERIMENTAL

Chemicals and reagents

Pharmaceutical grade (99.8%) chloramphenicol (CP), and chloramphenicol monoethanolamine succinate were donated by Pfizer Canada (Pointe Claire, Canada). Thiamphenicol (TP) was purchased from Sigma (St. Louis, MO, U.S.A.). For derivatization Trisil (standard mixture of hexamethyldisilazane (HMDS), trichloromethylsilane (TMS) and pyridine) was obtained from Pierce (Rockford, IL, U.S.A.). Solvents: ethyl acetate, methanol, light petroleum and benzene were distilled in glass (Caledon Laboratories, Georgetown, Canada).

Standard solutions

Standard solutions of CP and TP were prepared by dissolving 100 mg of either compound in 100 ml of methanol. These stock solutions were further diluted to produce an end concentration of 50 ng/ml. These standard solutions were then added to tissues for internal standardization (TP) and development of the calibration curve (CP).

Chromatographic conditions

A Varian Vista 401 gas—liquid chromatograph with dual electron-capture detectors (^{63}Ni) and dual automatic injectors was fitted with glass columns (1.83 m × 2 mm I.D.) packed with 3% OV-1 on Gas-Chrom Q, 100—120 mesh (Chromatographic Specialties, Brockville, Canada). Operating conditions were: injection port temperature, 230°C; column oven temperature, 220°C; ionization oven temperature 350°C; carrier gas high purity nitrogen, flow-rate 30 ml/min. Chromatograms were recorded on a 1-mV scale at detector range of 10, recorder attenuation 32, and the chart speed was 0.5 cm/min.

Procedure

A 5-g sample of ground frozen tissue (muscle, liver, kidney), was placed in a round bottom centrifuge tube (28 mm × 120 mm) fitted with a screw cap. A 2-ml aliquot of the standard solution (50 ng/ml) of TP in methanol, was added, stirred on a vortex mixer and allowed to stand for 15 min. Tissue was extracted twice by homogenizing, using a Tissumiser (Tek Mar, Cincinatti, OH, U.S.A.), in 10 ml ethyl acetate for 20 sec, then centrifuging at 700 *g* for 5 min. The ethyl acetate supernatants were pooled in a conical centrifuge tube (17 mm × 134 mm) and evaporated to dryness under a stream of dry nitrogen in an N-Evap (Organomation, South Berlin, MA, U.S.A.) at 60°C. The residue

was taken up in 0.2 ml methanol and 2.8 ml of 1 *N* hydrochloric acid were added. This solution was then washed three times with 1.5 ml of light petroleum. Sep-Pak C_{18} mini columns (Waters Assoc., Milford, MA, U.S.A.) were conditioned according to the manufacturer's directions by flushing with 2 ml of methanol, followed by 5 ml of double distilled water. Solution containing the residue was loaded onto the conditioned column and the initial fraction (3 ml) discarded. CP was eluted from the column using two 3-ml volumes of methanol—1 *N* hydrochloric acid (40:60). These fractions were collected in a screw-capped conical centrifuge tube (17 mm × 134 mm) and the methanol evaporated under a stream of dry nitrogen in a sandbath at 60°C. The drug residue was then extracted out of the aqueous phase with two 2-ml volumes of ethyl acetate.

The extracts were pooled in a clean conical centrifuge tube (17 mm × 134 mm) and evaporated to dryness under a stream of nitrogen as described above. The residue was redissolved by washing the tube with 1 ml of methanol. In a nitrogen atmosphere, methanol was evaporated and 400 µl of Trisil added. The tube was then stoppered, vortexed for a few seconds and reacted in a sandbath at 35°C for 30 min. After the Trisil was evaporated to dryness under a stream of dry nitrogen the residue was redissolved in 1 ml of benzene for injection into the gas chromatograph.

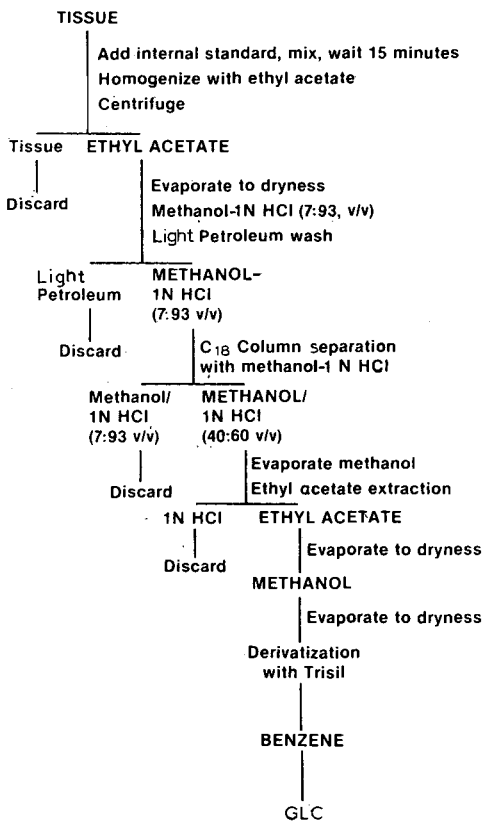

Fig. 1. Extraction and cleanup procedure for chloramphenicol (CP) from edible animal tissues.

Calibration graph

The calibration graph was developed by spiking tissue with standard solutions of CP and TP to yield concentrations of 5, 10, 20, 40, 60, 80 ng/g. The tissues were extracted using the above procedure and each level was assayed in triplicate. GLC analyses of spiked tissue were compared to those of derivatized standard solutions to calculate recovery rates. Peak areas were plotted against drug concentrations for the six levels of drug and a regression analysis carried out.

A summary of the extraction and cleanup procedure is shown in Fig. 1.

RESULTS AND DISCUSSION

Typical chromatograms of broiler chicken muscle and liver, and muscle and liver spiked with 10 ng/g of CP are shown in Fig. 2. The retention times for CP and TP varied from 5.9 to 7.4 and 11.9 to 15.0 min, respectively, with the retention time increasing over a run of fourteen samples. The small peak, which was eluted just after the CP, was present in all samples including the

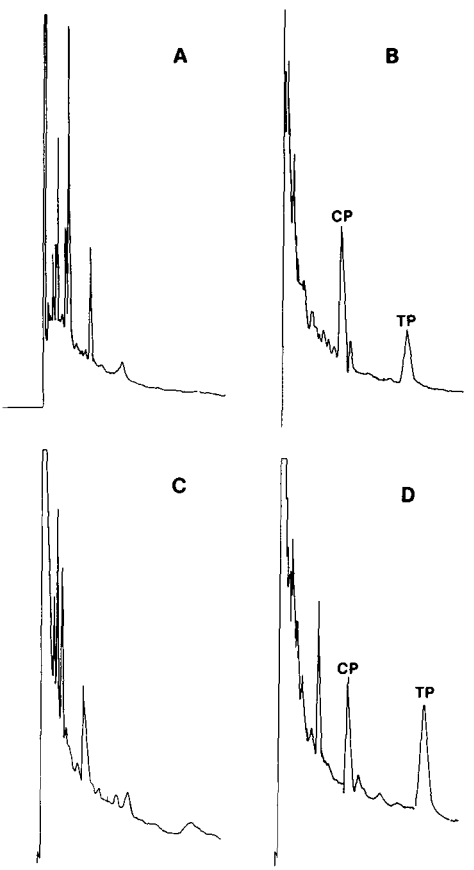

Fig. 2. Typical chromatograms of (A) broiler chicken muscle; (B) muscle with 10 ng/g chloramphenicol (CP) and 10 ng/g thiamphenicol (TP) added; (C) broiler chicken liver; (D) liver with 10 ng/g CP and 20 ng/g TP added.

derivatized blank. In liver a small peak occurred about 0.2 min before the CP. A similar but much larger peak was reported by Wal et al. [16] in milk when derivatized with heptafluorobutyrate. This peak represents about 9% of the area of chloramphenicol at 5 ng/g. A peak also occurs in liver, in the area of the TP but it could be differentiated from the TP. Chromatograms of chicken kidney were similar to muscle in that no interfering peaks were observed.

. TP as suggested by Least et al. [13] and Nakagawa et al. [14] proved to be a suitable internal standard to improve the accuracy of the test by correcting for variation in the extraction and injection procedure. This drug was selected also because it is commercially available in Canada and its extraction and chromatographic characteristics are similar to CP.

Calibration curves of CP in chicken muscle, liver and kidney were linear throughout the range of 5—80 ng/g with correlation coefficients of 0.974 for muscle, 0.939 for liver and 0.924 for kidney. This, plus the observation that the recovery rates of CP calculated at the points of the calibration curve were not significantly different, suggests that the level of drug did not affect recovery rate within the 5—80 ng/g range. Levels of less than 5 ng/g were readily detected.

Initially the recovery rate of TP in muscle, at every point on the calibration curve, was determined. Since there was no effect of drug concentration on the recovery rate, 20 ng/g was chosen as the level for internal standardization and for development of the calibration curves in liver and kidney.

Recovery rates (Table I) for CP and TP are similar to those reported by Least et al. [13] from blood serum. Liver values tended to be more variable than muscle or kidney. Kidney results were based on a smaller sample due to lack of tissue availability.

TABLE I

RECOVERY (PERCENT OF DERIVATIZED DRUG) OF CHLORAMPHENICOL (CP) AND THIAMPHENICOL (TP) FROM MUSCLE, LIVER AND KIDNEY OF BROILER CHICKENS

Tissue	No. of samples	Recovery of CP		Recovery of TP	
		Mean (%)	C.V. (%)*	Mean (%)	C.V. (%)*
Muscle	15	56.2	10.9	45.5	16.0
Liver	15	51.9	24.7	40.5**	12.8
Kidney	8	68.0	17.5	45**	14.6

*Coefficient of variation.

**Based on levels of 20 ng/g in tissue.

The method was applied to an experiment to determine CP residues in liver and muscle from chickens given a single oral dose of chloramphenicol monoethanolamine succinate (Fig. 3). The method showed good sensitivity in these tissues at 5 ng/g. This level of detection is 20 times lower than the presently used methods. After GLC analysis derivatized samples in benzene were sent to an independent analytical laboratory for analysis using GC—chemical ioniza-

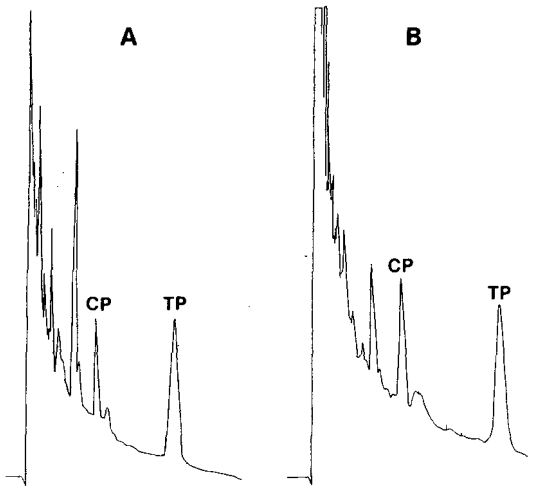

Fig. 3. Chromatograms of tissue extracts from broiler chickens treated with a single oral dose of chloramphenicol monoethanolamine succinate showing chloramphenicol (CP) at the level of 5.2 ng/g in muscle (A) and 6.4 ng/g in liver (B). Thiamphenicol (TP) was added as the internal standard at 20 ng/g.

tion mass spectroscopy (GC—CIMS). GC—CIMS analysis confirmed the presence of di-trimethylsilyl derivatives of CP and TP at concentrations as low as 1.4 ng/g. Since regulatory procedures require confirmatory tests for legal actions, the ability to use samples prepared for GLC without further extracting for GC—CIMS assays is a distinct advantage over existing tissue residue methodology.

CONCLUSIONS

The method described above provides a system which meets the generally accepted requirements for a tissue residue monitoring system. Inclusion of the cleanup steps, particularly the C_{18} mini columns, resulted in removal of many interfering tissue components and thus an improvement in the sensitivity of the test. The procedure is extremely sensitive (5 ng/g or less) and is capable of being carried out using standard analytical equipment and commercially available reagents. Further studies indicate that with minor modifications a lower limit of 1 ng/g may be possible.

Experience gained in residue studies with broiler chickens and swine tissues indicate that two technicians can process 70 samples in a week. Results of the above studies will be published elsewhere.

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Note

High-performance liquid chromatography of anticonvulsants — micro-assay for phenytoin and phenobarbital

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Currently available high-performance liquid chromatographic (HPLC) methods for the analysis of anticonvulsant drugs such as phenytoin and phenobarbital have mostly employed ODS-bonded phases [1—7]. The application of this mode of HPLC for the assay of anticonvulsants in biological fluids has a number of disadvantages which include: the requirement for special equipment such as a temperature block [1—5], the dependence of the separation on pH [5, 7], coelution of the hydroxylated metabolite of phenytoin and phenobarbital [4, 5], and in our experience the frequent loss of column performance due to build up of impurities on top of the column bed. In addition, no detailed studies have been performed to determine the best mode of chromatography for the separation of phenytoin compounds and phenobarbital.

In this paper we describe in detail the chromatography of several anticonvulsants by both normal-phase partition and reversed-phase modes of HPLC. Also included is a micro-assay for phenytoin and phenobarbital in plasma employing a polar-bonded phase of the cyano type.

EXPERIMENTAL

Chemicals

Phenytoin was obtained from Parke Davis and Co. (Detroit, MI, U.S.A.). 5-(*p*-Hydroxyphenyl)-5-phenylhydantoin (HPPH) and 5-(*p*-methylphenyl)-5-hydantoin (MPPH) were purchased from Aldrich (Milwaukee, WI, U.S.A.). Phenobarbital was a gift from Smith, Kline and French Labs. (Philadelphia, PA,

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U.S.A.). HPLC grade solvents were obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.), and phosphoric acid was from J.T. Baker (Phillipsburg, NJ, U.S.A.).

High-performance liquid chromatography

HPLC was carried out with a reciprocating pump (Model 6000A, Waters Assoc., Milford, MA, U.S.A.) coupled to a variable-wavelength UV detector (Model 450, Waters Assoc.). Anticonvulsants were detected at either 200 nm or 195 nm.

The column packings were either a cyano-propyl bonded (Zorbax-CN) or ODS-bonded (Zorbax-ODS) microparticulate silica purchased from DuPont (Wilmington, DE, U.S.A.). These were packed into columns (250 mm × 4.6 mm I.D.) by the suppliers. Samples were dissolved in the mobile phase and injected with an automated injection system (Model 710A, Waters Assoc.).

Procedure

To 50—100 μl plasma in a tapered tube were added 200 μl of 50% acetonitrile in propan-2-ol containing MPPH (10 mg/l) as internal standard. The contents of the tube were mixed thoroughly on a vortex mixer and then centrifuged (4000—4500 *g*) for 2 min.

The supernatant (30—100 μl) was injected directly into the chromatograph and eluted with a mobile phase of 7 m*M* phosphoric acid—methanol—acetonitrile (6:3:1) and a flow-rate of 1.5 ml/min.

Quantitation

Plasma phenytoin and phenobarbital were determined by comparison of the peak height ratios of drug to the internal standard with those given by standard solutions containing from 2.0 to 30.0 mg/l phenytoin, 5—50 mg/l phenobarbital and 10 mg/l internal standard (MPPH). Over this range the peak height ratio of drug to internal standard was related linearly to the concentration of phenytoin and phenobarbital.

RESULTS

Normal-phase partition HPLC

The chromatography of phenytoin and related compounds was examined on a commercial packing in which a cyanopropyl phase was bonded to 6-μm porous silica particles. Although a mobile phase mixture of 15% propan-2-ol in hexane resolved phenytoin from HPPH, the separation of phenytoin from MPPH was not possible. The addition of 5% acetonitrile to a mobile phase of hexane—propan-2-ol enabled the resolution of all test compounds (Fig. 1).

Although for the separation of phenytoin compounds and phenobarbital, normal-phase partition chromatography offered lower operating pressures and improved stability of the column bed compared to reversed-phase HPLC, this mode of chromatography was found unsuitable for the assay of phenytoin in plasma because injection of semi-aqueous extracts of serum resulted in poor peak shape.

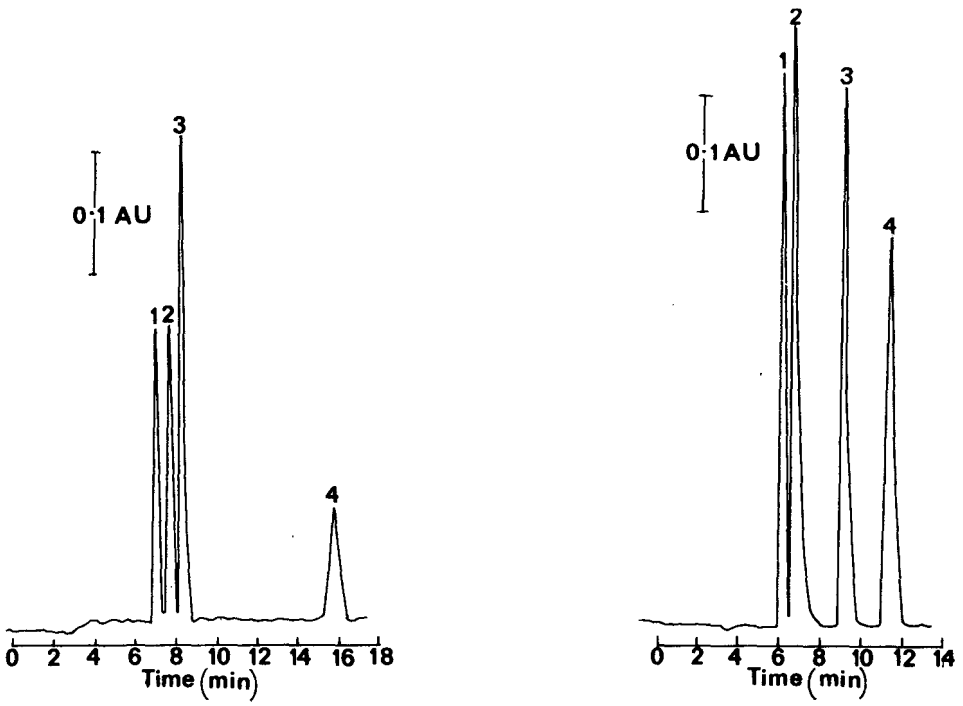

Fig. 1. Separation of anticonvulsants by normal-phase partition HPLC on Zorbax-CN with a mobile phase of 5% acetonitrile and 10% propan-2-ol in hexane and a flow-rate of 1.5 ml/min. Detection was at 200 nm. Peaks: 1 = MPPH; 2 = phenytoin; 3 = Phenobarbital; 4 = HPPH.

Fig. 2. Separation of anticonvulsants by reversed-phase HPLC on Zorbax-CN with a mobile phase of 10% acetonitrile and 30% methanol in 7 m*M* phosphoric acid and a flow-rate of 1.5 ml/min. Detection was at 195 nm. Peaks: 1 = phenobarbital; 2 = HPPH; 3 = Phenytoin; 4 = MPPH.

Reversed-phase HPLC

A typical resolution of phenytoin compounds and phenobarbital by reversed-phase HPLC (Zorbax-CN) employing mobile phase mixtures of phosphoric acid—methanol—acetonitrile is shown in Fig. 2. Although a similar separation could also be achieved on a non-polar bonded phase of Zorbax-ODS, this system retained phenytoin strongly and the HPPH weakly.

In general, for the resolution of phenytoin, HPPH, MPPH, and phenobarbital, it was found that to obtain reasonable capacity ratios, it was necessary to employ the Zorbax-CN column operated in the reversed-phase mode.

Assay of phenytoin and phenobarbital in plasma

Sample preparation and chromatography. Sample preparation was a simple protein precipitation described in the Experimental section. A mixture of 50% acetonitrile in propan-2-ol was used as an extractant because it gave more complete and faster protein precipitation than acetonitrile.

A typical chromatogram illustrating the assay of phenytoin and phenobarbital in plasma is shown in Fig. 3a. Extracts of drug-free plasma yielded no

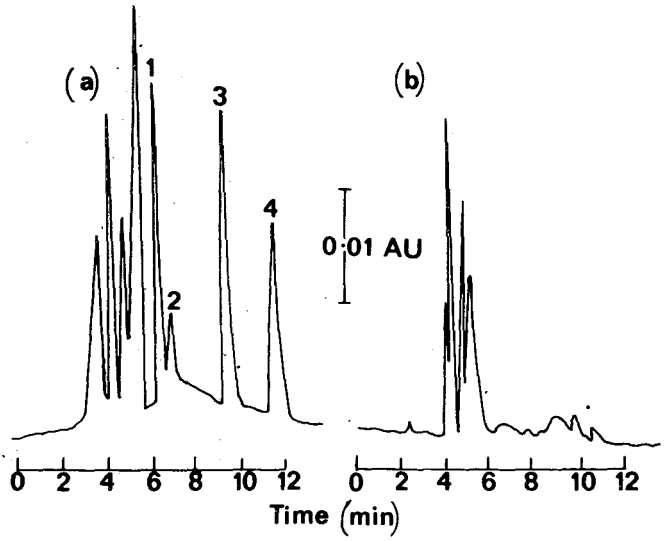

Fig. 3. (a) Chromatogram of a 50-μl injection of a serum sample from a patient on anticonvulsant therapy. Peaks: 1 = phenobarbital 10 mg/l; 2 = HPPH; 3 = phenytoin 15 mg/l; 4 = MPPH 10 mg/l. (b) Chromatogram of a drug-free serum sample. The chromatographic systems were the same as for Fig. 2.

significant interference from endogenous plasma components (Fig. 3b).

Reproducibility. Data on within-run precision were obtained by analyzing pooled human plasma spiked with phenytoin and phenobarbital at concentrations ranging from 2.0—25 mg/l and 8.0—50 mg/l, respectively. The mean coefficient of variation of ten replicates at each concentration was 3.4% for phenytoin and 2.8% for phenobarbital (Table I). The mean between-run precision for the assay of phenytoin and phenobarbital in plasma from patients undergoing anticonvulsant therapy was 5.3% and 5.5%, respectively (Table II).

Background. To obtain data on the amount of background appearing at elution times corresponding to those of the drugs of interest, we processed several drug-free plasmas with added internal standard (MPPH) through the complete procedure. Background interference was calculated at values from 0.00 to 0.4 mg/l.

TABLE I

WITHIN-RUN PRECISION OF ASSAY FOR PHENYTOIN AND PHENOBARBITAL (n = 10)

Phenytoin			Phenobarbital		
Concn. added (mg/l)	Mean measured value (mg/l ± S.D.)	C.V. (%)	Concn. added (mg/l)	Mean measured value (mg/l ± S.D.)	C.V. (%)
2.0	2.1 ± 0.10	4.8	8.0	8.4 ± 0.15	1.8
10.0	9.9 ± 0.24	2.4	25.0	24.8 ± 0.98	3.9
25.0	25.1 ± 0.76	3.0	50.0	52.4 ± 1.40	2.7

TABLE II

BETWEEN-RUN PRECISION OF ASSAY FOR PHENYTOIN AND PHENOBARBITAL (n = 10)

Plasma sample	Phenytoin		Phenobarbital	
	Mean concn. (mg/l ± S.D.)	C.V. (%)	Mean concn. (mg/l ± S.D.)	C.V. (%)
1	3.3 ± 0.34	10.3	9.4 ± 0.70	7.4
2	12.5 ± 0.28	2.2	12.2 ± 0.20	1.6
3	18.1 ± 0.80	4.4	21.4 ± 1.4	6.5
4	22.4 ± 0.97	4.3	30.8 ± 2.0	6.5

Sensitivity. The sensitivity of the procedure is limited largely by the serum volume, extraction efficiency and background. With a 0.1-ml sample volume, the compounds can be detected in concentrations as low as 1 mg/l.

Comparison with gas chromatography

To compare the results of the HPLC assay with those of gas chromatographic (GC) analysis we analyzed 40 specimens containing phenytoin and 40 samples containing phenobarbital by both methods. For phenytoin the coefficient of correlation was 0.914; slope, 0.912; y-intercept, +1.9. The standard estimated error was 0.068. Results of similar calculations for phenobarbital gave a coefficient of correlation of 0.894 (slope, 1.094; y-intercept, −1.71; estimated error, 0.089.

DISCUSSION

We have examined the chromatography of phenytoin compounds by both normal-phase partition and reversed-phase modes of HPLC. Although both modes of chromatography enabled the separation of a test mixture consisting of phenytoin, HPPH, MPPH and phenobarbital, normal-phase partition chromatography offered greater selectivity for the separation of phenobarbital and HPPH.

The only significant difference observed in the chromatography of phenytoin compounds on the two systems of reversed-phase HPLC examined was the much greater retention of phenytoin than its hydroxylated metabolite on the ODS-phase compared to the cyano-phase.

For the assay of phenytoin and phenobarbital in plasma we chose the less retentive Zorbax-CN column because it offered several advantages over the ODS-bonded phase. These were the close elution of phenytoin and its major hydroxylated metabolite allowing for their simultaneous quantitation, the improved stability of the column bed and the ability to inject solvent extracts of plasma directly into the column without further purification and evaporation.

The analysis time and resolution of the compounds during reversed-phase HPLC was found to be related to the concentration of acetonitrile in the mobile phase and, in practice, this provided a considerable opportunity to make the analysis optimal. The acetonitrile concentration (10%, v/v) used in

the assay was chosen because it adequately resolved phenobarbital and the hydroxylated metabolite of phenytoin. Increasing the amount of acetonitrile in the mobile phase to 20% resulted in decreased retention of all compounds of interest with co-elution of HPPH and phenobarbital.

Although the results obtained by HPLC and GC on the same samples correlated well for phenytoin and phenobarbital the sample requirement for GC (0.5—1.0 ml) was greater than for the HPLC method (50—100 μl). This was an important consideration in the design of our assay because the volume of plasma that can be obtained from children is often limited.

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Note

Micromethod for determination of thiopental in human plasma by high-performance liquid chromatography

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Since 1935, thiopental, an ultra-short-acting barbiturate, has been used to induce anesthesia. Lately, thiopental has been proposed for protection of the brain in comatose patients with head trauma [1—3]. In this indication, large doses of thiopental are given intravenously over a period of a few days and a control of plasma levels is necessary.

For the determination of thiopental in plasma, gas chromatographic [4] and high-performance liquid chromatographic (HPLC) [5—9] methods have been reported in the literature. The HPLC method described in this paper, requiring a minimum pretreatment of the plasma sample, presents the advantage of being rapid and simple.

EXPERIMENTAL

Chemicals

Acetonitrile (Burdick and Jackson Labs., Muskegon, MI, U.S.A.) used was of non-spectro grade. Stock thiopental (Specia, Paris, France) solution was 1 g/l in water. Flunitrazepam (Roche, Neuilly/Seine, France), used as internal standard, was 12.5 mg/l in acetonitrile.

Liquid chromatography

A liquid chromatograph Model 5040 (Varian, Orsay, France) combined with a Vista CDS 401 data system (Varian) was used throughout this work. The UV Spectromonitor III detector (LDC, Riviera Beach, FL, U.S.A.) was operated at 280 nm. The automatic injector was a WISP 710 B Model (Waters Assoc., Paris, France).

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Analysis was performed on a 5-μm Spherisorb C_6 column (20 cm × 4.6 mm) from Phase Sep (Queensferry, Great Britain), operating at 30°C. The analytical column was protected by a small precolumn (5 cm × 4.6 mm) packed with 30—40 μm Permaphase C_{18} (Dupont, Orsay, France).

The mobile phase was a mixture (70:30, v/v) of 0.01 *M* sodium acetate adjusted to pH 3.6 by concentrated acetic acid and acetonitrile. The flow-rate was kept at 1.5 ml/min.

Preparation of plasma samples

To 50 μl of plasma, in a 1.5-ml polypropylene microtube (Eppendorf, Hamburg, G.F.R.), were added 200 μl of the internal standard solution. The stoppered tube was shaken for 3 min on an Eppendorf shaker. After brief centrifugation, the supernatant was transferred to an automatic sampler vial and a 25-μl aliquot was injected.

Quantitation

Quantitation was done by the peak height ratio method with flunitrazepam as internal standard. Calibration curves were obtained by spiking control plasma with various amounts of stock thiopental solution (0, 12.5, 25, 50, 75, 100 mg/l) and a constant amount of internal standard (50 mg/l).

Within-run variation was determined by analyzing tenfold two plasma samples containing, respectively, 5 mg/l and 25 mg/l thiopental.

Stability

The stability of plasma thiopental was tested by using fresh control plasma surcharged with thiopental at the following concentrations: 12.5, 25, 50 and 100 mg/l.

Samples of 50 μl from each concentration were placed into separate 1.5-ml polypropylene microtubes and stored either at room temperature for 24 h or at −20°C for one to eight weeks.

RESULTS AND DISCUSSION

Typical chromatograms of a blank plasma and a normal plasma supplemented with thiopental are shown, respectively, in Fig. 1a and b. The retention times of thiopental and flunitrazepam are, respectively, 9.7 min and 11.1 min, which permit a chromatographic run of only 13 min. The chromatograms of patients receiving thiopental show a peak with a retention time of 3.0 min (Fig. 1c). We have not yet identified this peak which probably corresponds to a thiopental metabolite.

The linearity of the method is very good up to 100 mg/l. The detection limit for plasma samples is 0.5 mg/l. Reproducibility and accuracy of the method are illustrated in Table I. For concentrations of 25 mg/l as well as 5 mg/l, the coefficient of variation is below 2%.

Plasma thiopental is stable for at least 24 h at room temperature and no appreciable degradation was observed after storage for eight weeks at −20°C (Table II).

More than 500 patient plasma samples were analyzed and no interference

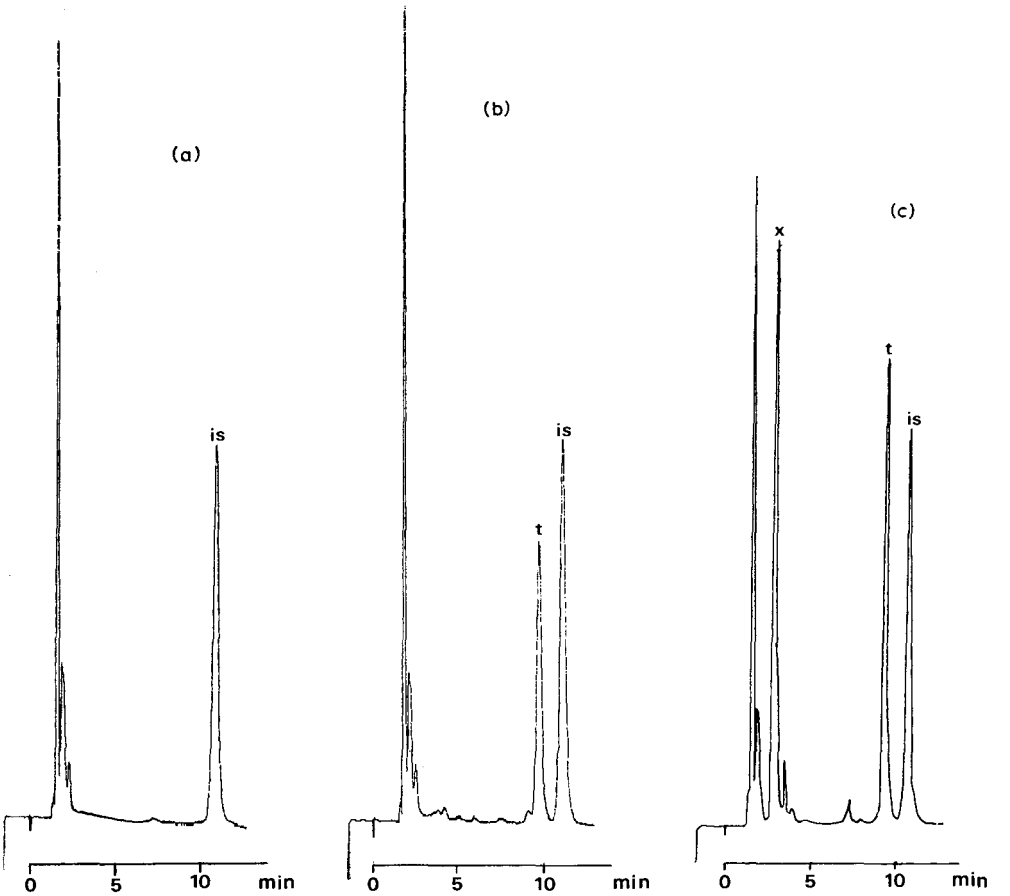

Fig. 1. Chromatograms of: (a) blank plasma, (b) normal plasma with 12.5 mg of thiopental added per liter, (c) a patient plasma (thiopental = 21 mg/l). x = unknown peak, t = thiopental, is = internal standard.

TABLE I

WITHIN-RUN VARIABILITY OF THE METHOD

Added (mg/l)	Found ($\bar{x}$ ± S.D.) (n = 10)	C.V.* (%)
5.00	5.03 ± 0.06	1.19
25.00	24.83 ± 0.34	1.37

*C.V. = coefficient of variation = (S.D./$\bar{x}$) × 100.

was encountered. Moreover, the barbiturates having a molecule without a sulphur atom, and in particular pentobarbital, a thiopental metabolite, do not absorb at 280 nm. The UV absorption spectra of thiopental and pentobarbital are shown in Fig. 2. In our laboratory, pentobarbital quantitation is carried out by a gas chromatographic method previously described [10].

TABLE II

STABILITY OF THIOPENTAL DURING PROLONGED STORAGE OF THE SAMPLES

Storage at 20°C (h)	Storage at −20°C (weeks)	Thiopental concentration found (mg/l)			
		12.5 mg/l added	25 mg/l added	50 mg/l added	100 mg/l added
0		12.5	23.5	52.0	95.0
4		13.0	26.0	53.0	99.0
24		12.5	26.0	54.0	99.5
	1	12.0	23.0	53.0	102.0
	2	12.0	23.0	52.0	109.0
	4	12.8	23.6	43.2	97.0
	8	12.0	24.5	45.5	106.0

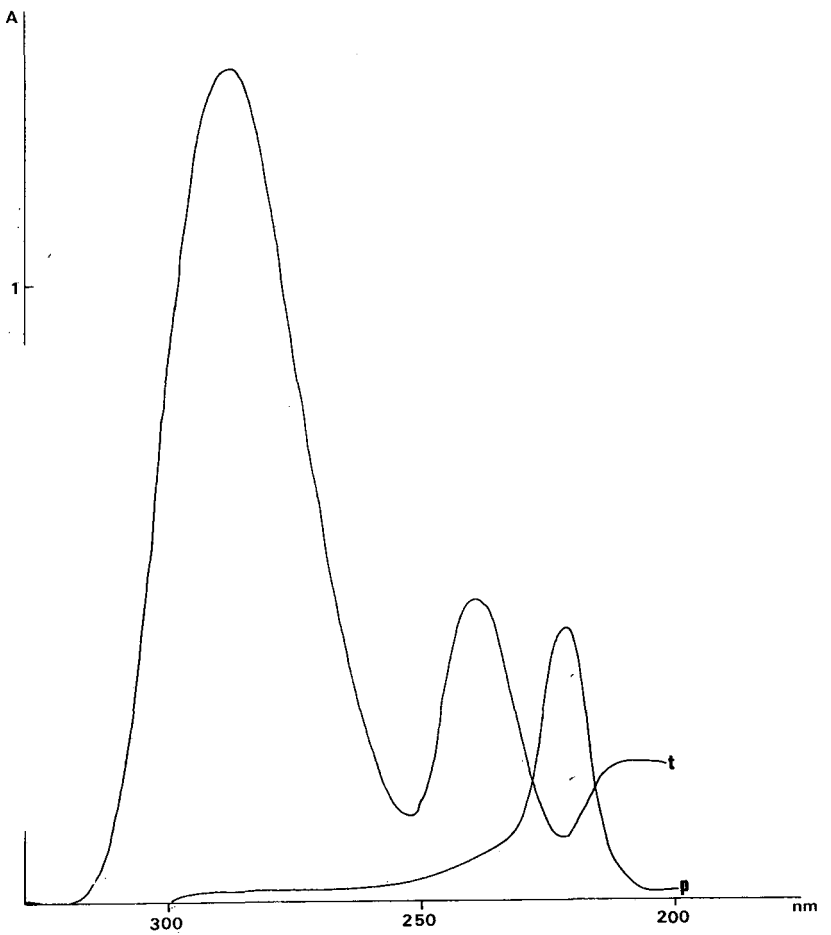

Fig. 2. UV absorption spectra of thiopental (t) and pentobarbital (p). The concentrations were 8 mg for thiopental and 16 mg for pentobarbital per liter of mobile phase.

In conclusion, this technique involving a small quantity of plasma, rapid sample preparation and automated liquid chromatography is highly suitable for routine analysis.

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Note

High-performance liquid chromatographic analysis of milrinone in plasma and urine

Intravenous pharmacokinetics in the dog

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Milrinone, 1,6-dihydro-2-methyl-6-oxo-(3,4′-bipyridine)-5-carbonitrile, is a new cardiotonic agent which has demonstrated inotropic activity in both laboratory animals and man. This novel drug is the subject of intensive clinical trials.

This report describes a high-performance liquid chromatographic (HPLC) method for the determination of milrinone in human plasma and human urine. The assay was used for the quantitation of milrinone in the plasma of dogs that had received milrinone by intravenous administration. The plasma concentration data were used to estimate pharmacokinetic parameters for milrinone in the dog.

EXPERIMENTAL

Chemicals

Milrinone and the internal standard for the assay, 1,6-dihydro-2-ethyl-6-oxo-(3,4′-bipyridine)-5-carbonitrile, were synthesized at Sterling-Winthrop Research Institute. The acetonitrile and ethyl acetate were chromatographic grade. Other chemicals were obtained commercially (reagent grade) and used without further purification.

Preparation of plasma and urine standards and samples

Plasma and urine standards were prepared by supplementing 1.0 ml of control human plasma (or urine) with 50-μl aliquots of stock solutions of

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milrinone in 0.01 *N* hydrochloric acid. The final concentrations of the standards were 0.0, 10, 20, 40, 60, 80, 100, 150 and 200 ng/ml of plasma and 0.00, 0.05, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0 and 10 μg/ml of urine. Duplicate standards at each concentration were prepared.

Four sets of randomized and coded samples, to be analyzed under single-blind conditions, were prepared as described above. Each plasma set contained triplicate samples at final concentrations of 0, 16, 32, 48, 120 and 180 ng/ml of plasma. Each urine set contained triplicate samples at final concentrations of 0, 0.06, 0.20, 2.40 and 9.0 μg/ml of urine. One set of plasma (or urine) samples was analyzed upon preparation. The other set was stored in the laboratory freezer for at least seven days before analysis.

Extraction procedure

To a tube containing 1.0 ml of sample (plasma containing potassium oxalate as the anticoagulant, or urine) were added 50 μl of internal standard solution (10 μg/ml for the plasma assay or 40 μg/ml for the urine assay). To the urine samples, 0.5 ml of 0.5 *M* phosphate buffer, pH 7.5, and 10 ml of ethyl acetate were added. To the plasma samples, 1.8 g of ammonium sulfate and 5 ml of ethyl acetate were added. Each sample was shaken for 10 min on a rotary shaker. The ethyl acetate layer was transferred to a clean 15-ml conical tube.

In the case of the plasma samples, it was necessary to repeat the extraction with a second 2.0-ml addition of ethyl acetate; the organic phases were combined. The combined extract was evaporated to dryness under a stream of nitrogen and the residue was dissolved in 250 μl of ethyl acetate.

For both plasma and urine, 0.1 *N* hydrochloric acid (0.1 ml for plasma and 0.4 ml for urine) was added to the ethyl acetate. After vigorous shaking, the organic layer was discarded. Residual ethyl acetate was evaporated by heating in a 55° C water bath, under a stream of nitrogen, for several minutes. Failure to eliminate traces of ethyl acetate can adversely affect the chromatography.

A neutralizing solution was prepared by adding 6 ml of 10 *N* sodium hydroxide to 94 ml of 0.5 *M* phosphate buffer, pH 7.0. For the urine assay, 50 μl of neutralizing solution were added to the residual acid phase. For the plasma assay, 15 μl of neutralizing solution were added. A 50-μl aliquot of the neutralized sample was injected into the chromatograph for analysis.

Chromatography

The HPLC system was operated isocratically at ambient temperature. The system consisted of an automatic injector, a pump (Milton Roy, Riviera Beach, FL, U.S.A.), a Partisil 10/25 ODS-3 column (10 μm particle size; Whatman, Clifton, NJ, U.S.A.) with a Waters Assoc. (Milford, MA, U.S.A.) 37—50 μm particle size Corasil C-18 precolumn, and a UV detector with a 340-nm filter. The mobile phase was a ternary mixture, tetrahydrofuran—acetonitrile—0.1 *M* phosphate buffer, pH 6.0 (28:260:1000). The flow-rates were 1.2 ml/min for the plasma and 1.5 ml/min for the urine assay.

Data processing

The output of the detector was interfaced with a Hewlett-Packard (Palo Alto, CA, U.S.A.) Model 3356 LAS computer system which calculated peak-

height ratios (milrinone:internal standard) for each standard and sample. A least-squares regression analysis was performed on the standards and used to determine the concentration of milrinone in the samples by inverse prediction [1]. The minimum quantifiable level of the assay was estimated as the concentration whose lower 80% confidence interval just encompassed zero [2].

The observed concentrations for the prepared, spiked samples in each biological media were expressed as percent differences from the nominal value. The range of these percent differences was used to define the accuracy of each assay. Precision was estimated from the standard deviation of the mean square error term derived from a two-way analysis of variance on the percent differences.

Extraction efficiency

The percent recovery of the extraction procedure for the internal standard was determined by comparing the peak heights of internal standard obtained from extracted samples with those obtained by injection of unextracted solutions. A similar procedure was used for milrinone over a range of concentrations; for plasma the concentrations were 20, 60 and 150 ng/ml and for urine the concentrations were 0.25, 2.5 and 10 μg/ml.

Animal study

Three female beagle hounds received single intravenous bolus doses of milrinone (5 mg/kg) via the saphenous vein. At appropriate intervals, blood samples were collected from the contralateral saphenous vein. Potassium oxalate was used as the anticoagulant. The blood samples were centrifuged and the plasma was transferred to clean tubes which were placed in the freezer (—20°C) until analyzed. Analysis was carried out as described above, with the exception that aliquots of plasma ranging from 0.25 to 1.0 ml were analyzed, depending on availability of sample and expected concentration. Those aliquots of less than 1.0 ml were supplemented with control dog plasma to 1.0 ml.

Pharmacokinetic calculations

The data obtained from the analysis of the dog plasma samples were fit to an open two-compartment body model by means of a nonlinear (NLIN) regression analysis using the Marquardt method [3]. The plasma concentrations were weighted as the squares of their reciprocals. The model is described by the equation:

$$C = Ae^{-\alpha t} + Be^{-\beta t}$$

where C is the concentration of milrinone in the plasma, α is the apparent first-order distribution rate constant, β is the apparent first-order disposition rate constant, t is time in hours, $A = D(\alpha - k_{21})/V_p(\alpha - \beta)$; $B = D(k_{21} - \beta)/V_p(\alpha - \beta)$; D is the administered dose in mg, V_p is the volume of the plasma compartment, and k_{21} is the apparent first-order rate constant from tissue to plasma.

The area under the curve of plasma concentration vs. time was estimated using the trapezoidal rule and extrapolated to zero concentration. The volume

of distribution at steady-state (V_{dss}) was calculated by a model-independent method [4] and by a regression-dependent method [5]:

$$V_{dss} = V_p\left(1 + \frac{k_{12}}{k_{21}}\right)$$

Other pharmacokinetic parameters were estimated by classical techniques [6].

RESULTS AND DISCUSSION

Analytical method

Representative chromatograms of an extracted plasma standard and an extracted plasma blank are shown in Fig. 1a and b, respectively. A plot of peak height ratios (milrinone:internal standard) vs. concentration of milrinone in the plasma standards was linear over the range of 0—200 ng/ml of plasma, as determined by linear regression analysis. The concentrations of the prepared plasma samples were estimated from the regression analysis by inverse prediction [1]. The accuracy of the assay, defined as the mean percentage difference from the nominal value, ranged from −8.2% to 1.7%. The mean (± S.E.M.) minimum quantifiable level (MQL) of the two sets was 3.7 ± 0.5 ng/ml using the full set of standards and 2.3 ± 0.05 ng/ml using a set of standards truncated to the range of 0–60 ng/ml. The estimated precision of the assay was ± 5.0%. Freezing and thawing of the plasma samples had no significant effect on the assay results.

The extraction efficiency studies in plasma indicated an apparent recovery of

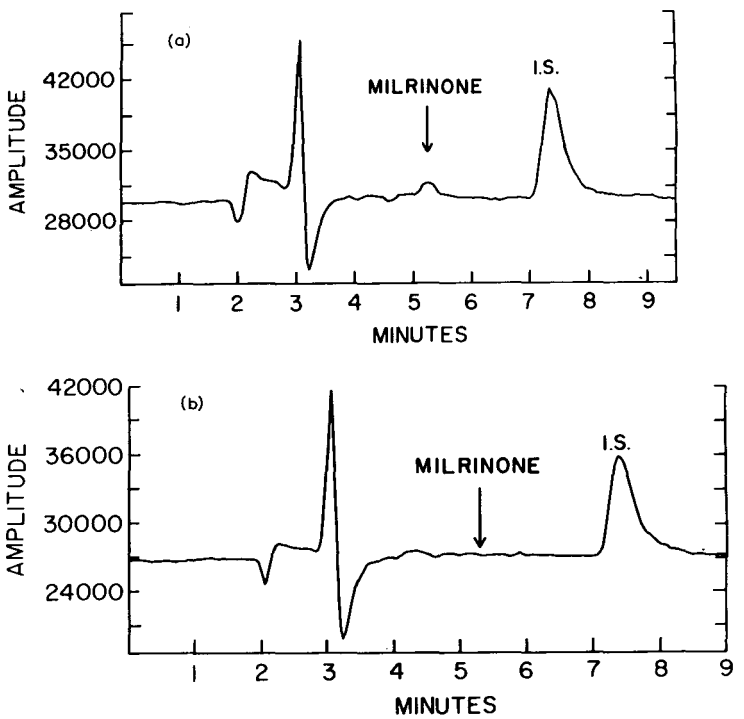

Fig. 1.

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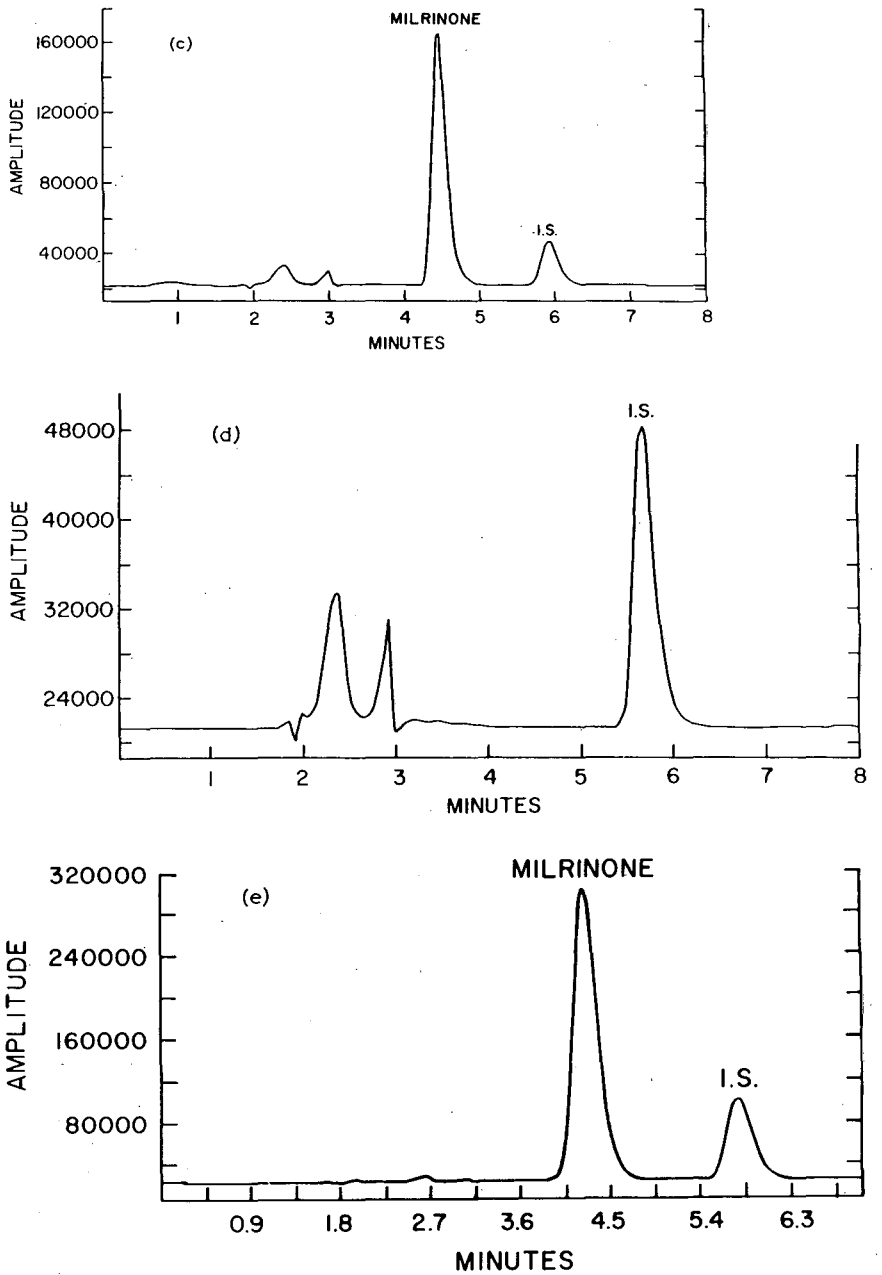

Fig. 1. Computer-reconstructed chromatograms of: (a) processed plasma standard containing 10 ng/ml of milrinone and 100 ng/ml of internal standard (I.S.); (b) processed plasma standard containing only the internal standard; (c) processed urine standard containing 10 μg/ml of milrinone and 2 μg/ml of internal standard; (d) processed urine standard containing only the internal standard; (e) processed plasma from a dog that had received a 5 mg/kg dose of milrinone, intravenously; specimen contains 6.2 μg of milrinone in 0.5 ml of plasma (5 min after medication).

104—105% for milrinone and the internal standard. After correction for the volume loss incurred during the evaporation of residual ethyl acetate, the recovery for both compounds was 93%.

Fig. 1c and d are representative chromatograms of processed urine standard and an extracted urine blank. A plot of the peak height ratios against concentration was linear over the range of 0 and 0.05—10 μg/ml.

The accuracy of the urine assay, defined as the mean percentage difference from the nominal value, ranged from −2.6% to 2.2%. The average MQL of the two sets of urine standards was 0.003 ± 0.001 μg/ml, using the set of standards which had been truncated to cover the range 0—0.5 μg/ml. The estimated precision of the assay was 2.6%. Freezing and thawing of the urine samples had no significant effect on the analytical results. The extraction efficiency for milrinone was 89%, and 99% for the internal standard.

Animal study

The milrinone levels in plasma, collected from three beagle hounds at various times after administration, are plotted against time in Fig. 2. Fig. 1e shows a chromatogram of plasma taken from a dog and processed as described above. The plasma data were fitted to an open two-compartment body model of intravenous administration, and the pharmacokinetic parameters were calculated (Table I). The predicted curves are represented as lines in Fig. 2. The mean half-lives of the distribution phase (α-phase) and the disposition phase (β-phase) were 0.48 and 3.6 h, respectively. The mean (± S.E.M.) regression-dependent

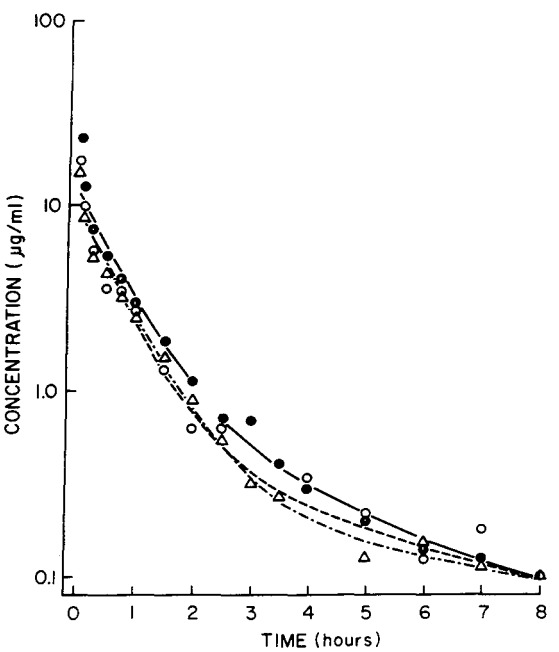

Fig. 2. Plasma concentration of milrinone in three dogs that had received a 5 mg/kg dose of milrinone, intravenously. Lines are those predicted by non-linear regression, see text. o——o, dog 51793; •——•, dog 52484; △ - - ∘ — △, dog ED26.

TABLE I

PHARMACOKINETIC PARAMETERS DERIVED FROM INTRAVENOUS PLASMA LEVEL DATA IN DOGS FOLLOWING A 5 mg/kg BOLUS DOSE OF [^{14}C]MILRINONE

Parameter	Dog 51793	Dog 52484	Dog ED26	Mean ± S.E.M.
Weight (kg)	8.0	10.2	8.4	8.9 ± 0.7
Dose (mg)	39.7	51.3	41.3	44.1 ± 3.6
V_p (l/kg)	0.52	0.42	0.55	0.50 ± 0.04
V_{dss} (l/kg)				
(1) Model-dependent	1.13	0.69	2.42	1.41 ± 0.52
(2) Model-independent	1.00	0.66	2.33	1.33 ± 0.51
k_{12} (h^{-1})	0.34	0.26	0.33	0.31 ± 0.03
k_{21} (h^{-1})	0.29	0.40	0.097	0.26 ± 0.09
k_{10} (h^{-1})	1.15	1.13	0.93	1.07 ± 0.07
α (h^{-1})	1.57	1.49	1.29	1.45 ± 0.08
β (h^{-1})	0.21	0.30	0.07	0.19 ± 0.07
A (μg/ml)	8.95	11.1	8.67	9.57 ± 0.77
B (μg/ml)	0.53	0.94	0.20	0.56 ± 0.21

volume of distribution was 1.41 (± 0.52) l/kg; the mean regression-independent volume of distribution was 1.33 (± 0.51) l/kg.

In summary, an accurate, sensitive and reproducible assay has been developed for the HPLC determination of milrinone in plasma and urine. This technique has been successfully applied to estimate pharmacokinetic parameters in dogs that had received an intravenous dose of milrinone.

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Note

High-performance liquid chromatographic determination of thiacetazone in body fluids

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Thiacetazone (TB1, thioacetazone, *p*-acetylaminobenzaldehyde-thiosemicarbazone) is the only oral companion drug to isoniazid that is commonly available for the treatment of tuberculosis in most Third World countries. Although TB1 was shown to have significant antileprosy activity as long ago as 1954 [1], it has been little used, primarily because when given as monotherapy the appearance of TB1-resistant *Mycobacterium leprae* caused many patients to relapse. However, in view of the problems posed by the wide-spread emergence of dapsone-resistant leprosy bacilli, there has been interest recently in the possibility of using TB1 as a cheap companion drug in the multi-drug treatment of lepromatous leprosy [2, 3]. TB1's minimal inhibitory concentration against *M. leprae*, calculated from experimental studies in the mouse footpad model, is approximately 0.2 μg/ml [2, 4]. Its antileprosy activity, like that against *M. tuberculosis*, is purely bacteriostatic [2, 4].

Since TB1 has been shown to be irregularly self-administered by both leprosy and tuberculosis patients [5, 6], it is probable that poor compliance may seriously limit its therapeutic efficacy when it is used as out-patient treatment of both diseases. To assess the likely importance of compliance, it is essential to determine the period for which inhibitory concentrations are maintained after the ingestion of standard daily doses of the drug. Few studies of the human pharmacology of TB1 have, however, been conducted, primarily because of the lack of sufficiently sensitive and specific methods to accurately measure blood levels of the drug. Methods based on the acid hydrolysis of TB1 followed by colorimetric determination of liberated *p*-aminobenzaldehyde by the Bratton and Marshall procedure [7—10] were sensitive to no better than 0.5 μg/ml and inevitably lacked specificity. More satisfactory UV and fluorimetric

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methods have been described but they are only capable of estimating concentrations of down to about 0.3 μg/ml TB1. Although peak TB1 serum concentrations after daily dosage with 150 mg of the drug could be measured with reasonable confidence by these methods, estimates of trough (24 h) concentrations were imprecise indicating the need for improved analytical methods for its estimation [11, 12].

This paper describes a sensitive and selective high-performance liquid chromatographic (HPLC) method for determining TB1 in plasma and urine. A preliminary study of the pharmacokinetics of TB1 in man using the method is also reported.

EXPERIMENTAL

Chemicals

All solvents and chemicals were of analytical grade. Distilled water of high purity was from a Fi-Stream glass still (Fisons, Loughborough, Great Britain). TB1 was donated by Smith and Nephew (Harlow, Great Britain) and was recrystallised thrice from ethanol (m.p. 227–231°C).

4-Propionylaminobenzaldehyde-thiosemicarbazone (PBT), the propionyl analogue of TB1, was synthesised for use as the internal standard. For this purpose *p*-propionylaminobenzaldehyde was first prepared following a procedure described for *p*-acetylaminobenzaldehyde [13]. Sodium sulphide nonahydrate (3 g) from a freshly opened bottle, 1.5 g flowers of sulphur and 2.6 g sodium hydroxide were dissolved by heating with 60 ml water. The solution was transferred to a round-bottomed flask containing a hot solution of *p*-nitrotoluene (5 g) in 30 ml ethanol and the mixture refluxed for 3 h. The product was rapidly steam distilled to leave about 50 ml of a reddish-coloured residue, which on cooling deposited *p*-aminobenzaldehyde as a mass of golden yellow crystals. These were rapidly filtered and immediately dissolved in 5 ml boiling propionic anhydride. After adding 5 ml water and boiling to reduce the volume, the solution was cooled in ice. The dark purple crystals obtained were purified by forming an adduct on boiling with 30% aqueous sodium bisulphite and then decomposed with 2 *M* sodium hydroxide to give a dense white precipitate. The *p*-propionylaminobenzaldehyde was filtered off, washed with water, dissolved in 75% aqueous ethanol and reacted by adding excess thiosemicarbazide in hot aqueous acetic acid. After cooling PBT precipitated out as a white solid which was washed with water and twice recrystallised from ethanol (m.p. 219—221°C). Its purity was confirmed by thin-layer chromatography on silica gel with ethyl acetate as the solvent and by HPLC. Stock solutions (1 mg/ml) of TB1 and PBT were prepared by dissolving the drugs in methanol and could be stored at 4°C for many months without appreciable decomposition. The stock solution of PBT was diluted with distilled water to give concentrations of either 15 or 60 μg/ml immediately prior to use as the internal standard.

Collection of urine, plasma and faecal samples and initial thiacetazone estimations

A preliminary study of the pharmacokinetics of TB1 was undertaken with

the help of a healthy male volunteer (G.A.E.) weighing 65 kg who ingested six consecutive daily doses of 150 mg of the drug on an empty stomach. Plasma samples were obtained immediately before and 2, 4, 6, 24 and 72 h after the final dose was swallowed. Complete pooled urine collections were made for each of the first four days and from 0—23 h on the fifth day. Two-hour collections were then made from 1 h before the last TB1 dose was ingested until 11 h afterwards. Further collections were then made from 11—23 h, 23—25 h, 25—47 h, 47—49 h, 49—71 h and 71—73 h, followed by 2-h collections at 24-h intervals from 95—97 h to 239—241 h, that is ten days after the ingestion of the final TB1 dose. Complete faecal collections were made up to ten days after the final TB1 dose. To arrive at suitable dilutions for the HPLC assays and to obtain evidence concerning the specificity of the formerly employed UV method [12], urinary concentrations of TB1 were first estimated by extracting 3-ml samples in a small separating funnel with 8 ml chloroform—propan-2-ol (4:1) after the addition of 1 ml of 1 *M* K_2HPO_4, drying the lower phase by filtration through anhydrous sodium sulphate and measuring the UV absorption at 333 nm.

Extraction procedure

Aliquots (3 ml) of plasma or appropriately diluted urine samples were pipetted into stoppered centrifuge tubes together with 0.1 ml of an aqueous solution containing 1.5 or 6 μg of PBT and 1 ml of 1 *M* phosphate buffer, pH 7.0, and extracted by shaking with 6 ml ethyl acetate on a vortex mixer for 15 sec. After centrifugation, the organic phase was decanted and washed by shaking with 0.5 ml of 0.1 *M* sodium hydroxide. Daily faecal collections were extracted by homogenisation with acetone (400 ml). After centrifugation and filtration, the extracts were diluted 50-fold with water, a 3-ml sample was taken and 6 μg PBT added. The extraction was carried out as above but with two washes of 0.5 ml of 0.1 *M* sodium hydroxide. The washed extracts were then transferred to a 10-ml tapered test tube and evaporated to dryness at 50°C under nitrogen. The dried residues could then be kept at 4°C prior to chromatography.

Liquid chromatography

Analyses were performed using a Waters Assoc. (Northwich, Great Britain) Model M6000A pump, a Cecil CE 212 variable-wavelength detector (Cambridge, Great Britain) set at 328 nm and a Waters U6K septumless universal injector. A reversed-phase system was used consisting of a Waters μBondapak column (30 cm × 4 mm I.D.; particle size 10 μm), which was eluted with a degassed, glass microfibre filtered (GF/F, Whatman, Maidstone, Great Britain) mobile phase of acetonitrile (Cambrian Chemicals, Croydon, Great Britain)—water (3:7) delivered at a flow-rate of 1.5 ml/min (ca. 12 MPa). The column was protected with a guard column (5 cm × 0.5 mm I.D.) containing dry-packed pellicular reversed-phase material (CO:Pell ODS, Whatman Reeve Angel, Maidstone, Great Britain). The dried urine extracts were dissolved in 100 μl of the mobile phase, duplicate 25-μl aliquots injected and the mean ratio of the peak heights for TB1 to that of the internal standard was calculated. The method was modified for the estimation of TB1 plasma concen-

trations to minimize the injection of extractable lipophilic components that might otherwise result in a rapid deterioration of the analytical column. Dried plasma residues were extracted by shaking with 100 μl of the mobile phase together with 100 μl of 2% ethanol in *n*-hexane. After centrifugation, 25-μl aliquots of the lower aqueous phase were then injected onto the column. Dried faecal extracts were treated in the same way.

Calibration curves

A calibration curve for estimating the maximal plasma TB1 concentrations expected after the ingestion of therapeutic doses of the drug was prepared by spiking blank plasma with TB1 to give concentrations of 0, 0.2, 0.5, 1 and 2 μg/ml. A similar calibration curve was prepared employing these concentrations of TB1 in normal urine, while a calibration curve was prepared with TB1 plasma concentrations of 0, 0.05, 0.1, 0.2 and 0.5 μg/ml, in order to determine levels near to its minimal inhibitory concentration against *M. leprae*. Duplicate 3-ml aliquots were then extracted and chromatographed as described above after the addition of the appropriate amount of PBT (6 μg or 1.5 μg). Calibration curves relating mean peak height ratio of duplicate injections to concentration to TB1 were shown to be linear, and the best straight lines and standard errors of slopes and intercepts were calculated by the least-squares method.

Selectivity

The selectivity of the method with respect to the most widely used antituberculosis and antileprosy drugs was evaluated by applying the analytical procedure to solutions containing 100 μg/ml ethambutol, isoniazid, *p*-aminosalicylic acid, pyrazinamide, rifampicin, streptomycin, clofazimine, dapsone, ethionamide and prothionamide in water or aqueous ethanol (9:1).

RESULTS

Analytical procedure

Representative chromatograms of urine and plasma extracts of samples obtained 72 h after the ingestion of the final 150-mg dose of TB1 are shown in Fig. 1a and b. Pretreatment urine and plasma samples contained no interfering peaks (Fig. 1c and d). For these analyses PBT was added as internal standard (6, 1.5 or 0.3 μg as appropriate). The retention times of TB1 and PBT were 3.2 and 4.1 min, respectively, giving baseline separation with a resolution factor (R_s) of 3.2. The TB1 concentrations were calculated from the peak height ratios of TB1 to that of the internal standard (PBT) and by reference to the equations of the calibration curves for the estimation of the drug in plasma and urine. These are given in Table I together with the standard errors of their slopes and intercepts. None of the intercepts was significantly different from zero and, as might have been expected, the slopes of the lines were inversely proportional to the amount of internal standard added. Replicate errors averaged about 1.5% at the higher concentrations and increased to about 7% when concentrations of 0.05–0.1 μg/ml TB1 were being determined in plasma. The overall recovery of TB1 in the extraction procedure was in excess of 90%.

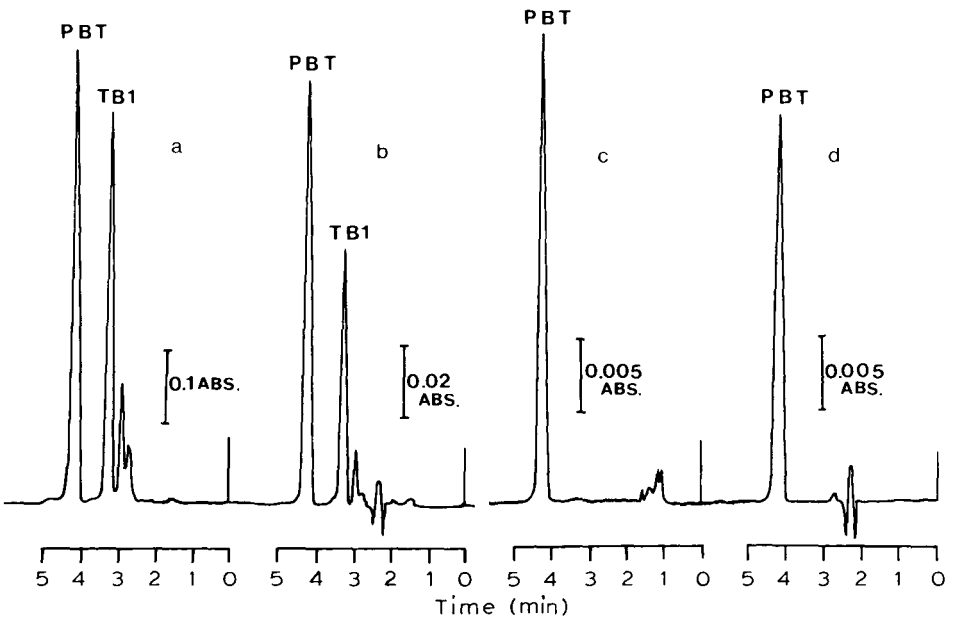

Fig. 1. (a) Chromatogram of an extract of a urine sample from a volunteer obtained 71—73 h after the ingestion of the final (sixth) daily dose of 150 mg TB1; 6 μg PBT was added as internal standard. (b) Chromatogram of an extract of a plasma sample obtained 72 h after the ingestion of the final daily dose of 150 mg TB1; 1.5 μg PBT was added as internal standard. (c) Chromatogram of an extract of a pretreatment urine sample; 0.3 μg PBT had been added to the sample. (d) Chromatogram of an extract of a pretreatment plasma sample; 0.3 μg PBT had been added as internal standard.

TABLE I

EQUATIONS OF CALIBRATION CURVES

Equation $y = mx + c$ where y is the ratio of the peak height of the drug to that of the internal standard, m the slope, x the concentration of TB1, and c the intercept.

Biological fluid	Concentration range (μg/ml)	Slope ± S.E.*	Intercept ± S.E.
Plasma	0.05—0.5	2.704 ± 0.024	0.014 ± 0.006
Plasma	0.2 —2.0	0.672 ± 0.005	0.006 ± 0.006
Urine	0.2 —2.0	0.684 ± 0.002	0.002 ± 0.002

*Standard error.

Detector response was linear over a wide range, direct injections of as little as 10 ng TB1 being easily measured. Calculations based on a signal-to-noise ratio of 3 indicated that the method could probably measure TB1 concentrations of down to about 3 ng/ml. None of the antituberculosis or antileprosy drugs tested was found to interfere with the method.

The urinary concentrations of TB1 estimated by the previous UV method [12] were consistently higher than those estimated by HPLC. While the ratios

of the two sets of measurements remained virtually constant (1.25 ± 0.08) during the first eight days of the pharmacokinetic study (up to three days after the administration of the final dose), they increased to about 2.2 from days 10—12. This finding indicated that extractable urinary metabolites of the drug had been estimated by the UV method, and two such metabolites were clearly visible in the HPLC scans with retention times of 2.7 and 2.9 min, respectively. Furthermore the ratio of their peak heights to that of TB1 remained virtually constant during the first eight days (0.12 ± 0.02 and 0.15 ± 0.04, respectively) but then increased to about 0.2 and 0.5 from days 10—12. Their retention times indicated that they were slightly more polar than TB1, but their nature is unknown. A previous study [12] showed that significant amounts of the potential metabolite *p*-aminobenzaldehyde-thiosemicarbazone (retention time 3.3 min) are not excreted after dosage with TB1. Another possible metabolite, *p*-acetylaminobenzaldehyde has a retention time of 3.7 min in the system used and was not found. This accords with the previous failure to detect its acid derivative, *p*-acetylaminobenzoic acid as a terminal metabolite of the drug [12].

Thiacetazone plasma concentrations, urinary and faecal excretion after oral dosage in man

The plasma concentrations and urinary excretion of TB1 are illustrated in Fig. 2. Immediately before the final 150-mg dose of TB1 was ingested, the plasma concentration of the drug was 0.64 μg/ml. Peak plasma TB1 concentrations were achieved after about 4 h (1.2 μg/ml) and thereafter fell to 0.62 μg/ml by 24 h and 0.22 μg/ml by 72 h. There was a strong suggestion that the elimination of the drug was biphasic with apparent half-lives for the decline from 6—24 h and 24—48 h of about 22 h and 33 h, respectively. The sensitivity

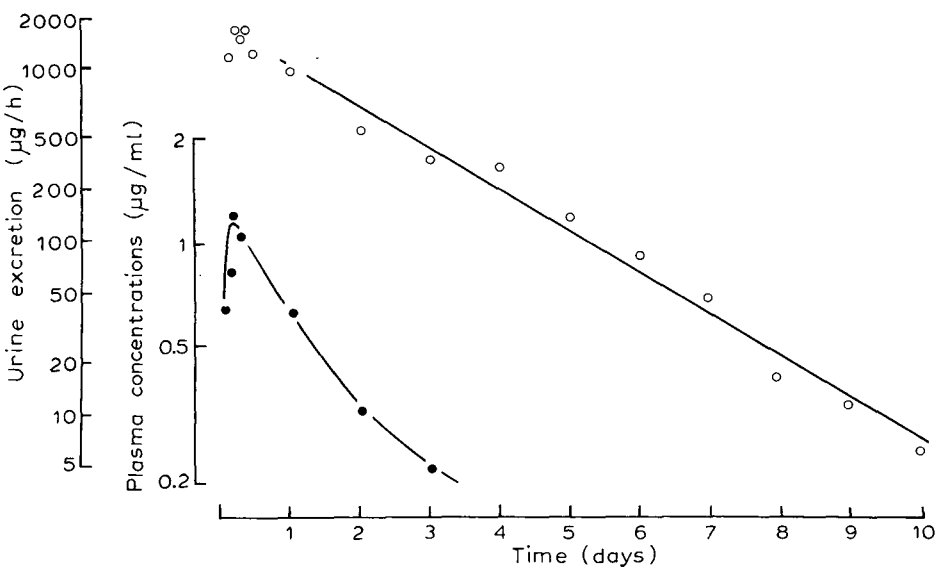

Fig. 2. Urinary excretion (○) and plasma concentration (●) of TB1 after the ingestion of the final (sixth) daily dose of 150 mg TB1.

of the HPLC method, together with the slow rate of elimination of TB1, was such that the urinary excretion could be accurately determined for at least ten days (Fig. 2). Over this period the urinary excretion of TB1 fell at a rate equivalent to a half-life of 29.8 ± 1.5 h. The urinary excretion rates correlated closely with the concomitant TB1 plasma concentrations ($r = 0.993$, $p < 0.001$) and gave a calculated renal clearance of 23 ml/min. The total cumulative excretion of unchanged TB1 in the urine over the whole period was equivalent to 15.4% of the dose. Significant amounts of TB1 were eliminated in the faeces up to six days after the ingestion of the final oral dose and totalled 28 mg or 3.1% of the administered doses.

DISCUSSION

The HPLC method described for the determination of TB1 in plasma and urine is sensitive, selective, precise and simple. Its sensitivity is about 50 times that of previous UV, colorimetric and fluorimetric procedures [7—12]. Furthermore, parallel estimations of urinary TB1 concentrations by a UV method which had been previously shown to be more specific than alternative colorimetric and fluorimetric procedures [12], provided evidence of the improved selectivity of the HPLC method. This was confirmed by the demonstration of extractable UV-absorbing urinary metabolites of TB1 in the HPLC traces. The accuracy of the HPLC method was such that replicate errors in the concentration ranges expected in the plasma of patients receiving standard treatment with the drug were equivalent to about ± 0.015 μg/ml TB1 in contrast to errors of ± 0.15 to 0.3 μg/ml encountered when fluorimetric or UV methods are used [12]. An additional advantage of the HPLC method is its rapidity, extraction and chromatography taking only about 15 min per sample.

The small proportion of the TB1 dose (3.1%) eliminated unchanged in the faeces confirms the previous conclusion, based on urinary excretion of the drug when single doses of 150—600 mg were ingested, that TB1 is well absorbed in man [12]. The peak and trough TB1 plasma concentrations determined in the volunteer after the administration of six consecutive daily doses of 150 mg of the drug were similar to those determined previously using UV and fluorimetric methods among tuberculosis patients from Kenya and Singapore [12].

The results of this preliminary pharmacokinetic study emphasize the inherent weakness of TB1, an essentially bacteriostatic drug, for potential use in the combined treatment of lepromatous leprosy [2—5]. Thus after the administration of standard daily doses of TB1, peak plasma concentrations of the drug only exceeded its minimal inhibitory concentration against *M. leprae* by about 6-fold and such inhibitory concentrations were only maintained for about three days after the final dose was ingested. Other more extensive studies of the blood levels and urinary excretion of TB1 in groups of healthy volunteers and tuberculosis patients using the HPLC method described in this paper will be reported elsewhere [14].

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Note

Determination of chlormethiazole in plasma by high-performance liquid chromatography

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Chlormethiazole, 5-(2-chloroethyl)-4-methylthiazole, a drug with sedative, hypnotic and anticonvulsant properties [1, 2] is becoming increasingly preferred by obstetricians for the treatment of pre-eclamptic toxaemia because, compared to alternative medications, associated side-effects are less severe [3]. Furthermore, these side-effects, which include muscular relaxation, hypothermia, respiratory depression and muscular hypotonia, are more easily managed since the short half-life of the drug and intravenous route of administration allow good dose control.

Chlormethiazole crosses the placenta easily [4, 5] and neonatal blood levels measured by gas—liquid chromatography (GLC) [6] indicate that the drug remains in the newborn much longer than in adults [5]. Cases of prolonged respiratory depression of the newborn have been reported following administration of chlormethiazole during labour [7, 8]. The possibility exists, therefore, that the differential diagnosis of neonatal respiratory depression together with muscular hypotonia should include excessive plasma chlormethiazole levels.

The frequent use of chlormethiazole in maternity hospitals specialising in the management of high risk pregnancies and the increasing sophistication of neonatal intensive care justify the availability of a simple method for monitoring the drug. In the absence of an established high-performance liquid chromatographic (HPLC) procedure we have developed such a technique to measure maternal, cord and neonatal drug levels following chlormethiazole therapy during labour.

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EXPERIMENTAL

Reagents

Methanol (Analar quality) was purchased from BDH Chemicals (Poole, Great Britain). Carbamazepine was generously donated by Geigy Pharmaceuticals (Horsham, Great Britain) and chlormethiazole edisylate was a gift from Astra Pharmaceuticals (St. Albans, Great Britain). Bovine serum albumin (30% solution) was supplied by Armour Pharmaceutical (Eastbourne, Great Britain).

Equipment

The Waters Assoc. high-performance liquid chromatograph consisted of a Model 6000A constant volume pump together with a U6K universal loop injector and a Model 440 UV detector set at 254 nm with an attenuation of 0.005 a.u.f.s. The detector output was connected to a 10-mV Linseis Model LS24/80/80 two-pen recorder operating with a chart speed of 200 mm/h.

Chromatography

Separation of chlormethiazole and the internal standard, carbamazepine (5-carbamyl-5H-dibenz[*b*,*f*]azepine), was achieved using methanol—water (45 : 55, v/v) in conjunction with a Waters Assoc. 30 cm × 3.9 mm I.D. μBondapak C_{18} analytical column (10-μm reversed-phase packing) protected by a guard column containing Bondapak C_{18}/Corasil. The mobile phase was prepared fresh daily, filtered through a 0.45-μm Millipore Filter (type HA), and used at a flow-rate of 1.8 ml/min.

Sample preparation

Heparinised plasma (500 μl) was diluted with an equal volume of methanol containing the internal standard, carbamazepine, at a concentration of 10 μg/ml. The sample was mixed for 30 sec on a Vortex mixer and centrifuged for 5 min at 2500 rpm (approximately 750 *g*). A 25-μl aliquot of the supernate was injected into the chromatograph.

Preparation of calibration standards

Two series of standards were compared. These were prepared in either heparinised plasma or 3% bovine serum albumin by making additions of aqueous chlormethiazole edisylate to give concentrations of 1, 2, 6, 10, 14 and 20 μg/ml. These samples were then subjected to the previously described sample preparation procedure and injected onto the chromatograph.

RESULTS

Fig. 1a shows the chromatogram obtained following injection of authentic chlormethiazole edisylate and the internal standard, carbamazepine. A chromatogram of extracted blank plasma is illustrated in Fig. 1b whilst Fig. 1c represents extracted plasma from a patient following intravenous administration of chlormethiazole. Comparison of Figs. 1b and 1c clearly indicates blank plasma to be free from any interfering compounds which may have coextracted along with chlormethiazole and the internal standard.

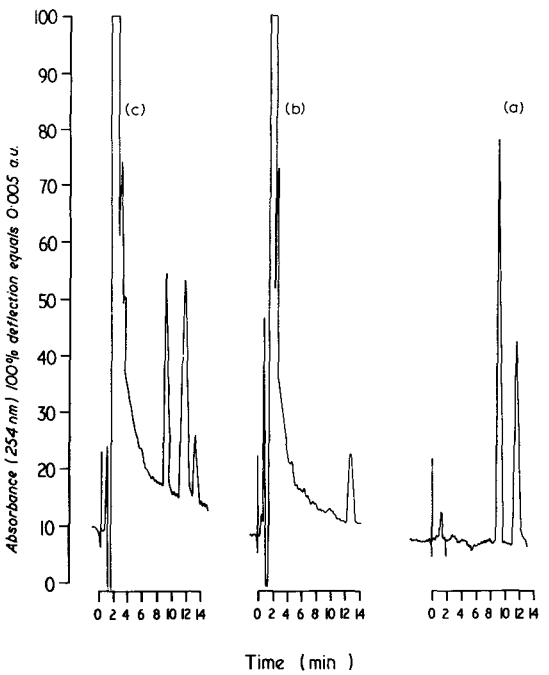

Fig. 1. (a) Chromatogram of authentic chlormethiazole edisylate and the internal standard, carbamazepine. The retention times are 9 min and 11.5 min, respectively, under normal assay conditions. Column, μBondapak C_{18} 30 cm × 3.9 mm I.D.; mobile phase, methanol—water (45:55); flow-rate, 1.8 ml/min; detection at 254 nm. (b) Chromatogram of extracted blank plasma showing absence of interference in the region of interest (8—12 min). (c) Chromatogram of extracted plasma from the same patient following intravenous administration of chlormethiazole. Peaks corresponding to carbamazepine (internal standard) and chlormethiazole are clearly visible with retention times of 11.5 min and 9 min, respectively.

TABLE I

THE REPRODUCIBILITY OF CHLORMETHIAZOLE DETERMINATIONS IN PLASMA AND 3% BOVINE SERUM ALBUMIN SHOWING THE EFFECTS OF STORAGE AT −20° C FOR 3 MONTHS

Sample	Concn. of chlormethiazole added (μg/ml)	Concn. of chlormethiazole determined at zero time (μg/ml)	Concn. of chlormethiazole determined after storage at −20° C for			Mean recovery (%) (n = 19)	Intra-batch coefficient of variation (n = 5)	Inter-batch coefficient of variation (n = 14)
			4 weeks	8 weeks	12 weeks			
Plasma	2	2.1	2.1	2.0	2.0	102.5	2.61	6.03
	14	14.1	13.3	14.0	13.7	98.4	3.45	3.19
3% BSA	2	2.2	1.8	1.8	2.0	97.5	2.03	5.8
	14	14.1	13.7	13.4	13.3	97.3	2.12	3.76

Calibration curves were obtained by comparing the peak height ratio (chlormethiazole/internal standard) with the actual concentration of chlormethiazole in spiked aliquots of plasma or 3% bovine serum albumin. In both cases the relationship was linear over the concentration range 0—20 μg/ml. Slope values are 0.09 and 0.088 with correlation coefficients (r) of 0.992 and 0.988 for plasma and 3% bovine serum albumin, respectively.

Storage trials were also carried out using both plasma and 3% bovine serum albumin. Samples, spiked with chlormethiazole to give concentrations of 2 and 14 μg/ml, were kept at −20°C and assayed at monthly intervals for up to three months. The results are presented in Table I. Five replicate samples measured at zero time gave values of 2.1 and 14.1 μg/ml with intra-batch coefficients of variation of 2.61 and 3.45 for plasma concentrations of 2 and 14 μg/ml, respectively. The same samples determined at monthly intervals over the 3-month storage period gave mean values of 2.03 and 13.67 μg/ml with inter-batch coefficients of variation of 6.03 and 3.19. Similar results (see Table I) were obtained for equivalent samples prepared in 3% bovine serum albumin.

The application of the method in clinical practice can be appreciated from the results listed in Table II which were obtained from patients treated with chlormethiazole during the later stages of labour. The plasma levels of chlormethiazole measured using this technique can be compared with those established using the alternative GLC and GLC—mass fragmentographic (MF) methods [4, 5, 7, 9—11] shown in Table III.

TABLE II

THE DETERMINATION OF CHLORMETHIAZOLE IN MATERNAL, CORD AND NEONATAL PLASMA USING HPLC

Sample	Mean plasma chlormethiazole concn.* (μg/ml)	Range of concn. (μg/ml)	S.D.	Number of determinations (*n*)
Maternal plasma	4.57	0.7 — 8.9	3.04	5
Cord plasma	5.37	0.6 —12.6	3.96	16
Neonatal plasma				
1 h	3.01	0.8 — 5.8	1.66	6
6 h	1.51	0.25— 2.9	1.09	4
12 h	0.975	0.6 — 1.3	0.29	4

*Mean plasma concentration of chlormethiazole edisylate (0.63 g of chlormethiazole base = 1 g of chlormethiazole edisylate).

TABLE III

CLINICAL RESULTS OBTAINED USING ALTERNATIVE METHODS SHOWING MEAN CHLORMETHIAZOLE PLASMA LEVELS

Literature source	Patient group	Analytical technique	Mean plasma concentration and range* (μg/ml)	Mean umbilical vein concentration and range (μg/ml)	No. of patients
Jostell et al. [9]	Healthy adults	GLC and GLC—MF	0.746 (0.370—1.350)	—	10
Jostell et al. [11]	Alcohol withdrawal	GLC MF	5.37 (3.12 —6.82) 5.81 —	— — — —	3 1
Tischler [5]	Maternity	GLC	11.5 (4.9 —20.4)	9.9 (7.2—15.3)	4
Duffus et al. [4]	Maternity	GLC	14.6 (4.7 —22.4)	10.8 (4.5—16.9)	11
Tunstall et al. [10]	Maternity	GLC—MF	1.59 —	1.30 —	1**
Young and Rasheed [7]	Maternity	GLC	3.1*** —	— —	1

*Mean plasma concentration of chlormethiazole base (0.63 g of base = 1 g of chlormethiazole edisylate).
**Single patient receiving intravenous chlormethiazole only.
***Neonatal level after 34 h.

DISCUSSION

The method described has proved useful for measuring drug levels in maternal, cord and neonatal plasma during studies of respiratory problems of the newborn following chlormethiazole therapy of pre-eclamptic toxaemia in labour. During twelve months of periodic use there has been no suggestion of interference from endogenous components which might invalidate the technique, Fig. 1b and c being typical of the many blank and patient plasma samples analyzed.

The advantage of this HPLC approach compared with the GLC methods of Jostell et al. [9] and Frisch and Ortengren [6] is the appreciably smaller sample requirement whilst maintaining a similar overall sensitivity. This is particularly important in paediatric applications. The detection limit of approximately 200 ng/ml may not facilitate pharmacokinetic studies but sensitivity is sufficient to enable the determination of chlormethiazole in neonates 12 h after delivery.

The possibility of improving overall sensitivity of the assay by alternative sample preparation exists since the method we have adopted, using methanol as a protein precipitant, causes dilution of the plasma due to mutual solubility of the two components. Solvent extraction into chloroform, followed by concentration of the drug, proved unsuccessful because although chlormethiazole is quantitatively extracted into the organic phase, recovery was poor due to simultaneous loss of sample during solvent evaporation under nitrogen at 55° C. Even when carried out at room temperature the recovery was only in the region of 20%. This phenomenon has also been observed in the case of ethchlorvynol [12] which is considered to be too volatile to permit concentration by solvent evaporation without significant loss.

Improvements in sample preparation, however, are unlikely to achieve the sensitivity of the GLC—MF method of Jostell et al. [11] which is capable of measuring plasma concentrations of 1 ng/ml but for those laboratories without access to such facilities, requiring only to monitor plasma chlormethiazole levels in excess of 200 ng/ml, the HPLC method is rapid and simple to operate.

Two pharmacologically active metabolites of chlormethiazole, 5-acetyl-4-methylthiazole (AMT) and 5-(1-hydroxyethyl)-4-methylthiazole (HEMT) [13], which have been measured in plasma using GLC—mass spectrometry [14] and more recently by GLC [15] do not interfere with the quantitation of the drug by this method. HMET, the major metabolite observed by Tsuei et al. [15], is not significantly retained on the column under the described conditions and elutes with the front material. According to the same authors, the maximum plasma level of the secondary metabolite only approaches 200 ng/ml. Since the lowest detectable concentration using our method exceeds this, any contribution from AMT would be expected to be minimal.

Although the GLC method of Tsuei et al. [15] is very sensitive and may be used in pharmacokinetic studies to estimate chlormethiazole and its two metabolites simultaneously, the HPLC approach for measuring chlormethiazole offers the advantage of a significant reduction in sample preparation time. Only a single addition of methanol is required in order to precipitate plasma proteins

compared with the relatively tedious three-stage extraction procedure employed by the GLC method.

Clinical results obtained using this technique (Table II) compare favourably with those gathered using alternative methods shown in Table III. Comparisons may be difficult to interpret since dosages are not standardised but based on individual clinical requirements and administered accordingly. However, at delivery the mean maternal plasma level (4.57 μg/ml) lies within the range of published values and the mean cord plasma level (5.37 μg/ml) is in close agreement with the maternal plasma concentration of chlormethiazole. In the neonate, mean plasma concentrations of the drug range from 3.01 μg/ml at 1 h after delivery decreasing to 1.51 μg/ml after 6 h and 0.975 μg/ml after 12 h.

For calibration purposes samples were prepared in both plasma and 3% bovine serum albumin. On comparison the results were so similar that, if preferred, the latter may be used for preparation of calibration standards.

The effects of storage on extraction efficiency and reproducibility were also examined using both plasma and 3% bovine serum albumin spiked with chlormethiazole. Again both series of samples behaved in a similar manner, the results (see Table I) clearly indicate that storage for three months at −20°C has no serious effect on recovery or reproducibility. The recovery of chlormethiazole using this method is predictably in the region of 100% since sample preparation involves dilution rather than extraction.

This technique was developed to permit quantitation of circulating chlormethiazole levels in mother and baby following treatment of pre-eclampsia during labour. It is being used in ongoing studies of neonates subsequent to the use of chlormethiazole during labour in order to examine relationships between circulating drug levels and symptoms of respiratory depression which may be encountered. The results of these studies will be published upon completion.

ACKNOWLEDGEMENT

The technical assistance of Mr. J.R. Cookman is gratefully acknowledged by the authors.

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Note

Determination of ampicillin, amoxicillin, cephalexin, and cephradine in plasma by high-performance liquid chromatography using fluorometric detection

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Several methods for the assay of aminopenicillins [1—8] and aminocephalosporins [9—16] in the body fluids have been studied. These utilize techniques such as fluorometory [1—6, 9—12] and high-performance liquid chromatography with UV detection [7, 8, 13—16]. These methods including our methods, however, are not sufficient for concentrations below about 50 ng/ml of plasma. And in the case of the experiments which study intestinal absorption behavior of these drugs, a more sensitive method of determination in the body fluids is required. Recently, Uno et al. [17], Lebelle et al. [18], and Barbhaiya et al. [19] showed that the structure of the fluorescent degradation products employed in some fluorometric assay procedures, including products obtained by our procedures, had a pyrazine ring as a common structural unit. In this study, we have developed a more sensitive and a reproducible high-performance liquid chromatographic (HPLC) assay for ampicillin, amoxicillin, cephalexin, and cephradine based on the common structure of these fluorescent degradation products obtained by our previous assay procedures.

EXPERIMENTAL

Materials

Ampicillin anhydrous, amoxicillin trihydrate, cephalexin monohydrate, and cephradine dihydrate were kindly supplied by Takeda Chemical Industries, Osaka, Japan, Kyowahakko Kogyo Co., Tokyo, Japan, Shionogi Co., Osaka, Japan, and Sankyo Co., Tokyo, Japan, respectively. Methyl anthranylate was purchased from Wako Pure Chemicals, Osaka, Japan. All the chemicals were of reagent grade and used without further purification.

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Buffer solution

A Sörensen buffer solution (pH 2.5) was prepared as follows. Citric acid (21.0 g) was dissolved in 200 ml of 1 *N* sodium hydroxide solution and the mixture was diluted to 1 liter with distilled water (0.1 *M* disodium hydrogen citrate solution). Then this solution was mixed with 0.1 *N* hydrochloric acid to adjust the pH of the solution to 2.5.

Formation of the fluorescent degradation products in plasma

Ampicillin. Plasma sample (0.5 ml) was added to 4 ml of distilled water in a 10-ml glass-stoppered centrifuge tube. Three milliliters of 10% trichloroacetic acid solution were then added to this diluted plasma sample and the mixture was centrifuged at 800—1000 *g* for 5 min. Three milliliters of the supernatant were pipetted into a test tube containing 0.5 ml of 2 *N* sodium hydroxide solution, then the mixture was allowed to stand for 5 min. Then 0.5 ml of 2 *N* hydrochloric acid was added. To this mixture, 1 ml of 0.1% (w/v) mercury bichloride solution prepared in Sörensen buffer solution (pH 2.5) was added. After 5 min, 2 ml of prewarmed 2/3 *M* disodium hydrogen phosphate solution were added to adjust the pH of the medium to 6.2. The mixture was then warmed at 40° C for 25 min. Then, 6 ml of ethyl acetate saturated with distilled water were added; the mixture was vigorously shaken for 5 min then centrifuged. Five milliliters of the organic layer were pipetted into a brown test tube and evaporated in vacuo. The residue was dissolved in 100 μl of methanol containing internal standard (methyl anthranylate); 20 μl of this solution were injected into the HPLC system.

For the determination in plasma of penicilloic acid, which is a metabolite of ampicillin, 1 ml of distilled water instead of sodium hydroxide and hydrochloric acid, and 0.5 *M* disodium hydrogen phosphate solution instead of 2/3 *M* solution were used; then the same method was followed as described above.

Amoxicillin. Three milliliters of the supernatant obtained from plasma were pipetted into a test tube containing 0.5 ml of 2 *N* sodium hydroxide solution and the mixture was allowed to stand for 5 min. Then 0.5 ml of 2 *N* hydrochloric acid was added. To this mixture, 2 ml of 0.002% (w/v) mercury bichloride solution prepared in 0.5 *M* disodium hydrogen phosphate solution were added to adjust the pH of this mixture to 6.0. A solution of the fluorescent degradation product was obtained by warming this mixture at 50° C for 25 min. After cooling, 6 ml of ethyl acetate saturated with distilled water were added and the mixture was vigorously shaken for 5 min and then centrifuged. Five milliliters of this organic layer were subjected to the method for the determination of ampicillin described above.

For the determination of the penicilloic acid of amoxicillin in plasma, 1 ml of distilled water instead of sodium hydroxide and hydrochloric acid was added to the 3 ml of supernatant. To this mixture, 2 ml of 0.002% (w/v) mercury bichloride solution prepared in 5/12 *M* disodium hydrogen phosphate solution were added to adjust the pH of the mixture to 6.0; then the same method as described above was followed.

Cephalexin. The procedure for the deproteinization of plasma was followed as described above. Three milliliters of the supernatant were pipetted into a

test tube containing 2 ml of 0.1 *M* disodium hydrogen citrate solution. One milliliter of 0.5% (w/v) hydrogen peroxide solution prepared in 0.1 *M* disodium hydrogen citrate solution was then added and the mixture (final pH 2.0) was heated in a boiling water bath for 70 min. This solution was cooled to room temperature and 2 ml of 0.5 *M* disodium hydrogen phosphate solution were added. Seven milliliters of the acetone–chloroform mixture (2:3, v/v) were added and the solution was vigorously shaken for 5 min and then centrifuged. Five milliliters of the organic layer were subjected to the method for the determination of ampicillin as described above.

Cephradine. The procedure for formation of the fluorescent degradation product of cephradine is similar to that of cephalexin except for a few details. Three milliliters of the centrifuged supernatant obtained from plasma were pipetted into a test tube containing 3 ml of 0.1 *M* disodium hydrogen citrate solution. Next, 1 ml of 0.6% (w/v) hydrogen peroxide solution prepared in 0.1 *M* disodium hydrogen citrate solution was added, and the mixture (final pH 2.5) was heated in a boiling water bath for 55 min. This solution was cooled to room temperature and 1 ml of prewarmed 1 *M* disodium hydrogen phosphate solution was added. The medium was then subjected to the method for the determination of cephalexin as described above.

Chromatographic conditions

A liquid chromatograph (Hitachi 638) equipped with a high-pressure sampling valve (638-0801, 1—150 μl) and fluorescence spectrometer (Hitachi 650-60) equipped with a flow cell (635-8001, 18 μl) was used. For the stationary phase, the reversed-phase column (Nucleosil C_{18}, 5 μm, 25 cm × 4 mm I.D., Macherey, Nagel & Co., Düren, G.F.R.) was used, and the column was warmed at 55°C using a constant-temperature water bath circulator. For the determination of ampicillin, cephalexin, and cephradine, a mixture of methanol–distilled water (3:2, v/v) was used as a mobile phase and the effluent was monitored at an excitation wavelength of 345 nm and an emission wavelength of 420 nm. For the determination of amoxicillin, the ratio of constituents of the mobile phase was modified to 55:45 (methanol–distilled water) and the fluorescence measurement was made with the excitation set at 355 nm and emission at 435 nm.

Calibration curve

Standard solutions containing 25, 50, 100, 150, and 250 ng/ml of each drug in distilled water were prepared. One milliliter of standard solution and 3 ml of distilled water were added to 0.5 ml of drug-free plasma and the samples were processed as described above. The ratios of the peak height of each drug to that of methyl anthranylate (internal standard) were used to construct a calibration graph.

RESULTS AND DISCUSSION

HPLC chromatogram

Fig. 1 shows a chromatogram of plasma spiked with 10 ng/ml of cephalexin compared to that of a plasma blank. The fluorescent degradation product of

cephalexin and the internal standard were well separated from endogenous substances. The retention time of the fluorescent degradation products obtained from ampicillin and cephradine were the same as that of the peak obtained from cephalexin. It was also found that the retention time of the fluorescent degradation product of amoxicillin was earlier than those of the other three fluorescent products, and the separation from the endogenous substances was carried out by modifying the ratio of constituents of the mobile phase.

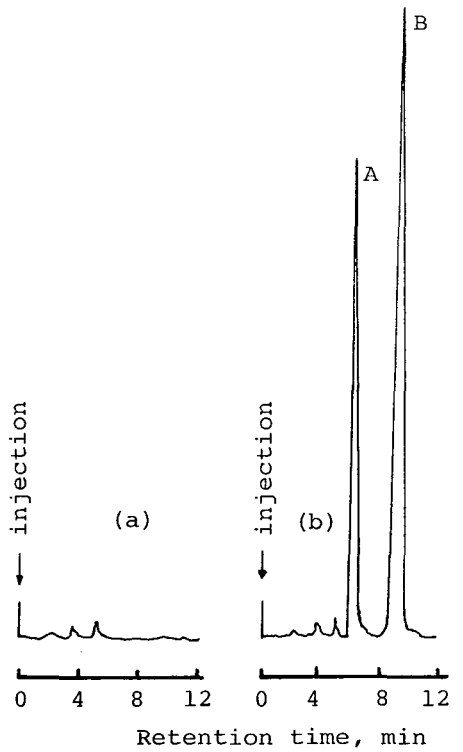

Fig. 1. HPLC chromatograms of (a) blank plasma, and (b) plasma spiked with 10 ng/ml cephalexin.

Selectivity

The calibration curves of peak height ratio for the four fluorescent degradation products were all linear with correlation coefficients of 0.998—0.999. The coefficients of variation of ampicillin and cephalexin at 2 ng/ml plasma were 5.1% (n = 5) and 3.5% (n = 9), respectively. The limit of the determination of the methods was 0.5 ng/ml for ampicillin, 2 ng/ml for cephalexin, and 10 ng/ml for amoxicillin and cephradine.

By using the rat intestinal loop technique for the absorption experiment [20] and this newly developed assay method the concentrations of cephalexin in plasma obtained from the mesenteric vein and carotid artery were determined. The time course of the plasma concentration of cephalexin in the

mesenteric vein after injection of the drug to the loop (150 μM) was 61.2 ng/ml (at 10 min), 80.0 ng/ml (at 15 min), 109.6 ng/ml (at 20 min), 231.4 ng/ml (at 25 min), and 259.5 ng/ml (at 30 min). The concentration of cephalexin in plasma obtained from the carotid artery was 41.9 ng/ml at 30 min.

It is possible to determine the low plasma concentrations of ampicillin, amoxicillin, cephalexin, and cephradine sensitively by the method described in this report. Our results suggest that the method is useful for studying intestinal absorption mechanisms of these drugs and for the determination of these drugs present in body fluids in very small quantities.

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Subject Index

Errata

J. Chromatogr., 273 (1983) 367—377
Pages 372 and 373, the retention times for flurazepam and pentobarbital should be read as 4.0 and 3.6 min, respectively.

J. Chromatogr., 274 (1983) 139—148
Table III: $ax \cdot (10^{-4})$ and $bx \cdot (10^{-4})$ should read $a \cdot 10^{-4}$ and $b \cdot 10^{-4}$ respectively.

corrected
A.J. 26/12/85

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JOURNAL OF CHROMATOGRAPHY
BIOMEDICAL APPLICATIONS

NEWS SECTION

MEETINGS

INTERNATIONAL SYMPOSIUM ON ANALYTICAL PROBLEMS AND METHODS IN BIOTECHNOLOGY

An International Symposium on Analytical Problems and Methods in Biotechnology will be held in Noordwijkerhout, The Netherlands, on April 17–19, 1984. The Symposium is organized under the auspices of the Analytical Division of the Royal Netherlands Chemical Society (KNCV) and the Netherlands Biotechnological Society (NBV).

The development of analytical methods for biotechnological applications is an area of growing importance. Analytical methods currently available are now being adapted for practical use in biotechnological research, development and industrial production. A large gap remains to be bridged between experts in analytical methodology and experts in biotechnology. It is the purpose of this Symposium to outline the problems faced in this field and to describe the rapid developments taking place. The Symposium is aimed at an interdisciplinary audience of those involved in industrial and academic biotechnology, as well as at analytical chemists themselves. Analytical tools will be presented for process control in industrial biotechnology, for environmental biotechnology and for fundamental research. Current research activity on all aspects of analytical chemistry related to biotechnology will be described.

Topics covered will include: *analytical strategies* (on-line versus discontinuous analysis, on-line sampling, on-line process analysis); *analytical techniques* for both gas-phase and liquid-phase analysis; and *process control* (computerized data evaluation and process control, biochips, process control strategies).

The scientific programme will consist of invited plenary lectures, invited and submitted research papers (both oral and poster presentations) and discussion sessions. The Symposium language will be in English, and the papers presented will be refereed for publication in a special issue of *Analytica Chimica Acta*. A selection of review papers will be considered for publication in *TrAC–Trends in Analytical Chemistry*.

A special Symposium package, including registration fee, accommodation for two nights, all meals and a copy of the Proceedings will be available at Dfl. 460 (approx. US$ 180).

Further information about the Symposium may be obtained from: W.A. Scheffers, Symposium Analytical Methods and Problems in Biotechnology, Delft University of Technology, Laboratory of Microbiology, Julianalaan 67A, NL-2628 BC Delft, The Netherlands. Tel: (015) 782411.

INTERNATIONAL SYMPOSIUM ON LIQUID CHROMATOGRAPHY IN THE BIOMEDICAL SCIENCES, RONNEBY, SWEDEN, JUNE 18–21, 1984

Liquid chromatography has had a strong impact on the development of the biomedical sciences, especially since the introduction of the high-performance mode some 15 years ago. This dynamic technique in analytical chemistry has opened possibilities to separate closely related compounds and to quantify them when present in very low concentrations in complex mixtures. Results based on liquid chromatographic determinations increase our understanding of various biological processes, where drugs and transmittor substances interact. Liquid chromatography is a very useful technique in the elucidation of the fate of drugs and other organic compounds in living systems.

New ideas are introduced continuously and it is the aim of this symposium to bring together scientists working with liquid chromatography in various respects in order to exchange ideas and to discuss common problems and impressions.

The following main areas will be covered: theory; ion-pair chromatography; microcolumns; separation of optical isomers; separation of peptides and proteins; new trends; detectors; and determination of bioactive compounds in biological material.

For further information contact: Swedish Academy of Pharmaceutical Sciences, P.O. Box 1136, S-111 81 Stockholm, Sweden.

PUBLICATION SCHEDULE FOR 1983

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

MONTH	1982	J	F	M	A	M	J	J	A	
Journal of Chromatography	252 253/1 253/2	254 255 256/1	256/2 256/3 257/1	257/2 258 259/1	259/2 259/3 260/1	260/2 261/1 261/2	261/3 262 263	264/1 264/2 264/3 265/1	265/2 266	The publication schedule for further issues will be published later
Chromatographic Reviews					271/1		271/2		271/3	
Biomedical Applications		272/1	272/2	273/1	273/2	274	275/1	275/2	276/1	

INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 244, No. 2, pp. 401–404. A free reprint can be obtained by application to the publisher.)

Types of Contributions. The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications:* Regular research papers (Full-length papers), Short communications and Notes. Short communications are preliminary announcements of important new developments and will, whenever possible, be published with maximum speed. Notes are usually descriptions of short investigations and reflect the same quality of research as Full-length papers, but should preferably not exceed four printed pages. For review articles, see page 2 of cover under Submission of Papers.

Submission. Every paper must be accompanied by a letter from the senior author, stating that he is submitting the paper for publication in the *Journal of Chromatography*. Please do not send a letter signed by the director of the institute or the professor unless he is one of the authors.

Manuscripts. Manuscripts should be typed in double spacing on consecutively numbered pages of uniform size. The manuscript should be preceded by a sheet of manuscript paper carrying the title of the paper and the name and full postal address of the person to whom the proofs are to be sent. Authors of papers in French or German are requested to supply an English translation of the title of the paper. As a rule, papers should be divided into sections, headed by a caption (*e.g.*, Summary, Introduction, Experimental, Results, Discussion, etc.). All illustrations, photographs, tables, etc., should be on separate sheets.

Introduction. Every paper must have a concise introduction mentioning what has been done before on the topic described, and stating clearly what is new in the paper now submitted.

Summary. Full-length papers and Review articles should have a summary of 50–100 words which clearly and briefly indicates what is new, different and significant. In the case of French or German articles an additional summary in English, headed by an English translation of the title, should also be provided. (Short communications and Notes are published without a summary.)

Illustrations. The figures should be submitted in a form suitable for reproduction, drawn in Indian ink on drawing or tracing paper. Each illustration should have a legend, all the *legends* being typed (with double spacing) together on a *separate sheet*. If structures are given in the text, the original drawings should be supplied. Coloured illustrations are reproduced at the author's expense, the cost being determined by the number of pages and by the number of colours needed. The written permission of the author and publisher must be obtained for the use of any figure already published. Its source must be indicated in the legend.

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