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SIMULTANEOUS DETERMINATION OF BETAMETHASONE, BETAMETHASONE ACETATE AND HYDROCORTISONE IN BIOLOGICAL FLUIDS USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(Received December 20th, 1979)

SUMMARY

A sensitive, specific, reproducible and rapid high-performance liquid chromatographic method for the simultaneous quantitation of betamethasone, betamethasone 21-acetate and hydrocortisone in biological fluids is described. Hydrocortisone acetate is used as an internal standard and the samples are extracted with dichloromethane before chromatographing on a reversed-phase system. Detection at two ultraviolet wavelengths (254 nm and 240 nm) was used to assess the specificity of the system, and the sensitivity was found to be greater than 10 ng for all steroids. The speed with which this assay can be performed makes it particularly useful for pharmacokinetic studies, and plasma concentration—time profiles resulting from the administration of betamethasone phosphate and betamethasone acetate are presented.

INTRODUCTION

Betamethasone (I) is commonly administered antenatally in an attempt to reduce the incidence of respiratory distress syndrome in premature infants. In this indication betamethasone is usually given intravenously or intramuscularly as the disodium salt of its 21-phosphate ester, or intramuscularly using a formulation which includes both the phosphate ester described and the 21-acetate ester of betamethasone (II). The phosphate and acetate esters are expected to be hydrolysed in vivo to betamethasone, the presumed active compound. The disposition of I in the maternal—foetal system is being investigated in this laboratory, and since pharmacokinetic studies of this nature involve the analysis of several hundred plasma, blood and urine samples they require a sensitive, specific, reproducible and rapid analytical method for the drug of interest. After administration of glucocorticoids it is also advantageous to be able to measure concentrations in biological fluids of endogenous hydrocortisone (III),

as well as the esters of betamethasone which are present in the commercially available dosage forms.

A high-performance liquid chromatographic (HPLC) technique which allows the simultaneous determination of I, II and III in blood and plasma, and I and II in urine has been developed. This method, which is reported here, is sensitive and precise, and allows very rapid analysis of samples. Betamethasone phosphate is water soluble and, using conventional solvent extraction, cannot be efficiently removed from biological samples with the other compounds of interest. Therefore its analysis is not described here.

EXPERIMENTAL

Reagents and materials

Betamethasone (I) and its 21-acetate ester (II) were gifts from Essex Laboratories (Sydney, Australia); hydrocortisone (III) and hydrocortisone 21-acetate (IV) were purchased from Sigma (St. Louis, MO, U.S.A.). Standard solutions of these compounds were made in distilled water (5 μg/ml) and stored at 4°C. The solutions of II and IV contained the disodium salt of EDTA at a concentration of 0.1% w/v, to stabilise the esters. Potassium fluoride (Ajax Unilab, Sydney, Australia) was kept as a 50% w/v solution in distilled water and sodium arsenate (Sigma) was stored as an aqueous solution (2 *M*). Dichloromethane (GR grade; Merck, Darmstadt, G.F.R.) was used without further purification.

Glass tubes were used for all steps in the analysis, and were cleaned in a 2% solution of Extran 300 (Merck). The glass extraction tubes (20 ml) were fitted with screw caps with PTFE liners.

A Varian Aerograph (Palo Alto, CA, U.S.A.) Model 8500 HPLC-pump equipped with a loop injection system (Valco, Houston, TX, U.S.A.) and a 250 mm × 4.6 mm I.D. RP-8 column (Brownlee, Santa Clara, CA, U.S.A.) with an average particle diameter of 10 μm was used. For all analyses a Waters Assoc. (Milford, MA, U.S.A.) Model 440 ultraviolet detector with a 254-nm filter was used and this was connected in series with a Spectra-Physics (Santa Clara, CA, U.S.A.) Model 770 variable-wavelength ultraviolet detector set at 240 nm when dual wavelength monitoring was required. A dual-pen recorder (Cole-Parmer, Chicago, IL, U.S.A.) with inputs set at 10 mV and a chart speed of 8 in./h was used. The mobile phase, HPLC grade methanol (Waters Assoc.)—filtered distilled water (60:40), was pumped through the column at a flow-rate of 1.25 ml/min, resulting in an inlet pressure of 700 p.s.i.g.

Collection of biological samples

The 21-acetate and 21-phosphate esters of betamethasone are unstable in biological fluids and steps must be taken to ensure that no hydrolysis to I occurs in vitro. Samples for plasma, blood and urine analysis were collected as follows.

Plasma. Blood samples (10 ml) were drawn by venipuncture or through a Dwellcath cannula (Tuta Lab., Lane Cove, Australia) using disposable plastic syringes (Pharmaplast, AHS/Australia, Balgowlah, Australia) and placed in

chilled plastic tubes (10 ml; Disposable Products, Sydney, Australia) containing 100 units of ammonium heparin and separation granules. These tubes also contained 100 μl of sodium arsenate solution which inhibited the hydrolysis of the phosphate ester. Each tube was placed in an ice bath, and centrifuged (1000 *g*, 5 min) within 5 min. The resulting plasma was immediately transferred to plain plastic tubes (Disposable Products) which contained 1% v/v of the potassium fluoride solution to inhibit hydrolysis of II.

Blood. Samples for whole blood analysis were collected exactly as for plasma samples, but instead of centrifuging, a measured quantity of blood was transferred immediately to the plain plastic tubes which contained 1% v/v of potassium fluoride solution. The blood was diluted with an equal volume of distilled water before freezing.

Urine. Urine was collected into large glass bottles containing sodium arsenate and potassium fluoride (6 ml of each solution per 12-h urine collection) and aliquots were transferred to plain plastic tubes for storage.

All samples were frozen at −22°C prior to analysis.

Extraction procedure

Samples (1 ml) of plasma and diluted blood were extracted directly, but urine was first diluted with an equal quantity of Sorenson's phosphate buffer pH 7.4 to standardise the pH of the samples. The internal standard, IV, (150 ng/30 μl) was added, then all samples were extracted with 5 ml dichloromethane by vortexing for 1 min. After centrifugation for 7 min at 1000 *g*, the upper aqueous layer was aspirated and the organic phase transferred to a 7-ml glass evaporation tube which had a 50-μl capillary base. A boiling chip (BDH Chemicals, Port Fairy, Australia) was added and the tube placed in a 45°C water bath. Immediately after dryness had been achieved, the tube was stoppered and placed in an ice bath to allow the dichloromethane vapour to condense and wash down the internal walls of the evaporation tube. Just before injection into the chromatograph the condensed dichloromethane was evaporated in the water bath, and the residue reconstituted with 100 μl of the mobile phase by vortex mixing for 20 sec. The whole extract was injected onto the HPLC column.

Calibration and reproducibility

Known quantities of I, II and III ranging from 10 ng to 300 ng were added to blank plasma, blood and urine samples. The samples were extracted and chromatographed as outlined above. Calibration curves were constructed by calculating the ratio of the peak height of each compound (I, II and III) to that of the internal standard (IV), and plotting the ratio against the amount of compound added to the sample. The calibration curves for III in plasma and blood were adjusted by subtracting the ordinate intercept, equivalent to the endogenous hydrocortisone in the sample, from all the peak height ratios calculated for III.

The reproducibility of the method was assessed for plasma and urine by spiking eight blank samples with 50 ng of I and II, and for blood by spiking six blank samples with 100 ng of each compound. Plasma and blood were analysed for endogenous hydrocortisone, I and II; urine was analysed for I and II only.

RESULTS

Stabilisation of the esters

Betamethasone phosphate, II and IV are all subject to both enzymic and chemical hydrolysis to their corresponding alcohols. The hydrolysis of the phosphate ester in vitro must be prevented so that the levels of I measured are not spuriously high. The conversion in biological fluids of betamethasone phosphate to I is rapid, but was prevented by the collection of samples into vessels which contained 1% v/v of a 2 *M* sodium arsenate solution [1]. The procedure did not cause haemolysis of red blood cells and did not alter the distribution of I between the blood cells and plasma.

The hydrolysis of II following the collection of samples was found to be considerable. When whole blood containing II (100 ng/ml) was left at room temperature for 30 min, 22% of II converted to I, and 15% conversion occurred if the sample was stored on ice for the same time. The addition of 1% v/v of sodium arsenate (2 *M*) did not fully prevent the hydrolysis, however the incorporation of 1% v/v of a 50% w/v potassium fluoride solution into the blood did inhibit the degradation [2]. Unfortunately, this was not suitable for blood samples which were drawn for plasma analysis since the potassium fluoride caused haemolysis of the red blood cells.

The collection methods described were found to be quite satisfactory. When blood was collected into chilled heparinised tubes containing sodium arsenate, placed on ice, centrifuged within 5 min, and the plasma immediately aspirated into tubes containing potassium fluoride, no detectable degradation of II occurred, even if the plasma remained at room temperature for 1 h. Similarly the collection methods for whole blood and urine were found to prevent any detectable hydrolysis of II. The addition of the potassium fluoride to plasma not only avoided the problem of haemolysis, but prevented any possible effects of the salt on the distribution of I, II and III between plasma and red blood cells.

Chemical hydrolysis of II and IV in stock aqueous solutions was prevented by adding 0.1% disodium salt of EDTA and storing them at 4°C. The chemical hydrolysis is base catalysed and therefore basification during the extraction procedure may result in degradation of the esters. No hydrolysis of the esters occurred during the analysis of spiked samples using the analytical method reported here.

Calibration and reproducibility

Chromatograms resulting from the analysis of blank human plasma, blood and urine are shown in Fig. 1, along with a chromatogram of blank plasma to which 50 ng of I, 100 ng of II and III and 150 ng of IV had been added. The retention times of III, I, IV and II were 5.3, 6.5, 7.2 and 9.0 min respectively. The small peak which chromatographs near the internal standard has been of the same magnitude in all samples studied and does not compromise the analysis since it is swamped by the internal standard peak.

The calibration plots obtained were linear over the concentration range from 10 ng to 300 ng for I and II and from 10 ng to 200 ng for III. The signal-to-noise ratio obtained when quantitating 10 ng was 30—40:1 which indicated

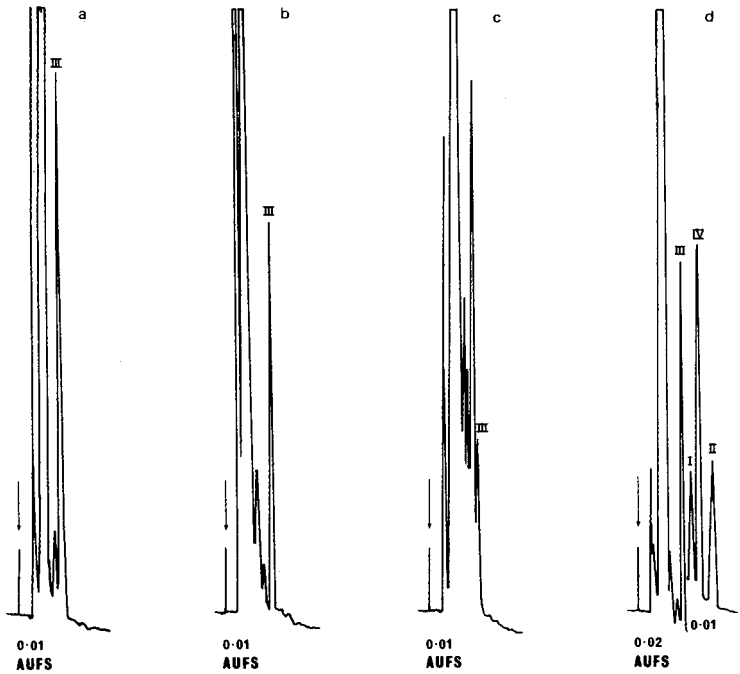

Fig. 1. Chromatograms of extracts of blank human (a) plasma, (b) blood, (c) urine and (d) plasma spiked with 50 ng betamethasone (I), 100 ng betamethasone acetate (II) and hydrocortisone (III) and 150 ng hydrocortisone 21-acetate (IV) as internal standard. The retention times of III, I, IV and II are 5.3, 6.5, 7.2 and 9.0 min respectively. The arrows mark the points of injection.

TABLE I

REPRODUCIBILITY DATA

	Coefficient of variation (%)		
	Plasma (n=8)	Blood (n=6)	Urine (n=8)
Hydrocortisone	2.4*	6.6*	—
Betamethasone	2.1**	4.2***	1.8**
Betamethasone acetate	1.6**	3.2***	2.8**

*Endogenous hydrocortisone assayed.
**Concentration of steroids 50 ng/ml.
***Concentration of steroids 100 ng/ml.

that quantitation to substantially lower levels of these compounds is possible on this system. The analytical recovery of the compounds was measured by comparing the chromatographic peak heights from the analysis of eight biological samples which were spiked with 100 ng of each compound, and the peak heights resulting from a direct injection of aqueous standards. The recovery of all compounds from plasma, blood and urine was 80—85%, when approximately 85—90% of the dichloromethane phase was available for evaporation.

Table I gives the results of the reproducibility study. The precision of this

method was very good as is illustrated by coefficients of variation which were less than 3% for all compounds in plasma and urine, and less than 7% for the blood samples.

Confirmation of the homogeneity of the hydrocortisone peak was particularly important since the absence of interfering compounds could not be shown directly using a blank sample. It was also necessary to ensure that no metabolites of I chromatographed simultaneously with any of the compounds of interest. To check the specificity of the method, the ratio of the absorbance of each peak of interest at 240 nm to that at 254 nm was calculated, and compared to the ratio observed when authentic standards were injected directly into the chromatograph. The ratio was computed for fifteen plasma samples which were collected over an 8-h period after the administration of an 8-mg dose of betamethasone phosphate. The ratio for each peak of interest was always within 3% of the ratio obtained for the authentic standard. A further check for specificity was carried out by examining the retention characteristics of several drugs commonly administered to women who are receiving glucocorticoids antenatally. Table II lists the drugs which were examined, none of which interfered with the analysis of any of the compounds of interest.

TABLE II

COMPOUNDS SHOWN NOT TO INTERFERE WITH THIS ASSAY

Salbutamol	Bupivacaine
Ritodrine	Lignocaine
Hyoscine	Phenobarbitone
Diazepam	Paracetamol
Pethidine	Salicylic acid
Promazine	Betamethasone phosphate

DISCUSSION

The analytical method reported here is a rapid, precise, selective and sensitive way of simultaneously determining I, II and III in biological fluids. This method not only allows the disposition of betamethasone to be examined after various dosage regimes, but facilitates an examination of the time course of levels of the acetate ester which is administered and the effect these steroids have on endogenous hydrocortisone. Fig. 2 shows the plasma concentration—time profiles of I and III after the administration of (a) 10.6 mg of betamethasone phosphate intravenously and (b) 6 mg betamethasone (3 mg as betamethasone phosphate and 3 mg as betamethasone acetate) intramuscularly to pregnant patients. Following dose (b) no II was detected throughout the sampling period, however this may not be the case when different doses, formulations and muscle sites for injection are studied. Chromatograms resulting from the analysis of plasma samples collected just prior to dosing and 60 min after the dose was given are shown in Fig. 3.

In the past, several methods have been used for the detection of I in biological fluids. These included radio-immunoassay (RIA) techniques which were preceded by column chromatography to reduce cross-reactivity problems [3]; and a combination [4] of a radioreceptor assay for total glucocorticoid activity

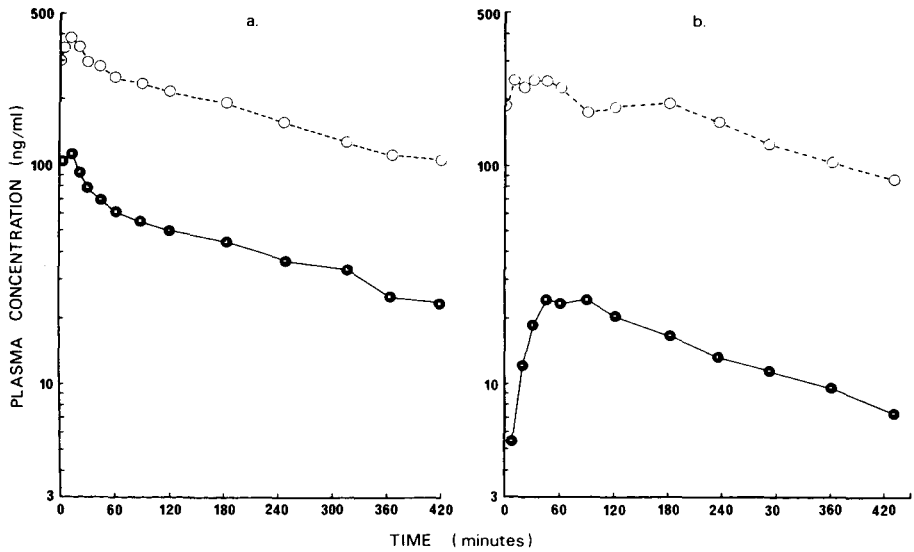

Fig. 2. Plasma concentration—time profiles of betamethasone (•——•) and hydrocortisone (○ — — ○) after the administration of (a) 10.6 mg betamethasone phosphate intravenously and (b) 3 mg betamethasone as the phosphate ester and 3 mg betamethasone as the acetate ester, intramuscularly, to pregnant patients.

[5] and the corticosteroid-binding globulin isotope assay of Murphy [6]. Similarly, III has been measured in plasma using competitive protein binding [6] and RIA [7] methods. However, these techniques cannot be used for simultaneous determination of steroids, and the time involved in the execution of these assays precludes their use in pharmacokinetic studies with large numbers of samples.

Recently, several HPLC assays for hydrocortisone and some synthetic steroids other than betamethasone in biological fluids have been reported [8—18]. The majority of these methods use normal-phase HPLC systems which are suitable for the analysis of the steroid alcohols, but the acetate esters are less polar and are not adequately retained on the column under these conditions. Additionally, these methods are relatively slow, involving several extraction steps, and usually include a basic wash with 0.1 *N* NaOH which is not suitable in this application since the chemical hydrolysis of II and IV is base catalysed.

Although the present method has been shown to be highly specific for the compounds of interest, it should be noted that one source of interference was encountered. When blood was collected in Monoject (Sherwood Medical Industries, Deland, FL, U.S.A.) or Terumo (Terumo Australia, Melbourne, Australia) disposable syringes, a large interfering peak eluted at a retention time of 6.5 min. Interference from this source has been reported before [19] and should be kept in mind since it is easy to erroneously label the peak as an endogenous biological substance. In this case the problem was solved by using Pharmaplast syringes for all dosing and sampling. An endogenous compound does elute after the steroids of interest but does not interfere with the subsequent injection provided that each sample is allowed to chromatograph for 12 min.

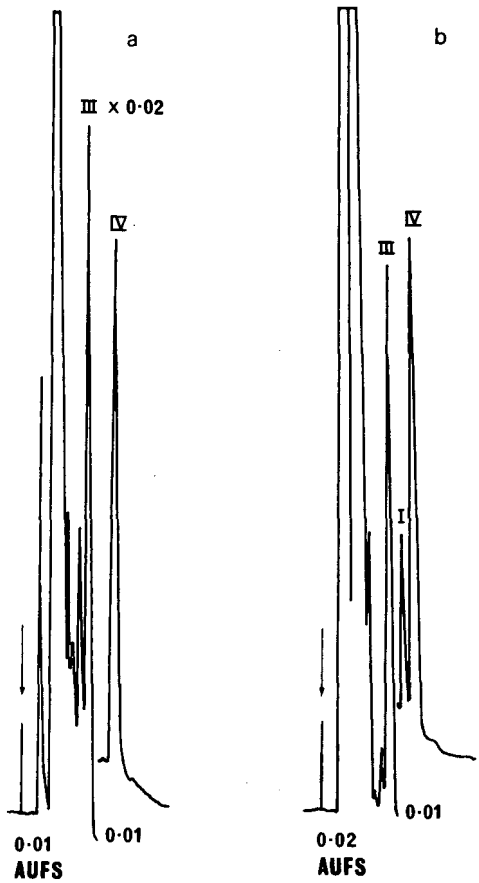

Fig. 3. Chromatograms of extracts of plasma from a pregnant patient (a) just before dosing and (b) 60 min after dosing with 10.6 mg betamethasone phosphate intravenously. Peaks: I, betamethasone; III, hydrocortisone and IV, internal standard (hydrocortisone 21-acetate). The arrows mark the points of injection.

This method is particularly suitable for pharmacokinetic studies since it is both rapid and sensitive. The ability to measure I, II, and III in plasma and blood is essential for studies where the distribution of drugs between plasma and the red blood cells is to be investigated. The amount of drug which is excreted by the kidneys as I and II can also be measured using this method.

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CHROMBIO. 587

CHEMICAL IONISATION MASS FRAGMENTOGRAPHIC MEASUREMENT OF DOTHIEPIN PLASMA CONCENTRATIONS FOLLOWING A SINGLE ORAL DOSE IN MAN

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SUMMARY

A method is described for the measurement of the antidepressant drug dothiepin in human plasma.

The procedure involves the addition of deuterodothiepin as an internal standard, extraction and measurement by chemical ionisation mass fragmentography. The minimum measurable concentration of 0.5 ng/ml enabled the pharmacokinetics of dothiepin in man to be studied after a single oral dose of 75 mg of dothiepin hydrochloride.

Unlike other tricyclic antidepressants the apparent half-life of elimination (approximately 24 h) showed very little intersubject variation. However, the apparent volume of distribution was much more variable and could account for the wide range of steady state concentrations which have been found in patients taking dothiepin.

INTRODUCTION

Although the tricyclic antidepressant, dothiepin hydrochloride or dosulepin hydrochloride, Prothiaden [11-(3-dimethylaminopropylidene)-6,11-dihydrodibenz[*b*,*e*]thiepin hydrochloride], has been in clinical use for a number of years, the pharmacokinetics after a single dose in man have not been studied, as a method with suitable sensitivity has not been available. This is because dothiepin, like other tricyclic antidepressants, is extensively metabolised in man resulting in very low plasma concentrations of unchanged drug.

We have predicted from studies in laboratory animals, that in order to study the single dose pharmacokinetics in man, an analytical method capable of measuring subnanogram amounts of dothiepin is required.

Whilst techniques using gas—liquid chromatography [1,2] or high-performance liquid chromatography [3] are suitable for the measurement of steady state levels of dothiepin (range 20—420 ng/ml) they are not sufficiently sensitive to enable a complete plasma profile to be obtained after a single dose of 25—75 mg of the drug.

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For the measurement of tertiary tricyclic amines, mass fragmentographic methods have been developed. Electron impact (EI) mass fragmentographic methods have been published for many tricyclic antidepressants [4] and more recently chemical ionisation (CI) methods have been reported [5]. However, there is no published mass fragmentographic method for dothiepin.

EXPERIMENTAL

Six healthy male volunteers within ± 10% of the Metropolitan Life Insurance tables of desirable weights for males and females (age 22—36 years, mean 26; weight 65—76 kg, mean 71.0; height 178.5—185.5 cm, mean 181.0) gave written consent to participate in the study. All volunteers undertook a screening procedure which included a full physical examination, electrocardiogram, urinalysis, haemoglobin, white blood cell and differential count, platelets, serum glutamic—oxaloacetic transaminase (SGOT), serum glutamic—pyruvic transaminase (SGPT), bilirubin and sodium and potassium levels.

Each volunteer received 75 mg (3 25-mg capsules) of dothiepin hydrochloride with 100 ml of water after an overnight fast. Breakfast was allowed 3 h after taking the dose. Blood samples were taken immediately prior to drug ingestion and after 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 6, 12, 24, 36, 48, 72 and 96 h. Samples were taken into heparinised containers, the first ten by indwelling cannula and the remaining samples by repeated venipuncture. For the first 8 h of the test the volunteers were under constant medical supervision. Plasma was separated by centrifugation at 850 *g* for 20 min and stored at −20°C until assayed.

Gas chromatography—mass spectrometry

All analyses were carried out on a Hewlett-Packard Model 5982A gas chromatograph—mass spectrometer fitted with a dual EI/CI source and coupled to a Hewlett-Packard Model 5947A multiple ion detector.

The gas chromatographic separation was carried out on silanised glass columns (1 m × 2 mm I.D.) packed with 3% OV 17 on 100—120 mesh Gas-Chrom Q (Chromatography Services, Merseyside, Great Britain).

The injection port, column oven and transfer lines were maintained at 250, 220 and 250°C respectively. Research grade methane (B.O.C., Derby, Great Britain) was used both as carrier gas and as CI reactant gas. The flow-rate of methane was adjusted to optimise the source pressure (80—93 Pa), which corresponded to a flow-rate of 8—10 ml/min through the column and gave satisfactory chromatography. The source temperature was 150°C, the electron beam energy was 250 eV and the filament current 200 μA. Other parameters were adjusted to optimise the performance of the instrument.

Reagents and standards

Internal standard. Deuterodothiepin HCl (2 mg) dissolved in distilled water (10 ml) diluted 1 to 100 with distilled water to give a solution containing 20 ng/10 μl.

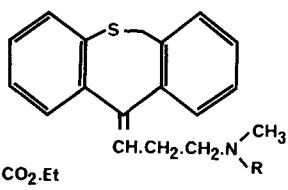
Carrier. Imipramine HCl (Courtine and Warner, Lewes, Great Britain) (25 mg) dissolved in distilled water (10 ml) diluted 1 to 10 with distilled water to give a solution containing 250 ng/10 μl.

Standard. Dothiepin HCl (The Boots Company, Nottingham, Great Britain) (11.2 mg) dissolved in distilled water (10 ml) diluted 1 to 1000 with distilled water to give a solution containing 1 ng/μl of dothiepin free base.

Reagents and solvents used were 1 *N* sodium hydroxide solution prepared from sodium hydroxide AR (B.D.H., Poole, Great Britain), Nanograde hexane (Camlab, Cambridge, Great Britain) and methyl acetate (B.D.H.).

Synthesis of internal standard [11-(3-N-trideuteromethyl-N-methylamino-propylidene)-6,11-dihydrodibenz[b,e] thiepin]

The N-methyl-N-ethoxycarbonyl derivative (I) (Fig. 1) of dothiepin was prepared by reaction of dothiepin (II) with ethyl chloroformate [6]. The carbamate was reduced to deuterodothiepin (III) with lithium aluminium deuteride and isolated as the hydrochloride.



I. R=CO_2.Et
II. R=CH_3
III. R=$C[^2H]_3$

Fig. 1. Structural formulae of deuterodothiepin and intermediates. I = N-methyl-N-ethoxycarbonyl derivative; II = dothiepin; III = deuterodothiepin.

Extraction and measurement

To 1 ml plasma in a 15-ml glass stoppered centrifuge tube were added 10 μl internal standard solution, 10 μl carrier solution, 200 μl 1 *N* sodium hydroxide solution and 10 ml hexane. Extraction was effected by rotation on an extraction wheel (Scientific Industries International, Loughborough, Great Britain) at 45 rpm for 30 min. After centrifugation (1500 *g* for 10 min), the hexane phase was transferred to a 10-ml pointed glass tube and evaporated to dryness at 45°C under a stream of nitrogen to concentrate the extract into the point of the tube. The residue was finally redissolved in 5 μl of methyl acetate and injected into the gas chromatograph.

To minimise adsorption losses all glassware was soaked overnight in 0.1 *N* sodium hydroxide solution, rinsed with distilled water and dried in an oven at 110°C.

The multiple ion detector was set to monitor approximately *m/e* 296 (M + 1 ion of dothiepin) and approximately *m/e* 299 (M + 1 ion of internal standard). Fractional mass settings required to give maximum response were dependent on the mass marker offset at the time of operation. Optimum mass settings and gain settings were therefore determined for each batch of samples by examining standard solutions of dothiepin and deuterated dothiepin.

The peak height for dothiepin (*m/e* 296) and deuterodothiepin (*m/e* 299) were measured and the ratio *m/e* 296/*m/e* 299 used to calculate the concentration of dothiepin relative to a standard calibration curve.

RESULTS AND DISCUSSION

Methodology

Electron impact ionisation results in the complete fragmentation of the dothiepin molecule such that no fragment ion greater than 2% of the base peak (*m/e* 58) is found (Fig. 2). Monitoring of the base peak was found to be insufficiently selective to allow the reliable measurement of subnanogram amounts of dothiepin in plasma or serum extracts. Chemical ionisation on the other hand gives rise to an abundant quasimolecular ion at *m/e* 296 (Fig. 3) and monitoring of this ion results in the specific detection of dothiepin with the minimum of sample preparation.

A calibration curve constructed from 40 measurements, over a period of 14 days, on control plasma to which had been added known amounts of dothiepin in the range 0—60 ng/ml is shown in Fig. 4. Regression analysis of these data gave a linear relationship between peak height ratio and dothiepin concentration, viz., dothiepin concentration (ng/ml) = (peak height ratio) × 2.095 − 2.225, with a relative standard deviation of 10.1%.

The known metabolites of dothiepin, which are present in blood at concentrations greater than the parent drug [7] and which interfere with the gas—liquid chromatographic assay, do not interfere with the mass fragmentographic assay of dothiepin when present at concentrations of 100 ng/ml.

The limit of detection of the method as reported is 0.5 ng/ml.

The main limitation on the sensitivity of the method described is the contribution at *m/e* 296 from the deuterated internal standard which is responsible for the positive intercept of the least squares plot. This ion at *m/e* 296 does not reflect the isotopic purity of the internal standard. One possible explanation is the exchange of the $-C[^2H]_3$ label with methane reactant gas in the mass

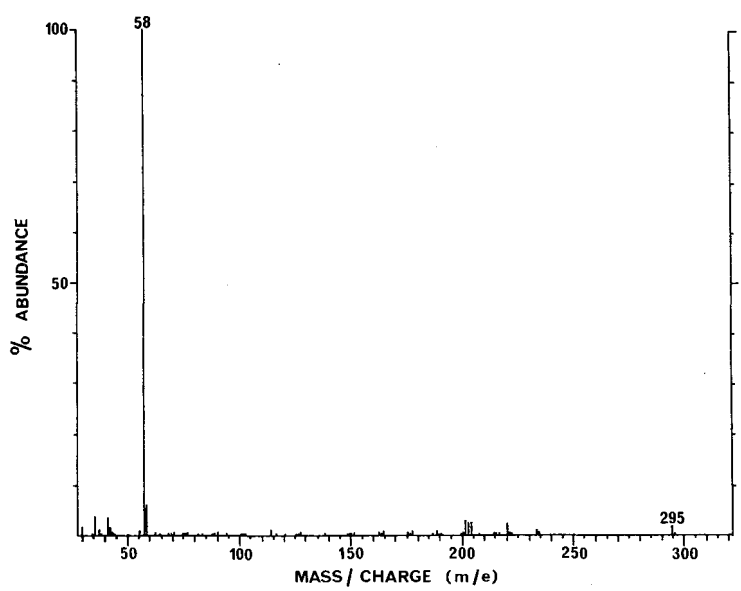


Fig. 2. Electron-impact mass spectrum of dothiepin hydrochloride.

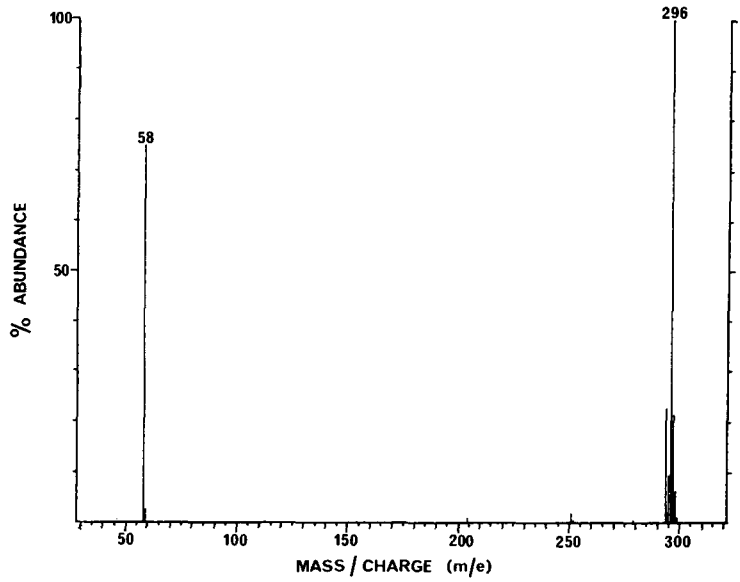


Fig. 3. Chemical ionisation (methane) mass spectrum of dothiepin hydrochloride.

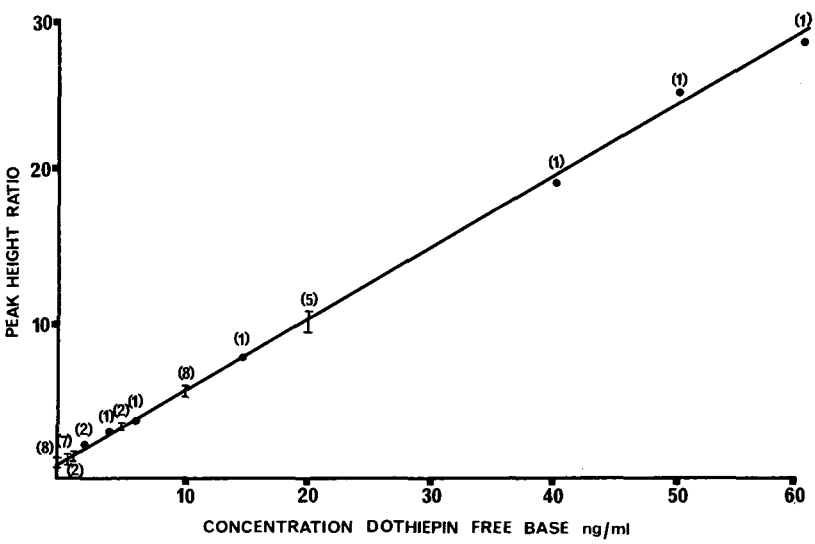


Fig. 4. Calibration curve for dothiepin in plasma. Numbers in parentheses indicate number of measurements. Vertical bars indicate the range of replicate determinations — single or identical values. The full line gives the least squares best fit.

spectrometer source, a phenomenon first reported by Ford et al. [8]. This effect remained constant at a fixed source temperature and was not affected by small changes in source pressure.

The use of isobutane, or preferably ammonia, as reactant gas eliminates the interference at *m/e* 296 and could form the basis of a much more sensitive assay if this was required.

TABLE I

PLASMA CONCENTRATIONS (ng/ml) OF DOTHIEPIN IN VOLUNTEERS AFTER A SINGLE 75-mg ORAL DOSE OF DOTHIEPIN HYDROCHLORIDE

Dothiepin hydrochloride in 3 25-mg Prothiaden capsules. Peak values are underlined. S.E.M. = standard error of the mean; N.D. = not detected (< 0.5 ng/ml) taken for calculation purposes as zero.

Volunteer	Time (h)															
	0	0.5	1	1.5	2	2.5	3	3.5	4	6	12	24	36	48	72	96
1	N.D.	1.4	103.6	69.1	87.5	81.3	95.1	81.5	76.3	49.5	58.0	36.5	31.3	20.1	13.3	7.7
2	N.D.	N.D.	1.2	5.0	7.1	9.8	25.2	29.6	22.3	20.0	19.1	10.4	5.8	3.3	1.8	1.2
3	0.9	1.5	39.1	92.3	91.1	80.4	78.4	85.7	73.2	49.5	37.8	28.6	13.0	9.3	4.6	2.6
4	N.D.	3.6	69.9	92.4	68.5	70.7	61.9	56.5	48.7	37.0	31.8	16.2	7.2	5.2	1.6	0.7
5	N.D.	N.D.	1.7	16.1	25.6	27.1	38.2	35.4	31.4	18.5	17.3	8.7	7.4	4.2	1.9	1.4
6	N.D.	1.5	33.3	53.9	70.9	42.2	70.6	58.3	54.3	41.3	36.7	26.8	19.0	12.6	7.4	2.5
Mean	0.2	1.3	41.5	54.8	58.5	51.9	61.6	57.8	51.0	36.0	33.5	21.2	14.0	9.1	5.1	2.7
± S.E.M.	0.2	0.5	16.3	15.3	14.0	12.3	10.6	9.4	8.9	5.6	6.1	4.5	4.0	2.6	1.9	1.0

TABLE II

PHARMACOKINETIC PARAMETERS OF SIX VOLUNTEERS AFTER A SINGLE 75-mg ORAL DOSE OF DOTHIEPIN HCl (EQUIVALENT TO 66.7 mg FREE BASE)

$V_d = \frac{\text{dose}}{\text{body weight}} \times \frac{1}{A+B}$; $t_{1/2} = \frac{\ln 2}{\beta}$; $Cl = \frac{\text{dose}}{\text{A.U.C.}_{\infty}}$; $\text{A.U.C.}_{\infty} = \frac{A}{\alpha} + \frac{B}{\beta}$; $\text{A.U.C.}_{96\text{ h}}$ measured using trapezoidal rule.

Volunteer	Body weight (kg)	Apparent volume of distribution of central compartment (l/kg) V_d	Terminal elimination half-life $t_{1/2}$ (h)	Clearance (l/h) Cl	Area under curve to ∞ (calculated) (h·ng/ml) A.U.C._{∞}	Area under curve to 96 h (measured) (h·ng/ml) $\text{A.U.C.}_{96\text{ h}}$	Fraction of dose not metabolised at first-pass
1	76	2.9	29.6	21.8	3065	2662	0.81
2	73.5	24.5	23.1	90.4	738	630	0.50
3	73	6.4	21.3	36.2	1842	1672	0.71
4	65	11.8	23.1	61.7	1082	1128	0.59
5	69.3	12.2	23.5	87.4	764	672	0.51
6	69.3	9.2	23.1	35.8	1865	1713	0.72
Mean	71.0	11.2	23.9	55.6	1560	1413	0.64
± S.E.M.	1.6	3.0	1.2	11.8	363	314	0.05

Pharmacokinetics

No serious adverse effects were observed in any of the volunteers after administration of 75 mg dothiepin hydrochloride. Side effects were limited to slight drowsiness in all subjects and a dry mouth in one individual. Pulse and blood pressure recordings taken throughout the study did not change to any clinically significant extent.

The plasma concentrations in six healthy male volunteers who had each received 75 mg of dothiepin hydrochloride are given in Table I and mean results shown graphically in Fig. 5. The results show that there was a large intersubject variation in peak plasma concentrations of dothiepin which occurred from 1 to 3.5 h after the dose. In four of the six subjects secondary peaks occurred up to 4 h after administration of the dose. We have also observed this effect, which is attributed to biliary recycling, in baboons. One consequence of this observation was that absorption parameters could not be accurately calculated.

Non-linear regression analysis of the elimination phase showed this to be biphasic, and the data could be fitted to a bi-exponential equation $C_t = Ae^{-\alpha t} + Be^{-\beta t}$ where C_t (ng/ml) is the concentration at time t (h) and $A = 210$, $\alpha = 0.71$, $B = 42$ and $\beta = 0.029$ for the composite curve. The closeness of fit between experimental data and the computer generated curve ($r = 0.999$) strongly supports the applicability of a two-compartment model to describe the elimination pharmacokinetics of dothiepin in man. The composite apparent distribution and elimination half-lives were calculated as 2.9 and 23.7 h respectively.

Using equations derived for a two-compartment open model [9] individual data were used to predict the predose steady state levels of dothiepin which would be expected during a 50-mg three times a day dose regimen. The pre-

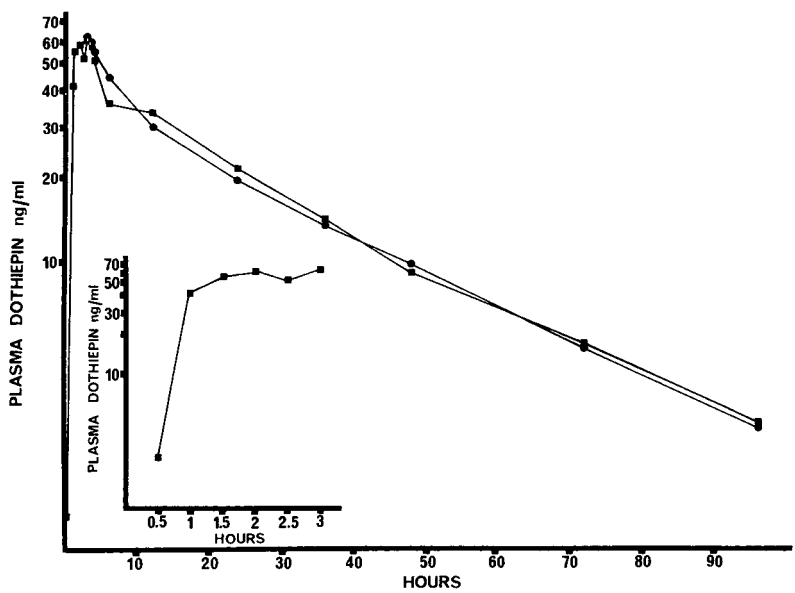


Fig. 5. Mean plasma concentrations of dothiepin after a single oral dose of 75 mg dothiepin hydrochloride.

dicted value of 107 ± 28 (S.E.M.) ng/ml is in good agreement with the mean value of 106 ± 12 (S.E.M.) ng/ml found in 16 patients receiving dothiepin hydrochloride 50 mg three times a day [10].

Volumes of distribution, areas under plasma concentration versus time curves (0—∞) and clearances were calculated from the bi-exponential constants of the individual profiles (Table II). There were large intersubject variations in these parameters consistent with a drug that is extensively metabolised [11]. However, there was very little intersubject variation in elimination half-lives of unchanged dothiepin in contrast to reports published for other tricyclic antidepressants [12—14].

The variations in steady state concentrations of tricyclic antidepressants have been attributed to variations in half-life of elimination and theoretical volume of distribution [15, 16]. If the elimination kinetics of dothiepin in patients are similar to those in normal subjects, then the range of steady state concentrations achieved clinically (20—420 ng/ml) is more likely to be a consequence of variations in apparent volume of distribution. Since there is a linear correlation (r = 0.84) between apparent volume of distribution and the calculated [17] fraction of drug metabolised at first-pass, it is suggested that steady state levels of dothiepin are controlled primarily by the extent of first-pass metabolism.

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TECHNICAL CONSIDERATIONS IN THE GAS CHROMATOGRAPHIC ANALYSIS OF DESIPRAMINE

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SUMMARY

Procedures are presented for minimizing variability in and interferences with the gas chromatographic determination of desipramine in plasma. Careful consideration of procedures for sample collection and storage, drug separation from matrix components, and chromatography appears to be a prerequisite for avoiding inaccurate and imprecise determinations of this antidepressant, especially at levels below 20 μg/l. Numerous pitfalls are examined and optimal conditions for obviating them are presented.

INTRODUCTION

The determination of tricyclic antidepressant levels in biological specimens has received much attention in recent years. These levels have been utilized in studies of product bioavailability, patient compliance, toxic overdose, and in relating dose or plasma level to clinical response. The validity of conclusions drawn from such studies depends heavily upon the accuracy of the drug level determinations utilized.

Numerous assay techniques for the tricyclics have been described [1—6]. Several popular procedures utilize gas—liquid chromatography with nitrogen-specific detection [7—12]. This technique is relatively inexpensive, offers adequate sensitivity in concentration ranges encountered during clinical use of the tricyclics, and is adequate for many single-dose pharmacokinetic studies where plasma levels as low as a few micrograms per liter may be encountered.

Attempts to implement published nitrogen-detector gas chromatographic assay procedures for the tricyclic antidepressant desipramine in our laboratory have met with a variety of difficulties. Drug adsorption onto glassware and column materials has led to decreased sensitivity, non-linear standard curves and unacceptable reproducibility. As a result of the nitrogen detector's high

sensitivity to certain nitrogenous compounds, extraneous peaks from biological and chemical contaminants have complicated analyses at low drug concentrations. Our objective in undertaking the present study was to identify and isolate sources of variability and interference and develop a gas chromatographic method for accurately and reproducibly determining desipramine at levels approaching 1 μg/l in as small a plasma sample as possible.

This report systematically presents observations on the various aspects of a viable chromatographic assay for the tricyclics. It considers each step in the analysis in turn and provides negative as well as positive findings in an attempt to assist readers who may encounter one or more technical difficulties in the course of implementing and/or routinely performing such assays. It concludes with a presentation of those conditions found to be most satisfactory and their application to drug level determinations in human volunteers.

METHODS AND RESULTS

Reagents and chemicals

Methanol, hexane and *n*-butanol were purchased distilled in glass (Burdick & Jackson Labs., Muskegon, MI, U.S.A.) and were used without further purification. Isoamyl alcohol (Mallinckrodt, St. Louis, MO, U.S.A.) was redistilled in glass (b.p. 128.5° C). Water was double-distilled in glass and washed with hexane before use in extractions. Desipramine·HCl and amitriptyline·HCl were gifts of USV Pharmaceutical Corp., Tuckahoe, NY, U.S.A. and Merck, Sharp and Dohme, West Point, PA, U.S.A., respectively. [^{3}H]Desipramine (38.68 Ci/mmole) was a 7.7 μg/l solution in ethanol (New England Nuclear, Boston, MA, U.S.A.). All other reagents were analytical reagent grade and were used without further purification.

General approach

A number of techniques are available for isolating tricyclics from plasma*. The basic approach consists of extraction with an organic solvent at an alkaline pH, back-extraction into acid to remove interfering biological components and realkalization. The drug is then prepared for injection by either extracting into a small volume of organic solvent or extracting with a larger volume followed by evaporation and subsequent reconstitution with organic solvent. Typically a tricyclic of minimal therapeutic relevance to the study at hand is used as an internal standard.

Sample collection and storage

Adsorptive losses of desipramine must be considered whenever the drug is placed in contact with glass surfaces, especially in the low μg/l concentration range. These losses are most frequently overlooked, not in the assay procedure itself, but rather in the collection and storage of patient samples and during the preparation of serum or plasma standards.

Vacutainer® tubes must be avoided because of their well-documented

*Serum and heparinized plasma behaved identically in our hands and will be used synonymously throughout this report. EDTA and oxalate produced interfering peaks and were not suitable as anticoagulants.

plasticizer interferences [13]. The plasticizer, tris(2-butoxyethyl)phosphate, interferes with the binding of the tricyclics to α_1-acid glycoprotein, thereby effecting a redistribution of the drug into erythrocytes. The apparent plasma level of the drug is thus altered and the plasticizer must, therefore, be avoided even when it produces no chromatographic interference per se. Our samples were drawn into plastic syringes. The needle was then removed and the syringe emptied into a silanized and heparinized glass tube. The separated plasma was immediately frozen at −20° C in a silanized glass tube and not permitted to thaw until assay. Once thawed, we found significant drug losses at concentrations of less than 20 μg/l in as little as 3 h at 25° C.

Preparation of standards

In the course of establishing recoveries of drug from plasma at low concentrations, discrepancies were noted which led us to evaluate our procedure for the addition of known quantities of drug. Recovery and reproducibility appeared to depend upon the solvent utilized to prepare drug stock solutions. Serum standards were therefore prepared using desipramine stock solutions in methanol, water and serum. Of these, water was judged most acceptable. Inaccurate pipetting of small volumes of serum resulted in poor reproducibility. When methanol was used as the "spiking" solution vehicle, reproducibility was also diminished. This may have been due to a localized protein precipitation observed upon addition of the methanolic solution, an effect not observed with the other vehicles.

To determine whether stock solution solvent affects adsorptive losses in the standards, thereby decreasing recoveries, a solution of [^{3}H]desipramine was prepared in each of the three aforementioned solvents. These stock solutions were then used to prepare serum samples at a concentration of 20 μg/l. Twenty-five microliters of stock solution were added, with constant stirring, to 2.5 ml of serum in a narrow, cylindrical silanized glass tube. The geometry was such that sampling could be achieved while maintaining an essentially constant surface area/volume ratio. Samples were withdrawn for analysis at 5 min intervals over a period of about 60 min. All specimens appeared stable over this relatively brief time period and adsorption was ruled out as the source of variable recoveries.

As a result of these findings, all subsequent studies were performed using a 1 g/l primary stock solution of desipramine hydrochloride in water. This solution is stable for at least three months at 4° C. At the time of assay a 2 mg/l secondary stock solution is prepared from which plasma standards are then derived.

Internal standard

Amitriptyline was chosen as the internal standard for our assay, but other tertiary tricyclics, such as imipramine, were also suitable. Protriptyline was evaluated because of its use by other authors, but it produced unacceptable peak shapes under our chromatographic conditions. An aqueous solution (600 μg/l) of internal standard was added to the serum sample prior to the first extraction step. More reproducible results were obtained by this method than by the addition of internal standard to the first extraction solvent.

Sample size

In selecting a sample for extraction, our objective was to minimize the sample size while ensuring adequate volume for repeated measurements. Sample sizes of 0.5—3.0 ml were evaluated. The smaller volumes limited sensitivity while the larger sample sizes yielded disproportionately large contaminant peaks on the chromatogram. Samples of 1 ml seemed to provide sufficient sampling accuracy and adequate sensitivity with minimal interferences.

Sample cleanup

Because serum and plasma contain numerous interfering nitrogenous components, the original plasma was washed with hexane as were subsequent aqueous phases of the sample workup. While this failed to remove all extraneous peaks, it did eliminate all interferences at the retention times of interest.

Alkalinization

Since desipramine is a weak base ($pK_a = 10.2$), at higher pH the drug is more extractable and recoveries are higher. Specifically, recovery from plasma increased from 35% at pH 9.0 to 65% at pH 11.5. Unfortunately, more alkaline conditions increased the extraction of interfering serum constituents as well. Alkalinization with ammonium hydroxide to a sample pH of 11.5 provided adequate recovery with minimal contamination.

Extraction

Various extraction solvents were evaluated using a variety of phase volume ratios and extraction times. Hexane containing a small percentage of isoamyl alcohol was found most efficient. Systematically increasing the alcohol content from 2 to 10% produced only slight improvement in recovery. As the alcohol content increased, gel formation at the interface began to interfere with phase separation. Interfering peaks also became more pronounced. Identical recoveries were subsequently obtained when isoamyl alcohol was replaced by *n*-butanol. Glass-distilled *n*-butanol could be used as purchased whereas redistillation of commercial isoamyl alcohol was deemed essential. A hexane—*n*-butanol (98:2) extraction solvent was, therefore, selected.

Extraction efficiency plateaued at a phase volume ratio of 1:1, with higher volumes of extractant yielding little improvement. Equilibration times were a function of the agitation imposed on the system. Samples vortexed at a high speed equilibrated within 3 min, while samples placed in a mechanical shaker (Eberbach Corp., Ann Arbor, MI, U.S.A.) at 200 oscillations per min required at least 15 min.

Back-extraction

Insignificant differences were noted among phosphoric, hydrochloric and sulfuric acids when each was utilized for the back-extraction of tricyclic into acid, and 0.1 N H_2SO_4 was selected.

Concentration of final extract

Two methods were considered for achieving a final concentrated tricyclic

extract suitable for injection. Extraction into volumes of hexane–butanol ranging from 50 to 200 μl were evaluated. Volumes of less than 100 μl resulted in poor extraction efficiency because of unfavorable phase volume ratios. Above 100 μl sensitivity was limited by our 10 μl injection volume which dictated that less than 10% of extracted drug could be placed on the column using this method. Alternatively, the final extraction was performed with a more favorable phase ratio of about 1:1 followed by subsequent evaporation of the extractant and reconstitution into a small volume of methanol prior to injection. This procedure worked well as long as precautions were taken to avoid several potential sources of adsorptive loss during the evaporation step.

Adsorptive losses during evaporation

Large decreases in recovery were observed when samples were evaporated from glass surfaces (Reacti-Vials, Pierce, Rockford, IL, U.S.A.). The observation of a proportionality between evaporative surface area and drug loss is consistent with adsorption onto the glass surface. Neither the use of polypropylene or polystyrene containers nor the addition of *n*-butanol, isoamyl alcohol or glacial acetic acid to the evaporating solution alleviated the problem. Silanization (Siliclad, Clay Adams, Parsippany, NJ, U.S.A.) reduced adsorption, and silanized glassware was utilized for collection, storage, and workup of samples. Such glassware was not useful in the evaporative step, however, because a contaminant peak with a retention time identical to that of desipramine appeared on the chromatogram. Instead, the glass vials were cleaned by washing in a non-phosphate detergent (Labtone, VWR Scientific, Boston, MA, U.S.A.), soaking consecutively in chromic acid and 10% NaOH and then sonicating. To deactivate adsorption sites, vials were then soaked in 20% triethylamine in methanol and rinsed consecutively with methanol and hexane—*n*-butanol (98:2) to remove excess amine. Using this procedure, little adsorptive loss occurred during the evaporation step and assay sensitivity and reproducibility were ensured.

Chromatographic considerations

A Varian Model 3740 gas chromatograph equipped with nitrogen/phosphorus detector (Varian, Palo Alto, CA, U.S.A) was used. The carrier gas flow-rate and oven temperature (see below) were adjusted to provide maximum resolution of serum components from the peaks of interest. Oven temperature was elevated after the peaks of interest eluted to clean the column between injections. The detector and injector temperatures were maintained as high as drug stability would permit to prevent accumulation of sample components at these locations. The silanized glass-wool plug at the head of the column was replaced every 30 injections, a procedure found essential if reproducible peak shapes and retention times were to be achieved.

Initially, separations were performed using 3% OV-17 on Gas-Chrom Q, 100–200 mesh (Applied Science Labs., State College, PA, U.S.A.). Peak tailing and variable peak shape, probably due to adsorption, rendered this packing unacceptable. "Doping" the column with a concentrated drug solution prior to the analysis did reduce drug adsorption, but it was effective only for short and quite variable periods of time. A commercially available clinical packing, 3%

SP-2250 on 80—100 Supelcoport (Supelco, Bellefonte, PA, U.S.A.), advertised as specially deactivated toward tricyclics, was found to minimally adsorb desipramine and not to require "doping".

Derivatizations with acetic and trifluoroacetic anhydrides were attempted in an effort to improve response. Although detector response was modestly increased, the increase was not sufficiently large to offset the fact that extraneous peaks also increased in magnitude and number to the point where interference became a problem. Consequently, derivatization was abandoned.

Optimal assay procedure

After due consideration of the aforementioned, the following assay procedure was adopted. One milliliter of plasma or serum is pipetted into a 10-ml silanized glass tube fitted with an extraction tube plug (Oxford Laboratories, Foster City, CA, U.S.A.). Twenty microliters of an aqueous internal standard solution, containing 12 ng of amitriptyline·HCl, are added. The sample is acidified with 200 μl of 1 *N* HCl, washed for 1 min with 1 ml of hexane, centrifuged (1686 *g*) for 5 min, and the upper organic phase discarded. The aqueous phase is then alkalinized with 300 μl of concentrated ammonium hydroxide and shaken mechanically for 15 min with 1 ml of hexane—*n*-butanol (98:2). The upper phase is transferred to a clean 10-ml silanized glass tube and 1 ml of 0.1 *N* H_2SO_4 added. The tube is shaken mechanically for 15 min, centrifuged, and the upper organic phase discarded. The acidic aqueous phase is washed with 2 ml of hexane (5 min agitation), transferred to a 10-ml silanized glass tube, alkalinized with 100 μl of ammonium hydroxide, and extracted for 15 min with 1.5 ml of hexane—*n*-butanol (98:2). The organic phase is transferred to a clean, deactivated Reacti-Vial® and evaporated under a stream of nitrogen at 25° C. It should be noted that evaporated samples stored overnight at either 25°C or −20°C display unacceptable variability. If the assay is to be interrupted, samples should be stored just prior to the evaporation step. The residue is reconstituted with 20 μl of methanol and 6 μl are chromatographed under the following conditions: column temperature 243°C, injector 300°C, detector 300°C, carrier gas (ultra high purity nitrogen) 17 ml/min, air 175 ml/min, and hydrogen 4.5 ml/min. Rubidium bead current is set according to manufacturer's recommendations. Plasma concentrations are determined by comparison of peak height ratios (desipramine/amitriptyline) to comparable ratios for extracted standards.

Standard curves

Desipramine serum standards ranging from 5 to 200 μg/l were prepared using the aqueous stock solutions discussed above. Peak height ratios and concentrations were linearly related over this concentration range. The slopes and intercepts of five standard curves obtained over a period of about two months were compared in an analysis of variance [14] in order to assess their variation with time. The data (Table I) indicate that neither the intercepts nor the slopes of the standard curves varied with time ($p > 0.05$). We interpret this to mean that changes in column performance were not significant over the time span considered and matrix components present in the various lots of serum used to prepare the standards did not introduce significant variability.

TABLE I

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

F statistic (slope) (4,14) = 0.176 N.S. ($p > 0.05$); F statistic (intercept) (4,14) = 0.014 N.S. ($p > 0.05$).

Curve No.	r^2	Slope	Variance of slope	Intercept	Variance of intercept
1	0.9947	0.03267	2.18×10^{-6}	−0.1128	0.00716
2	0.9961	0.03322	4.43×10^{-6}	−0.0764	0.01840
3	0.9861	0.03382	8.16×10^{-6}	−0.0597	0.02600
4	0.9924	0.03398	8.73×10^{-6}	−0.0833	0.02800
5	0.9852	0.03106	1.46×10^{-5}	−0.0879	0.04700

Reproducibility

Six replicate analyses of the same 5 μg/l serum sample on a single day yielded a coefficient of variation of 5.3%. Six measurements of the same 50 μg/l sample over a period of one month yielded a between-day coefficient of variation of 6%. Analytical recovery from plasma or serum was 65% as determined by comparison of peak heights from extracted samples with those from an equivalent amount of drug in methanolic solution injected directly. This was in contrast to an 80% recovery from water. Extraction efficiency was also diminished by prolonged storage (−20° C, 3 months or more) of either the serum used to prepare standards or the patient samples themselves. Other than speculating that plasma protein binding may somehow account for the discrepancies, the reasons for these observations remain obscure.

APPLICATION TO PATIENT SPECIMENS

In order to evaluate the assay procedure on actual plasma samples, a single

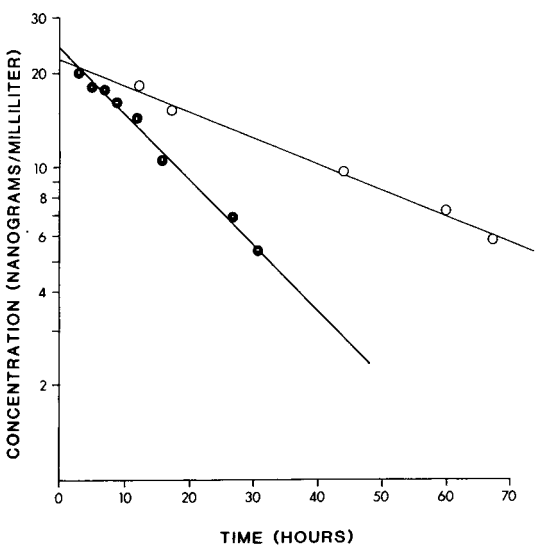

Fig. 1. Log plasma concentration versus time curves for desipramine following single 75-mg oral doses to young (•) and elderly (○) volunteers.

75-mg oral dose of desipramine·HCl (Norpramin, Merrell-National, Cincinnati, OH, U.S.A.) was administered to both a young (29 years) normal volunteer and an elderly (67 years) depressed patient. Plasma levels of desipramine over time are shown in Fig. 1. Postabsorptive levels declined monoexponentially with apparent half-lives of 14.7 and 36.5 h in the young and elderly subjects, respectively. The assay is currently being utilized to perform pharmacokinetic studies on depressed patients following both single and multiple oral doses. Our plasma level versus time curves have remained log-linear down to desipramine concentrations of 1 μg/l, confirming our estimate of that level as our lower level of quantitative sensitivity.

DISCUSSION

Many studies have been conducted in an attempt to relate clinical response to tricyclic therapy to plasma levels. In addition, several pharmacokinetic studies have been undertaken following both single and multiple oral doses [15, 16] and others are certain to follow. It has recently become clear that sample collection techniques may have influenced the results of a number of these reports. For example, the use of Vacutainers® causes the inadvertent introduction of tris(2-butoxyethyl)phosphate, a plasticizer present in the stoppers, which displaces basic drugs from binding sites of α_1-acid glyco protein, thus enhancing diffusion of drug into erythrocytes and reducing plasma levels. The present report illuminates several other areas where inaccuracies may have gone unnoticed in previous studies. Consideration must be given to careful removal of contaminants and adsorptive losses during sample storage, workup and chromatography. The significance of these contributing factors will increase dramatically as one approaches the "nanogram barrier" during low-dose pharmacokinetic studies of these heavily tissue-distributed drugs. Hopefully, the results reported herein will assist analysts in avoiding the numerous pitfalls that confront them during the analysis of the tricyclic antidepressants, especially at low concentration.

ACKNOWLEDGEMENTS

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CHROMBIO. 591

GAS CHROMATOGRAPHIC METHOD FOR THE SIMULTANEOUS DETERMINATION OF HYDRALAZINE AND ITS ACETYLATED METABOLITE IN SERUM USING A NITROGEN-SELECTIVE DETECTOR

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SUMMARY

A relatively simple gas chromatographic method has been developed for the quantitative determination of hydralazine simultaneously with its acetylated metabolite, 3-methyl-*s*-triazolo[3,4-α]phthalazine (MTP). The proteins were removed by means of sulfosalicylic acid and Sure-Sep®. On treatment with formic acid, hydralazine and its internal standard were converted into their formylated derivatives. These derivatives, MTP and its internal standard were extracted with toluene and determined by gas chromatography with a nitrogen-selective detector. The lower limits of detection for hydralazine and MTP were 0.13 and 0.27 μmol/l, respectively.

INTRODUCTION

N-Acetylation seems to be one of the major metabolic pathways for hydralazine in man [1]. Measurement of both hydralazine and the acetylated metabolite, 3-methyl-*s*-triazolo[3,4-α]phthalazine (MTP) is therefore of interest.

Since hydralazine is not extractable from biological material with organic solvents a method based on derivatization directly in the biological milieu is needed. Many papers have been published on the measurement of hydralazine, but only a recently published gas chromatographic (GC) method has sufficient specificity and sensitivity. In this GC method hydralazine is converted into tetrazolo[1,5-α]phthalazine and detected by an electron-

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capture detector [2]. A slight modification of this procedure allows simultaneous determination of MTP [3].

We present a GC method using a nitrogen-selective detector for the simultaneous determination of hydralazine and MTP. Hydralazine is analysed as its formylated derivative, *s*-triazolo[3,4-α]phthalazine (TP) (see Fig.1), and detected by a nitrogen-selective detector. The described method is relatively simple and therefore well suited for the routine determination of hydralazine in serum from patients under treatment with this drug.

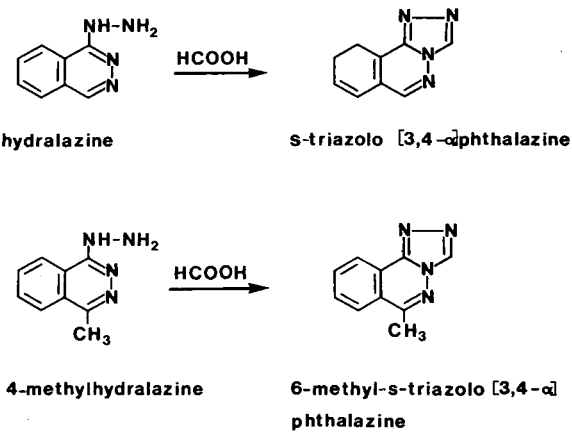
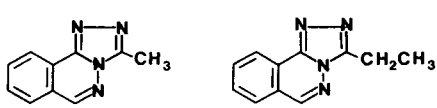


Fig.1. Transformation of hydralazine and 4-methylhydralazine (internal standard) to their formyl derivatives.

EXPERIMENTAL

Materials

Hydralazine hydrochloride, 4-methylhydralazine hydrochloride (internal standard) and 3-methyl-*s*-triazolo[3,4-α]phthalazine (MTP, Fig.2) were a gift from Ciba-Geigy (Basle, Switserland). 3-Ethyl-*s*-triazolo[3,4-α]phthalazine [ETP (Fig.2), internal standard] was synthesized as described for the analogous methyl derivative by Haegele et al. [1].



MTP ETP

Fig.2. Structural formulae of the acetylated metabolite of hydralazine (MTP) and its internal standard (ETP).

Serum standards were prepared every day of analysis and 10% ascorbic acid solution added as described below for the serum samples. Stock solutions of hydralazine hydrochloride (0.5 mmol/l of 0.1 *N* HCl) and 4-methylhydralazine hydrochloride (1.7 mmol/l of 0.1 *N* HCl) were kept at 2–8°C for two and four weeks, respectively. Stock solutions of MTP and ETP (1.3 mmol/l of ethanol) were kept at −20°C for several months. All reagents were of analytical quality. Sure-Sep® (plasma/serum separators) was purchased from General Diagnostics (Morris Plains, NJ, U.S.A.).

Serum samples

Within 15 min of blood collection the serum was separated, 5 μl of 10% ascorbic acid solution per ml of serum were added and the samples stored at −20°C.

Procedure

To 1.0 ml serum were added 100 μl of 4-methylhydralazine solution (16.6 μmol/l), 125 μl of ETP solution (12.6 μmol/l) and 100 μl of 50% sulfosalicylic acid solution. The reagents were whirlimixed for 5 sec and centrifuged (5 min at 1300 *g*). After adding Sure-Sep® the centrifugation was repeated. The supernatant was decanted, 100 μl of formic acid were added, then the test-tube was sealed and placed at 100°C for 20 min. After cooling at room temperature (about 3 min), 400 μl of 5 *M* NaOH, 1.5 ml of 1 *M* carbonate buffer (pH 10.5) and 3 ml of toluene were added. The mixture was shaken for 5 min, centrifuged for 5 min (1300 *g*) and the toluene phase transferred to a new test-tube. The extraction procedure was repeated and the combined toluene extracts were evaporated under nitrogen at 50°C. The evaporated samples were stored at −20°C until the GC analysis could be carried out.

The dry residue was dissolved in 30 μl of toluene and 2 μl were analysed by GC. The serum concentrations of hydralazine and MTP were calculated on the basis of the peak-height ratios of hydralazine/4-methylhydralazine and MTP/ETP by reference to the graphs obtained by analysing serum standards simultaneously.

Gas chromatography

A Model 5830A gas chromatograph equipped with a nitrogen-selective detector (Hewlett Packard, Palo Alto, CA, U.S.A.) was used with the following operating conditions: a 2.0 × 2 mm I.D. glass column was packed with 1% SP 1000 on Chromosorb W 80—100 mesh; the carrier gas (helium) flow-rate was 30 ml/min, the air flow-rate 50 ml/min and the hydrogen flow-rate 3 ml/min; injector temperature was 220°C, detector temperature 300°C. The oven was programmed from 220 to 250°C at 16°C/min. The voltage of the rubidium bead was set at 16—20 V.

RESULTS

Evaluation of the analytical procedure

Fig. 3 shows chromatograms obtained from serum analysis. Plots of the standard curve of hydralazine over the range 1—15 μmol/l were linear and

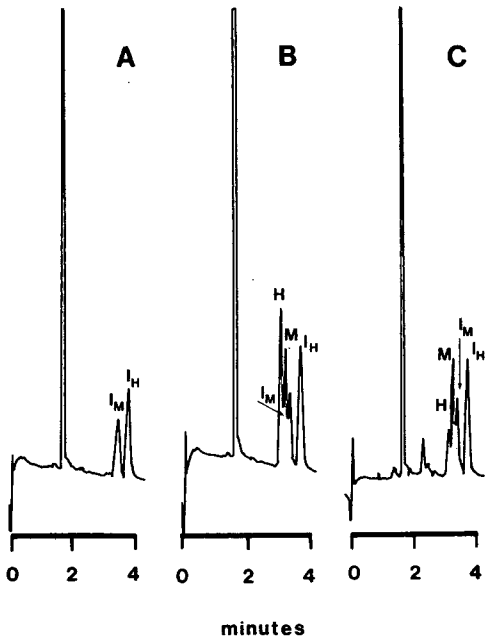


Fig. 3. Chromatograms of human serum extracts, analysed as described in the text. A, Serum; B, serum to which hydralazine and MTP were added (2.5 and 1.2 μmol/l, respectively); C, serum from a patient, therapeutically treated with hydralazine. H = Hydralazine, M = MTP, I_M = internal standard for MTP, I_H = internal standard for hydralazine.

passed through the origin. The standard curve of MTP was linear over the same range but did not pass through the origin. The blank was from 0.05 to 0.15 μmol/l.

The lower limits of detection for hydralazine and MTP were 0.13 and 0.27

TABLE I

REPRODUCIBILITY OF REPLICATE ANALYSES OF HYDRALAZINE AND MTP ADDED TO HUMAN SERUM

The serum samples were stored at −20°C from one day to seven weeks and analysed at random on different days.

Compound	Serum concentration* (μmol/l)	Coefficient of variation (%)
Hydralazine	9.92	4.9
	3.30	3.4
	0.50	7.7
MTP	14.64	6.4
	4.86	4.8
	0.88	9.4

*Mean values from eight duplicate samples of each concentration.

TABLE II

STABILITY OF HYDRALAZINE AND MTP IN SERUM SAMPLES AFTER STORAGE AT −20°C

	Hydralazine (μmol/l)				MTP (μmol/l)			
	1st determination	2nd determination	Time between 1st and 2nd determination	Deviation (%)	1st determination	2nd determination	Time between 1st and 2nd determination	Deviation (%)
Patient samples	1.17	1.11	6 weeks	−5.1	< 0.27	< 0.27	6 weeks	—
Patient samples	1.32	1.34	6 weeks	+1.5	0.31	0.34	6 weeks	+9.7
Patient samples	3.28	3.46	6 weeks	+3.1	0.53	0.54	6 weeks	+1.9
Spiked samples	2.38	2.60	6 months	+9.2	1.24	1.25	6 months	+0.8
Spiked samples	4.87	4.98	7 months	+2.2	2.53	2.47	7 months	−2.4

μmol/l, respectively (25 and 50 μg/l). The reproducibility was determined using spiked serum samples analysed at random on different days. These spiked serum samples were stored at −20°C from one day to seven weeks. The reasonable reproducibility shown in Table I demonstrates that hydralazine and MTP are stable for weeks under the conditions mentioned above. The stability of hydralazine at −20°C is confirmed by the results in Table II, where both patient serum samples and spiked samples were determined twice with a time interval of from six weeks to seven months.

Table III compares results from analyses of patient serum samples by our GC method and another GC method [4].

Application of the analytical procedure

Venous blood samples from 32 patients, therapeutically treated with 50—200 mg (0.25—1.0 mmol) of hydralazine per day, were collected just before and 1 h after the first morning dose.

The ranges of the serum levels of hydralazine were found to be < 0.13—7.6 and 0.50—12.7 μmol/l, respectively (< 25—1500 and 100—2500 μg/l).

The MTP levels were < 27 and < 0.27—8.2 μmol/l, respectively (< 50 and < 50—1500 ng/ml).

TABLE III

COMPARISON OF RESULTS OF ANALYSIS OF PATIENT SERUM SAMPLES BY OUR GC METHOD AND ANOTHER GC METHOD [4]

Sample No.	Our results	Results from another GC method [4]
	Hydralazine concentration (μmol/l)	"Apparent" hydralazine concentration (μmol/l)
1	4.6	5.3
2	5.5	3.9
3	1.9	2.9
4	4.8	3.2
5	13.1	9.2
6	0.6	0.2

DISCUSSION

Several authors have mentioned the instability of hydralazine in human serum during storage [2,5]. In consequence, Jack et al. [2] recommend derivatization before cold storage. We found that, after the addition of ascorbic acid, serum samples were stable at −20°C for at least seven months (Table II). Furthermore, we have discovered that the ascorbic acid prevents cleavage of hydralazine in the analytical procedure. The use of sulfosalicylic acid as denaturating agent and separation of the denaturated proteins by means of Sure-Sep® have been shown to remove the proteins so completely, that no interfering peaks appeared in the chromatogram.

We have tested many derivatives of hydralazine such as tetrazolo[1,5-α]-phthalazine [2] and formaldehyde, acetaldehyde and acetone hydrazones of hydrazine, but we found neither as sensitive at the nitrogen-sensitive detector as the formyl derivative of hydralazine (TP).

The yield of the derivatization was found to be about 50% by comparison with TP, prepared on an analytical scale as described by Haegele et al. [6]. Different reaction conditions have been studied to optimize the derivatization of hydralazine. An elevated temperature was necessary; 100°C was chosen to complete the reaction within 10—20 min. Neither a longer reaction time (60 min) nor more formic acid (500 μl) improved the yield. Both TP and MTP appeared to be rather stable. No loss of TP or MTP was observed in evaporated samples, neither after standing at 50°C for 15 min nor after storage for one week at −20°C. The yield of one extraction with toluene was only about 50%. Consequently, two extractions raised the yield to about 75%.

SP 1000 turned out to be the best stationary phase since phases as OV-17 and SE-30 resulted in extreme tailing. Although the peaks are rather close (Fig. 3), the separation has proved satisfactory to obtain a reasonable precision (Table I). A column 3 m long improved the separation, but not the precision.

Earlier published GC methods [2,3] used an electron-capture detector, but recently a method using a nitrogen-selective detector has been published [7]. It is our opinion that a nitrogen-sensitive detector is more suited for routine analysis. In our laboratory nitrogen-sensitive detectors have been used without problems by different technicians during the past four years.

TP has been identified as a metabolite in rat urine [1] and recently in human urine, too [6]. But like Haegele et al. [6] we did not find any detectable amount in the serum from patients therapeutically treated with hydralazine.

Some authors have postulated the existence of some acid-hydrolysable conjugates of hydralazine [4,7,8]. These findings are confirmed by Haegele et al. [6], who identified acetone, pyruvate, and α-ketoglutarate hydrazones. In vitro activity studies, however, have shown that these hydrazones are at least as active as parent hydralazine [6,9]. During the last few years it has been shown [4,7,8] that earlier published methods [2,5] involving acidic treatment of the samples do not distinguish between hydralazine and its acid-hydrolysable conjugates, but recently Degen [7] has described a GC method for the specific determination of unchanged hydralazine.

In view of the acid conditions in our derivation procedure (pH < 1) these conjugates are most likely co-determined in our analysis. A comparison of our method with another GC method (Table III) indicates that this actually is the case, because our results are in reasonable agreement with the "apparent" hydralazine concentrations (hydralazine with co-determination of the acid-hydrolysable conjugates).

Great individual variations in serum concentrations of hydralazine and MTP were found in patients therapeutically treated with almost the same dose of hydralazine. It has already been shown that the different extent of acetylation of hydralazine in man [3,10] is an important determinant of the

plasma level of hydralazine, but many other factors may equally be the causes of the great variations in bioavailability of hydralazine.

Earlier investigators [10] have shown that the hypotensive effect of hydralazine is proportional to its plasma concentration. Our preliminary results from blood pressure measurements and serum concentration determinations seem to show correlation, too [1]. This indicates that the described method may be a useful guide to a more rational and safe dosage of hydralazine.

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CHROMBIO. 601

DETERMINATION OF HYDRALAZINE METABOLITES: 4-HYDRAZINOPHTHALAZIN-1-ONE AND N-ACETYLHYDRAZINOPHTHALAZIN-1-ONE BY GAS CHROMATOGRAPHY AND *s*-TRIAZOLO[3,4-*a*]PHTHALAZINE AND PHTHALAZINONE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Methods are described for the determination of 4-N-acetylhydrazinophthalazin-1-one, 4-hydrazinophthalazin-1-one, phthalazinone and *s*-triazolo[3,4-*a*]phthalazine in human urine.

4-Hydrazinophthalazin-1-one and 4-N-acetylhydrazinophthalazin-1-one (following acid hydrolysis) are reacted with acetylacetone to give a distinctive pyrazole derivative which can be determined by gas chromatography using a nitrogen-specific detector.

Phthalazinone and *s*-triazolo[3,4-*a*]phthalazine are measured underivatised by high-performance liquid chromatography.

INTRODUCTION

Hydralazine (1-hydrazinophthalazine; H) is a potent vasodilating drug, which in combination with a suitable β-blocking drug represents a most effective and widely used antihypertensive regime [1]. However, associated with the use of hydralazine is a lupus erythematosus-like syndrome, an adverse effect almost exclusively confined to the slow acetylator phenotype as determined by the acetylation of sulphamethazine [2].

The metabolism of hydralazine is complex and is known to involve more than one acetylation pathway (Fig. 1) [3]. It has recently been shown that one of these acetylation pathways, leading to 3-hydroxymethyltriazolophthalazine (HOMTP) is under the same genetic control as the acetylation of sulphamethazine [4]. However, this metabolic route is not a major pathway, only accounting for 20% of the dose in man. It was therefore important to investi-

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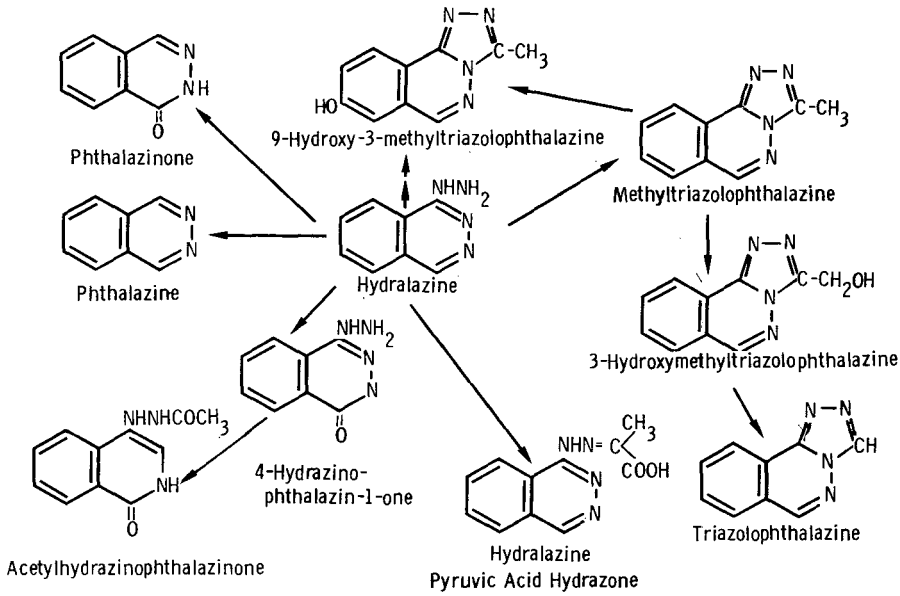

Fig. 1. The metabolism of hydralazine in man.

gate the alternative acetylation pathway, leading to 4-N-acetylhydrazinophthalazin-1-one (NAcHPZ), to define further the effect of the acetylator phenotype on hydralazine metabolism.

It was therefore necessary to devise a method for the measurement of NAcHPZ, and its precursor 4-hydrazinophthalazin-1-one (HPZ). *s*-Triazolo-[3,4-*a*]phthalazine (TP) is the terminal product of the primary acetylation pathway and its measurement is necessary for complete quantitation of this pathway.

As the lupus erythematosus syndrome is confined almost exclusively to slow acetylators, alternative pathways to acetylation are important. One such pathway is the production of phthalazinone (PZ) and a method for quantitation of this metabolite was therefore necessary.

Measurement of these metabolites may also be used for determination of the acetylator phenotype.

EXPERIMENTAL

Chemicals

Hydralazine hydrochloride was obtained from Koch-Light Labs. (Colnbrook, Great Britain); acetylacetone from BDH (Poole, Great Britain). 4-Methylhydralazine (MeH), 4-N-acetylhydrazinophthalazin-1-one, *s*-triazolo[3,4-*a*]-phthalazine, phthalazinone and 4-hydrazinophthalazin-1-one were generously supplied by Ciba-Geigy (Basle, Switzerland).

General methods

Gas chromatography (GC) was carried out on a Perkin-Elmer F17 instrument

fitted with a nitrogen—phosphorus detector. The glass column (2 m × 1.75 mm I.D.) was packed with 10% OV-17 on Gas-Chrom Q, 100—200 mesh (Applied Science Labs., State College, PA, U.S.A.). The detector/injector temperature was set at 300°C and the nitrogen detector used a setting of 6.0 giving a rubidium bead temperature of approximately 600°C. The oven temperature was maintained at 250°C. Nitrogen (carrier gas) flow-rate was set at 35 ml/min.

Gas chromatography—electron impact mass spectrometry (GC—EIMS) was performed on a Finnigan 320 instrument fitted with a 5 ft. × 2 mm I.D. glass column, packed with 10% OV-17 on Gas-Chrom Q, 100—200 mesh, with helium as carrier gas; oven temperature 190°C. An ionisation current of 400 μA and electron energy of 25 eV were used.

High-performance liquid chromatography (HPLC) was carried out using a Waters Model 6000A pump and U6K injector. Reversed-phase chromatography was effected on a column (25 cm × 5 mm I.D.) packed with ODS-Hypersil (octadecyl functional groups bonded to spherical silica particles of 5—7 μm in diameter), supplied by Shandon (Runcorn, Great Britain). The UV detector, a Pye-Unicam LC-UV model, was used at a wavelength setting of 254 nm.

Synthesis of 4-methylphthalazin-1-one

4-Methylphthalazin-1-one (MePZ) used as the internal standard (I.S.) in the determination of PZ and TP, was synthesised in the manner described previously for the preparation of PZ [5]. To 2-acetylbenzoic acid (3.4 g) were added 250 ml of distilled water, 2.06 g of sodium bicarbonate and 4.0 ml of hydrazine hydrate. The mixture was refluxed for 45 min, then acidified by the dropwise addition of concentrated HCl and refluxed for a further 30 min. The hot solution was then filtered and evaporated, and the residue extracted with five 50-ml aliquots of boiling chloroform. The pooled filtered extracts were then filtered and evaporated to leave a white powder which was recrystallised from chloroform. GC—EIMS gave a single peak which yielded the following mass spectrum: *m/e* 160 (M^+ parent ion and base peak); 132 (M−28; M−CO); 131 (M−29); 105 (M−55); 104 (M−56); 103 (M−57). This analysis revealed a molecular ion and fragmentation pattern consistent with the structure of MePZ.

Assay procedures

4-Hydrazinophthalazin-1-one. As the determination of NAcHPZ (see below) involves hydrolysis to HPZ, any free HPZ existing in the urine sample prior to hydrolysis, will also be determined. The assay for HPZ is based on derivatisation with acetylacetone to form a pyrazole which can be assayed by GC as previously described [6,7].

Duplicate urine samples (10 ml) were adjusted to pH 3 (at this pH there is no hydrolysis of NAcHPZ to HPZ) and MeH (I.S.) 0.04 mg added. After reaction with excess acetylacetone (0.5 ml) for 1 h at room temperature the urine was adjusted to pH 9.5 and extracted with methylene chloride (30 ml). The extract was filtered through phase separating paper, the filtrate evaporated to dryness and the residue dissolved in ethyl acetate (1.0 ml). Aliquots (1—5 μl) of the ethyl acetate solution were then injected into the gas chromatograph as described above. The ratio of the peak heights of HPZ to MeH (I.S.) derivatives was determined.

A standard curve was constructed by spiking blank urine samples with HPZ and MeH, reacting with acetylacetone at pH 3, extracting and analysing the extracts as described above.

4-N-Acetylhydrazinophthalazin-1-one (NAcHPZ). This metabolite was determined after acid hydrolysis to yield HPZ which is simultaneously reacted with acetylacetone to form a pyrazole derivative as described above (Fig. 2).

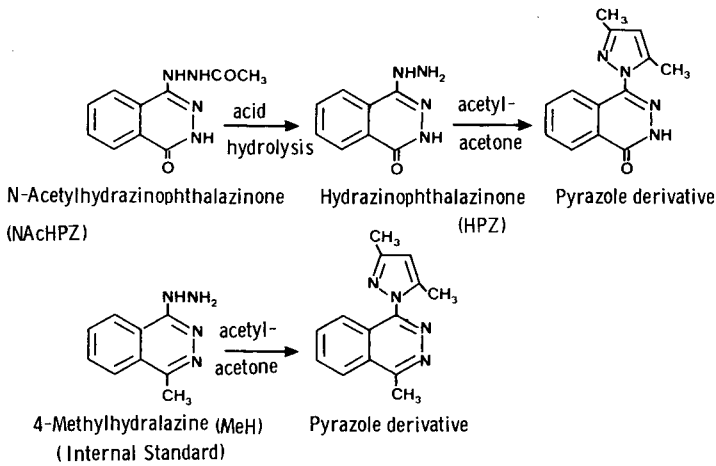

Fig. 2. Structure of hydralazine metabolites, internal standard and their respective pyrazole derivatives necessary for GC.

To duplicate urine samples (10 ml) were added MeH (I.S.) (0.05 mg), excess acetylacetone (0.5 ml) followed immediately by concentrated hydrochloric acid (5.0 ml) with immediate vortexing to ensure adequate mixing. After reaction for 2 h at room temperature with periodic vortexing the samples were adjusted to pH 9.5 and processed as described for the determination of HPZ.

The ratio of the peak heights HPZ to MeH were determined by GC using the conditions described above.

A standard curve was constructed by spiking blank urine samples with NAcHPZ and MeH (I.S.) and assaying as described above.

s-Triazolo[3,4-a]phthalazine (TP) and phthalazinone (PZ). These metabolites were determined underivatised by HPLC.

To duplicate urine samples (10 ml) MePZ (I.S.) (0.1 mg) was added and the pH adjusted to pH 9.5. The samples were then extracted with methylene chloride (30 ml), the extract filtered through phase separating paper and then reduced to dryness. The residue was dissolved in methanol (1.0 ml) and was assayed by HPLC. Aliquots (1.5 μl) of this extract were injected into the instrument and eluted with methanol—water (15:85), 1.8 ml/min. PZ, TP and MePZ (I.S.) were detected by UV absorption at 254 nm and the ratio of the peak heights of PZ/MePZ and TP/MePZ were determined. Standard curves were constructed by spiking blank urine with known amounts of PZ, TP and MePZ (I.S.).

RESULTS AND DISCUSSION

The methods described for the determination of NAcHPZ, HPZ, TP and PZ are straightforward and specific. Adequate separation is achieved between each metabolite under investigation and the respective internal standards, and no interfering peaks are present in blank urine (Fig. 3).

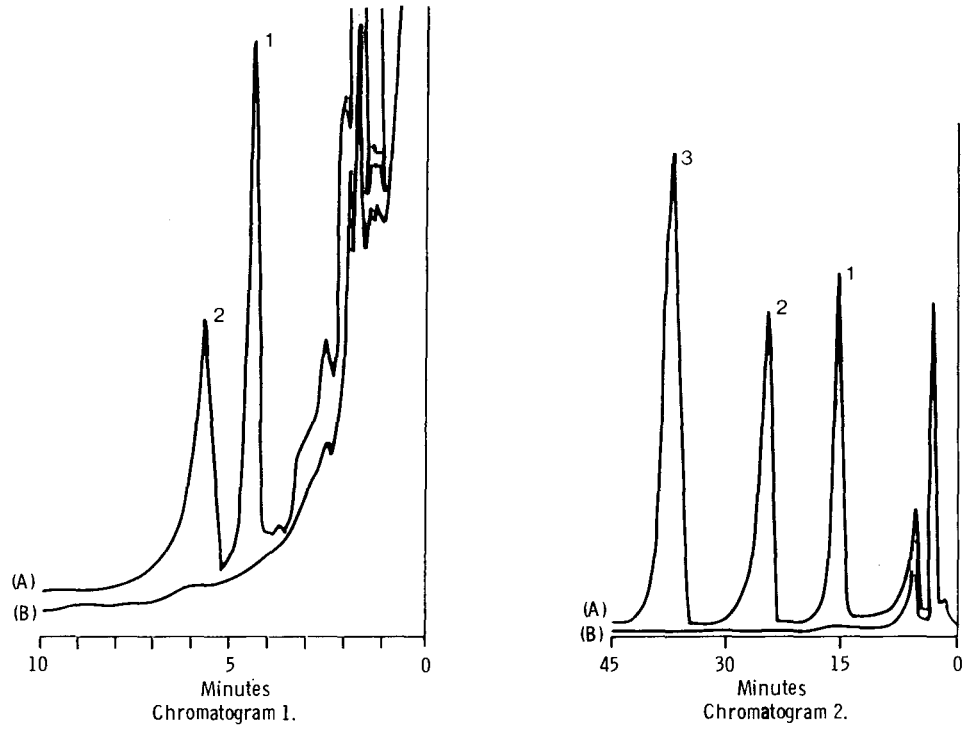

Fig. 3. Chromatogram 1: (A) extract of urine containing NAcHPZ or HPZ and MeH (I.S.). Peaks: 1 = 1-(3′,5′-dimethylpyrazole)4-methylphthalazine (I.S.); 2 = 1-(3′,5′-dimethylpyrazole)phthalazin-4-one; (B) extract of blank urine. Chromatogram 2: (A) extract of urine containing PZ (peak 1), TP (peak 2) and MePZ (I.S.) (peak 3); (B) extract of blank urine.

The standard curves, prepared by spiking blank urine with various known amounts of the compounds in question and the respective internal standards, are linear over the concentration ranges assayed. The curves were fitted by regression analysis, and parameters of accuracy and sensitivity are shown in Table I. Preliminary studies of the urine from patients given hydralazine have indicated that the methods described are suitable for the determination of these metabolites.

TABLE I

ACCURACY AND SENSITIVITY OF THE ASSAY METHODS

Compound	Concentration range of standard curve (μg/ml)	Regression coefficient	Standard error* (μg/ml)	Slope** ± S.E.	Intercept** ± S.E.	Sensitivity (μg/ml)
HPLC assay						
Phthalazinone	2—15	1.0	(2) ± 0.10	15.64 ± 0.80	−0.02 ± 0.01	0.5
s-Triazolo[3,4-*a*]phthalazine	2—15	0.99	(2) ± 0.25	12.89 ± 0.40	−0.03 ± 0.03	0.5
GC assay						
4-Hydrazinophthalazin-1-one	1—6	0.99	(1) ± 0.01	27.12 ± 1.89	−0.05 ± 0.07	0.25
4-N-Acetylhydrazinophthalazin-1-one	4—20	1.0	(4) ± 0.15	8.86 ± 0.27	−0.09 ± 0.03	1.0

*Standard error calculated for one point on the curve (4 determinations), concentration in brackets.

**Standard error of the slope and intercept calculated from the five points of the calibration curve by linear regression.

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CHROMBIO. 599

ELECTRON-CAPTURE GAS CHROMATOGRAPHIC ASSAY FOR METOCLOPRAMIDE IN PLASMA

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SUMMARY

An original electron-capture gas chromatographic assay has been developed for the quantitation of metoclopramide in human plasma. The method involves derivatization with heptafluorobutyryl imidazole after alkaline extraction, acid backwash, and a further alkaline extraction. Plasma levels of metoclopramide as low as 5 μg/l can be measured using 1 ml of plasma, and no interference from related substances or commonly prescribed drugs has been found.

The percentage recovery of drug from plasma ranges from 88% to virtually 100%, and the between-run variation in the assay is 4.3%.

The assay has been used for the study of metoclopramide pharmacokinetics in man following intravenous single-dose administration. The resultant plasma concentration vs. time curve was biexponential, with a terminal half-life of 5.0 h, and a distribution half-time of 0.3 h.

INTRODUCTION

Metoclopramide [Maxolon, 4-amino-5-chloro-N-(2-diethylaminoethyl)-2-methoxybenzamide] is a potent anti-emetic and antispasmodic agent, structurally related to procainamide. Though metoclopramide has been in use for over ten years, and many clinical trials of the drug have been conducted [1—3], detailed human pharmacokinetic studies of metoclopramide have been hampered by the lack of a sufficiently sensitive and specific assay.

Various spectrophotometric and thin-layer chromatographic assays for the drug are available [4,5]; however, such assays suffer both from a lack of sensitivity in the nanogram range required for human studies and from interference from structurally related compounds.

Recently a mass fragmentographic assay for metoclopramide has been de-

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scribed. However, 5-ml sampling volumes were necessary to achieve adequate sensitivity [6]. A recent high-performance liquid chromatographic assay also requires similar large sampling volumes for the same reason [7].

The present paper describes an original electron-capture gas chromatographic (GC) assay for metoclopramide in plasma which is both sensitive and specific enough for pharmacokinetic studies in man; plasma levels of metoclopramide up to 200 μg/l can be measured using 1 ml of plasma, and no interference from related drugs has been noted. This method was developed independently of the somewhat similar method described by Tam and Axelson [8]. Their technique, though simpler, had already proved insufficiently sensitive in our hands while the present method was being developed. Preliminary pharmacokinetic data obtained with the method are included in this report.

EXPERIMENTAL

Reagents

Metoclopramide hydrochloride was provided by Beecham Research Laboratories (Melbourne, Australia) and maprotiline, the internal standard, by Ciba-Geigy (Sydney, Australia). The chemical structures of these compounds are shown in Fig.1.

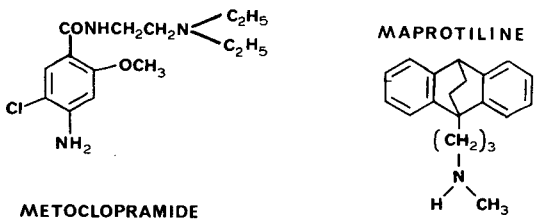

Fig. 1. Chemical structures of metoclopramide and maprotiline, the internal standard.

Stock solutions (1000 μg/l) of both drugs were prepared in absolute methanol and stored at 3—4°C. Chloroform, hexane and methanol were all glass-distilled prior to use. Anhydrous diethyl ether (Mallinckrodt, St. Louis, MO, U.S.A.) was obtained in 450-g cans and used only within 48 h of opening. Bicarbonate—carbonate buffer (pH 10), sodium hydroxide solution (1.0 mol/l) and hydrochloric acid (0.1 mol/l) were prepared in distilled water. The derivatizing reagent, heptafluorobutyryl imidazole (HFBI) Pierce (Rockford, IL, U.S.A.) was obtained in ampoules and stored under nitrogen in glass septum-sealed containers at 4°C after opening. Unused reagent was discarded after 4 days.

Preparation of plasma standards

A 1000 μg/l stock solution of metoclopramide hydrochloride in absolute methanol was prepared and stored at 3—4°C. Appropriate aliquots were removed and transferred to assay tubes. Solvent was removed by gentle evaporation under nitrogen, and immediately, residues were redissolved and equilibrated in 1.0 ml drug-free plasma. These samples were then extracted and assayed by the technique described below. Ten different concentrations of metoclopramide

hydrochloride within the range 5—200 μg/l were used in the preparation of the standard curve. Initial estimates were done in triplicate. The standard curve was checked at least every two weeks using ten single-point estimates over the range 5—200 μg/l. As well, daily checks of the precision and reproducibility of the assay were carried out by including two spiked plasma samples in each group of patient samples being analysed.

Procedure

To a 1.0-ml human plasma sample (spiked or from dosed subjects) in a 20-ml screw-top test-tube were added 250 μl of a 1000 μg/l solution of the internal standard in methanol. 1.0 ml bicarbonate—carbonate buffer (pH 10), and 8.0 ml diethyl ether were also added. The tube was capped and drug and standard were extracted by gentle mixing for 15 min. After centrifugation at 2000 *g* for 4 min, the ether phase was transferred to another screw-cap tube and the aqueous phase re-extracted with a further 8.0 ml ether for 15 min. Following centrifugation, the two ether phases were combined and extracted twice with 2.0 ml 0.1 mol/l hydrochloric acid for 15 min. After centrifugation and subsequent combination of the two aqueous phases in another test-tube, the samples were made alkaline with 1.0 ml sodium hydroxide solution (1.0 mol/l) and extracted into 10.0 ml chloroform for a further 15 min. Layers were separated by centrifugation, and the aqueous phase was discarded, while the organic phase was transferred to a 15-ml glass-stoppered test-tube.

The organic extract was evaporated under nitrogen using gentle heat (50—60°C). To the residue were immediately added 20 μl HFBI, and derivatization proceeded at 75°C for 90 min. After cooling, the sample was alkalinized using 2.0 ml bicarbonate—carbonate buffer (pH 10) and then extracted into 1.0 ml hexane for 2 min.

The hexane phase, after centrifugation, was transferred to a 10-ml centrifuge tube and evaporated to dryness. Immediately prior to chromatography, the residues were reconstituted in 50 μl hexane. A 3—4-μl aliquot was then injected into the gas chromatograph.

Gas chromatographic analysis

The instrument used was a Varian 2700 gas chromatograph fitted with a scandium tritide electron-capture detector. The coiled glass column (1.2 m × 2 mm I.D.) was packed with 3% OV-101 on acid-washed, DMCS treated Gas-Chrom Q (80—100 mesh) (Applied Science Labs., State College, PA, U.S.A.). The column was conditioned at 250°C for 18 h prior to use. Chromatographic conditions were as follows: injector port, foil and oven temperatures were 275°C, 275°C and 200°C respectively; nitrogen carrier gas flow-rate was 37 ml/min; attenuation setting, $128 \cdot 10^{-10}$. Standing current varied between 90% and 50% during the study.

Peak height ratios were calculated by dividing the height of the peak due to metoclopramide by the height of that due to maprotiline. Calibration curves were constructed by plotting peak height ratio as a function of metoclopramide hydrochloride concentration (μg/l of plasma) using known concentrations of metoclopramide hydrochloride in plasma. All assays were carried out in triplicate. Least squares linear regression analysis of the calibration curves was

carried out on a Hewlett-Packard programmable desk calculator. The equation for the regression line was subsequently used to calculate unknown concentrations of metoclopramide hydrochloride in plasma from peak height ratio data.

Derivatization procedure

Aliquots (100 μl) of a 1000 μg/l methanolic solution of metoclopramide hydrochloride were mixed with 250-μl aliquots of a 1000 μg/l solution of maprotiline, and the solvent removed by gentle evaporation. Residues were derivatized with 20 μl HFBI at 75°C for varying time periods up to 5 h, in order to determine the optimal reaction time for derivatization. To stop the reaction instantly, 2 ml bicarbonate—carbonate buffer (pH 10) were added and then the compounds were extracted into hexane. The achievement of optimal conditions was reflected, as will be explained later, in a 1:1 peak height ratio of metoclopramide to maprotiline, as evidenced by GC. Each point in this section of the study was the mean of two determinations.

The derivatives of both metoclopramide and maprotiline were subjected to chemical ionization gas chromatography—mass spectrometry (GC—MS) on a

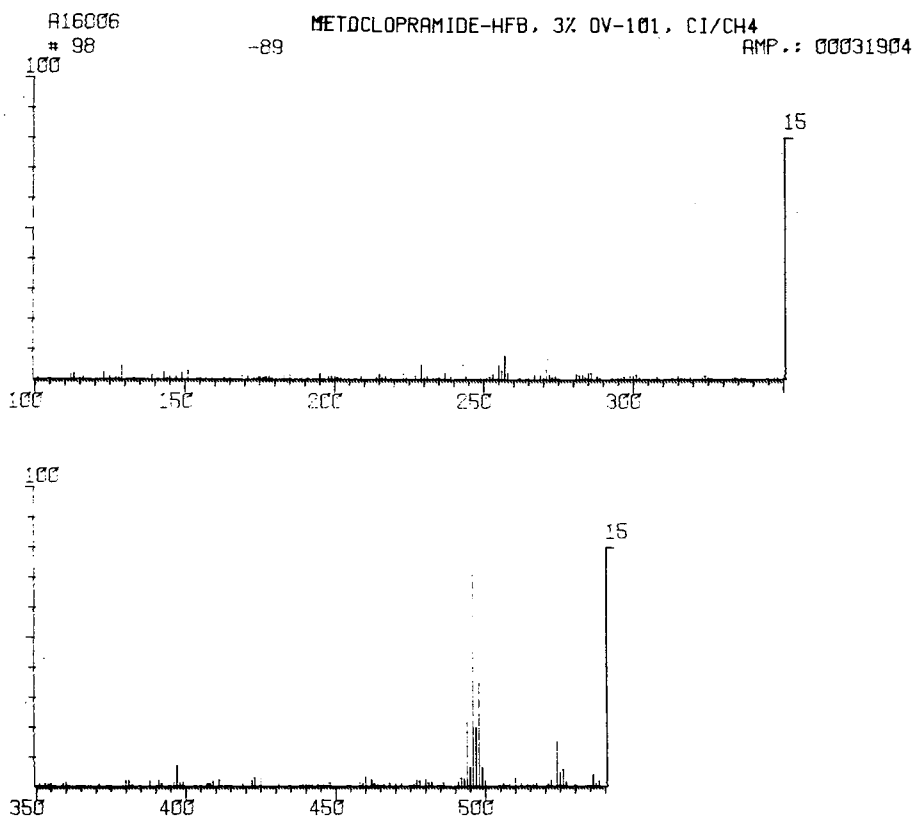

Fig. 2. Chemical ionization mass spectrum of heptafluorobutyric metoclopramide. Carrier gas, methane (20 ml/min); column packing, 3% OV-101 on Gas-Chrom Q (100—120 mesh); oven temperature, 270°C; injector temperature, 250°C; MH^+, *m/e* 496.

Finnigan 3200 gas chromatograph—mass spectrometer and were confirmed as the respective monoheptafluorobutyryl derivatives (Fig. 2).

Precision studies

Samples (1.0 ml) of drug-free human plasma were spiked with appropriate aliquots of a methanolic solution of metoclopramide hydrochloride (1000 μg/l) to give plasma concentrations of 10, 20, 50, 100 and 200 μg/l. Samples were mixed thoroughly and then extracted and assayed as described. Six replicates were analysed at each concentration.

Recovery

Plasma standards of metoclopramide hydrochloride were prepared using appropriate aliquots of a methanolic solution of metoclopramide hydrochloride (1000 μg/l); the solvent was evaporated and the residues reconstituted in 1.0 ml drug-free plasma. These standards were extracted and assayed as described. The plasma standards were then compared with aqueous standards (prepared similarly, with the residues being reconstituted in 1.0 ml distilled water) and with derivatized standards, directly injected (i.e. not extracted). Concentrations of 5, 20, 50, 100, 150 and 200 μg/l were studied in triplicate.

Interference

Over 30 substances, including commonly prescribed drugs, were tested for interference in the assay procedure. Plasma samples (1.0 ml) from treated patients, or plasma samples spiked with therapeutic concentrations of the substances, were extracted and assayed as described above, or methanolic solutions of some compounds at appropriate concentration levels were derivatized with HFBI (after removal of solvent) and chromatographed as described previously.

Pharmacokinetic studies

A 10-mg dose of metoclopramide hydrochloride was administered by intravenous injection over 1 min to a volunteer. The subject, a 37-year old male weighing 67 kg, had been fasting prior to dosing. A PTFE catheter was inserted into an antecubital vein for the collection of blood samples. Twenty 10-ml blood samples were collected at appropriate intervals over a 24-h period, with six samples being taken in the first hour. Samples were centrifuged immediately, the plasma being removed and stored at −20°C prior to analysis.

RESULTS AND DISCUSSION

Acetylation has often proved to be a useful derivatization procedure prior to GC, and with the advent of the fluorinated acylimidazoles as derivatizing agents, the potential applications of such reactions have increased enormously. Several investigators have employed these reagents to facilitate quantitation of phenols, and primary and secondary amines, especially by electron-capture GC [8—10].

Derivatization with HFBI has been used in the present study to quantitate metoclopramide in plasma. The addition of seven fluorine atoms to both metoclopramide and its internal standard, maprotiline, provides excellent sensitivity

for electron-capture GC; as well, the derivatization procedure overcomes problems associated with excessive adsorptive losses on column.

Fig. 3 illustrates the time dependency of the derivatization procedure. The relative molar electron-capture response of the derivatives of metoclopramide and maprotiline is such that 100 ng of metoclopramide and 250 ng maprotiline are responsible for a 1:1 peak height ratio upon chromatography. Plateau conditions are reached at 75 min; 90 min was the time chosen for optimal reaction conditions. Monoheptafluorobutyryl derivatives of both the drug and its internal standard are formed (as determined by GC—MS).

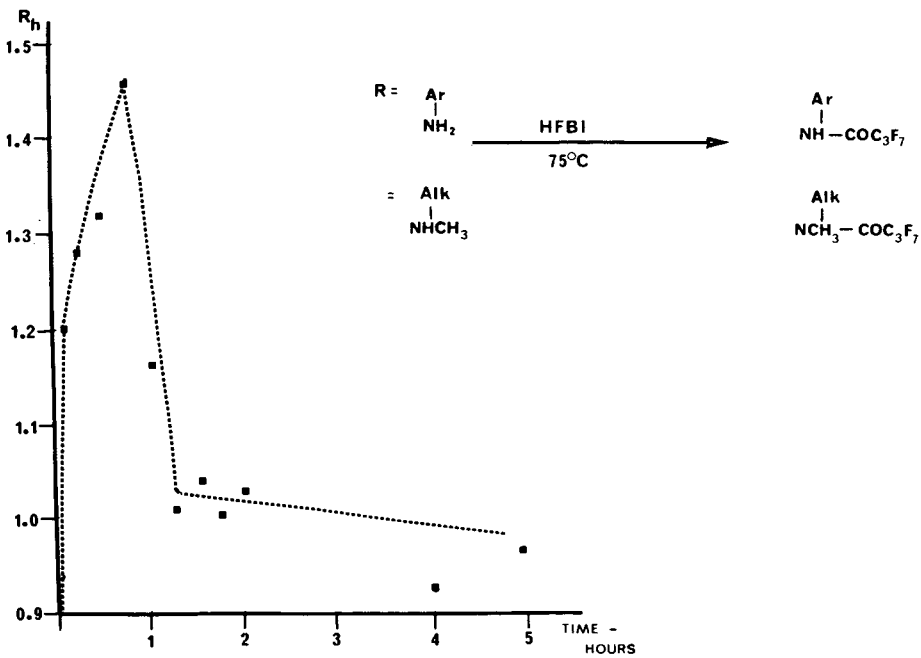

Fig. 3. Time dependency of the formation of the HFB-amides of 100 ng metoclopramide and 250 ng maprotiline, shown as the peak height ratio of the derivatives of the two substances.

The derivatizing agent is unstable in the presence of moisture and every effort must be made to ensure as anhydrous an environment as possible during the actual derivatization procedure. The derivatized samples themselves do not have an extremely long bench-life and must be chromatographed within 2—3 days of preparation.

The extraction procedure described consists essentially of an alkaline extraction, followed by an acid backwash, then a further alkaline extraction. The final organic phase is evaporated to dryness and the residues derivatized. An alkaline wash is then used to remove excess reagent and to convert any diperfluoroacyl derivatives to monoperfluoroacylamines. A second extraction step is employed in the early stages to maximize recovery of the drug.

A typical gas chromatogram obtained with the method is illustrated in Fig. 4. Excellent resolution of peaks due to maprotiline and metoclopramide is achieved at the operating conditions described, and there is no interference

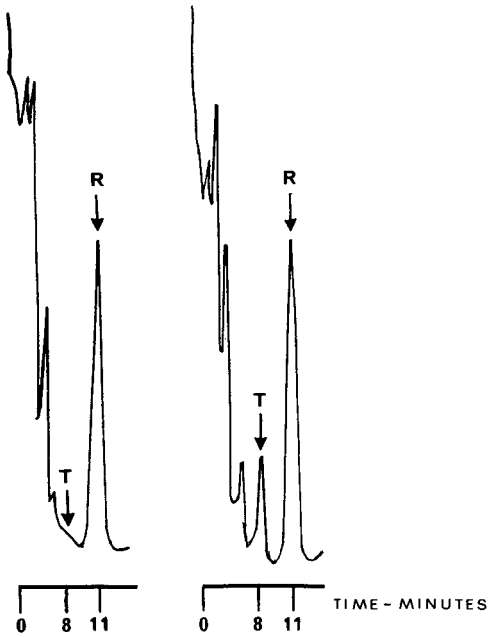

Fig. 4. Gas chromatograms obtained with extracts from 1.0-ml plasma samples containing no metoclopramide (left) and 30 μg/l metoclopramide (right). 250 ng maprotiline was used per ml of plasma. Derivatized metoclopramide (T) is eluted at 8 min and derivatized maprotiline (R) at 11 min.

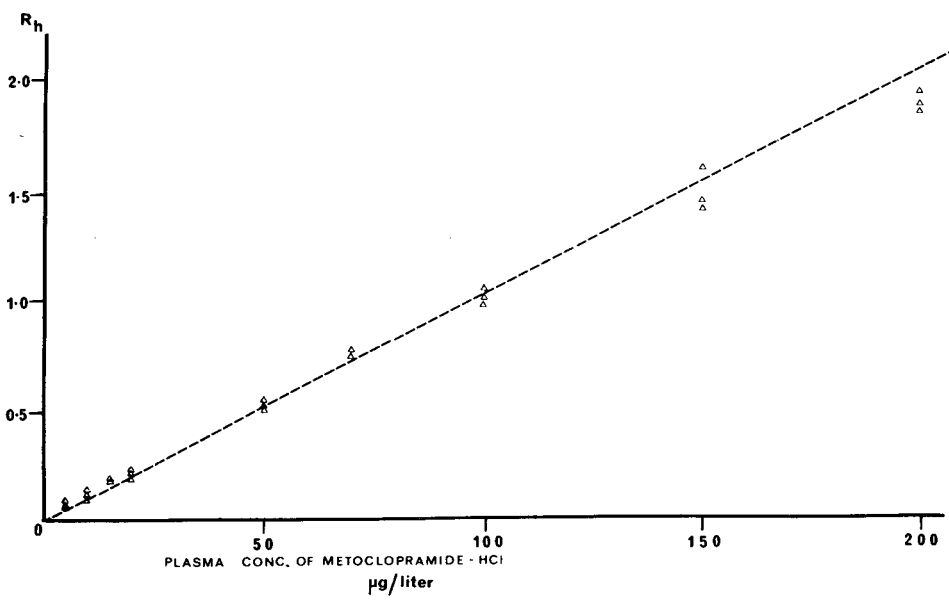

Fig. 5. Calibration curve for the quantitation of metoclopramide hydrochloride in plasma from 5 to 200 μg/l. Peak height ratio of drug to standard is related to plasma concentration of metoclopramide hydrochloride by $R_h = 0.009$ (concn.) + 0.032 ($r^2 = 0.998$).

from extracted plasma components. The retention times for metoclopramide and maprotiline are 8 and 11 min respectively.

The assay is quantitated by expressing the peak height ratio of metoclopramide to maprotiline as a function of plasma concentration of metoclopramide hydrochloride. Standard curves are linear up to 200 μg/l with a minimal detectable concentration of 5 μg/l based on 1.0-ml sampling volumes. Coefficient of determination (r^2) values, as determined by linear regression analysis, have ranged from 0.985 to 0.999 (see Fig. 5). Within-run (Table I) and between-run precision studies have proved the reliability and reproducibility of this method; the average variation from day to day is 4.3%. As well, a study conducted to assess percentage recovery from plasma, compared with directly injected derivatized standards, and with recovery from distilled water, indicated that mean percentages recovered ranged from 88% to virtually 100% (Table II).

TABLE I

WITHIN-RUN PRECISION

n = 6 at each concentration studied.

Concentration added (μg/l in plasma)	Mean concentration recovered (± S.D.) (μg/l)
10	10.15 ± 1.95
20	21.77 ± 2.87
50	53.15 ± 2.36
100	100.08 ± 7.94
200	208.20 ± 8.28

TABLE II

RECOVERY (%)

n = 3 at each concentration studied.

Concentration (μg/l)	Recovery (%)*	Recovery (%)**
5	101.4	101.3
20	108.8	105.9
50	106.3	94.9
100	100.1	89.5
150	102.7	90.5
200	104.1	100.7

*Compared with directly injected, derivatized standards.
**Compared with recovery from distilled water.

Specificity of the assay was established for over 30 drugs (Table III) that were thought likely to interfere, or which might possibly be taken concurrently with metoclopramide. No such interference was observed at normal therapeutic concentrations of these drugs. This method, in our experience, proved superior to that of Tam and Axelson [8] which we found insufficiently sensitive for pharmacokinetic studies in man. The assay for metoclopramide developed personally, uses a different internal standard, maprotiline, which is added prior

TABLE III

DRUGS AND METABOLITES TESTED FOR INTERFERENCE IN ASSAY

Acetylsalicylic acid	Methdilazine
Carbamazepine	Methysergide
Chlordiazepoxide	Nitrazepam
Chlorimipramine	Nortriptyline
Clonazepam	Oxazepam
Cyproheptadine	Phenobarbitone
Desipramine	Phenytoin
Desmethyldiazepam	Prednisolone
Diazepam	Prednisone
Dicyclomine	Procainamide
Ergotamine	Prochlorperazine
Ethosuximide	Propranolol
Fluphenazine	Salicylic acid
Haloperidol	Trifluoperazine
Imipramine	Valproic acid
Levodopa	

to the first extraction, rather than prior to derivatization. It also uses a double extraction technique which results in higher recoveries and better sensitivity. HFBI, the derivatizing agent, also gave higher yields of metoclopramide-HFB and maprotiline-HFB than did heptafluorobutyric anhydride.

The assay has been used in preliminary pharmacokinetic studies in man; it appears sufficiently sensitive to determine blood levels of metoclopramide after an intravenous dose of 10 mg. The relevant plasma concentration—time profile is shown in Fig.6, and the pharmacokinetic parameters calculated from these data are tabulated in Table IV.

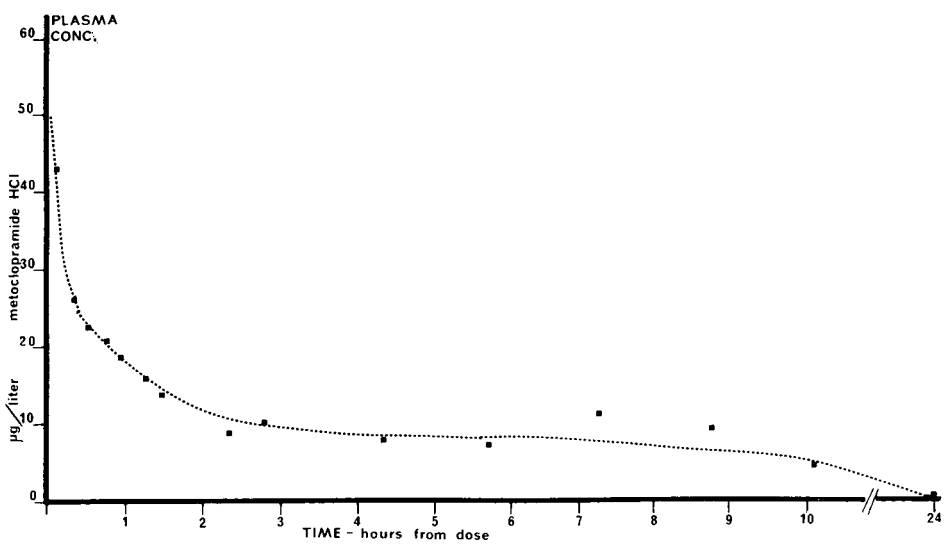

Fig.6. Concentrations of metoclopramide hydrochloride in human plasma after administration of a 10-mg intravenous dose to a healthy male volunteer. Plasma levels were followed for 24 h.

TABLE IV

PHARMACOKINETIC PARAMETERS IN ONE SUBJECT

Subject I (intravenous metoclopramide 10 mg)	
$t_{\frac{1}{2}}$ (β)	5.0 h
k_β	0.14 h^{-1}
Clearance (area)	0.94 l/kg/h
Volume of distribution	6.79 l/kg
AUC (trapez)	158.21 μg/l·h
$t_{\frac{1}{2}}$ (α)	0.33 h
k_α	2.1 h^{-1}

The plasma concentration—time profile was biexponential; data were found to fit a two-compartment open model with terminal half-life of the order of 5 h. This would appear to differ from preliminary work published by Teng et al. [7] which suggested that data were best fitted to a one-compartment model with half-life of about 4.0 h, though further pharmacokinetic studies are required.

From the results collected in Table IV, it would appear that metoclopramide is fairly rapidly and extensively distributed throughout the body. Clearance is high, indicating rapid elimination of this drug, most likely by conjugation and N-dealkylation [4,11,12].

Side effects experienced by the subject included dry mouth, irritability and mild disorientation (all in first 60 min) and very marked sedation at about 3 h after dosing. No attempt was made to correlate these effects with metoclopramide plasma levels.

ACKNOWLEDGEMENTS

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CHROMBIO. 598

ASSAY OF HA-966 IN RAT PLASMA BY CAPILLARY GAS—LIQUID CHROMATOGRAPHY WITH NITROGEN-SELECTIVE DETECTION

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SUMMARY

A gas—liquid chromatographic method for the determination of the γ-aminobutyric acid-like drug 1-hydroxy-3-aminopyrrolidone-2 (HA-966) in plasma is described. HA-966 was converted into its diacetyl derivative Ac_2HA-966 with acetic anhydride. This compound could be suitably eluted from a capillary OV-17 support-coated open tubular column. A sensitive detection method was achieved by making use of nitrogen—phosphorus-selective flame ionization.

INTRODUCTION

The experimental drug 1-hydroxy-3-aminopyrrolidone-2 (HA-966) [1], synthesized by Havinga et al. [2] in 1959, has proved to be a valuable agent in the study of central dopaminergic systems [3—6]. Unfortunately little information is available as yet on the fate or metabolism of the drug because of the lack of a suitable analytical method. Hence pharmacokinetics of HA-966 are unexplored and it is even unknown whether HA-966 itself or a metabolite elicits the central depressant effects. In order to improve evaluations of the actions of this drug [4] it was necessary to develop an assay of HA-966 in biological materials. In this article a rapid and sensitive determination of HA-966 by capillary gas—liquid chromatography (GLC) with nitrogen—phosphorus (N—P) selective detection is reported.

MATERIALS AND METHODS

Apparatus

A Hewlett-Packard 5830A gas chromatograph equipped with a dual nitrogen—phosphorus flame ionization detector Model 18789A was used. A solid

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phase injector was assembled from a Hoke ball valve [7—9]. For the derivatization Pierce Reacti-Vials (Hicol, Rotterdam, The Netherlands) were used. Heating of vials was carried out in a Tecam Dri-Block DB-3 (Salm and Kipp, Breukelen, The Netherlands). ^{1}H- and ^{13}C-NMR spectra were recorded with a Jeol-Ps-100 NMR spectrometer equipped with a Jeol-JNM-Pft-100 pulse unit and an EC-100 computer. For ^{13}C-NMR spectroscopy the Fourier transform mode was used. Mass spectra were measured with an LKB 2091-2130 gas chromatograph—mass spectrometer with a PDP 11 computer system.

Solvents, standards and reagents

The solvents, analytical grade, were obtained from Baker (Deventer, The Netherlands) and Merck (Darmstadt, G.F.R.). Deionized, glass distilled water was used. PPE-21 and OV-17 were purchased from Chrompack (Middelburg, The Netherlands). Tullanox was from Cabot Corporation (Boston, MA, U.S.A.). Racemic HA-966 was a gift from Organon (Oss, The Netherlands); elemental analysis gave the following percentages: C found 41.38 (calculated 41.37), H 6.94 (6.94), N 27.55 (27.56) and O 23.95 (24.13). Lidocaine was provided by the Rijksmagazijn van Geneesmiddelen (Amsterdam, The Netherlands). Acetic anhydride was from Baker.

Animals

Male Wistar derived rats (SPF, 225—275 g) were used. The animals were allowed free access to food and water.

Isolation procedure

To 0.1 ml of rat plasma were added 4 μg of lidocaine in 40 μl of ethyl acetate and 0.3 ml of methanol. After centrifuging for 10 min (1650 *g*) the supernatant was transferred to a Pierce 1.0-ml Reacti-Vial. To the residue 0.05 ml water and 0.3 ml methanol were added and the mixture was centrifuged. The aqueous methanol supernatants were combined and evaporated at 50°C in a stream of dry nitrogen.

Derivatization procedure

To the residue thus isolated, 50 μl acetic anhydride were added. The mixture was allowed to react at room temperature for 20 min. Then the solution was evaporated for about 0.5 h at 50°C in a stream of dry nitrogen. The residue was dissolved in 0.4 ml ethyl acetate, transferred to another vial and evaporated at 50°C under nitrogen.

GLC procedure

The residue obtained in the derivatization procedure was reconstituted in 100 μl ethyl acetate. A 1-μl aliquot of this solution was used for each solid phase injection on the capillary OV-17 support-coated open tubular (SCOT) column [7—9]. Tullanox was applied as a support material for the capillary column and was subsequently coated twice with PPE-21 and OV-17 [7,8]. The experimental GLC conditions were: column, 10 m × 0.45 mm I.D., temperatures: oven, 195°C, injection, 280°C; detection, 300°C; flow-rates: carrier gas, helium 2.5 ml/min; auxiliary gas, helium 27.5 ml/min; detection gases:

hydrogen, 3 ml/min; air, 50 ml/min. Representative retention times were: 1-acetoxy-3-acetylaminopyrrolidine-2 (Ac_2HA-966) 3.4 min, lidocaine 4.0 min.

Calibration curve

For the construction of a calibration curve amounts of 1, 2, 5, 10, 20 or 50 μg of HA-966 and 4 μg of lidocaine were spiked to 0.1 ml of rat plasma. The samples were processed as described above. The resulting ratios of the peak areas of Ac_2HA-966 and lidocaine were plotted against the amounts of HA-966 spiked to the rat plasma (10—500 ng). For the assay of rat plasma samples the calibration curve was determined at least twice daily. Each reference or rat sample was measured at least in duplicate.

Preparation and characterization of Ac_2HA-966

Pure samples of Ac_2HA-966 (ca. 200 mg) were prepared by heating HA-966 for 1 h in 2 ml acetic anhydride and evaporating the solution for 2 h in a stream of dry nitrogen. Ac_2HA-966 was obtained as a colourless oil; it was characterized by ^{1}H- and ^{13}C-NMR. Mass spectrometry (MS) with electron ionization showed only the $[M-\overset{O}{\overset{\|}{C}}-CH_2]^{\cdot+}$ fragment with *m/e* 158. MS with chemical ionization (NH_3) clearly displayed the $[MH]^{\cdot+}$ ion with *m/e* 201, where M denotes Ac_2HA-966.

RESULTS AND DISCUSSION

Up to now a considerable amount of information is available on the pharmacological action of the GABA-like experimental drug HA-966 (see, for example, refs. 3—6). In order to obtain insight into the actual fate of the drug it was necessary to develop a suitable analytical method. It seemed promising to take advantage of the presence of two nitrogen atoms in HA-966 and apply nitrogen-selective detection in the GLC analysis. Preliminary experiments however indicated that HA-966 in its underivatized form was not suitable for GLC. Therefore it was decided to prepare a derivative of HA-966 with good GLC properties. A second problem offered the isolation of HA-966 from biological material. HA-966 is soluble in rather polar solvents such as water, but only slightly soluble in solvents such as methanol. Since no satisfactory extraction procedure could be developed, a capillary column of high separation power was selected. For the derivatization of HA-966 acylation was expected to lead to a product which could be readily manipulated in GLC (see ref. 7). Trifluoroacetylation proved to be too aggressive, but acetylation yielded promising results. HA-966 reacted smoothly with acetic anhydride under a variety of experimental conditions. Fig. 1 presents the course of the reaction; the structural proof of the formation of the compound Ac_2HA-966 was given by NMR and MS (see Materials and Methods).

As water is the best solvent for HA-966, which is insoluble in most other solvents, we were not successful in developing an extraction procedure. The removal of protein was sufficient for the correct performance of a GLC analysis after derivatization.

An initial purification step, namely extraction of the evaporated plasma

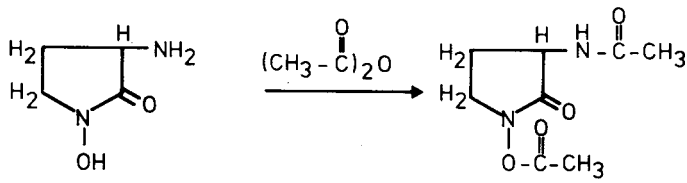

Fig. 1. Conversion of HA-966 with acetic anhydride into Ac_2HA-966.

sample with ethyl acetate, could be omitted. The recovery of the isolation procedure was estimated at 70% (S.D. 10%, n = 5) on the basis of absolute peak areas with respect to a sample of derivatized pure HA-966.

In order to find an adequate GLC column for the analysis of HA-966 as the derivative Ac_2HA-966, several types of capillary columns were tested. Excellent results were obtained with a capillary SCOT OV-17 column, the support material Tullanox first being coated with PPE-21. Chromatograms recorded with this column showed the desired separation of Ac_2HA-966 and lidocaine from other compounds present (Fig. 2); peaks were sharp and the sensitivity achieved with N—P-selective detection was very high. Lidocaine proved to be a useful internal standard. Representative examples of chromatograms are given in Fig. 2. The calibration curve in Fig. 3 determined in triplicate from spiked rat plasma samples shows linearity in the range of 10—500 ng per injection.

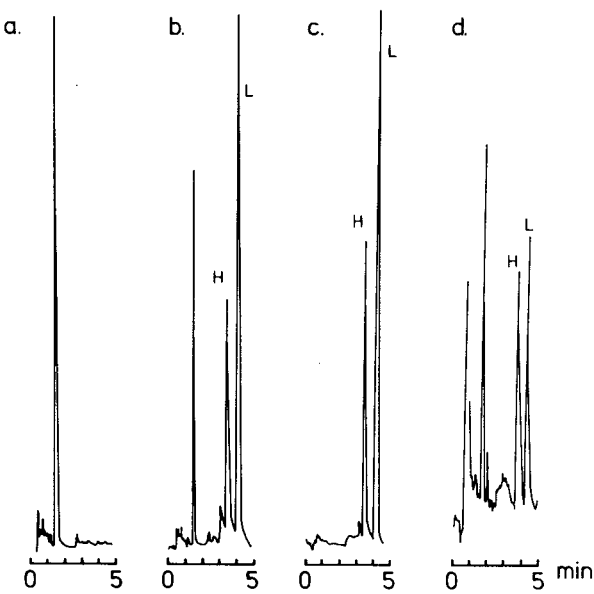

Fig. 2. Representative chromatograms of the assay of HA-966 as Ac_2HA-966 on the capillary OV-17 SCOT column with lidocaine as internal standard: (a) blank rat plasma; (b) 20 ng of HA-966, converted into Ac_2HA-966, and 40 ng of lidocaine spiked to rat plasma; (c) reference sample of 20 ng of HA-966, converted into Ac_2HA-966, and 40 ng of lidocaine; (d) 43 ng of HA-966 as Ac_2HA-966 and 40 ng of lidocaine in an authentic rat plasma sample. Peaks: H = Ac_2HA-966; L = lidocaine.

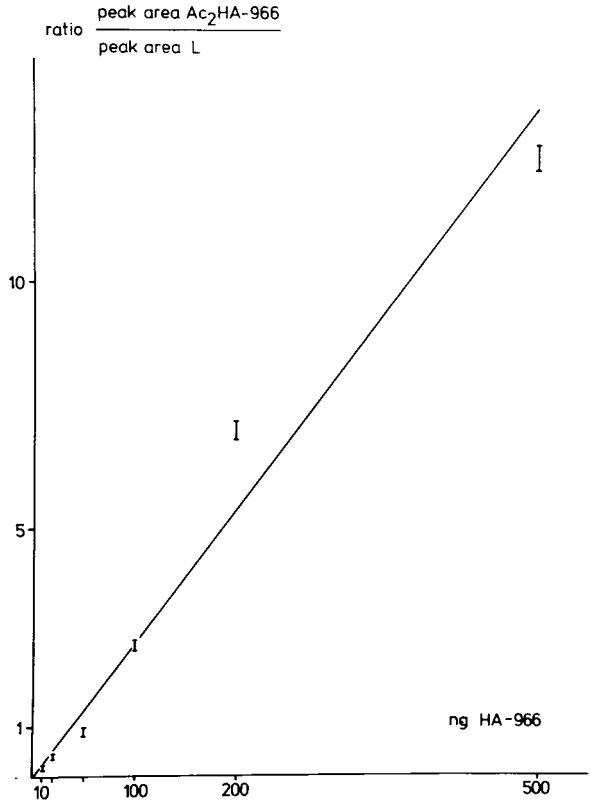

Fig. 3. Calibration curve for the determination of HA-966 as the derivative Ac_2HA-966 in the range of 10—500 ng measured from plasma extracts. The ratio of the peak areas of Ac_2HA-966 and the internal standard lidocaine (L) is plotted against the amount of HA-966 (n=3, correlation coefficient, 0.999).

TABLE I

PHARMACOKINETIC DATA OF HA-966 INTRAPERITONEALLY ADMINISTERED TO RATS

Data calculated according to a one-compartment model; n = 5.

Dose	100 mg/kg
$t_{\frac{1}{2}}$	40.2 ± 4.8* min
Co extrap.	111 ± 29 μg/ml
V	226 ± 62 ml
$Cl_{tot.}$ **	4.0 ± 1.3 ml/min
AUC***	$66 \cdot 10^2 \pm 20 \cdot 10^2$ μg/ml·min
$Cl_{tot.}$ §	3.9 ± 1.3 ml/min

*Standard deviation.
**$Cl_{tot.} = k \cdot V$.
***Calculated by the trapezoidal rule.
§$Cl_{tot.}$ = dose/AUC.

For the GLC method described a small amount of plasma of only 0.1 ml was needed. A further advantage is the rather low detection limit which was found to be about 5 ng of HA-966 on a fresh column. When before starting the analysis, some blank plasma samples were injected, an additional decrease of the detection limit to 1 ng was reached, probably owing to deactivation of the column.

As an illustrative example the plasma decay of HA-966 in a male Wistar rat after an intraperitoneal injection of HA-966 (100 mg/kg) is presented in Fig. 4. Some pharmacokinetic data for HA-966 are given in Table I.

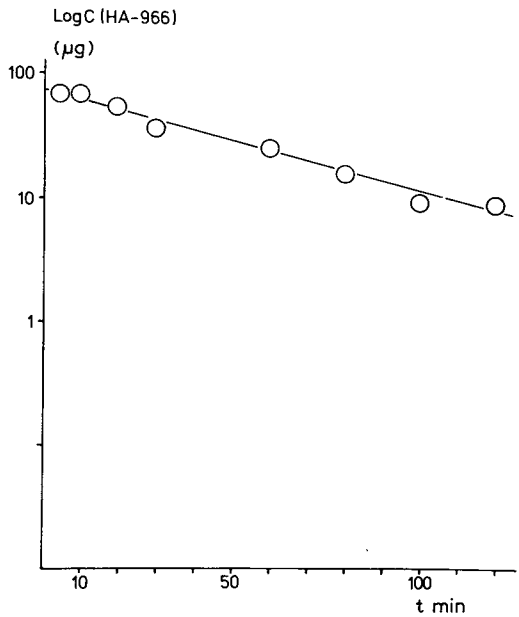

Fig. 4. Plasma decay of HA-966 in a male Wistar rat after intraperitoneal injection of 100 mg/kg of HA-966.

In conclusion it can be stated that HA-966 can be determined in a rapid and sensitive way. The pharmacokinetics of HA-966 in relation to its pharmacodynamics are under current research.

ACKNOWLEDGEMENTS

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REVERSED-PHASE, ION-PAIR LIQUID CHROMATOGRAPHY OF QUATERNARY AMMONIUM COMPOUNDS

DETERMINATION OF PYRIDOSTIGMINE, NEOSTIGMINE AND EDROPHONIUM IN BIOLOGICAL FLUIDS

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SUMMARY

A reversed-phase, ion-pair liquid chromatographic method for the quantitative determination of quaternary acetylcholinesterase inhibitors is described. The method uses an ion-pair extraction to isolate the drugs from biological material prior to liquid chromatographic separation and online UV detection at 214 nm. Quantitation down to 5 ng/ml and within-day precision with coefficient of variation (C.V.) of 1.5% (n=10, $\bar{x}$ = 100 ng/ml) for neostigmine, C.V., 1.7% (n=10, $\bar{x}$ = 80 ng/ml) for pyridostigmine and C.V., 1.5% (n=10, $\bar{x}$ = 100 ng/ml) for edrophonium have been achieved. The assay was designed for pharmacokinetic studies of these drugs in anesthetized patients.

INTRODUCTION

The acetylcholinesterase inhibitors pyridostigmine, neostigmine and edrophonium are used extensively in anesthesiology to reverse non-depolarizing neuromuscular blockade [1] and in the treatment of myastenia gravis, a neuromuscular disorder [2].

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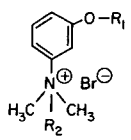
In order to study relationships between dose, serum level and effect, reliable quantitative methods to estimate the levels of these drugs are necessary. Methods available to quantitate these quaternary ammonium compounds include a spectrophotometric assay for pyridostigmine [3] and gas—liquid chromatographic assays for neostigmine and/or pyridostigmine [4—7]. The chromatographic assays depend on thermal dequaternization followed by thermionic detection [4,5] or selected ion monitoring [7] of the released tertiary amine or by electron-capture detection of the methyl iodide [6] formed in the process. Extraction of the drugs to be assayed is based on complexation with ion-pairing reagents [3—5] or with potassium triiodide [6]. Edrophonium, a reversible acetylcholinesterase inhibitor, can be assayed enzymatically [8]. These methods are tedious and/or limited in terms of sensitivity and reliability.



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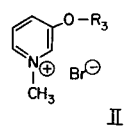

II

Ia : $R_1 = -\overset{O}{\overset{\|}{C}}-N(CH_3)_2$; $R_2 = -CH_3$ Neostigmine bromide

Ib : $R_1 = -H$; $R_2 = -CH_3$ 3-Hydroxytrimethylammonium bromide

Ic : $R_1 = -H$; $R_2 = -C_2H_5$ Edrophonium bromide

IIa : $R_3 = -\overset{O}{\overset{\|}{C}}-N(CH_3)_2$ Pyridostigmine bromide

IIb : $R_3 = -H$ 3-Hydroxy-N-methylpyridinium bromide

This paper describes a reversed-phase, ion-pair liquid chromatographic method with absorption detection at 214 nm to quantitate the analytes and a modified ion-pair extraction procedure with picrate anion as counter ion for the isolation of the analytes from biological material. Retention behavior of pyridostigmine, neostigmine, their 3-hydroxy metabolites and edrophonium are examined. The assay was used to determine pharmacokinetic parameters of edrophonium, neostigmine and pyridostigmine. These results will be published in forthcoming papers.

EXPERIMENTAL

Chromatographic equipment

The liquid chromatograph consisted of a Varian 5020 pump (Varian Aerograph, Walnut Creek, CA, U.S.A.), an LDC 214-nm UV-III monitor Model 1203 with a Zn-lamp (Laboratory Data Control, Riviera Beach, FL, U.S.A.), a Valco CV-6-UHPA-N60 sampling valve with a 50-μl loop and a linear potentiometric recorder (Varian 9176).

Four reversed-phase columns were used in this study, two homemade and two from commercial sources. The homemade columns (15 × 0.32 cm) were packed with LiChrosorb RP-8 5 μm and LiChrosorb RP-18 10 μm (Merck, Darmstadt, G.F.R.) using a slurry technique. The commercial columns were

a Varian (30 × 0.4 cm) MCH 10-μm column and an Altex (15 × 0.46 cm) Ultrasphere Octyl 5-μm column (Altex, Berkeley, CA, U.S.A.). In order to avoid contamination of the analytical column, a pre-column (5 × 0.32 cm) tap-filled with Perisorb RP-2 (Merck, particle size 30—40 μm) was placed between the injector and the separation column.

Chemicals and reagents

Acetonitrile and dichloromethane were of liquid chromatographic purity (Burdick and Jackson Labs., Muskegon, MI, U.S.A.). The water used for all solutions and mobile phases was doubly deionized. Heptanesulfonic acid sodium salt ($C_7H_{15}SO_3^-Na^+$) and tetramethylammonium chloride (TMA^+Cl^-) were obtained from Eastman-Kodak (Rochester, NY, U.S.A.). Tetrabutylammonium hydrogen sulfate ($TBA^+HSO_4^-$) was obtained from Aldrich (Milwaukee, WI, U.S.A.). Sodium dihydrogen phosphate (NaH_2PO_4) and picric acid were Baker analyzed reagents (Baker Chemical, Phillipsburg, NJ, U.S.A.).

Crystalline neostigmine (I_a) and pyridostigmine (II_a) were obtained from Hoffman-LaRoche (Nutley, NJ, U.S.A.); edrophonium (I_c) was obtained from an intravenous injection solution (Tensilon® Hoffman-LaRoche). Hydrolysis in 2 *N* sodium hydroxide at 50°C for 4 h gave the corresponding 3-hydroxy metabolites (I_b = neostigmine metabolite; II_b = pyridostigmine metabolite) which were shown to be pure by liquid chromatographic analysis under conditions described for pyridostigmine and neostigmine (see Fig. 3). Neutralized solutions were used.

Chromatography

The influence of the following parameters on the chromatographic behavior of each quaternary ammonium compound was examined: TMA^+Cl^- concentration, $C_7H_{15}SO_3^-Na^+$ concentration, pH, acetonitrile concentration and support type.

Procedures for bioanalysis

Extraction from biological fluids. The internal standard used for the determination of pyridostigmine and neostigmine was edrophonium; neostigmine served as internal standard for the edrophonium assay. Standard solutions of these compounds (0.5 μg/ml) were prepared in water. To a PTFE-lined screw-cap culture tube (150 × 16 mm) were added 1.0 ml of serum, 100 μl of appropriate internal standard and 0.5 ml of 0.1 *M* picric acid in 0.1 *M* sodium hydroxide (pH adjusted to 7). Sodium dihydrogen phosphate (0.4 ml, 0.1 *M*) was then added. The resulting mixture was extracted with water-saturated dichloromethane (12.0 ml) by shaking vigorously for 5 min. Following centrifugation (2000 *g*, 10 min) the aqueous phase, protein pellet and emulsified interface were removed with the aid of a Pasteur pipet. The organic phase (10.0 ml) was then transferred to a PTFE-lined screw-capped conical centrifuge tube (134 × 17 mm) and 10^{-3} *M* tetrabutylammonium hydrogen sulfate (200 μl) was added. After vigorous shaking for 30 sec the mixture was centrifuged (2000 *g*, 2 min) and the majority of the lower organic phase was removed by means of a Pasteur pipet leaving approximate-

ly 400 μl. Care was exercised to avoid disturbing the aqueous layer. The remaining mixture was recentrifuged (2000 *g*, 1 min) and 50 μl of the aqueous phase were subjected to liquid chromatographic analysis.

Chromatography of biological extracts. The Altex 5-μm Ultrasphere Octyl column was used with the following mobile phases: for neostigmine and edrophonium assays, 0.01 *M* heptanesulfonic acid sodium salt, 0.01 *M* sodium dihydrogen phosphate, and 0.0025 *M* tetramethylammonium chloride in acetonitrile—water (20:80, v/v); for pyridostigmine assays, acetonitrile—water (17:83, v/v). The pH of the mobile phase was adjusted to 3 with concentrated sulphuric acid. The assays were performed at ambient temperature with a flow-rate of 2 ml/min. Detection was at 214 nm.

Quantitation. The procedure was standardized by analyzing drug-free serum samples spiked with known amounts of the analytes. Peak height ratios of analytes vs. internal standard were used to establish calibration curves. Serum concentrations in the unknown samples were determined using these calibration curves.

Recovery. Absolute overall recoveries from serum were estimated by comparison of peak heights obtained from the injection of known quantities of the analytes with peak heights obtained from the injection of extracts of serum samples spiked with the analytes.

Reproducibility. Within-day precision was determined by performing ten replicate analyses of spiked serum samples.

Treatment of glassware. The culture tubes used in the extraction were "conditioned" by storing them filled with a 0.1 *M* tetramethylammonium chloride solution. Before use the solution was poured off and the tubes rinsed five times with deionized water. The conical centrifuge tubes used in the back extraction were pretreated in the same way but were dried before use.

RESULTS AND DISCUSSION

Chromatographic behavior

Preliminary experiments revealed that pyridostigmine and neostigmine could not be eluted from the Varian MCH reversed-phase column (monomeric C_{18}, not end-capped) with acetonitrile—water mixtures unless TMA^+Cl^- was added to the mobile phase. These observations may be explained by the irreversible adsorption of the drugs to residual silanol groups and deactivation of adsorption sites by TMA^+. A similar observation was reported in the chromatography of curare alkaloids [9]. These investigators reported, however, that the concentration of TMA^+ did not affect retention volumes. We found that the capacity ratio of pyridostigmine and neostigmine could be regulated by altering the concentration of TMA^+ in the mobile phase (Fig. 1); increasing the concentration of TMA^+ decreased the retention volume. This was true for the MCH column employing a mobile phase free of ion-pair reagent and for all columns with heptanesulfonic acid in the mobile phase. This effect can be explained if the TMA^+ binds not only to the reactive silanol groups but also to the bonded stationary phase, resulting in a repulsion or a decreased availability of binding sites for pyridostigmine and

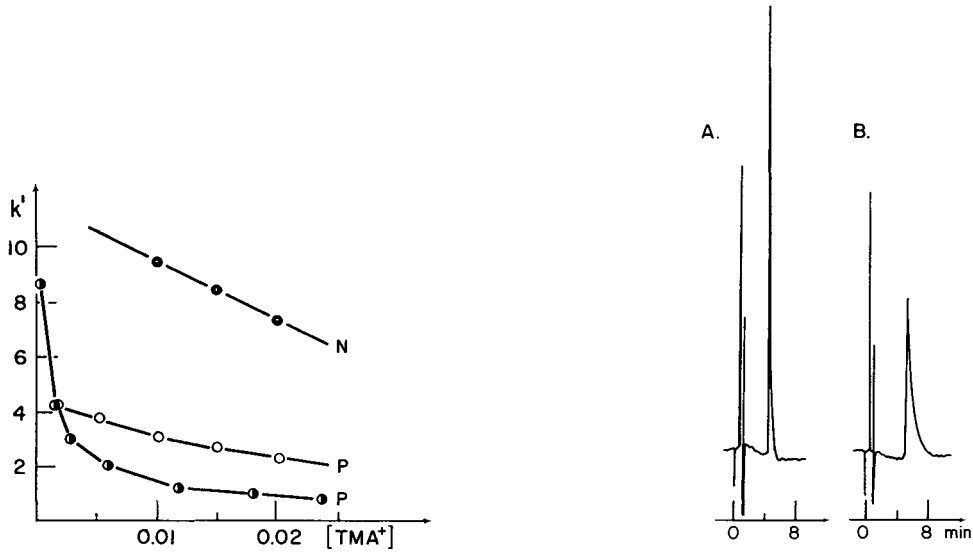

Fig. 1. Relationship between capacity factors (k') and tetramethylammonium (TMA^+) concentration. N = Neostigmine, P = pyridostigmine. ○ and ●: LiChrosorb RP-18 column with 0.005 *M* $C_7H_{15}SO_3^-Na^+$, 0.005 *M* NaH_2PO_4 and variable TMA^+ concentration in acetonitrile—water (15:85, v/v). ◑: MCH column with 0.01 *M* NaH_2PO_4 and variable TMA^+ concentration in acetonitrile—water (33:67, v/v).

Fig. 2. Comparison of peak shape from a 100-ng pyridostigmine injection (A) with 0.0025 *M* TMA^+ and (B) without TMA^+ in the mobile phase (0.01 *M* $C_7H_{15}SO_3^-Na^+$ and 0.01 *M* NaH_2PO_4 in acetonitrile—water (15:85, v/v). Column: 5 μm LiChrosorb RP-8 (15 × 0.32 cm); flow-rate, 1 ml/min.

neostigmine interaction. The addition of TMA^+ also had a pronounced effect on the peak shape. It reduced dramatically the tailing of the peaks obtained on the MCH 10-μm and the RP-8 5-μm column (Fig. 2). No effect on peak shape was noted for the RP-18 and the Ultrasphere Octyl columns.

The capacity ratios could be regulated further by changing the acetonitrile concentration and/or the ion-pairing reagent concentration (heptanesulfonic acid). A deviation from linearity with increasing concentration of the ion-pair reagent was observed and could have been due to the high concentrations used [10].

The effect of the pH was of dual nature. Not only was a decrease in retention volume observed at higher pH, but also a dramatic decrease in plate count. For neostigmine on the RP-18 column there were 600 plates at pH 5.7 vs. 2800 plates at pH 3.0.

The separation of pyridostigmine, neostigmine and their 3-hydroxymetabolites on the RP-18 column is demonstrated in Fig. 3.

We had some problems with the longevity of our home-packed columns. The RP-18 10-μm columns could be used for only two weeks before the plate count deteriorated. Also, the RP-8 5-μm columns developed a dramatic increase of back pressure after two days of use due to partial clogging of the bottom frit (2 μm) by fines. Therefore, we used an Altex Ultrasphere Octyl

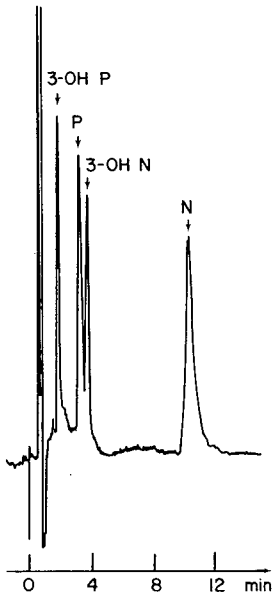

Fig. 3. Separation of neostigmine (N), pyridostigmine (P) and their 3-hydroxy metabolites (3-OH N, 3-OH P) on the 10-μm LiChrosorb RP-18 column. Mobile phase: 0.01 *M* $C_7H_{15}SO_3^-Na^+$ and 0.01 *M* NaH_2PO_4 in acetonitrile—water (15:85, v/v) adjusted to pH 3.0.

5-μm column for the work with biological materials. This column proved to be very stable and also had very good height equivalent to a theoretical plate (HETP) values for our samples. A value of 11.5 μm for neostigmine at a flow-rate of 1 ml/min without pre-column was achieved (15 μm with pre-column). To detect the low levels of compounds present in serum during our pharmacokinetic studies, a fixed-wavelength 214-nm detector was used. Neostigmine absorbs weakly at 254 nm ($\epsilon < 400$) compared with its absorption at 214 nm ($\epsilon > 6000$). Higher sensitivities for pyridostigmine also could be achieved at this low wavelength ($\epsilon_{254nm} < 2500$, $\epsilon_{214nm} > 7000$).

Bioanalysis

Iodide ion [4,5], dipicrylamine [3] and tetraphenylborate [11] have been reported as ion-pairing reagents for the extraction of neostigmine and pyridostigmine. Extraction recoveries with iodide were only 30% compared with the near-quantitative recoveries with the two other reagents. The use of picrate anion to extract a variety of quaternary ammonium compounds is well documented [12]. Due to its ready availability and utility over a broad pH range we examined picrate anion as a counter ion for biological extractions. To remove the picrate anion, which gives an interfering peak on the liquid chromatographic tracings, and to obtain cleaner extracts, a back-extraction step with 0.001 *M* TBA^+ HSO_4^- was employed. The TBA^+ helps to partition the analytes into the aqueous phase and keeps the picrate anion into the organic phase. Using untreated glassware, analysis of spiked (100 ng/ml) serum or aqueous samples afforded low recoveries and irrepro-

ducible results. Recoveries ranged from 42 to 78% for neostigmine and from 56 to 85% for pyridostigmine. Substitution of picrate anion with dipicrylamine at pH 9 did not improve recoveries. These low and variable recoveries can be explained by losses due to adsorption to glassware and/or interphase. Treatment of the glassware to deactivate adsorption sites could minimize this effect. As silanization was reported ineffective [12], another approach was used. Deactivation with TMA^+Cl^-, as described in the experimental section, increased extraction reproducibility and recovery (Table I) to a satisfactory level. Variation of extraction pH (4 to 9) had no pronounced effect on extraction behavior.

TABLE I

EXTRACTION RECOVERY

Substance	$\bar{x}$ (%)	S.D. (%)	C.V. (%)	n	Concentration (ng/ml)
Pyridostigmine	96.4	3.8	3.9	9	80
Neostigmine	99.1	2.4	2.5	9	100
Edrophonium	87.8	2.7	3.1	9	100

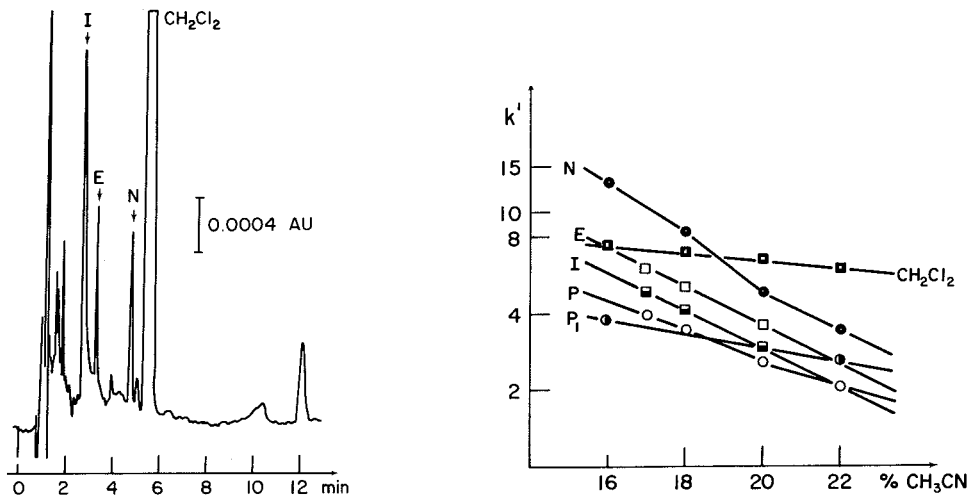

Fig. 4. Liquid chromatographic trace from the injection of an extract from a serum sample containing 53 ng/ml neostigmine. Eluent: 0.01 *M* $C_7H_{15}SO_3^-Na^+$, 0.01 *M* NaH_2PO_4 and 0.0025 *M* TMA^+Cl^- in acetonitrile—water (20:80, v/v), pH 3.0. Column: 5-μm Ultrasphere Octyl (15 × 0.46 cm). Flow-rate, 2 ml/min. Detection at 214 nm 0.004 a.u.f.s. Peaks: I = interference, E = edrophonium (internal standard), N = neostigmine and CH_2Cl_2 = dichloromethane present in the injection solution.

Fig. 5. Separation possibilities of analytes (N, neostigmine; P, pyridostigmine; E, edrophonium) and interference (I, CH_2Cl_2, Pi = picrate). Relationship between capacity ratios (k') and acetonitrile content of mobile phase. Column: 5-μm Ultrasphere Octyl (15 × 0.46 cm); eluent: 0.01 *M* $C_7H_{15}SO_3^-Na^+$, 0.01 *M* NaH_2PO_4 and 0.0025 *M* TMA^+Cl^- in acetonitrile—water adjusted to pH 3.0.

Peak height ratios and concentration were linearly related over the range of 0—400 ng/ml for neostigmine, 0—1000 ng/ml for pyridostigmine and 0—1500 ng/ml for edrophonium. The lowest points on the calibration graphs were 10 ng/ml for neostigmine, 30 ng/ml for pyridostigmine and 10 ng/ml for edrophonium. Quantitation down to 5 ng/ml (signal-to-noise ratio > 4) was achievable for all analytes.

Within-day precision of the assay was coefficient of variation (C.V.) 1.5% (n=10, $\bar{x}$ = 100 ng/ml) for neostigmine, C.V. 1.7% (n=10, $\bar{x}$ = 80 ng/ml) for pyridostigmine and C.V. 1.5% (n=10, $\bar{x}$ = 100 ng/ml) for edrophonium.

Chromatography of biological extracts showed several peaks on the liquid chromatographic tracing other than those from the analytes (Fig. 4). The dichloromethane dissolved in the aqueous back-extraction phase gave rise to a major peak (CH_2Cl_2 in Fig. 4). A large peak interfered with pyridostigmine but was not always present (I in Fig. 4). No interferences were found at the elution volumes of neostigmine and edrophonium. There was a selectivity difference for the dichloromethane peak and the large interference compared to the analytes. The concentration of acetonitrile in the mobile phase could be used to regulate the separation of analytes from these interfering peaks

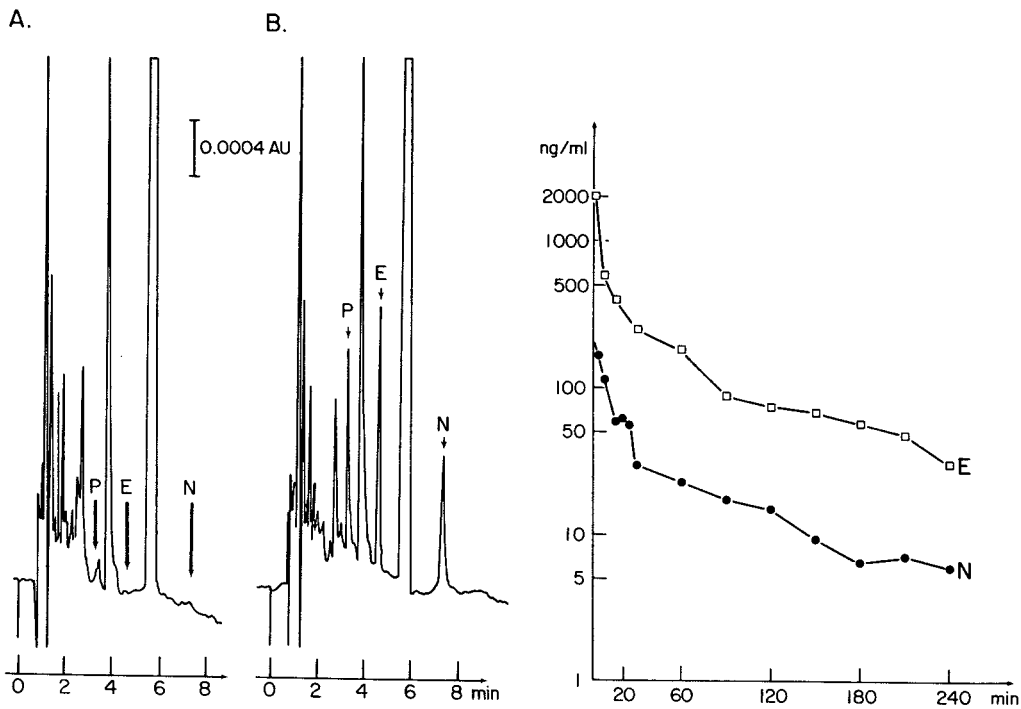

Fig. 6. Liquid chromatographic trace of extracts from blank (A) and spiked (B) serum samples using acetonitrile—water (17 : 83, v/v) in the mobile phase. Other conditions as in Fig. 4.

Fig. 7. Concentration versus time curves for neostigmine (N), and edrophonium (E) after intravenous administration. Infusion, 2 min; 0.5 mg/kg for edrophonium and 0.05 mg/kg for neostigmine.

(Fig.5). Use of 20% acetonitrile for neostigmine and edrophonium assays (Fig.4) and 17% for pyridostigmine assays (Fig.6) provided the optimum resolution. No attempts were made in this study to optimize parameters for the detection of the 3-hydroxy metabolites of pyridostigmine and neostigmine in biological fluids.

The procedure was employed to examine the pharmacokinetics of neostigmine, pyridostigmine and edrophonium. Examples of concentration versus time curves for neostigmine and edrophonium are shown in Fig. 7. Serum concentrations could be followed up to 4 h after administration for neostigmine and beyond for edrophonium. The procedure also may be used to detect these drugs in urine.

CONCLUSION

The proposed method provides several advantages over methods currently available. An improved extraction procedure is used that gives better efficiencies and greater reproducibility. The method does not rely on thermal dequaternization processes that are difficult to control. It is a versatile method that can be used to analyze edrophonium and urine samples as well. Minor modifications would allow analysis of the 3-hydroxy metabolite of neostigmine. The method has the potential to monitor drug levels of pyridostigmine and neostigmine in myasthenia gravis patients.

ACKNOWLEDGEMENTS

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CHROMBIO. 595

THE USE OF A PRE-COLUMN FOR THE DIRECT HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF THE ANTI-DEPRESSANTS CLOVOXAMINE AND FLUVOXAMINE IN PLASMA

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SUMMARY

A high-performance liquid chromatographic system for the direct determination of the antidepressants clovoxamine and fluvoxamine in plasma is described. The primary amines are derivatized with the fluorogenic reagent fluorescamine in order to increase the sensitivity and selectivity, but also to decrease the polarity of the amines. The band broadening of some combinations of pre-column and analytical column is compared for the fluorescamine derivative of fluvoxamine. The combination of a pre-column containing RP-2 and an analytical column containing RP-8 has been successfully used for the determination of the two antidepressants in plasma. At relatively low concentrations of the drugs, a simple step-gradient elution is required for the removal of a large proportion of the more polar components of the samples. Concentrations in the range of 10—1000 ng clovoxamine per ml plasma were determined by means of external standardization and show a good correlation with the data of a more laborious gas chromatographic method. The detection limits for clovoxamine and fluvoxamine are approximately 3 ng/ml in plasma.

INTRODUCTION

During the last decade, high-performance liquid chromatography (HPLC) has become an important method of analysis, especially in the field of pharmaceutical chemistry. Here, a simple gas chromatographic separation is often not possible because of the nonvolatility, thermolability, and polarity of the substances. At this moment, many HPLC papers are also devoted to the determination of drugs and their metabolites in biological samples. Generally, the combined problems of a complex matrix and the trace level make this type of analysis rather difficult. For pre-concentration and/or clean-up, a more or less extensive and time-consuming pretreatment of the samples is often necessary. Moreover, during this step, considerable losses

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may occur, so that the choice of a good method of internal standardization is very critical. Therefore, one of the present research topics in HPLC is the reduction of the pretreatment of biological and other samples by use of selective and sensitive detection modes. Another interesting development is the application of pre-columns for clean-up [1—3] and enrichment [4—8]. Further, the pre-columns also act as so-called guard columns and thus increase considerably the life of the main column.

The preparation of such a pre-column must be as simple and economic as possible in order to limit the time and cost of renewal. In addition it is essential that the pre-column has sufficient capacity and that it should make only a small contribution to the band broadening. For minimization of the band broadening in the enrichment mode, back-flushing of the pre-column has been described [5,7]. However, the filter effect of the pre-column is then less efficient. The contributions of the pre-column (p) and analytical column (a) to the total variance are additive, i.e. from the equation for the total variance,

$$\sigma^2_{\text{total}} = \left(\frac{t_R^2}{N}\right)_p + \left(\frac{t_R^2}{N}\right)_a, \quad (1)$$

it can be seen that for a pre-column dry-packed with relatively large, and thus inexpensive, particles, the low plate number (N) must be compensated by a relatively small retention time (t_R). This can be effected by a small length of the pre-column [8], but this may lead to an insufficient capacity. Alternatively, one can use a pre-column packing material different from that of the analytical column. This principle has been reported by Eisenbeiss et al. [6] for the determination of polycyclic aromatic hydrocarbons in water. The pre-column and analytical column were packed with a non-specified Merck packing and RP-18, respectively.

In the present study, some combinations of stationary phases for a relatively long pre-column (5 cm) and the analytical column have been compared for the analysis of the fluorescamine derivative of the antidepressant fluvoxamine. Subsequently, a system with a relatively small band broadening was investigated for the determination of fluvoxamine and clovoxamine in plasma after a pre-chromatographic derivatization with fluorescamine.

EXPERIMENTAL

Materials

Fig. 1 shows the structures of the antidepressants clovoxamine and fluvoxamine* (Philips-Duphar, Weesp, The Netherlands), which are now under clinical investigation. These were dissolved in distilled water at a concentration of 250 mg/l. Dilutions of this stock solution were made as required. The fluorogenic reagent fluorescamine (Fluram, Hoffmann-La Roche, Nutley, NJ, U.S.A.) was dissolved in acetone (analytical-reagent grade) at a

*Actually, the drugs are the fumarate and maleate salt, respectively, of the amines in Fig. 1.

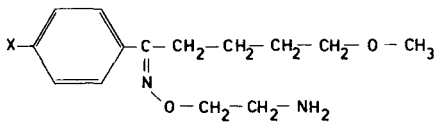

Fig.1. Structures of clovoxamine (X = Cl) and fluvoxamine (X = CF_3).

concentration of 1 mg/ml. The pre-column was a stainless-steel tube (5 cm × 4.6 mm I.D.) dry packed with different LiChrosorb and LiChroprep RP-materials (Merck, Darmstadt, G.F.R.). The analytical column was a stainless-steel tube (15 cm × 4.6 mm I.D.) packed by a slurry technique with LiChrosorb RP-8 (Merck) of average particle size 7 μm. All other chemicals were of analytical-reagent grade.

Apparatus

The HPLC pump was an Orlita DMP-AE-10.4 pump (Orlita, Giessen, G.F.R.); the injection port was a Rheodyne six-port valve (Rheodyne, Berkeley, CA, U.S.A.) with a 200-μl loop. For on-line/off-line switching, a second Rheodyne six-port valve was inserted between the pre-column and the main column. Detection was carried out with a Perkin-Elmer Model 204A fluorescence spectrophotometer ($\lambda_{exc.}$ = 380 nm, $\lambda_{em.}$ = 470 nm). For comparison purposes, a variable-wavelength UV detector (Schoeffel Spectroflow Monitor SF 770, Schoeffel Instruments, Westwood, NJ, U.S.A.) was used.

Procedure

The human and canine plasmas were stored frozen. After thawing and centrifugation, 1 ml of plasma was spiked with a known amount of clovoxamine or fluvoxamine, and subsequently 1 ml of a 0.01 *M* phosphate buffer pH 7 and 1 ml of the fluorescamine solution were added. After centrifugation, 200 μl of the supernatant were injected into the HPLC system. Methanol—0.01 *M* phosphate buffer pH 7 (62:38, v/v) was used as the mobile phase at a flow-rate of approximately 1 ml/min. After approximately five injections, the system was purged for about 5 min with methanol.

However, after injection of sample solutions with concentrations of the amines below approximately 100 ng/ml, the pre-column containing 32-μm RP-2 was first flushed for about 5 min with methanol—phosphate buffer pH 7 (50:50, v/v) with the switching valve in the waste position. Next the valve between the two columns was switched on-line and the derivatives of the amines were eluted with methanol—phosphate buffer pH 7 (62:38, v/v), i.e. a mobile phase containing a higher proportion of methanol. At the end of each analysis, the pre-column and analytical column were flushed for about 5 min with methanol. Finally, the columns were equilibrated for about 10 min with methanol—buffer (50:50, v/v) and methanol—buffer (62:38, v/v), respectively. With real plasma samples, the same procedure was followed.

RESULTS AND DISCUSSION

Fluorescamine reaction

The fluorescence signal of the fluorescamine derivatives of primary amines is influenced by the kinetics of the derivatization reaction and also by dielectric effects and some other effects of the medium [9—11]. For the direct injection of a polar sample such as plasma, a reversed-phase chromatographic system is necessary. Fortunately, the results of Frei et al. [10] with a similar system, i.e. oxytocin—fluorescamine, point to the fact that the net fluorescence decreases with a decrease in solvent polarity, so that it is advantageous to use a polar mobile phase. Data published for different primary amines indicate that the pH optimum is between about 6 and 10.5 [10,12,13]. For the present system, the maximum fluorescence intensity was obtained at pH 7—8. Because the life of RP-8 columns appeared to be longer at pH 7 than at pH 8, the mobile phase was buffered to pH 7. A phosphate buffer was preferred to a borate buffer, since borate ions may complex with the fluorophore and, in this way, suppress the fluorescence [14].

For the excess of fluorescamine, very different values have been reported, the required molar excess varying from 3.6 [15] to approximately 1000 [13]. For concentrations of 0.5—1 μg/ml clovoxamine and fluvoxamine, a molar fluorescamine-to-amine ratio of approximately 300—400 was found to be sufficient. Although fluorescamine itself was known to be non-fluorescent [9,16], the fluorescamine solution was observed to be fluorescent. Probably, this is due to one or more hydrolysis products. For a derivatization reaction prior to separation, this does not interfere, because the excess of reagent and its hydrolysis products are separated from the derivative in the chromatographic system. It is true that the reaction rate is dependent on the concentrations of the amine and fluorescamine; with the conditions used in this study the maximum signal was always obtained within a few minutes.

Packing material of the pre-column

As already described in the introduction, the choice of the pre-column is rather critical. Briefly, the following demands have to be met: (1) rapid and economic preparation; (2) sufficient capacity; (3) small contribution to band broadening, and (4) suitable for clean-up and pre-concentration.

A relatively large pre-column (5 cm × 4.6 mm I.D.) can easily be filled by a simple dry-packing technique and the capacity of this column was found to be sufficient for at least 250 μl plasma. For the present analytical system, i.e. RP-8 with methanol—0.01 *M* phosphate buffer pH 7 (62:38, v/v) as the mobile phase, the use of RP-8 as the packing material for this pre-column causes an intolerable band broadening because of too long a retention time. With the use of RP-2, the retention time of the derivatives of clovoxamine and fluvoxamine and, consequently, the band broadening caused by the pre-column are much less. Retention times and capacity factors of the analytical column and some pre-columns are compared in Table I.

Plate numbers for the analytical column and the pre-column were typically about 4000 and 200, respectively. Using eqn. 1, one can now calculate that for the RP-8 pre-column $\sigma_{total} = 1.9\sigma_{anal.}$ and for the 32-μm RP-2 pre-

TABLE I

RETENTION TIMES AND CAPACITY FACTORS OF THE FLUORESCAMINE DERIVATIVE OF FLUVOXAMINE FOR SOME COLUMNS

Mobile phase: methanol—0.01 *M* phosphate buffer pH 7 (60:40, v/v).

Column	Retention time (min)	Capacity factor
Analytical column* RP-8 7 μm	16.8	15.8
Pre-column** RP-8 25—40 μm	6.2	15.9
Pre-column RP-2 32 μm	2.2	8.5
Pre-column RP-2 10 μm	1.7	5.6

*15 cm × 4.6 mm I.D.
**5 cm × 4.6 mm I.D.

column $\sigma_{total} = 1.1\sigma_{anal.}$. The behaviour of the much more expensive 10-μm RP-2 is similar to that of 32-μm RP-2. It should be noted that the observed total standard deviation is often higher than the theoretical one, because connections and the valve between the columns can contribute to band broadening [17—19]. A possible disadvantage of RP-2 with respect to the more apolar RP-8 is the smaller recovery of a pre-concentration step. However, for 1-ml sample solutions with concentrations in the nanogram range, the recovery with water as the mobile phase during the pre-concentration was found to be 100%. Therefore RP-2 is also useful for the pre-concentration of the derivatives of clovoxamine and fluvoxamine. It should be noted that the derivatization of the amines with fluorescamine makes them much less polar and therefore suitable for pre-concentration in reversed-phase systems. Next to the use of chemical reactions in liquid chromatography as an aid to improving the detection properties and selectivities for certain groups of compounds, the modification of the polarity seems also a very useful aspect of derivatization.

Determination of clovoxamine and fluvoxamine in plasma

The system of pre-column 32-μm RP-2 and analytical column 7-μm RP-8 with as the mobile phase methanol—buffer pH 7 (62:38, v/v) was used for the determination of clovoxamine and fluvoxamine in plasma. This system is suitable for the direct analysis of plasma; a chromatogram of a plasma sample is presented in Fig. 2a. For the concentration range of 100—2500 ng of fluvoxamine per ml, a linear calibration curve was obtained ($r = 0.9999$).

The detection limit (signal-to-noise ratio 2:1) for this procedure is approximately 25 ng/ml plasma. By means of repeated analyses of a plasma sample containing approximately 600 ng of fluvoxamine per ml, the reproducibility was shown to be 3% (rel. S.D.; $n = 6$). The efficiency of the analytical column decreases slowly, but the column can be used for several weeks. As for the pre-column, because of deterioration it is necessary to replace this

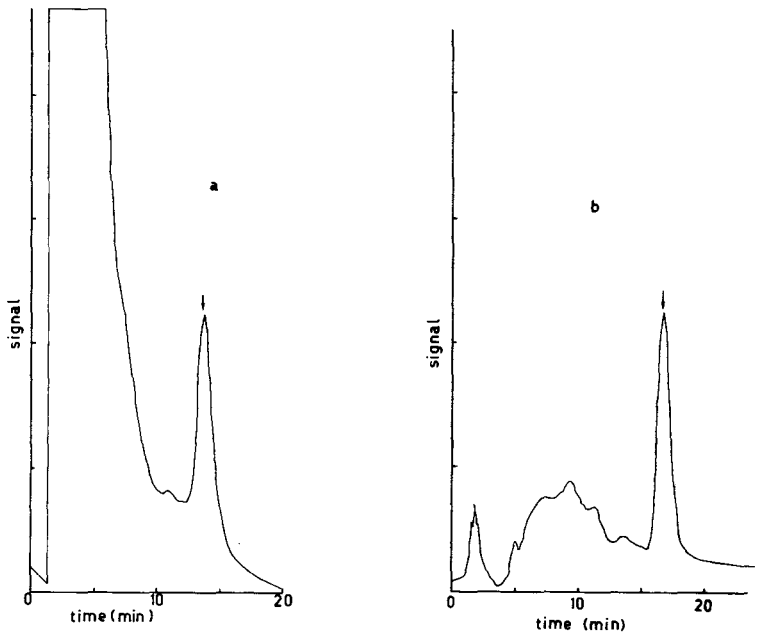

Fig. 2. HPLC chromatograms of a canine plasma sample containing 652 ng fluvoxamine per ml. Chromatographic conditions: pre-column, 32-μm RP-2, 5 cm × 4.6 mm I.D.; main column, 7-μm RP-8, 15 cm × 4.6 mm I.D.; mobile phase, (a) methanol—0.01 *M* phosphate buffer pH 7 (62:38, v/v), (b) methanol—0.01 *M* phosphate buffer pH 7 (62:38, v/v) after previous elution for 5 min with methanol—0.01 *M* phosphate buffer pH 7 (50:50, v/v); flow-rate, 1 ml/min. Detection: pre-separation reaction with fluorescamine; fluorescence detector, $\lambda_{exc.}$ = 380 nm and $\lambda_{em.}$ = 470 nm. The retention time of the fluvoxamine derivative in chromatogram (b) is higher, because the first part of the elution takes place in a gradient.

column after 5—10 samples. Moreover, it is useful to flush the whole system regularly with, for example, methanol in order to ensure the elution of relatively lipophilic substances.

The fluorescence signal of a spiked plasma sample is lower than that of the standard solution in water with the same concentration. Confirmation was obtained that the partial deproteination of plasma by the addition of the acetonic solution of fluorescamine did not cause any loss of the amine of interest. The reduction of the signal is due to the decrease of the excess of fluorescamine brought about by its reaction with biogenic primary amines, especially amino acids. This was proved by an experiment whereby the plasma was added after the reaction between fluvoxamine and fluorescamine; the peak heights of the standard and the plasma sample were then equal. It is true that the excess of fluorescamine can be adjusted, but this also widens the band in the front of the chromatogram. Fortunately, the excess concentration of fluorescamine had no noticeable influence on the linearity of the calibration curve.

The above-mentioned detection limit is determined by the tailing band in the chromatogram, which is caused by polar endogenous compounds and to

a large extent, as demonstrated, by compounds formed during the derivatization reaction. Therefore, for concentrations below approximately 100 ng/ml, a simple step gradient is required in order to remove a large proportion of the more polar compounds from the pre-column prior to the elution of the derivatives of clovoxamine or fluvoxamine. It was found to be possible to flush the pre-column for about 5 min with methanol—buffer pH 7 (50:50, v/v) without the derivatives being eluted. Fig. 2b shows the chromatogram of the same sample as in Fig. 2a obtained after the use of this step gradient. The latter procedure enables the use of the higher sensitivity ranges of the fluorescence detector; consequently, it is then necessary to flush the columns with methanol each time after the analytical step, i.e. elution with methanol—buffer (62:38, v/v). It is evident that this procedure is more time-consuming than the isocratic method, especially in consequence of the equilibration times of the columns. The detection limit then becomes approximately 3 ng/ml plasma for both clovoxamine and fluvoxamine and a further decrease was shown to be possible by means of larger injection volumes. Plasma samples have almost always a higher concentration, but then such a preconcentration step can be useful for increasing the precision of the concentrations just above the detection limit. The detection limit of clovoxamine and fluvoxamine with UV detection at 254 nm was improved about twenty times by the use of fluorescence detection after derivatization with fluorescamine; also the selectivity of the latter method is considerably superior.

TABLE II

COMPARISON OF CONCENTRATIONS IN ng/ml OF CLOVOXAMINE IN PLASMA SAMPLES AS FOUND BY GAS AND LIQUID CHROMATOGRAPHY

The HPLC data of samples No. 1—3 have been obtained with the isocratic method and the data of samples No. 4—7 with the step-gradient method. For experimental details, see text.

Sample No.	Method	
	GC	HPLC
1	803	803
2	686	703
3	561	604
4	72	79
5	23	26
6	23	21
7	11	15

Table II compares some typical data of the method described in this paper with the results obtained by the gas chromatographic analysis used in our laboratory [20]. Similar results have been obtained for fluvoxamine samples. If it is remembered that the relative standard deviation of the gas chromatographic analysis generally is about 3—15% (depending on the range), the correlation between the two methods can be considered good. The gas chromatographic method is rather time-consuming as compared with the HPLC method; it includes extraction with isooctane, two re-extractions with

phosphoric acid, hydrolysis at 90°C, and again extraction with isooctane. The recovery is about 60% and, therefore, internal standardization is necessary. In conclusion it can be stated that the HPLC method is simpler and more suitable for automation. As for the analysis time, for the gas chromatographic analysis many samples can be pretreated at the same time. Therefore, the saving of time is only considerable if one or a few samples are analysed.

CONCLUSION

For the analysis of plasma by means of HPLC, direct injection of the (diluted) sample is possible if a suitable pre-column, and a selective derivatization and/or detection mode are used. Probably, this is also true for the analysis of other complex samples. The actual choice of the packing materials of pre-column and analytical column depends on the polarity of the compound (or derivative) to be assayed, on the ratio of the capacity factors of the two columns and on the nature of the sample. The principle of using different materials in pre-column and analytical column appears to be promising for certain applications of pre-columns for clean-up and pre-concentration of biological and other samples. The application of step gradients can enhance the potential of such systems (see also ref. 21).

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CHROMBIO. 600

DETERMINATION OF PROPRANOLOL AND ITS MAJOR METABOLITES IN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITHOUT SOLVENT EXTRACTION

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SUMMARY

Fast, reliable, specific and sensitive methods are reported to accurately quantitate unchanged propranolol in plasma, and its major metabolites in plasma and urine after enzymatic hydrolysis without the need for solvent extraction. These methods enable the analyst to process a large number of propranolol samples in one working day and should prove valuable to clinical laboratories demanding both speed and specificity in an assay.

INTRODUCTION

Propranolol, a drug widely used in the treatment of hypertension, angina, cardiac arrhythmias and thyrotoxicosis is almost completely metabolized in man [1]. The major metabolites identified in plasma and urine include: conjugates of propranolol and 4-hydroxypropranolol and free α-naphthoxylactic acid [2—7]. Methods presently available to measure these metabolites use either spectrofluorometry [8], thin-layer chromatography [2,9], gas chromatography [10—13], gas chromatography—mass spectrometry [3,14,15] or high-performance liquid chromatography (HPLC) [4,16—20]. All these methods require extensive sample work-up. We report for the first time an assay procedure that requires only a simple protein precipitation step yet allows one to measure all these compounds in one chromatographic run. For clinical laboratories not concerned with measuring metabolites, a method is reported which affords an accurate measure of unconjugated propranolol in plasma.

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EXPERIMENTAL

Standards and reagents

Propranolol HCl, 4-hydroxypropranolol HCl, α-naphthoxylactic acid, propranolol glycol and N-desisopropylpropranolol were kindly supplied by I.C.I. (Macclesfield, Great Britain). α-Naphthol and α-naphthoxyacetic acid were obtained from Aldrich (Milwaukee, WI, U.S.A.) and Trans World Chemicals (Washington, DC, U.S.A.), respectively.

The internal standard N-ethylpropranolol was prepared according to the method of Wood et al. [21]. However, the crude product contained an impurity that interfered with the assay and could not be separated by solvent extraction. Therefore it was necessary to purify the product by reversed-phase HPLC and solvent extraction. The recovered N-ethylpropranolol was stored in methanol, which was used to make up three concentrations for use as internal standards: one in acetonitrile, for unchanged propranolol in plasma and two in dilute phosphoric acid, for hydrolyzed plasma and at five-fold concentration for use in the urine assay.

Acetonitrile (UV grade) and methanol were supplied by Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). All other chemicals were analytical grade.

Instrumentation

A Varian Model 8500 high-performance liquid chromatograph equipped with a Perkin-Elmer 650-10 LC fluorescence spectrophotometer, a LiChrosorb RP-8 column (25 cm × 4.6 mm I.D.; 10 μm particle size; Altex Assoc., Berkeley, CA, U.S.A.) and a LiChrosorb RP-2 precolumn (4 cm × 3.2 mm I.D.; 10 μm particle size; Altex) were used. The fluorescence output was recorded on a dual-channel recorder (Linear Instruments, Irvine, CA, U.S.A.). Injections were made with a 100-μl Hamilton syringe through a Valco CV-6-UHPa-N60 sweep-flow injector equipped with a 100-μl loop.

Method 1: Measurement of unconjugated propranolol in plasma

Daily standard curves were prepared as follows. A 1.5 μg/ml propranolol (free base) solution in water was prepared from a 50 μg/ml propranolol HCl aqueous stock solution. A 0.5-ml aliquot of this standard solution was added to 2 ml of drug-free human plasma to make up a 300 ng/ml standard. It was then serially diluted with plasma to yield concentrations of 150, 75, 30, 20, 10 and 5 ng/ml.

Plasma samples were processed by transferring a 0.2-ml quantity into an Eppendorf polypropylene 1.5-ml micro test tube (Brinkmann No. 2236411-1, Brinkmann Instruments, Westbury, NY, U.S.A.), and 0.4 ml of the N-ethylpropranolol solution in acetonitrile was added. After the sample was vortexed for 15 sec it was centrifuged for 2 min at 12,800 *g* using an Eppendorf Microcentrifuge, Model 5412. The clear supernatant was transferred to a disposable glass culture tube (13 × 100 mm) and evaporated to an approximate volume of 0.1—0.2 ml under a gentle stream of nitrogen. After adding 0.2 ml of 0.05 *M* phosphoric acid and brief vortexing, a 50—90-μl aliquot was injected onto the column. The mobile phase was composed of 360 ml acetonitrile, 180 ml methanol and 70 ml of 0.0871 *M* phosphoric acid diluted to one liter with glass

distilled water. The flow-rate was 100 ml/h. The fluorometer was set at an excitation wavelength of 230 nm and an emission wavelength of 340 nm. Both slit openings were set at 20 nm. The fluorometer was operated at a sensitivity range of 0.1 and normal power gain. The output was 1 V and sensitivity varied by changing the voltage spans on the dual-pen recorder. One pen was always fixed to measure the internal standard.

Method 2: Measurement of propranolol, 4-hydroxypropranolol and α-naphthoxylactic acid in plasma and urine after enzymatic hydrolysis

The plasma and urine standards were made up as follows: the most concentrated plasma standard containing 2 μg/ml of α-naphthoxylactic acid, 1 μg/ml of propranolol and 4-hydroxypropranolol was prepared by evaporating to dryness 0.5 ml of 20 μg/ml α-naphthoxylactic acid in methanol. Then 0.5 ml of propranolol (10 μg/ml in water) and 0.25 ml of 4-hydroxypropranolol (20 μg/ml in 0.01 *M* phosphoric acid including 5 mg/ml ascorbic acid to minimize oxidation) were added. These were diluted with 4.25 ml of human (drug-free) plasma. Additional standards were prepared by serial dilution with more blank plasma. Urinary standards were prepared slightly differently because of the instability of 4-hydroxypropranolol in urine. A urine sample containing 20 μg/ml of propranolol and α-naphthoxylactic acid was first prepared and then serially diluted with more blank urine. A series of disposable culture tubes were prepared each containing 0.1 ml of a 200 mg/ml solution of ascorbic acid. Then 0.025, 0.05, 0.1, or 0.2 ml of 4-hydroxypropranolol (20 μg/ml protected by ascorbic acid) was added. To each of the tubes was added 0.2 ml of the appropriate propranolol—α-naphthoxylactic acid mixture and a 0.2-ml quantity of the 5-fold concentrated internal standard solution.

The assays were performed as follows: a 0.2-ml quantity of urine or a 0.4-ml quantity of plasma were mixed with 0.2 ml aqueous internal standard in a disposable glass culture tube. The urine sample was diluted with 0.2 ml of water. A 0.1-ml quantity of ascorbic acid (200 mg/ml), 0.04 ml of acetate buffer (1.4 *M*, pH 5.5) and 25 mg of β-glucuronidase/aryl sulfatase (400 units/mg, Sigma G 0751, St. Louis, MO, U.S.A.) were added. The mixture was incubated at 37°C for 90 min. After precipitating the protein with 0.8 ml of acetonitrile, the resulting mixture was transferred to a 1.5-ml microcentrifuge tube and centrifuged for 3 min at 12,800 *g*. A 0.6-ml amount of the clear supernatant was removed and 0.3 ml of 0.05 *M* phosphoric acid was added. It should be noted that no evaporation was necessary. A 40—50-μl aliquot was injected onto the column. The eluent was composed of 300 ml of acetonitrile, 90 ml of methanol and 66 ml of 0.0871 *M* phosphoric acid diluted to one liter with glass distilled water. The flow-rate was 100 ml/h. Since 4-hydroxypropranolol fluoresces differently when compared to propranolol and α-naphthoxylactic acid, the fluorometer's emission wavelength was first set to 430 nm to measure 4-hydroxypropranolol. The excitation wavelength was fixed at 310 nm. After the elution of 4-hydroxypropranolol (about 6 min), the emission was then changed to 350 nm for the detection of propranolol and α-naphthoxylactic acid. Slit widths were set at 20 nm and the sensitivity range set at 0.1. The recorder output was set at 1 V and a normal power gain was used. The spans of the recorder were used to vary sensitivity.

By omitting the enzymatic hydrolysis procedure one can directly measure α-naphthoxylactic acid and unconjugated propranolol. For these assays the fluorescent excitation wavelength was set at 230 nm and the emission at 340 nm.

RESULTS AND DISCUSSION

Fig. 1 includes chromatograms of blank plasma and a typical patient plasma obtained by the direct injection assay. The total elution time per assay is 8 min. All the known metabolites of propranolol elute before propranolol and do not interfere with the assay. The limit of detection using 0.2 ml of plasma is 2 ng/

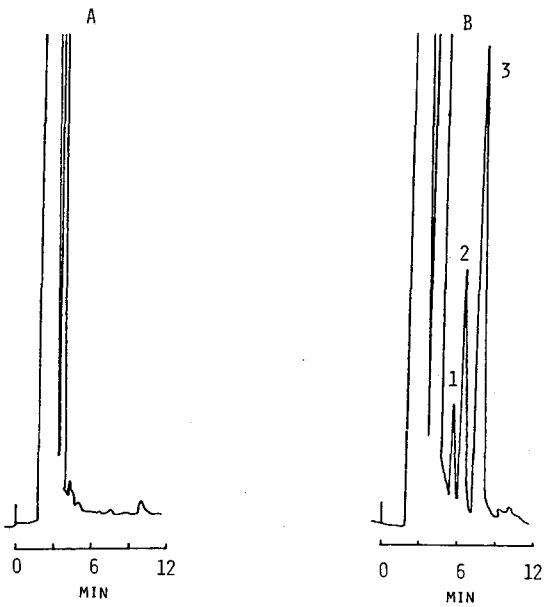

Fig. 1. Assay of unchanged propranolol in plasma (see Method 1). (A) Drug-free human plasma. (B) 2-h plasma sample obtained from an angina patient taking 20 mg of propranolol every 6 h. Peaks: 1 = α-naphthoxyacetic acid; 2 = propranolol, 34 ng/ml; 3 = N-ethyl-propranolol (internal standard). Fluorometer settings: excitation = 230 nm; emission = 340 nm.

TABLE I

INTRA- AND INTER-ASSAY VARIATION OF PROPRANOLOL IN PLASMA

Spiked concentration (ng/ml)	Intra-assay C.V. (%) (n = 5)	Inter-assay C.V. (%) (n = 3, over 2 weeks)
10.05	1.5	3.9
30.14	n.d.*	3.8
75.35	0.9	3.9
150.69	2.2	n.d.*
301.38	1.1	5.5

*Not determined.

ml. The precision and accuracy of the assay are shown in Table I. Intra- and inter-assay coefficients of variation (C.V.) were 2.2%, and 5.5%, respectively, over the concentration range of 10—300 ng/ml. Our method was compared with a double extraction technique developed in this laboratory earlier [22]. It involved alkalinizing a plasma sample containing propranolol with sodium

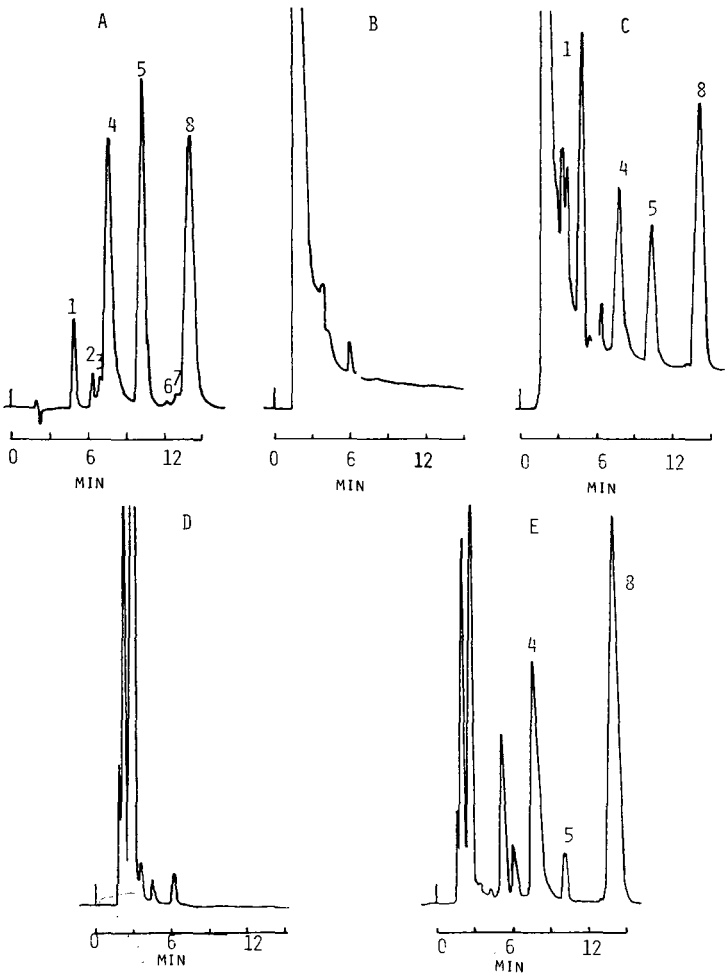

Fig. 2. Assay of propranolol and metabolites in plasma (see Method 2). (A) Standard mixture. Peaks: 1 = 4-hydroxypropranolol; 2 = N-desisopropylpropranolol; 3 = propranolol glycol; 4 = α-naphthoxylactic acid; 5 = propranolol; 6 = α-naphthol; 7 = α-naphthoxyacetic acid; 8 = N-ethylpropranolol (internal standard). (B) Hydrolyzed human plasma blank. (C) Hydrolyzed 1-h plasma sample from an angina patient taking 80 mg of propranolol every 6 h. Peaks: 1 = 490 ng/ml 4-hydroxypropranolol; 4 = 770 ng/ml α-naphthoxylactic acid; 5 = 490 ng/ml propranolol; 8 = N-ethylpropranolol (internal standard). (D) Unhydrolyzed human plasma blank (enzyme omitted). (E) Unhydrolyzed patient sample, same as C. Peaks: 4 = 770 ng/ml α-naphthoxylactic acid; 5 = 128 ng/ml propranolol; 8 = N-ethylpropranolol (internal standard). Fluorometer settings: B and C, excitation = 310 nm; emission = 430 nm for the first 6 min, then changed to 350 nm. D and E, excitation = 230 nm; emission = 340 nm.

carbonate buffer and extracting it with diethyl ether. The propranolol was then extracted into phosphoric acid and injected onto the column. Comparison of the values obtained using the two techniques to assay three samples from angina patients differed from one another by 6.4%, −3.7% and 1%, respectively, even though the analyses were done over a six-month period.

Drugs tested for interference by direct injection onto the column included hydralazine, hydrochlorthiazide, triamterene, furosemide, procainamide and quinidine. Only quinidine was found to interfere.

Approximately 75% of propranolol in plasma, virtually all propranolol in urine and 4-hydroxypropranolol in plasma and urine appear as their conjugates. Enzymatic hydrolysis is therefore required to liberate the compounds. Fig. 2A shows how 4-hydroxypropranolol, α-naphthoxylactic acid, propranolol and the internal standard N-ethylpropranolol are separated from the minor metabolites of propranolol, namely N-desisopropylpropranolol, propranolol glycol, α-naphthol and α-naphthoxyacetic acid. Chromatograms of patient plasma and urine samples and blanks after enzymatic hydrolysis are shown in Fig. 2B, C and Fig. 3. It can be seen that there is no interference from normal biological constituents. The same patient plasma sample was analyzed before hydrolysis for α-naphthoxylactic acid and unconjugated propranolol and the chromatogram is shown in Fig. 2E, again there is no interference from blank plasma (Fig. 2D).

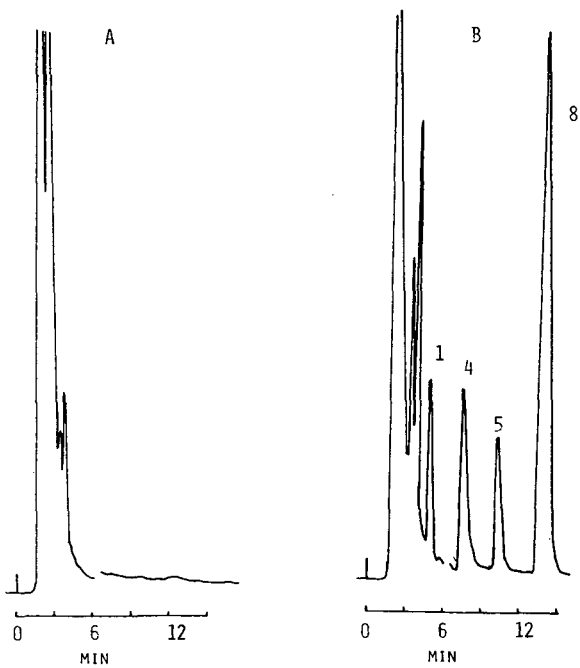

Fig. 3. Assay of propranolol and metabolites in urine (see Method 2). (A) Hydrolyzed human urine blank. (B) Hydrolyzed 2—3-h urine collection from a hypertensive patient taking 10 mg of propranolol every 6 h. Peaks: 1 = 3.3 μg/ml 4-hydroxypropranolol; 4 = 3.27 μg/ml α-naphthoxylactic acid; 5 = 2.36 μg/ml propranolol; 8 = N-ethylpropranolol (internal standard). Fluorometer settings: excitation = 310 nm; emission = 430 nm for the first 6 min, then changed to 350 nm.

Concentration of propranolol conjugates can thus be determined by the difference of the propranolol level measured before and after hydrolysis (Fig. 2C and E). The total chromatographic analysis time is 15 min. This method allows determination of all three compounds even though they have widely different acid—base characteristics. A kinetic study examining the rate of enzymatic hydrolysis revealed that 90 min was sufficient for complete hydrolysis of the conjugates using 10,000 units of enzyme. Pritchard et al. [4] used sodium metabisulfite as an antioxidant during hydrolysis. In contrast, the present method utilizes ascorbic acid. A comparison of these two antioxidants as well as sodium bisulfite was made using metabolites-spiked plasma and urine.

TABLE II

PERCENT OF PROPRANOLOL AND METABOLITES REMAINING IN PLASMA AND URINE FOLLOWING ENZYMATIC HYDROLYSIS* USING ASCORBIC ACID, SODIUM METABISULFITE, AND SODIUM BISULFITE AS ANTIOXIDANTS

Compound	Antioxidant**		
	Ascorbic acid	Sodium metabisulfite	Sodium bisulfite
Propranolol			
Plasma (0.5 μg/ml)	99.4	95.9	94.6
Urine (5.0 μg/ml)	96.7	88.3	88.6
4-Hydroxypropranolol			
Plasma (0.5 μg/ml)	99.9	76.1	72.5
Urine (5.0 μg/ml)	99.2	39.2	46.3
α-Naphthoxylactic acid			
Plasma (1.0 μg/ml)	99.6	91.3	91.5
Urine (5.0 μg/ml)	91.9	71.6	66.3

*10,000 units of enzyme, 90 min, 37°C.
**20 mg of each antioxidant used.

TABLE III

INTRA- AND INTER-ASSAY VARIATION OF PROPRANOLOL AND ITS METABOLITES IN PATIENT PLASMA AND URINE SAMPLES* FOLLOWING ENZYMATIC HYDROLYSIS**

	Propranolol	4-Hydroxypropranolol	α-Naphthoxylactic acid
Plasma			
Intra-day C.V. (%) ($n=5$)	1.6	2.2	2.3
Inter-day C.V. (%) ($n=3$)	1.3	7.1	1.9
Urine			
Intra-day C.V. (%) ($n=5$)	1.4	1.4	0.9
Inter-day C.V. (%) ($n=3$)	1.3	3.9	4.2

*Determinations were made over a 2-week, and 2-month period for plasma and urine samples, respectively.
**10,000 units, 37°C, 90 min.

Duplicate plasma standards containing 1 μg/ml of α-naphthoxylactic acid, 0.5 μg/ml of propranolol and 4-hydroxypropranolol, and urine standards containing 5 μg/ml of all three compounds were incubated with enzyme for 90 min using 20 mg of each of the three antioxidants. The percent of each compound remaining was calculated by comparison to an unincubated standard. The results shown in Table II suggest that ascorbic acid is the best antioxidant for the protection of all three compounds.

Intra- and inter-day assay variations of propranolol and its metabolites determined by assaying patient plasma and urine samples over a period of two weeks for plasma and two months for urine are shown in Table III. The limits of detection of the three compounds are about 20 ng/ml using 0.4 ml plasma and 100 ng/ml using 0.2 ml urine. Lower levels can be measured by use of larger volumes of samples.

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Note

Determination of specific urinary hydroxyproline-containing peptides in patients with lung cancer

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Urinary hydroxyproline is usually taken as a marker of collagen metabolism which has been shown to be altered in a number of diseases (for review see ref. 1). In urine both free and peptide-bound hydroxyproline can be found; this is because some of the hydroxyproline-containing sequences occurring in collagen are not susceptible to cleavage by liver proteases. Early papers [1] were restricted to the estimation of total urinary hydroxyproline. Considerable progress was achieved when, as well as free hydroxyproline, the hydroxyproline-containing urinary peptides were separated by chromatographic methods. Since a very complex mixture is being dealt with, one step separations like gas chromatography failed [2], and several subsequent liquid column chromatographic separations have been used instead [3]. According to the present point of view, however, urinary hydroxyproline does not strictly reflect collagen metabolism since it has been shown that this amino acid is present in other proteins as well; for example, in the first complement subcomponent C1q. These findings resulted in the effort to determine the sequences of urinary hydroxyproline-containing peptides so that they could be attributed to some part of the collagen molecule. At the beginning of these studies it was anticipated that the hydroxyproline-containing sequence most likely to occur in urine would be Gly-Pro-Hyp.

Later, Dubovský and Meyer [4], using molecular sieving on a Bio-Gel P-2 column, demonstrated that urinary hydroxyproline-containing peptides can be categorized into two groups differing in their molecular weight. In a very detailed study by Szymanowicz et al. [3] it was shown that the fraction of low molecular weight peptides represents about 85% of all hydroxyproline-containing peptides while the remaining 15% are bound into much larger

sequences. In the same paper the sequences of 96% of the low molecular weight peptides were established. However, none of the efforts to separate and purify the high molecular weight peptides were successful Also it has been shown that the difference in the profile of hydroxyproline-containing peptides in healthy individuals and in patients with Paget's disease is limited to a change in the ratio of the low and high molecular weight fractions. Concomitantly it was shown that the low molecular weight fraction is devoid of the peptide Gly-Pro-Hyp.

In the present paper we have compared the fraction of urinary low molecular weight hydroxyproline-containing peptides in patients with lung cancer with that present in the urine of healthy individuals.

MATERIALS AND METHODS

Urine was collected from patients over a period of 24 h; control urine samples (24-h collections) were obtained from the laboratory staff. The filtered urine was stored frozen at −20° C until used for chromatography. All urine samples were concentrated ten times on a rotary evaporator at 50° C. The concentrate was acidified by adding acetic acid to a final concentration of 2.5 *M*.

Bio-Gel P-2 chromatography

A 2.5-ml portion of the concentrated sample was loaded on a Bio-Gel P-2 (Bio-Rad, Richmond, CA, U.S.A.) column (50—100 mesh, 40 cm × 2.5 cm; 0.2 *M* acetic acid was used as mobile phase and 2.5-ml fractions were collected. A flow-rate of 1 ml/min was ensured by a low-pressure piston pump (Mikrotechna, Prague, Czechoslovakia). From every fraction 0.5 ml was taken for the hydroxyproline assay using the Hypronosticon test (Organon, Oss, The Netherlands) [5].

QAE-Sephadex A-25 chromatography

Pooled fractions 25—29 from seven subsequent Bio-Gel runs containing small molecular weight peptides were subjected to further fractionation according to the procedure of Szymanowicz et al. [6]. The sample was taken almost to dryness and redissolved in 5 ml of γ-picoline—morpholine—pyridine—water (80:60:40:3820, v/v) mixture to which concentrated acetic acid was added to reach the final pH 9.4. A 1-ml aliquot of this solution was layered on top of a 40 cm × 2 cm QAE-Sephadex A-25 column (Pharmacia, Uppsala, Sweden) that had previously been washed with the above solvent mixture. A complex Varigrad system was used for elution. Chambers 1—3 were filled with 140 ml of the γ-picoline buffer the pH of which was adjusted with concentrated acetic acid to 9.5, 8.5 and 6.5, respectively. Chambers 4 and 5 contained the same volume of 0.5 *M* and 2.0 *M* acetic acid. Fractions of 5 ml were collected. The fractions were evaluated for hydroxyproline content and total ninhydrin value [6]. The flow-rate of 1 ml/min was ensured by a low-pressure piston pump (Mikrotechna).

Chromatography on Dowex 50 M 82

Peptides emerging from the QAE-Sephadex A-25 column between 210 and

300 ml of the eluate were accumulated from repeated runs of the lung cancer urine and separated further on the Dowex 50 M 82 column (Beckman, Munich, G.F.R.).

Amino acid analyses were done on an automated amino acid analyzer (Mikrotechna), using a 50 cm × 1 cm column. Elution was done with a stepwise gradient as follows: 0.2 *M* citrate buffer pH 3.23 for 120 min, 0.2 *M* citrate buffer pH 4.1 for 65 min, and 0.2 *M* citrate buffer 1 *M* with respect to NaCl pH 5.0 for an additional 110 min. The column was operated at 40° C for the first 40 min, then the temperature was raised to 55° C. The flow-rate was kept at 70 ml/h.

Sequence analysis

This was done using the original procedure of Gray and Hartley [7] with the identification of the N-terminal amino acid in the form of the dansyl derivative [8] on silica gel thin layers (Eastman Chromatogram sheets K301R). Standard solutions of dansyl amino acids were prepared by adding 1 ml of the dansyl reagent (6 mg of dansyl chloride in 1 ml of acetone) to an equal volume of amino acid (or peptide) solution. The concentration of the amino acids was 6.5 μmol/ml and the sample was dissolved in 1 ml of 0.1 *M* $NaHCO_3$ to ensure the alkaline reaction of the mixture. The mixture was left overnight; then 8 ml of acetone were added and the diluted sample was centrifuged. The supernatant was spotted directly onto the chromatogram. Dansyl derivatives of peptides were prepared in a similar way. After the reaction was completed, peptides were hydrolyzed in 6 *N* hydrochloric acid for 12 h and the resulting hydrolysate was chromatographed. For two-dimensional development the following solvents were used [8]: first run: benzene—pyridine—acetic acid (16:4:1); second run: chloroform—benzyl alcohol—acetic acid (70:30:3).

The nature of the C-terminal amino acid in tripeptides and hexapeptides was determined enzymatically [9].

RESULTS AND DISCUSSION

As expected the peptide mixture was separated by chromatography on Bio-Gel P-2 into two complex fractions. The low molecular weight fraction showed a clear tendency to separate further into two subfractions (Fig. 1). Of these two overlapping peaks, material occurring in the faster fraction was accumulated and subjected to further investigation.

The peptidic material obtained in this way was transferred to a QAE-Sephadex A-25 column. The result of the separation is shown in Fig. 2. The profile of hydroxyproline-containing peptides obtained from the urine of patients with lung cancer differed from that of controls in the presence of two unusual peaks emerging with the retention volume around 250 ml. This material was accumulated again (as shown by the solid bar in Fig. 2) and subjected to chromatography on a Dowex 50 M 82 column (Fig. 3). The separation of the fraction with the lower retention time is shown in the upper part of the figure, while beneath the profile of the more delayed material is shown.

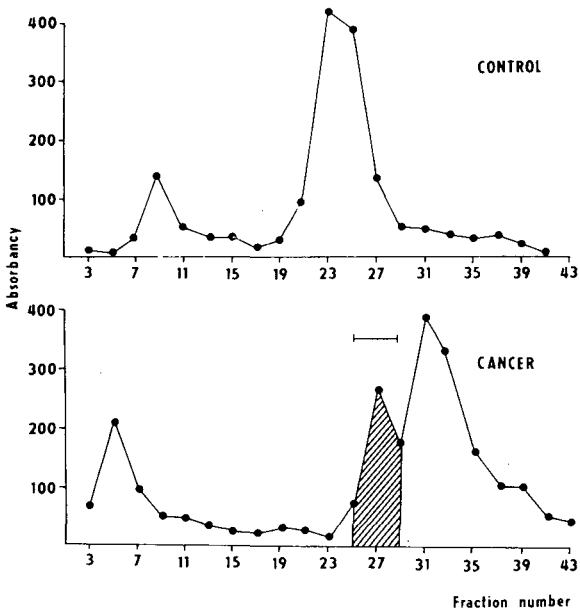

Fig. 1. Chromatographic separation of hydroxyproline-containing peptides on Bio-Gel P-2. Evaluation according to the hydroxyproline content in individual fractions by the hypronosticon test.

Material of all peaks that appeared in this last separation step was accumulated and subjected to routine amino acid analysis and to manual Edman's sequencing procedure. Appearance of a single N-terminal amino acid during this procedure as well as simple ratios obtained during amino acid analysis was considered sufficient for proving the homogeneity of the analysed peptide.

From the data presented the two urinary tripeptides occurring in patients with lung cancer were assigned the sequence Pro-Hyp-Gly and Ala-Hyp-Gly. The sequence of the hexapeptide (Fig. 3, upper part) is assumed on the basis of the obtained results and the data published by Szymanowicz et al. [3] (Ala-Hyp-Gly-Ala-Hyp-Gly).

It can be concluded that among hydroxyproline-containing peptides two additional tripeptides are formed in the urine of patients with lung cancer, namely Ala-Hyp-Gly- and Pro-Hyp-Gly. The presence of these two previously undetected urinary hydroxyproline-containing peptides was assayed in five controls and ten patients with bronchogenic carcinoma. While in healthy individuals all assays were negative, in the set of patients followed 0.8 –1.4% of peptide-bound hydroxyproline was recovered in these peptides. These figures are, however, rather semiquantitative due to the complex separation procedure. It has to be stressed that both these tripeptides belong to the category of sequences frequently occurring in all collagen types that have so far been described in vertebrates. This finding is not in contradiction with the previously expressed hypothesis that normal collagen types are synthesized in neoplastic tissues, albeit, in different ratios than unaffected tissues. On the

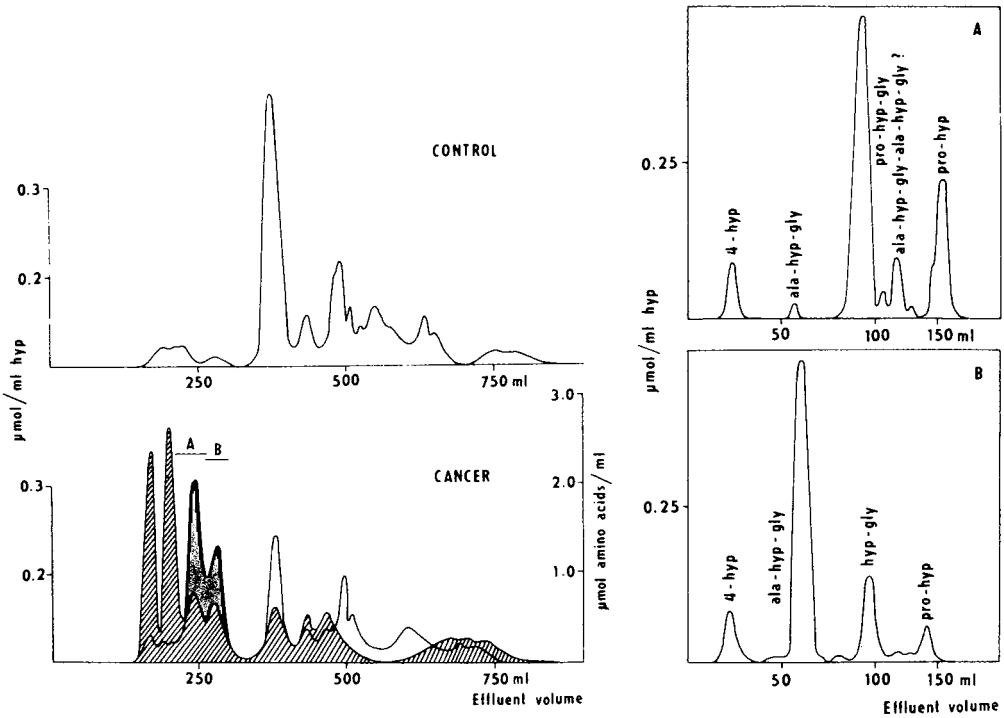

Fig. 2. Chromatography on QAE-Sephadex A-25 of the pooled fractions 25—29 from the Bio-Gel P-2 separation. The shaded area represents ninhydrin-reacting substance concentration in µmol/ml isoleucine; non-shaded urea represents hydroxyproline concentration in µmol/ml. Solid bars indicate pools used for further fractionation.

Fig. 3. Dowex 50 M 82 chromatographic profile of peptides present in fractions A and B from the QAE-Sephadex A-25 separation (Fig. 2) indicating the presence of Ala-Hyp-Gly and Pro-Hyp-Gly.

other hand, it can be concluded that in lung cancer considerable changes occur in collagen catabolism, which are reflected in alterations of hydroxyproline-containing urinary peptides.

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Note

Isotachophoretic analysis of some sulfur-containing amino acids in human urine

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S-(1,2-Dicarboxyethyl)cysteine (DCEC) [1], S-(carboxymethyl)cysteine (CMC) [2] and S-(2-methyl-2-carboxyethyl)cysteine (isobuteine) [3] have been found in normal human urine. They are known to be present in several animal tissues, but their biosynthesis and physiological role are as yet not entirely understood.

So far we have used an automatic amino acid analyzer for the determination of these sulfur-containing amino acids, after preliminary fractionation of fairly large volumes of urine on different ion-exchange resins [4]. The isotachophoretic analysis [5—8] presented here has made possible the rapid determination of these compounds.

METHOD

A 50-ml sample of urine was hydrolyzed in 6 *N* HCl for 20 h on a sandbath. The hydrolyzate was evaporated to dryness under reduced pressure to remove excess HCl. The residue was applied on a column containing 100 ml of Diaion SK-1 (sulfonated cation exchanger (H^+, 100 mesh) produced by Mitsubishi Kasei Co., Tokyo, Japan), washed with deionized water and eluted with 2 *N* ammonia solution. The eluate was evaporated under reduced pressure. The residue was dissolved in about 10 ml of 0.2 *M* acetic acid and filtered. The filtrate was transferred to a column containing 100 ml of Amberlite IRA-68 (CH_3COO^-, 50 mesh) and fractionated with 800 ml of 2 *M* acetic acid and 2 *M* HCl. Each fraction was evaporated to dryness under reduced pressure.

The residues of each fraction were fractionated on a column containing 100 ml of Diaion SK-1 with 0.2 *M* HCl, and each 100 ml of eluate was dried and tested by high-voltage paper electrophoresis [9].

The fractions containing DCEC, CMC and isobuteine were analyzed by isotachophoretic analyzer and amino acid analyzer.

INSTRUMENTATION

The capillary apparatus was a Shimadzu IP-1B isotachophoretic analyzer [10,11] (Shimadzu Seisakusho, Kyoto, Japan). The separations were carried out in a capillary tube, 20 cm × 0.5 mm I.D., which was maintained at a constant temperature of 20°C. The detector cell had an I.D. of 0.5 mm and length of 0.05 mm. The migration current was 50 μA. The leading electrolyte consisted of 0.01 *M* hydrochloric acid and β-alanine (pH 3.1). The terminal electrolyte was 0.01 *M* caproic acid.

The amino acid analyzer used was an Hitachi Model 835 liquid chromatograph. The chemicals used were analytical grade. Authentic DCEC, CMC and isobuteine were synthesized in our laboratory.

RESULTS AND DISCUSSION

It was possible to detect 1 nmol amounts of sulfur-containing amino acids by partial separation of a urine sample of 50—100 ml. The fraction containing DCEC was almost pure (Fig. 1B). The fractions containing CMC and isobuteine contained a couple of other amino acids, but it was possible to detect CMC and isobuteine in each fraction.

The standard curves prepared by plotting zone length against concentration for standard CMC, DCEC, isobuteine and cysteic acid were linear from 1 to 80 nmol.

Isotachophoretic analysis of acidic amino acids is shown in Fig. 1A. When DCEC, CMC, aspartic acid and isovalthine were run separately under only 100 μA, good resolution was obtained, but a mixture of these compounds did not give good separation in the same migration current. Eventually, we obtained good results, as shown in Fig. 1A, by changing the migration current from 100 to 50 μA.

Determination of DCEC, CMC and isobuteine in human urine by isotachophoresis and by amino acid analyzer is compared in Table I.

The recovery of these sulfur-containing amino acids during these column chromatographic procedures was approximately 95—100%. The pretreatment for determination of the amino acids in the isotachophoretic analyzer was more

TABLE I

COMPARISON OF THE DCEC, CMC AND ISOBUTEINE CONTENT DETERMINED FROM THE SAME SAMPLE BY THE ISOTACHOPHORETIC ANALYZER AND THE AMINO ACID ANALYZER

Each value represents the mean ± S.D. obtained from three separate determinations.

	Isotachophoresis (nmol/ml)	Amino acid analyzer (nmol/ml)
DCEC	9.84 ± 1.7	10.32 ± 1.2
CMC	0.56 ± 0.21	0.52 ± 0.12
Isobuteine	5.58 ± 1.5	5.39 ± 0.9

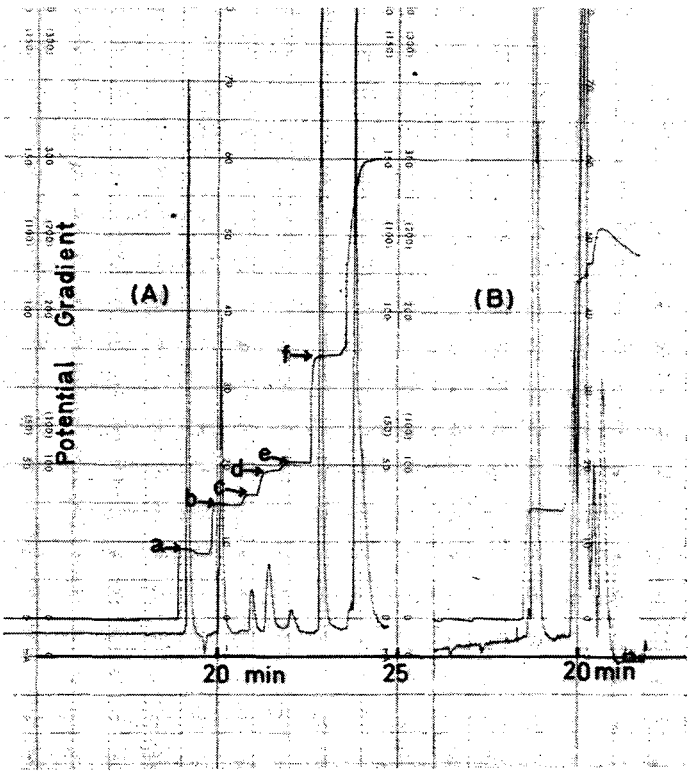

Fig. 1. Isotachophoretic runs of authentic samples (A) and the DCEC fraction (B). The leading electrolyte was 0.01 *M* HCl and β-alanine (pH 3.1) and the terminator was 0.01 *M* caproic acid. Migration current, 50 μA; chart speed, 10 mm/min; temperature of electrolyte, 20°C. Synthetic samples: (a) cysteic acid, (b) DCEC; (c) CMC, (d) isovalthine, (e) aspartic acid, (f) isobuteine.

simple than for the amino acid analyzer as described previously [1], and the method was very useful for detecting metabolites such as those described above.

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Note

Determination of propiomazine and its N-demethyl metabolite in plasma by gas chromatography with alkali flame ionization detection

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Propiomazine, Fig. 1, is a compound of the phenothiazine type which has found a widespread use as sedative and hypnotic, particularly in geriatric practice. Although the drug has been on the market for more than thirty years, little is known about its disposition in animal or human. For an optimal dosage regimen, it is essential to evaluate the basic pharmacokinetics of the drug after different ways of administration. Lack of sufficiently sensitive and selective analytical procedures has, however, hampered the quantitative determination of the drug and its main metabolites in plasma and urine.

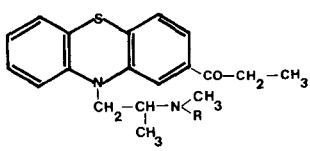

Fig. 1. Structure of propiomazine (R = CH_3) and N-demethylpropiomazine (R = H).

Propiomazine has been positively identified in urine using thin-layer chromatography [1,2]. Spectrophotometric methods have been developed for the urinary analysis of the drug and its sulfoxide metabolite [3,4]. Obviously these methods have an insufficient sensitivity and selectivity for the determination of plasma concentrations of propiomazine.

This paper describes an analytical procedure for the measurement of plasma propiomazine and its N-demethyl metabolite comprising selective extraction, trifluoroacetylation of the secondary amine and determination by gas chromatography with alkali flame ionization detection.

EXPERIMENTAL

Gas chromatography

A Pye series 104B chromatograph equipped with flame ionization and alkali flame ionization detectors was used. The glass column (150 cm × 0.2 cm I.D.) was filled with 3% OV-17 on Gas-Chrom Q(100—120 mesh) and operated at 280°C. Injector and detector temperatures were 280 and 320°C, respectively. The flow-rate of nitrogen carrier gas was 30 ml/min. The flow-rates of hydrogen and air were 30 and 300 ml/min, respectively.

Reagents and chemicals

Propiomazine maleate, N-demethylpropiomazine chloride and propiomazine sulfoxide (base) were kindly supplied by Pharmacia (Uppsala, Sweden). Heptane, trifluoroacetic anhydride and triethylamine were of highest analytical quality and purchased from E. Merck (Darmstadt, G.F.R.). Anhydrous diethyl ether was supplied by Mallinckrodt (St. Louis, MO, U.S.A.). For standardization, propiomazine maleate and N-demethylpropiomazine chloride were dissolved in 0.1 *M* phosphoric acid and diluted with water. Aliquots of this solution were diluted with plasma to contain 20—400 ng/ml of each of the amines. Internal standard was prochloroperazine maleate (Leo, Helsingborg, Sweden). It was diluted with water to 0.5 μg/ml. One ml of this solution was used in the analytical method.

Determination of partition ratio

The amine as base ($2 \cdot 10^{-3}$ *M*) was dissolved in diethyl ether together with an internal standard (octacosane). The peak height ratio of the amine to the internal standard was determined by gas chromatography with flame ionization detection. Five ml of the diethyl ether phase were shaken with an equal volume of phosphate buffer (μ=0.1) pH 4.5—6.0 at 25°C for 1 h. After phase separation, the peak height ratio of amine to internal standard was determined in the diethyl ether phase. The partition ratio, K_D, was determined from the peak height ratio after extraction/(initial peak height ratio minus peak height ratio after extraction).

Determination of propiomazine and N-demethylpropiomazine in plasma

To 2 ml of plasma, 1.0 ml of internal standard solution was added together with 1 ml of 0.5 *M* sodium hydroxide and water to 5 ml. The mixture was shaken with 5 ml of diethyl ether for 1 h at room temperature. After centrifugation for 10 min at 500 *g*, the organic phase was transferred to another tube and shaken for 10 min with 1 ml of 0.1 *M* sulphuric acid.

After removal of the organic layer and alkalinization with 0.5 ml of 0.5 *M* sodium hydroxide, the aqueous phase was shaken with 1 ml of diethyl ether for 10 min. The organic phase was transferred to another tube and evaporated in a stream of nitrogen. Ten microlitres of a mixture of trifluoroacetic anhydride and triethyl amine (1:1) was added to the residue and after 15 min reaction time at room temperature, 0.5 ml of heptane was added. Excess reagent was removed by washing with 1 ml of 0.1 *M* sodium hydroxide. Two to four microlitres of the organic phase were analysed by gas chromatography with alkali flame ionization detection.

A standard curve was prepared in parallel by treating six standard samples in the concentration range 20—400 ng/ml of propiomazine or N-demethylpropiomazine, according to the procedure above.

RESULTS AND DISCUSSION

Extraction conditions

Diethyl ether was chosen for the extraction of the amines from alkalinized plasma samples owing to its good extraction power and for its low tendency for formation of emulsions. An estimation of the extraction degree of propiomazine and its N-demethyl metabolite from buffered aqueous solutions was obtained by determination of partition coefficients. The partition coefficients, K_D, between diethyl ether and buffered aqueous solution are given in Table I, the value of $-\log(K_D \times K_{HA})$ corresponds to the pH at which the amine is present in equal concentration in the phases using equal phase volumes. A quantitative extraction of propiomazine, N-demethylpropiomazine and the internal standard, prochlorperazine, might be obtained at pH > 7.5.

The extraction degree of propiomazine sulfoxide to diethyl ether was shown by gas chromatographic analysis to be low (<1%) even at pH 10. The relative retention of the sulfoxide to propiomazine was 3.1.

In the procedure, a purification step was included by re-extraction of the amines to acidic aqueous phase followed by alkalinization and extraction to a small volume of diethyl ether. It was verified that the yield of the amines through the extraction steps was the same with and without the presence of plasma.

TABLE I

PARTITION COEFFICIENTS OF PROPIOMAZINE, METABOLITES AND THE INTERNAL STANDARD

$K_D = A_{org}/A_{aq}$ = partition coefficient of the amine. K_{HA} = acid dissociation constant of the amine. Aqueous phase: phosphate buffer pH 4.5—6.0 (μ=0.1); organic phase: diethyl ether; equal phase volumes.

Compound	$\log(K_D \times K_{HA})$	pH for 99% extraction	pH for 1% extraction
Propiomazine	—4.4	>6.4	<2.4
N-Demethylpropiomazine	—5.5	>7.5	<3.5
Propiomazine sulfoxide	—	— *	
Prochlorperazine, internal standard	—3.9	>5.9	<1.9

* Less than 1% extraction at pH 8—10.

Reaction conditions

The polar character of the N-demethyl metabolite and its poor gas chromatographic resolution from propiomazine made it necessary to prepare the trifluoroacetyl derivative from the secondary amine. A quantitative acylation was achieved within 5 min in the presence of base. The relative retention of the derivative to propiomazine was 1.25.

Selectivity of the method

The metabolism of propiomazine is poorly investigated. The in vitro biotransformation has been studied using the microsomal fraction of rat liver, and only a ring-hydroxylated metabolite could be identified [5]. An extensive metabolism must, however, be anticipated in accordance with other drugs of the phenothiazine class. Possible routes of metabolism beside ring hydroxylation are sulfoxidation and N-demethylation. Owing to the polar properties of the sulfoxide of propiomazine, this metabolite was excluded in the extraction procedure. Phenolic metabolites were also probably excluded in the alkaline extraction of the plasma sample. Interference from unknown metabolites of propiomazine has not been observed. The selectivity of the method was verified for the following phenothiazine drugs: promazine, promethazine, thioridazine, methopromazine and levomepromazine.

Sensitivity, yield and precision

The detection limit of the method, defined as three times the background noise level, was 10—20 ng/ml of each of propiomazine and the N-demethyl metabolite. Rectilinear standard curves through the origin in the concentration range 20—400 ng/ml were obtained for propiomazine (r = 0.996) and for N-

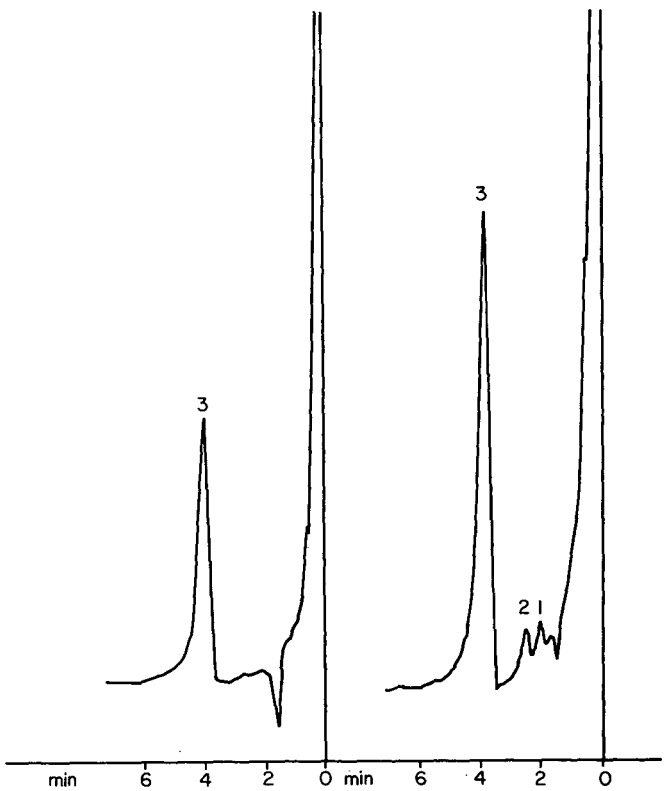

Fig. 2. Gas chromatogram of plasma sample containing propiomazine (25 ng/ml) (1) and N-demethylpropiomazine (25 ng/ml) (2) run through the method (right panel) and of a blank plasma sample containing internal standard prochlorperazine (3) (left panel).

demethylpropiomazine (r = 0.995). Usually five or six standard samples at different concentration levels were processed.

The relative recovery and precision of the method with spiked plasma samples at the 100 ng/ml level were 100 ± 7.6% and 98 ± 4.6% (n = 8) for propiomazine and the N-demethyl metabolite, respectively. The absolute recovery in the method was determined by comparison to pure compound injected in the gas chromatograph with alkali flame ionization detector. The yield was 89% for both compounds.

The amines chromatographed without indication of adsorption losses. A chromatogram of a plasma sample run through the method is shown in Fig. 2. Prochlorperazine was used as internal standard owing to its similar extraction properties and suitable gas chromatographic retention.

Application to biological samples

The method was applied to the determination of propiomazine in plasma samples after therapeutic doses of the drug. Peak plasma concentrations in the range 150—300 ng/ml occurred 2—4 h after oral administration. The plasma concentrations 24 h after administration were still in the range 40—80 ng/ml. Minute amounts of N-demethylpropiomazine were seen.

A full description of the pharmacokinetics of propiomazine will be given.

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Note

Plasma and urine concentrations of methapyrilene by nitrogen—phosphorus gas—liquid chromatography

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Methapyrilene is an antihistamine with sedative effects which is used in numerous over-the-counter sleeping aids and cold preparations. Since methapyrilene is widely available, it is commonly encountered during the analysis of human body fluids for toxicological purposes.

Several previously reported analytical methods for methapyrilene levels in blood and urine dealt with overdose situations [1—3]. These methods lack the sensitivity and specificity needed for detection of therapeutic levels of the drug.

Two other methods were reported with sensitivity for methapyrilene in the ng/ml range. One involved gas chromatography using a sulfur-specific flame photometric detector [4], sensitive enough to detect as little as 10 ng of methapyrilene on-column. However, it was claimed that a metabolite of methapyrilene, rather than methapyrilene itself, was detected in the plasma and urine samples. The other technique was designed as a general procedure for basic drugs in postmortem blood using a nitrogen—phosphorus detector [5]. Its sensitivity limit for methapyrilene, however, was not tested beyond 100 ng/ml since the method was not specifically concerned with any one drug.

The purpose of this communication is to present a rapid, gas—liquid chromatographic (GLC) procedure for determination of therapeutic concentra-

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tions of methapyrilene, to describe in quantitative terms the human urinary excretion of the drug, and to qualitatively follow its plasma profile over several hours.

EXPERIMENTAL

Chemicals and reagents

Methapyrilene HCl and brompheniramine maleate were supplied by Schering Corporation (Kenilworth, NJ, U.S.A.). A stock solution of methapyrilene was prepared by dissolving an appropriate amount of the salt in methanol to make a 1.0 mg/ml solution as the free base. A plasma standard was prepared by adding 5 μl of the stock solution to 50 ml of drug-free human plasma, resulting in a 100 ng/ml standard. Serial dilutions of this standard were made to prepare the 50 and 10 ng/ml standards. Urine standards of 50, 100 and 300 ng/ml concentration were prepared in a similar manner. The internal standard consisted of a 40 μg/ml aqueous solution of the maleate salt of brompheniramine.

Sample preparation

A 2-ml aliquot of either plasma or urine was transferred to a 15-ml glass-stoppered centrifuge tube. Exactly 25 μl of internal standard was added and the mixture vortexed. Four drops of concentrated ammonium hydroxide were added to the plasma while three drops were added to the urine to ensure that a pH 9 medium resulted. After vortexing, 4 ml of heptane—isoamyl alcohol (98.3:1.7) were added and the tube was capped and shaken by hand for 1 min. Following centrifugation for 5 min at 2000 rpm, 3 ml of the upper solvent layer were removed by aspiration, with care taken so as not to disturb the interface area, and transferred to a clean 15-ml graduated centrifuge tube. The solvent was evaporated to dryness under a stream of dry air while the tube was in a 40°C water-bath. Methanol (50 μl) was used to reconstitute the residue immediately after drying and a 2 μl aliquot injected into the gas chromatograph.

Instrumental conditions

A Hewlett-Packard Model 5711A gas chromatograph with dual nitrogen—phosphorus detectors was used. The instrument was fitted with a 0.6 m × 2 mm I.D. glass column containing 2% OV-1 on 100—120 mesh Chromosorb G HP (liquid phase and solid support purchased separately from Applied Science Labs., State College, PA, U.S.A.). The carrier gas, extra dry nitrogen, was set to a flow-rate of 60 ml/min; hydrogen flow-rate was 3 ml/min and air flow-rate was 50 ml/min to the nitrogen—phosphorus detectors. Instrument temperatures were maintained at injector, 250°C; oven, 230°C; and detector, 300°C. Retention times under these conditions for methapyrilene and brompheniramine were 2.5 and 3.3 min, respectively.

Calculation

Once linearity was established for methapyrilene using the plasma and urine standards, the quantitation of unknown samples was estimated from a

standard curve by means of the drug/internal standard peak height ratio.

Experimental subject

A non-fasting adult male volunteer, 73 kg, received a 25-mg oral dose of methapyrilene HCl (21.9 mg of free base) at approximately 9:00 a.m. Blood was collected in heparinized tubes over the ensuing 6 h and urine was collected over a 24-h period. After the plasma was obtained by centrifuging the blood and after the urine pH was determined with a Beckman Model 3500 pH meter, the samples were refrigerated and analyzed within 4 days.

Thin-layer chromatography

Thin-layer chromatographic separation of the drugs was accomplished following extraction of 8—10-ml volumes of urine using a procedure similar to that described above, excluding the addition of internal standard. The extracts were applied to thin-layer plates coated with a 250-μm thick layer of silica gel G (J.T. Baker, Phillipsburg, NJ, U.S.A.). The plates were developed first to a height of 7.5 cm in ethyl acetate—methanol—ammonia (82:13:5), removed and air-dried, and then redeveloped to a height of 15 cm in ethyl acetate—methanol (98:2). After final drying each plate was sprayed with acidified iodoplatinate reagent. The R_F values under these conditions were 0.52 for methapyrilene and 0.33 for an apparent metabolite.

RESULTS AND DISCUSSION

Methapyrilene and brompheniramine were well separated from each other and the solvent front under the described GLC conditions (Fig. 1). There was relatively little background from the plasma sample, although an interfering substance found to be persistent in drug-free plasma eluted just after methapyrilene and was twice the height of the drug peak of the 10 ng/ml standard. One possible source of this interference was the plastic bag in which the drug-free plasma was stored. No significant background interference was found in the urine extracts.

The extracts were reasonably stable when stored overnight in the refrigerator in 1 ml of methanol. Results of the stability and reproducibility studies of a 10 ng/ml plasma standard were found to be acceptable. The coefficient of variation based on 5 determinations over a 15-day period was 13.8% (Table I).

TABLE I

DAY-TO-DAY REPRODUCIBILITY AND STABILITY OF PLASMA METHAPYRILENE MEASUREMENTS BASED ON 5 SEPARATE DETERMINATIONS OVER A 15-DAY PERIOD

Concentration (ng/ml)*		
Mean	S.D.	C.V. (%)
9.74	1.34	13.8

*A 10 ng/ml methapyrilene plasma standard was prepared with each run and used as the reference.

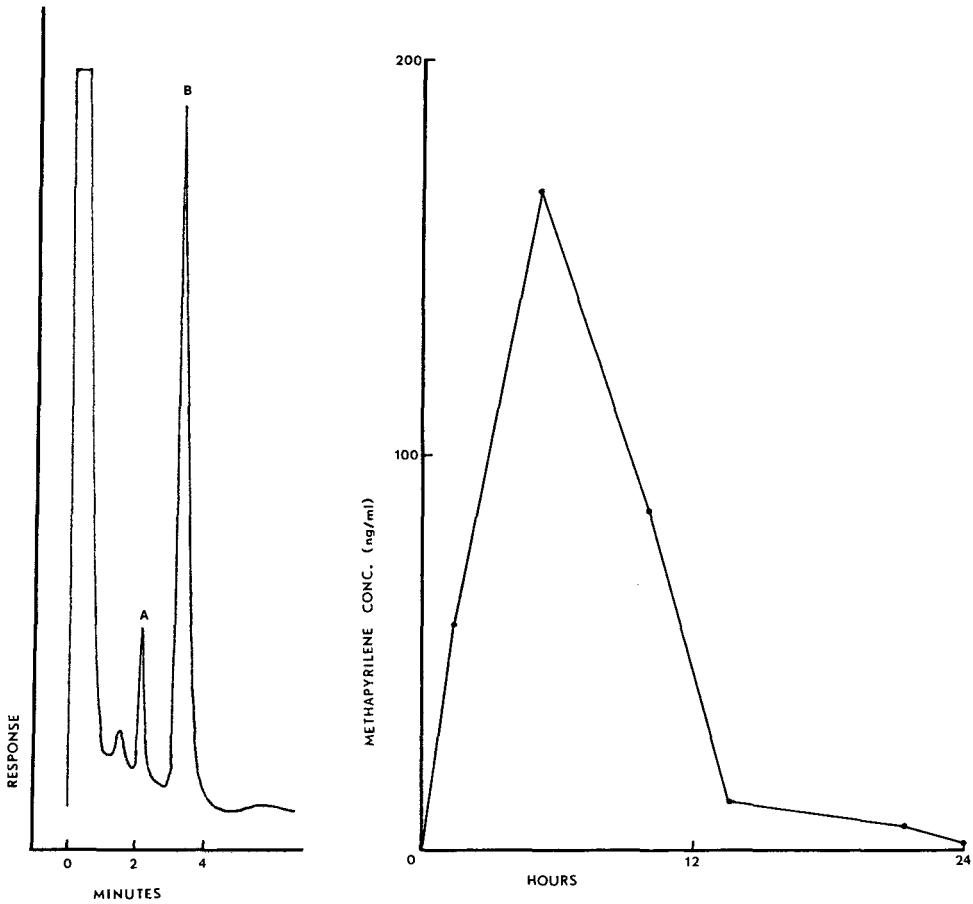

Fig. 1. Gas chromatogram of an extract of a urine sample from a subject who ingested 25 mg of methapyrilene HCl. A, Methapyrilene; B, internal standard (brompheniramine).

Fig. 2. Urine methapyrilene concentration profile showing range of concentrations for adult subject following ingestion of 25 mg of methapyrilene HCl.

Results obtained from a graph of the plasma drug/internal standard peak height ratio vs. concentration showed the volunteer's plasma concentration to be within a range lower than the 10 ng/ml plasma standard. Although the method is inaccurate at this range, we estimate that methapyrilene plasma levels were between 2 and 7 ng/ml over the 5 h following drug ingestion, with the peak concentration occurring 3 h after ingestion. The volunteer experienced no subjective feelings of drowsiness at this dosage.

The procedure was found to be linear for the urine methapyrilene concentrations over a range of 50—300 ng/ml. The urine methapyrilene concentration for the volunteer was at its maximum of 172 ng/ml at 5.3 h after oral administration of 25 mg of the drug (Fig. 2). The concentrations then fell rapidly and averaged less than 2 ng/ml at 24 h after administration.

The 24-h urinary excretion of unchanged methapyrilene amounted to

TABLE II

URINARY EXCRETION OF METHAPYRILENE*

Urine pH	Time elapsed (h)	Volume (ml)	Drug concentration (μg/ml)	Drug excreted (μg)	Urine flow-rate (ml/min)	Excretion rate (μg base/min)
6.4	1.6	90	0.054	4.9	0.9	0.051
5.8	5.3	145	0.172	25.0	0.7	0.110
5.6	10.0	175	0.080	14.0	0.6	0.049
6.1	13.8	136	0.009	1.2	0.6	0.005
6.2	21.7	335	0.005	1.7	0.7	0.004
5.8	23.2	87	0.002	0.2	1.0	0.002
5.8	24.0	53	0.002	0.1	1.1	0.002
	Total 1021 ml			Total 47.1 μg		

*In 24-h urine specimens following a single ingestion of 21.9 mg of methapyrilene base.

only 0.2% of the administered dose (Table II). The rate of excretion of methapyrilene, urinary pH, and urine flow over the 24-h period are also shown in Table II. The maximum rate of excretion was observed approximately 6 h after ingestion of the drug, while the urinary pH was steadily dropping.

In reviewing the amount of methapyrilene excreted, it is evident that either a large portion of the administered dose had not been absorbed or that the drug was extensively metabolized. Thin-layer chromatography performed on the urine sample taken 5 h after dosing showed an unknown spot of lesser intensity than the unchanged methapyrilene. It was found that this spot had the same R_F as acid-hydrolyzed methapyrilene. Thus, a portion of the methapyrilene that is absorbed may be hydrolyzed or oxidized in vivo. This is further supported by the thin-layer chromatographic properties of the apparent metabolite, which suggest that it is more polar than methapyrilene. This metabolite may be the hydroxylated compound that Schirmer and Pierson [4] detected with their GLC technique for methapyrilene. This compound was not observable during our GLC analysis of plasma or urine, however.

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Note

Specific high-performance liquid chromatographic method for estimation of the *cis*(*Z*)- and *trans*(*E*)-isomers of clopenthixol and a N-dealkyl metabolite

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(First received December 21st, 1979; revised manuscript received February 29th, 1980)

Clopenthixol, a neuroleptic drug of the thioxanthene group, exists as two geometric isomers, a *cis*(*Z*)- and a *trans*(*E*)-isomer. It has been shown, that while the *cis*(*Z*)-isomer has a strong neuroleptic activity in pharmacological tests, the *trans*(*E*)-isomer is practically without any effect [1]. To prolong the neuroleptic effect *cis*(*Z*)-clopenthixol was esterified with decanoic acid, dissolved in an oil and administered as an intramuscular depot. Pharmacokinetic studies in animals indicated rapid hydrolysis of the ester after liberation from the depot and showed significant drug levels for considerably longer periods of time after clopenthixol decanoate injection than after orally given clopenthixol [2]. These data correlate well with the long lasting pharmacological effect of clopenthixol decanoate demonstrated in animals [3] and they are in agreement with studies in patients administered clopenthixol decanoate in Viscoleo with intervals of two and four weeks [4, 5].

Analytical methods for determination of clopenthixol in biological material have been developed by Fredricson Overø [4], who used fluorometry after thin-layer separation and oxidation, and Muusze et al. [6], who worked with thin-layer scanning. The former method estimates clopenthixol decanoate and a N-dealkyl metabolite in addition to clopenthixol, but does not distinguish between the isomers. The method by Muusze et al. [6] supplies a separation of the isomers of clopenthixol, but the separation is unsatisfactory and the isomers of the N-dealkyl metabolite are not separated at all. The ester is not estimated by this method. High-performance liquid chromatographic separation of the isomers of clopenthixol has been performed by Li Wan Po and Irwin [7], who used pure solutions of rather high concentrations.

In the present paper a specific high-performance liquid chromatographic method for determination of the concentrations in serum of the *cis*(*Z*)- and *trans*(*E*)-isomers of clopenthixol and N-dealkylclopenthixol is presented.

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EXPERIMENTAL

Apparatus

The liquid chromatographic system consists of a Rheodyne Model 7120 syringe loading sample injector, a Spherisorb S 5 W (5-μm spherical silica particles from Phase Separations, Queensferry, Great Britiain) column (25 cm × 4.6 mm), a Waters Model 6000A pump, a Waters Model 440 UV-absorbance detector with a measuring wavelength of 254 nm, and a Kipp & Zonen Model BD9 two-channel recorder.

The eluent was *n*-heptane—2-propanol—concentrated ammonia—water (85:15:0.4:0.2, v/v), and the flow-rate 1 ml/min. The chromatograph was operated at room temperature and at a pressure of about 70 bar.

Chemicals

Glass distilled water and organic solvents of analytical grade were used in the analysis. The hexane was from Mallinckrodt (St. Louis, MO, U.S.A.); the other solvents, hydrochloric acid and the NaOH pellets from Merck (Darmstadt, G.F.R.). The isopropylamine was distilled every fortnight.

Reference substances

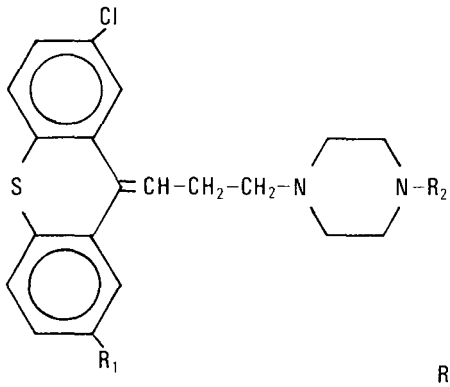
The dihydrochloride of the *cis*(*Z*)- and the *trans*(*E*)-isomers of clopenthixol and the dimaleate of the N-dealkylated clopenthixol consisting of 44% *cis*(*Z*)- and 56% *trans*(*E*)-isomer were used as reference substances. The percentages of *cis*(*Z*)- and *trans*(*E*)-N-dealkylclopenthixol were estimated from the peak areas. It was necessary to make the assumption that the *cis*(*Z*)- and *trans*(*E*)-isomers of N-dealkylclopenthixol have equal molar UV-absorption as the pure isomers cannot be produced. Stock standard solutions were made in ethanol (1 mg/ml) and stored in a refrigerator for 1 month. Dilutions were made every day. The substances were all synthesized in our laboratories.

Internal standard

A compound, Lu 9-215, structurally related to clopenthixol, but without the double bond in the side chain was used as internal standard (Fig. 1). Solution and dilutions were made in the same way as for the reference substances. The substance was synthesized in our laboratories and is available on request.

Extraction procedure

To a serum sample of 2 ml in a stoppered glass tube were added 25 ng of Lu 9-215, 300 μl ethanol, 100 μl of 7 *N* NaOH solution and 8 ml of hexane containing 0.1% isopropylamine. The samples were shaken for 15 min and then centrifuged at 2400 *g* for 5 min. The hexane phase was transferred to another tube, and 2 ml of 0.1 *N* HCl were added, the samples were shaken for 15 min and centrifuged for 5 min. The hexane phase was discarded, and to the HCl phase were added 200 μl of 7 *N* NaOH and 4 ml of hexane with 0.1% isopropylamine. The samples were again shaken and centrifuged for 15 and 5 min, respectively. Thereafter the hexane phase was transferred to a conical glass tube and was evaporated to dryness under a stream of air at 30°C. The sample was redissolved in 1 ml of hexane, evaporated and redissolved in 100 μl of hexane



		R_1	R_2
Clopenthixol	(CPT)	H	$-(CH_2)_2-OH$
Dealkyl clopenthixol	(CPT-NH)	H	H
Internal Standard	(Lu 9-215)*	CF_3	$-(CH_2)_2-OH$

*Without the double bond in the side chain to the ring structure

Fig. 1. Structures of clopenthixol, N-dealkylclopenthixol and the internal standard.

containing 0.1% isopropylamine. A 70-μl aliquot of the final solution was injected on the chromatograph.

RESULTS AND DISCUSSION

Identification and quantitation

The drug, the metabolites and the internal standard were identified by their retention times.

Standard curves were evaluated by measuring the peak heights relative to that of the internal standard. As the variations in the daily standard curves obtained for *cis*(*Z*)-clopenthixol and *cis*(*Z*)- and *trans*(*E*)-N-dealkylclopenthixol were small, three mean standard curves were made for these compounds on the basis of the data from the individual curves. The standard curves did not differ significantly from linearity ($p > 0.10$). The standard curve used for determination of *cis*(*Z*)-clopenthixol was also used for *trans*(*E*)-clopenthixol as the peak heights of these two isomers were not significantly different for the same amount of drug. The standard curve used for calculation of *cis*(*Z*)- and *trans*(*E*)-clopenthixol is shown in Fig. 2. The line is based on 206 determinations as indicated on the figure. The equation for the line is $y = 0.713\ x + 0.0054$. The corresponding standard curves for *cis*(*Z*)- and *trans*(*E*)-N-dealkylclopenthixol are based on 208 and 198 determinations and the equations are $y = 0.0415\ x - 0.0482$ and $y = 0.0270\ x + 0.0390$, respectively.

Every day standards of *cis*(*Z*)-clopenthixol and *cis*(*Z*)/*trans*(*E*)-N-dealkylclopenthixol added to blank serum samples are run through the procedure in order to check that the standard curves are still valid (standards are within the 95% confidence limits). The reason for not adding *trans*(*E*)-clopenthixol to the samples is to be sure of the degree of isomerization of *cis*(*Z*)- to *trans*(*E*)-clopenthixol during the analytical procedure. The *cis*(*Z*)-clopenthixol standard

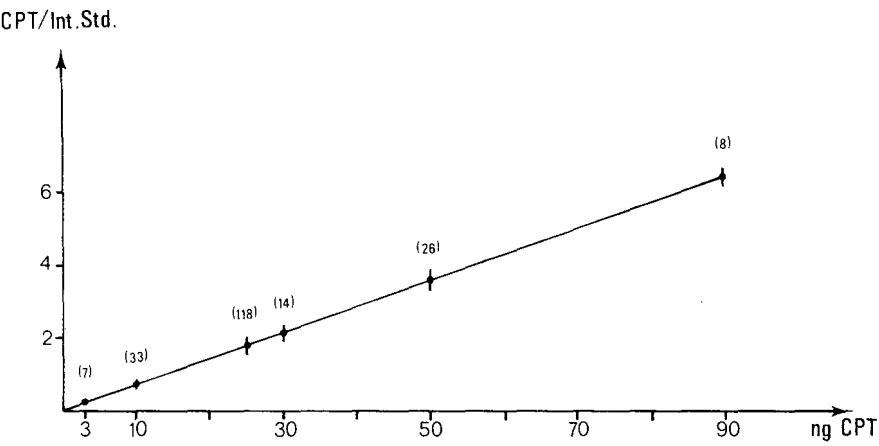


Fig. 2. Standard curve for calculation of the amounts of *cis*(*Z*)- and *trans*(*E*)-clopenthixol (CPT) in a serum sample. Mean ± S.D. is given for each concentration. The figures in the brackets indicate the number of observations.

contains 1—2% of *trans*(*E*)-clopenthixol, and during the extraction procedure this normally increases to about 6%. If the samples are handled in an inexpedient way (heat, light), further isomerization can take place.

Sensitivity and precision

The lower limit of sensitivity was for clopenthixol about 0.5 ng/ml and for N-dealkylclopenthixol about 2.5 ng/ml in a 2-ml sample. Because of this high sensitivity it is possible to follow the drug concentrations in serum for a relatively long time, even after a single dose as low as 10 mg of *cis*(*Z*)-clopenthixol. The method is thus more sensitive than the other methods described for clopenthixol and N-dealkylclopenthixol [4, 6].

Based on the assay of 4—6 identical samples containing 3, 10, 30, and 90 ng of *cis*(*Z*)-clopenthixol and 10, 30, 90, and 270 ng of *cis*(*Z*)/*trans*(*E*)-N-dealkylclopenthixol, coefficients of variance of 3—7 were found. The coefficients of variance were independent of the drug concentration.

Specificity

The assay of clopenthixol and the metabolite is not disturbed by compounds from the serum as seen in Fig. 3A.

Other relevant drugs have been assayed in the chromatographic system described in this paper, to investigate a possible interference with the assay. The tricyclic antidepressants amitriptyline, nortriptyline, and imipramine do not interfere with the assay, the same is true for the antiparkinsonian drugs orfenadin, procyclidin, and biperidin, whereas most of the benzodiazepines interfere with the assay. Estazolam does not interfere with clopenthixol, but is very close to the *cis*(*Z*)-isomer of the metabolite in the chromatogram. Metabolites of the above-mentioned drugs have not been investigated and it therefore cannot be excluded that some of them will interfere with the clopenthixol assay. However, until now no peaks from other compounds have disturbed the assay.

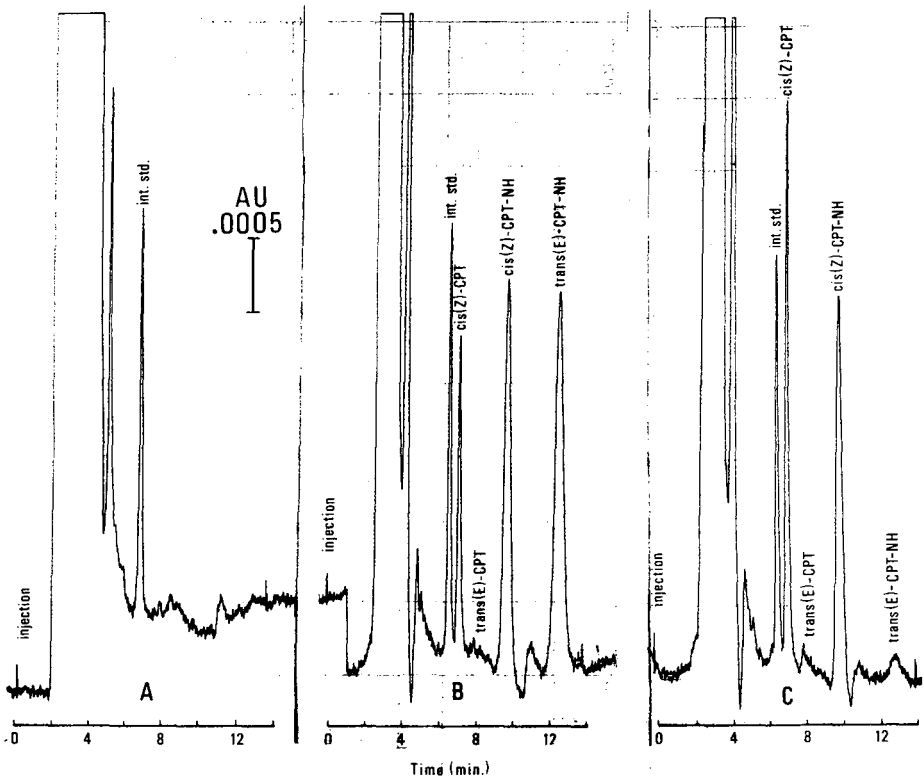


Fig. 3. Chromatograms of (A) blank serum with 25 ng internal standard; (B) blank serum with 25 ng internal standard, 10 ng *cis*(*Z*)-clopenthixol [*cis*(*Z*)-CPT], 22 ng *cis*(*Z*)-N-dealkylclopenthixol [*cis*(*Z*)-CPT-NH], and 28 ng *trans*(*E*)-N-dealkylclopenthixol [*trans*(*E*)-CPT-NH]; (C) serum sample from a patient given daily dosages of *cis*(*Z*)-clopenthixol, 25 ng of internal standard were added to the sample containing 19.8 ng *cis*(*Z*)-clopenthixol and 24.9 ng *cis*(*Z*)-N-dealkylclopenthixol, traces of *trans*(*E*)-clopenthixol and *trans*(*E*)-N-dealkylclopenthixol were also present.

Serum samples

The method has until now only been used for serum samples from rat, dog, and man. In Fig. 3 are shown examples of chromatograms of extracts of human serum. Fig. 3A shows a blank serum sample to which are added 25 ng of internal standard; the same is seen in Fig. 3B, but with the addition of *cis*(*Z*)-clopenthixol (10 ng) and *cis*(*Z*)- and *trans*(*E*)-N-dealkylclopenthixol (22 and 28 ng, respectively). Fig. 3C shows a serum sample from a patient with added internal standard (25 ng). The amounts of *cis*(*Z*)-clopenthixol and *cis*(*Z*)-N-dealkylclopenthixol are 19.8 and 24.9 ng, respectively. The corresponding *trans* isomers are present in amounts below the limit of detection.

Fig. 4 shows the levels of *cis*(*Z*)- and *trans*(*E*)-clopenthixol in a human volunteer given a single dose of 30 mg Sordinol®, i.e. about 10 mg *cis*(*Z*)- and 20 mg *trans*(*E*)-clopenthixol. The peak levels are obtained 3 and 4 h after administration. Thereafter the curves decline slowly and the drug is still measurable after 48 h. The biological half-lives are almost one day. The isomers of the dealkylated metabolite were only seen as traces.

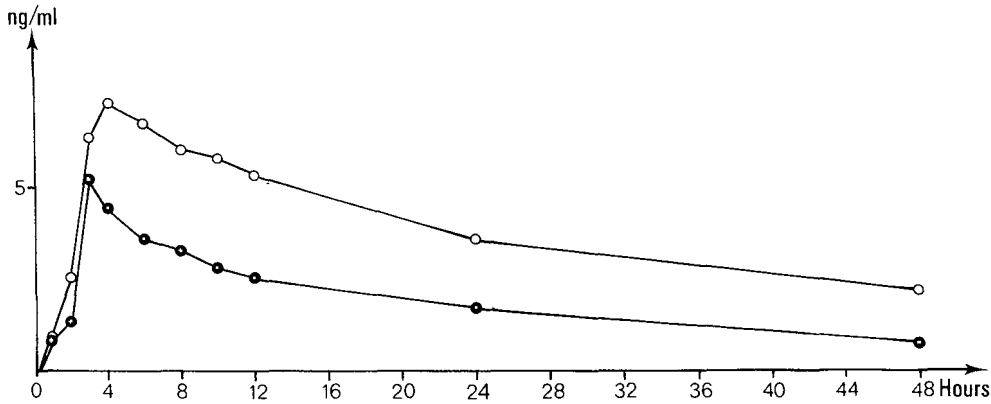


Fig. 4. Concentrations of *cis*(*Z*)-clopenthixol (•) and *trans*(*E*)-clopenthixol (○) in serum from a human volunteer given a single dose of 30 mg Sordinol®.

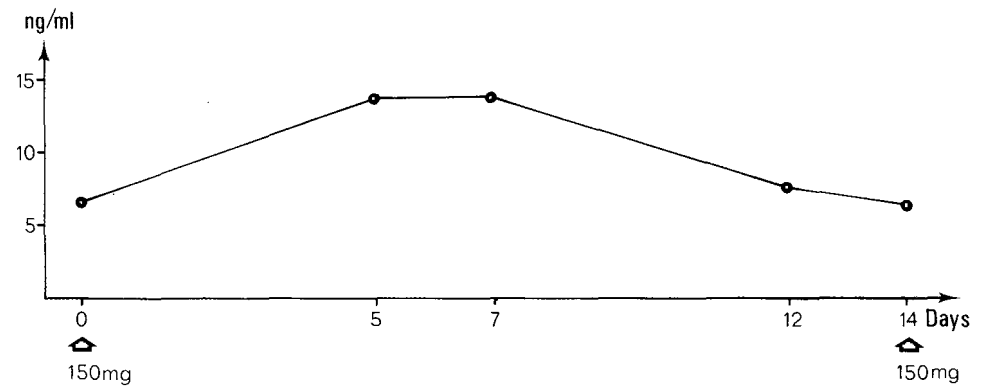


Fig. 5. Concentrations of *cis*(*Z*)-clopenthixol in serum from a patient given *cis*(*Z*)-clopenthixol decanoate intramuscularly in Viscoleo, 150 mg fortnightly.

Another example of the application of the method is seen in Fig. 5 showing the concentration of *cis*(*Z*)-clopenthixol after intramuscular injection of *cis*(*Z*)-clopenthixol decanoate in Viscoleo to a patient in fortnightly doses of 150 mg. It is seen that the curve shows a maximum between days 5 and 7 and that a maximum/minimum fluctuation of about 2 occurs. Although *cis*(*Z*)-clopenthixol decanoate and not *cis*(*Z*)-clopenthixol has been administered to the patient, only *cis*(*Z*)-clopenthixol has been measured as this seems to be the compound responsible for the clinical effect. This assumption is based on animal studies [2] which showed rapid hydrolysis of the ester, clopenthixol being the dominating compound in the tissues, and on earlier investigations in patients [4, 5] in whom the ester was hardly detectable in serum. In addition to *cis*(*Z*)-clopenthixol *trans*(*E*)-clopenthixol was observed in trace amounts. *cis*(*Z*)-N-Dealkylclopenthixol was seen in a concentration of about half of the *cis*(*Z*)-clopenthixol concentration, while the *trans*(*E*)-N-dealkylclopenthixol was seen in trace amounts.

The present paper describes a method for the estimation of the two isomers of clopenthixol and the two isomers of the clopenthixol metabolite, N-dealkyl-

clopenthixol. Of these compounds *cis*(*Z*)-clopenthixol is by far the most important compound, as it is the compound exerting the pharmacological and clinical activity also after ester administration. Measurement of *trans*(*E*)-clopenthixol is of less importance since the compound is almost without pharmacological activity [1], but it gives an indication of the degree to which the *cis*(*Z*)-isomer is transformed to the *trans*(*E*)-isomer. Also the measurement of the isomers of N-dealkylclopenthixol is of less importance as the compounds have very low pharmacological activity [8]. However, the measurement of the metabolite in serum may give an indication as to the metabolic capacity of the animal or the human from whom the serum sample originates.

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CHROMBIO. 590

Note

Determination of carpipramine in plasma by high-performance liquid chromatography

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Carpipramine, 1′-[3-(10,11-dihydro-5H-dibenz[*b*,*f*]azepin-5-yl)-propyl]-[1, 4′-bipiperidine]-4′-carboxamide (CPP) (Fig. 1), is a currently used psychotropic agent [1]. CPP is usually administered orally and is absorbed rapidly from the gastrointestinal tract. Several preparations containing its dihydrochloride monohydrate have been used clinically.

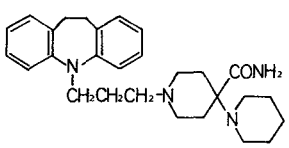


Fig. 1. Structure of carpipramine.

The pharmacological characteristics of CPP [2] and its metabolic fate in rats and rabbits [3, 4] have been reported. In these early studies, CPP was determined spectrophotometrically or fluorimetrically in extracts from tissue homogenates and body fluids [5].

For a comparison of the bioavailabilities of CPP and its preparations and for

drug monitoring during therapy, a more specific and sensitive method of assay was required. High-performance liquid chromatography (HPLC) was found to meet this demand. In this paper, we describe the HPLC determination of CPP in the plasma of dogs, though the method can be applied to human plasma and tissue homogenates.

EXPERIMENTAL

Materials

Carpipramine dihydrochloride monohydrate, and its various preparations, and chlorpromazine hydrochloride were the products of Yoshitomi Pharmaceutical Industries Ltd., Fukuoka, Japan. All solvents and chemicals were of reagent grade.

HPLC instrumentation

An Hitachi Model 635 liquid chromatograph equipped with a universal injector and an Hitachi variable-wavelength UV effluent monitor operated at 250 nm was used. The column was a Zorbax-SIL (Du Pont, Wilmington, DE, U.S.A.; particle size 5 μm; 150 mm × 3.9 mm I.D.). The flow-rate of the mobile phase was 0.4 ml/min.

Extraction procedure

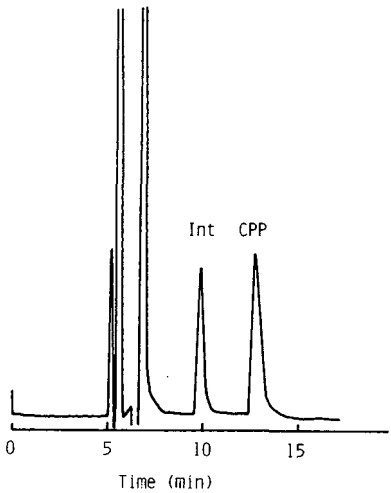
Plasma (0.3—3.0 ml), 10% NaOH (1.0 ml), water (2.0 ml), and *n*-heptane containing 1.5% isoamyl alcohol (25 ml) were added to a 50-ml test-tube with a ground-glass stopper. The amount of plasma was varied according to the CPP content and available volume. The sample was extracted for 20 min with vigorous shaking. The organic layer (20 ml) was separated by centrifugation and shaken for 10 min with 10 *M* HCl (5.0 ml).

After the heptane layer was discarded, the aqueous HCl layer (4.5 ml) was transferred to a glass-stoppered test-tube. The aqueous solution (1.0 ml) containing chlorpromazine hydrochloride (200 ng/ml) as an internal standard and 40% NaOH (0.5 ml) were added. The resulting solution was extracted with chloroform (100 μl) for 10 min with vigorous shaking. After the removal of the aqueous layer, a 30-μl volume of the chloroform solution was injected into the liquid chromatograph.

For the preparation of the calibration curve, CPP was dissolved in water so as to contain 100—700 ng/ml. Drug-free plasma (3.0 ml), CPP solution (2.0 ml), and 10% NaOH (1.0 ml) were mixed. These standards were carried through the procedure described above.

RESULTS AND DISCUSSION

Fig. 2 shows a chromatogram of an extract of plasma of a dog administered 100 mg of CPP orally. The mobile phase used was dichloromethane containing 10% methanol and 0.2% aqueous ammonia. The retention times were 12.6 min for CPP and 9.7 min for chlorpromazine, the internal standard. The ratio of the peak height of CPP to that of the internal standard was plotted against the



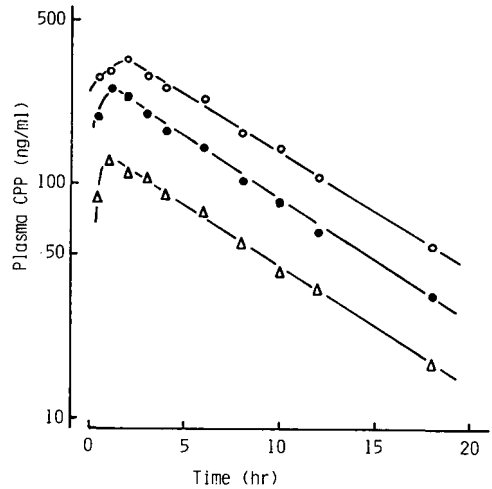


Fig. 2. Chromatogram of an extract of plasma of a dog administered 100 mg of CPP orally. Int = chlorpromazine.

Fig. 3. Plasma concentration of CPP at various times after oral administration to dogs. Doses of CPP per animal were 150 mg (○), 100 mg (●), and 50 mg (△). The analytical data presented are the averages of three animals.

amounts of CPP added to the standard. The calibration curves thus obtained were linear up to at least 700 ng and passed through the origin.

It was verified that the extraction of CPP and chlorpromazine into chloroform from aqueous alkaline solution were virtually complete in their wide ranges of concentration. In the determination of CPP in plasma containing 100 ng/ml, the standard deviation was 3 ng (n = 10; the volume of plasma used, 3 ml).

The present method permits the accurate determination of CPP in plasma at concentrations as low as 9 ng/ml (the volume of plasma, 3 ml) and is suited for monitoring the drug in the therapeutic dose range (50—300 mg/day per person).

Fig. 3 shows examples of the time—concentration curves in the plasma of dogs administered CPP. The CPP concentration in plasma increased immediately after the oral administration, reached a maximum in 1—2 h, and then decreased at a first-order rate. The biological half-life was about 6.0 h. These measurements give the basis for comparing the bioavailabilities of CPP and its various preparations. The details of the pharmacokinetics will be discussed elsewhere.

Since the present assay method was introduced in our laboratories, unfailing analytical results have always been obtained.

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Note

High-performance liquid column chromatography of fenoprofen in serum

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Fenoprofen is a nonsteroidal anti-inflammatory drug which has been shown to be effective in reducing inflammation in osteoarthritis [1, 2] and rheumatoid arthritis [3, 4] in adults.

Recently Nash et al. [5] elucidated the pharmacokinetics of oral fenoprofen in adults, but no such information is available for children. Factors contributing to this lack are the large sample size required for gas chromatographic analysis by the procedure of Nash et al. [6] and the fact that the gas—liquid chromatographic procedure is tedious and time-consuming. The purpose of this paper is to report a high-performance liquid chromatographic (HPLC) method which requires as little as 50 μl of serum, making it particularly well suited for pediatric studies and therapeutic monitoring. The method is fast, simple and reliable.

MATERIALS AND METHODS

All reagents were Baker reagent grade (J.T. Baker, Phillipsburg, NJ, U.S.A.). Chloroform, methanol and acetonitrile were distilled in glass (Burdick and Jackson Labs., Muskegon, MI, U.S.A.). No additional purification was carried out. Sodium fenoprofen and valeric acid were supplied by the Eli Lilly Company (Indianapolis, IN, U.S.A.).

A Perkin-Elmer Model 601 high-performance liquid chromatograph equipped

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with an LC55 UV/VIS variable-wavelength detector and interfaced to a Perkin-Elmer Sigma 10 data system was used for the chromatography and data analysis.

The samples were chromatographed on a 25 cm × 0.26 cm HC-ODS-Sil-X column. This is a high-efficiency, high-capacity octadecyl silane (10 μm) packing. The column was maintained at 40°C during the chromatography and the compounds were detected at 272 nm. The mobile phase was acetonitrile—distilled water—glacial acetic acid (50:50:2); the flow-rate was 1.5 ml/min.

A 50- or 100-μl volume of serum is placed in a 1.5-ml Eppendorf centrifuge tube and 100 μl 1 *N* HCl are added; the tube is then vortexed. A 500-μl aliquot of chloroform, containing 20 μg/ml valeric acid (internal standard) is added, vortexed vigorously for 2 min and then centrifuged for 5 min in a Brinkmann table-top centrifuge.

The chloroform layer is placed in a clean glass tube and evaporated to dryness with nitrogen (40°C). The sample is reconstituted with either 20 or 50 μl of methanol, vortexed vigorously and 5 μl are injected into the HPLC column.

RESULTS AND DISCUSSION

Under these conditions, fenoprofen and valeric acid have retention times of 1.50 and 2.45 min, respectively. Fig. 1 shows typical chromatograms obtained from blank serum, control serum and patient's serum. Concentration is determined by the integrated area under the peak, relative to the internal standard (valeric acid). The two early peaks are unidentified artifacts which do not interfere with the analysis. The peak heights and areas remain relatively constant and are not appreciably influenced by increasing concentrations of fenoprofen.

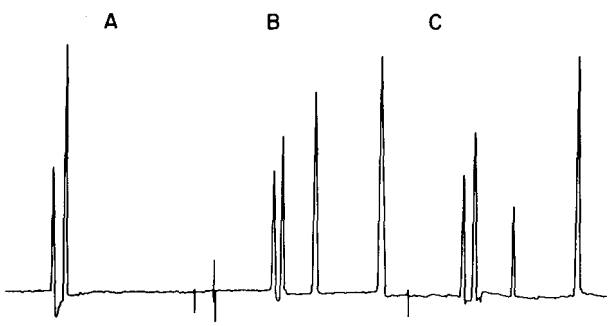

Fig. 1. Typical serum chromatograms: (A) blank, drug-free serum; (B) drug-free serum fortified with 10 μg/ml fenoprofen; (C) patient's serum determined as 4.7 μg/ml. The first two peaks in the chromatogram are unidentified artifacts; the third peak is fenoprofen; the last peak is the internal standard (valeric acid).

As mentioned in the Methods section, either 50 or 100 μl of serum can be used, with a corresponding reduction of the methanol reconstitution step from 50 to 20 μl when 50 μl of serum is used. The 100-μl sample size is preferred because it allows greater accuracy and reproducibility.

Reproducibility and day-to-day variation studies were carried out by

preparing fresh samples of 10 and 20 μg of fenoprofen per ml in drug-free serum. The results are shown in Table I. All results presented are averages of at least duplicate analyses.

TABLE I

REPRODUCIBILITY AND DAY-TO-DAY VARIATION OF ANALYSIS

Day	Fenoprofen (μg/ml)					
	Calculated	Observed	Mean (± S.D.)	Calculated	Observed	Mean (± S.D.)
1	20	20.7, 20.2, 19.1, 20.0, 19.0	19.98 (0.58)	10	10.0, 10.0, 9.9, 8.9, 9.5	9.66 (0.47)
2	20	20.0, 20.0, 20.2, 19.6, 20.3	20.02 (0.27)	10	10.9, 10.2, 10.1, 9.9, 9.7	9.98 (0.19)
3	20	20.0, 20.4, 18.9, 19.9, 20.5	19.94 (0.63)	10	10.3, 10.4, 9.9, 9.8, 10.0	10.08 (0.26)
4	20	20.6, 19.8, 19.9, 20.1, 19.7	20.02 (0.36)	10	10.0, 9.7, 8.9, 10.0, 10.5	9.82 (0.59)
5	20	21.0, 20.5, 18.9, 19.9, 19.5	19.96 (0.82)	10	10.2, 10.3, 9.9, 10.0, 10.6	10.20 (0.27)
Total (n = 25)			19.98 (0.52)			9.95 (0.40)

Analyses of prepared serum samples containing fenoprofen concentrations of 5—100 μg/ml were performed. The results indicate that the relationship between the serum concentration and the peak area is essentially linear over this range with a greater variability at the upper concentrations (Fig. 2). For example, at 100 μg/ml the range for five samples was 92—107, mean 98 μg/ml. This should present no problem because the recent work by Nash et al. [5] indicates a maximum serum concentration of 20 μg/ml in adults under therapeutic dosing conditions.

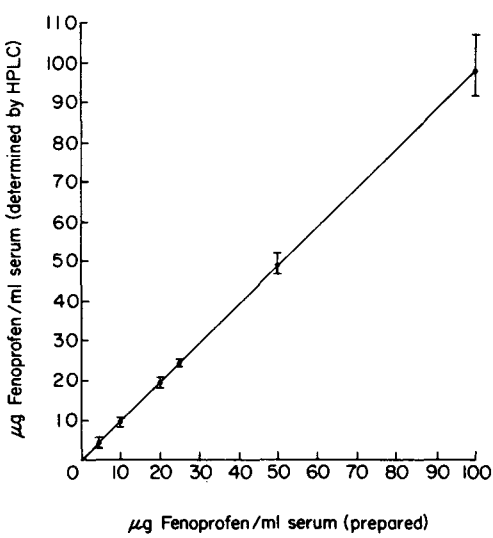

Fig 2. Fenoprofen standard curve. Mean (•) and range (n = 5 at each concentration).

Recovery studies were carried out by preparing a standard serum concentration of 20 μg/ml. An aliquot of this sample was analyzed as such. Another aliquot was fortified with an additional 20 μg/ml to yield a total concentration of 40 μg/ml The results (Table II) indicate virtually 100% recovery (Table I also indicates 100% recovery of samples studied).

TABLE II

RECOVERY STUDY OF STANDARD SERUM SAMPLES

Theoretical amount	Amount measured	Range	Mean ± S.D.
40 μg/ml	38.0, 38.65, 39.43, 39.8, 40.6, 40.6, 41.6	38—41.6	39.81 ± 1.24

Refrigerator and freezer stability studies were carried out by preparing 20 μg/ml fenoprofen in serum which was analyzed on the day of preparation. Part of the sample was stored frozen and part placed in the refrigerator. Analysis of the refrigerated samples over the next four days indicated a gradual decline of assayable fenoprofen (Table III). Samples were removed from the freezer on days 7, 10, and 14 and analysis indicated essentially no loss of fenoprofen (Table III). It is not necessary, therefore, to have major concern about sample stability; if samples are to be analyzed within a week they can be stored in the refrigerator.

TABLE III

STORAGE STABILITY STUDY OF STANDARD SERUM SAMPLES

Refrigerator		Freezer	
Day	Measured value* (μg/ml)	Day	Measured value* (μg/ml)
1	20.0	7	19.8
2	20.7	10	20.1
3	19.6	14	19.9
4	19.0		
5	18.2		
Mean (±) S.D.	19.5 (0.95)		19.3 (0.15)
Range	20—18.2		19.8—20.1

*Theoretical amount 20 μg/ml.

The method presented here is simple, fast (50 samples can conveniently be analyzed per day), accurate and reliable. In addition, the use of small sample sizes (50 or 100 μl of serum) makes the assay ideal for pediatric studies and therapeutic monitoring.

ACKNOWLEDGEMENTS

The authors thank Dr. Nash from the Eli Lilly Co. for his helpful comments and discussions about the development of this assay. This work was supported by the Eli Lilly Company.

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CHROMBIO. 602

Note

Determination of pindolol in human plasma by high-performance liquid chromatography

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(Received January 25th, 1980)

Pindolol, *d,l*-4-(2-hydroxy-3-isopropylamino-propoxy)-indole is a potent β-adrenoceptor blocking agent for the treatment of hypertension [1,2] and angina [3]. Fluorimetry, after reaction with *o*-phthalaldehyde, is the only published method sensitive enough to estimate therapeutic concentrations of this drug [4]. This method is sensitive to approximately 20 ng pindolol and is relatively simple to perform [5,6]. However, its specificity particularly in relation to interference by other drugs is questionable and could have contributed to the very large scatter in pindolol plasma concentrations of hypertensive subjects taking additional medication [7].

We report here a procedure for determining plasma pindolol concentrations utilising high-performance liquid chromatography (HPLC). The procedure involves a three-step solvent extraction of pindolol from plasma combined with the separative capability of HPLC and the sensitivity of fluorescence detection.

EXPERIMENTAL

Reagents

Acetonitrile, HPLC 190-nm grade was purchased from Waters Assoc. (Milford, MA, U.S.A.). Diethyl ether (Ajax Chemicals, Sydney, Australia) and *n*-heptane (BDH Chemicals, Liverpool, Great Britain) were washed successively with 1 *M* sodium hydroxide, 1 *M* hydrochloric acid, water and then distilled prior to use. Water for HPLC was distilled from alkaline potassium permanganate before use. Pindolol was obtained from Sandoz, Sydney, Australia. All other reagents were of analytical grade obtained from commercial sources.

Chromatographic system

A 5000 series liquid chromatograph fitted with a universal loop injector (Varian Assoc., Palo Alto, CA, U.S.A.) was used in conjunction with a column

(300 × 4 mm I.D.) packed with Micropak MCH-10 octadecylsilane (particle size 10 μm). A guard column (40 × 4 mm I.D.) was packed with pellicular C_{18} material (particle size 40 μm, Vydac SC reversed-phase, Varian Assoc.). The detector was a Schoeffel FS-970 fluorimeter (Schoeffel, Westwood, NJ, U.S.A.) fitted with a deuterium arc source. Pindolol was detected by excitation at 220 nm ($\lambda_{max.}$ for pindolol 219 nm) and its fluorescence emission was selected by a Corning 7-60 glass filter with an approximate band pass of 320—400 nm.

Plasma samples

Drug-free venous blood was obtained from healthy human subjects. Blood was also obtained from (i) clinic out-patients on chronic pindolol therapy 2—4 h after taking 15 mg pindolol once daily and (ii) clinic out-patients taking medication which did not include pindolol. These patients served as controls for evaluating possible drug interferences in the assay. Blood was collected into plastic tubes containing lithium heparin and centrifuged at 1000 *g* for 10 min in a refrigerated centrifuge. Plasma was stored in plastic tubes at −20°C until analysed for pindolol.

Extraction of pindolol and HPLC estimation

Plasma (2 ml) was placed into 25-ml stoppered glass tubes in ice. To each tube were added 0.5 ml 1 *M* sodium hydroxide and 8 ml diethyl ether. Pindolol was extracted into the ethereal layer by mechanically vortexing the phases for 2 min. The phases were separated by centrifugation (3 min at 1000 *g*) and 6 ml of the ethereal extract transferred to clean 10-ml tapered glass-stoppered tubes containing 200 μl of 0.1 *M* sodium dihydrogen phosphate (pH 3.0). Pindolol was extracted into the aqueous phase by mechanically vortexing the solution for 20 sec. After separation of the phases by centrifugation the aqueous phase was frozen by immersion of the tubes into a dry ice—ethanol mixture and the ethereal phase aspirated. The aqueous phase was then washed with 2 ml of *n*-heptane by mechanically vortexing the solution for 20 sec. After centrifugation and freezing of the aqueous phase, the *n*-heptane was aspirated and discarded. Accurate aliquots (50—100 μl) of the acidic phase were injected directly onto the HPLC column. The mobile phase was 0.01 *M* perchloric acid—acetonitrile (3 : 2) at a flow-rate of 2.0 ml/min. A calibration curve was prepared by treating either water or plasma (2 ml) containing known concentrations (25, 50, 75 and 150 ng/ml) of pindolol in an identical manner. There was no difference in calibration curves prepared by substituting water for plasma.

RESULTS AND DISCUSSION

Pindolol isolated from plasma chromatographed with a retention time of 3.6 min and is well separated from any analytical artefacts (Fig. 1). The fluorescence (y) due to pindolol expressed in μA of detector current is linearly related to its plasma concentration (x) according to the equation: $y = 0.426x + 0.167$ ($r = 0.996$) up to a concentration of 150 ng pindolol per ml of plasma. Higher concentrations were not examined. Variations in the calibration curve from day to day were small, the coefficient of variation in the slope being 8.5%. Recoveries of pindolol from plasma or water were identical and after allowing for

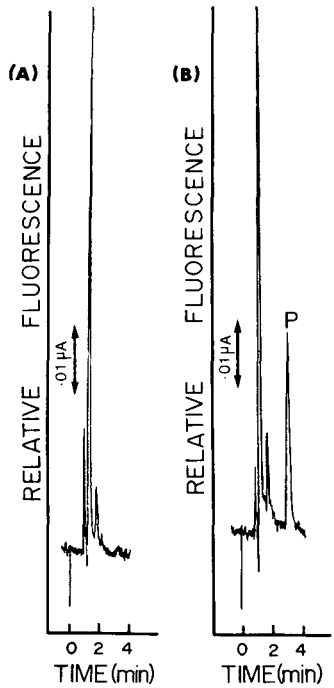

Fig. 1. High-performance liquid chromatograms of extracts from plasma. (A) Control drug-free plasma; (B) plasma containing 67 ng/ml pindolol; peak P = pindolol. A volume equivalent to 25 ng pindolol was injected onto the HPLC column.

TABLE I

PRECISION OF THE HPLC PINDOLOL ASSAY

Plasma pindolol concentration (ng/ml)		
Pindolol added	Pindolol recovered (mean ± S.D.)	*n*
2	2.5 ± 0.4	3
5	5.2 ± 0.5	6
10	10.4 ± 1.3	7
25	26.0 ± 1.0	4
75	76.7 ± 2.7	3
100	101.7 ± 4.8	4

aliquoting this was in excess of 96%. Hence calibration curves were usually prepared using water instead of drug-free plasma. The reproducibility of the assay together with the standard deviations determined for various plasma concentrations of pindolol are given in Table I. The sensitivity of the assay is about 2 ng pindolol per ml plasma when 2 ml of plasma is used in the assay. This is about a four-fold increase in sensitivity over what we have observed [4] for the fluorimetric procedure developed by Pacha [6]. In this assay we chose not to include an internal standard so as to minimise the probability of potential drug interferences when analysing plasma obtained from out-patients taking additional medication.

TABLE II

DRUGS EXAMINED IN VIVO FOR POSSIBLE INTERFERENCE IN THE HPLC DETERMINATION OF PINDOLOL

Allopurinol	Indomethacin
Amiloride	Isosorbide dinitrate
Chlordiazepoxide	Methyldopa
Chlorothiazide	Nitrazepam
Clonidine	Prazosin
Cyclopenthiazide	Quinidine
Diazepam	Salbutamol
Dicoumarol	Sulphinpyrazone
Digoxin	Thyroxine
Disopyramide	Trifluorperazine
Frusemide	

TABLE III

PLASMA PINDOLOL CONCENTRATIONS OF 9 OUT-PATIENTS DURING A PERIOD OF 12 MONTHS

Patients were taking 15 mg pindolol once daily. Blood was collected for pindolol analysis 2—4 h after taking pindolol. Results are the means ± standard error of mean.

	Months after commencing pindolol medication			
	1	3	6	12
Plasma pindolol concentration (ng/ml)	77.3 ± 10.9	73.9 ± 11.3	73.7 ± 7.8	59.6 ± 8.0
Time of sampling after dose (h)	2.69 ± 0.15	3.15 ± 0.26	3.47 ± 0.29	3.45 ± 0.19

Analysis of plasma samples from out-patients taking medication which did not include pindolol served as a useful method for investigating possible interferences by other drugs and their metabolites on the estimation of plasma pindolol concentrations. Therapeutic concentrations of drugs which were investigated for possible interference in the assay are listed in Table II. None of these drugs interfered in the estimation of pindolol. However, plasma extracts of patients taking quinidine or prazosin had extra peaks in their chromatograms. In both instances these drugs prolonged analysis time. After quinidine administration two intense peaks were observed with retention times of about 4 and 9 min whilst after prazosin, a single intense peak appeared at 6 min.

Pindolol concentrations determined by this method from plasma of nine out-patients over a 12-month period are presented in Table III. Mean plasma pindolol concentrations observed in these patients are in agreement with the known pharmacokinetics of this drug [4]. Differences in plasma concentration observed over this 12-month period of therapy are small as would be expected from a drug with a small degree of first pass metabolism [4].

In summary, these results indicate that the HPLC method described for estimating plasma pindolol concentrations is of sufficient sensitivity and specificity for use in pharmacokinetic studies and clinical trials. We have docu-

mented its specificity in relation to interference by other drugs which are often co-administered. The lack of interference by these drugs suggests that the method is also applicable to determining plasma pindolol concentrations in outpatients taking additional medications.

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CHROMBIO. 589

Book Review

Biological/biomedical applications of liquid chromatography II, edited by G.L. Hawk, Marcel Dekker, New York, 1979, XIV + 504 pp., price Sfr. 100.00, ISBN 0-8247-6915-5.

The second volume of the above monograph represents 24 papers which were presented at the Second Liquid Chromatography Symposium held in Boston, MA, U.S.A., October 5 and 6, 1978. As in the first volume [for review see J. Chromatogr., 181 (1980) 516] the published papers can be categorized into three groups:

1. Clinico-chemical applications (original papers dealing with catecholamines, steroids, bile acids, prostaglandins, purines and pyrimidines, LDH isoenzymes, vitamins and monitoring of hemodialysis, and a review by Altshuler et al. on HPLC in the hospital clinical laboratory).
2. Diverse biological and biochemical applications (fatty acids in micro-organisms, amino acids, peptides, proteins, peptide hormones, endorphines, purines, dyes, and a review on carbohydrates by Schwarzenbach).
3. Drug monitoring and pharmacokinetic studies (original papers dealing with methylphenidate, ritalinic acid and chlorambucil, and a review by Anhalt on antibiotic assays).

Apart from these categories there is a paper by P. Brown et al. dealing with the methods of peak identification in HPLC. The end of the book is devoted to a glossary of liquid chromatography terms, and author and subject index.

The advantages and drawbacks of this volume are about the same as those of volume I. In comparison to the previously published volume the number of papers devoted to clinico-chemical applications is increased on account of drug monitoring. This, however, does not mean that such a distribution reflects the present trends in the literature. From the published papers one can trace the continuously increasing application of reversed phases in the biomedical field. About 75% of published papers exploit silica gel with C-18 chemically bonded phase. Including a glossary of liquid chromatography terms is certainly not a bad idea. One can, however, criticize the selection of terms. There are elementary terms on the one hand (but even these are sometimes defined erroneously; for example, the liquid chromatography definition involves column techniques only); on the other hand, several important terms from the biomedical field are missing.

In spite of some criticism, Hawk's book can be welcomed because it offers a fair amount of information for those involved in biochemistry and clinical chemistry.

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Vol. 183, No. 2

JOURNAL OF CHROMATOGRAPHY
BIOMEDICAL APPLICATIONS

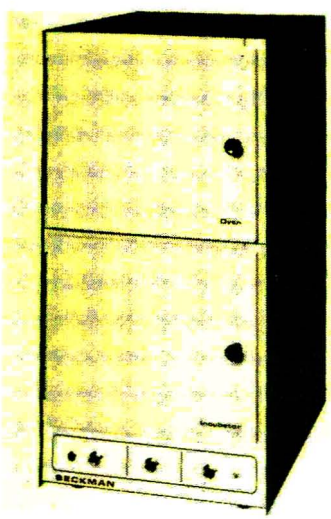
NEWS SECTION

APPARATUS

N-1504

OVEN/INCUBATOR

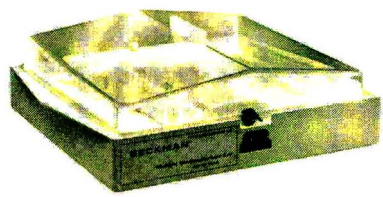
A compact oven/incubator from Beckman Instuments provides a fast method for drying down agarose and polyacrylamide gels and incubating protein separations. The upper oven compartment and the lower incubation compartment accommodate large loads. The temperature variations are limited to no more than ±1°C. The maximum oven temperature is 90°C. The forced-draft incubator offers two fixed temperatures (37°C and 45°C), that can be selected by a switch.



N-1505

ELECTROPHORESIS CELL AND BUFFERS

Beckman Instruments has introduced the Multizone™ cell and two electrophoresis buffers for protein analysis. The cell aids in separating a large number of samples at one time. The electrophoresis chamber has space for three cellulose acetate membranes or three agarose gels. The Multizone cell enables the separation of about three times as many samples as the usual electrophoresis chambers. The Beckman B-1 Buffer is a universal buffer than can be used with both agarose gels and cellulose acetate membranes; the B-4 TEB Hemoglobin Buffer is designed to provide sharp separations for hemoglobin screening.



For further information concerning any of the news items, apply to the publisher, using the reply cards provided, quoting the reference number printed at the beginning of the item.

N-1484

AMINO ACID ANALYZER

The new LKB 4400 amino acid analyzer completes a protein hydrolysate analysis in a little over an hour. The instrument can detect amounts less than 100 picomoles of amino acids. The amount of sample required is 5–80 μl per sample capsule. The built-in sample autoloader has 72 capsule positions. The integral refrigeration unit preserves the samples and the reagents. The columns and the reaction coil are heated by a solid-state unit, which provides a fast temperature rise capability and removes the need for a water bath.

N-1475

ELECTROPHORESIS SYSTEM

The Model 600 Electrophoresis Chamber from Shandon Southern Instruments Ltd. can be combined with a range of power supplies and accessories in order to form an electrophoresis system for immunoelectrophoresis, thin-layer electrophoresis, cellulose acetate electrophoresis and iso-electric focusing. The Shandon 600 is designed to produce rapid, reliable results with a minimum of preparation time and a minimum of work for the user. Protection of the operator is a feature of the 600 with an interlocking system that instantly shuts off power in the chamber when the cover is raised.

N-1499

BROCHURES FROM PHARMACIA FINE CHEMICALS

A number of brochures describing apparatus and chemicals for electrophoresis and ion-exchange chromatography are available from Pharmacia Fine Chemicals. The first one, an 8-page brochure, describes Pharmalyte™, a carrier ampholyte for use in isoelectric focusing. A 6-page brochure describes the principles of ion-exchange chromatography and can be used as a guide for the selection of methods and applications. Another leaflet gives compact information on Pharmacia's ECPS 3000/150 power supply for isoelectric focusing and electrophoresis. Finally, a 2-page brochure gives essential information on Pharmacia's 214-nm UV monitor.

N-1472

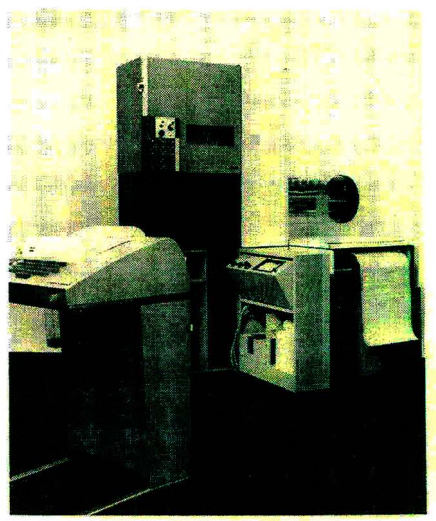
CLINISCAN

Helena Laboratories have introduced a microprocessor-based densitometer system, Cliniscan, for the evaluation of electrophoresis plates. The system is said to have positive sample indentification and can scan 48 serum proteins in about 9 minutes. It is equipped with a video display for manual editing and it has keyboard control with an alphanumeric display. The system can be operated in fluorescence, visible and quenching modes. It can give peak height and/or peak area results and it is compatible with most in-lab computer systems.

N-1508

AMINO ACID ANALYZER

Dionex introduced their Model D-502, as an addition to the D-500 Series of amino acid analyzers. The Model D-502 is an automatic, high-performance analyzer for the analysis of amino acids and other ninhydrin positive compounds in peptide–protein hydrolyzates and biological fluids. Single column ion-exchange chromatography is controlled and monitored by a dedicated minicomputer having 16 K core memory. The software package, Dionex Operating System II, also provides data acquisition capability for a dual channel ratio photometer. Output data are presented in both analog and alphanumeric format. Typical analysis times are 30 min for peptide–protein hydrolyzates and 180 min for complex biological fluids.



CHEMICALS

N-1460

COLUMNS FOR PROTEIN GEL FILTRATION

The Bio-Sil® TSK packings from Bio-Rad Laboratories make it possible to apply HPLC technology to the gel filtration separation of proteins and other bio-polymers. The packings combine the properties of classical GFC media, viz. stability, efficiency and inertness, with those of silica packings, viz. mechanical strength and capability for high-speed, high-pressure operation. The Bio-Rad packings are said to allow resolutions comparable to that of classical gels in less than one hour in many cases. The TSK packings have a particle size of 10 μm and are available in two molecular weight operating ranges. Recoveries of protein from Bio-Sil TSK packings are said to be quite high; values over 95% are not uncommon.

N-1496

CELLULOSE ACETATE MEMBRANES

Beckman Instruments' Microzone® PLUS membranes are said to offer pinpoint resolution, good transparency and high sensitivity for small monoclonal protein detection. The Microzone membranes can be used in most of the electrophoretic methods including lipoprotein and isoenzyme procedures.

N-1481

SEPARATION NEWS, NO. 1, 1980

Separation News, a periodical issued by Pharmacia Fine Chemicals AB, opens with an article on SPDP, a new reagent for preparing intermolecular conjugates. The article is followed by an instructive illustrated story on preparative iso-electric focusing. Furthermore Separation News contains abstracts of articles in the literature about work done with chemicals and apparatus from Pharmacia.

N-1495

REACTI-GEL™

Reacti-Gel™, N,N-carbonyldiimidazole activated agarose, is available from Pierce Chemical in a new 10-ml "trial size" package. Reacti-Gel is a new product in the rapidly expanding field of affinity chromatography. Features of this product include a leak resistant, charge-free linkage to N-nucleophiles, good stability and the efficiency of coupling leashes, ligands and proteins. Reacti-Gel has an activation level of 20–25 micromoles per ml of gel.

PROCEDURES

N-1497

ISOELECTRIC FOCUSING ON AGAROSE

Quick gel preparation and short staining and destaining times, are advantages of using agarose as the support medium in isoelectric focusing. It is also possible to focus large molecules, apply zymogram techniques and quickly dry the gels to be able to record the results permanently. Pharmacia's Agarose IEF is a purified agarose, optimized for isoelectric focusing. Pharmacia has a booklet available which describes the properties and the applications of Agarose IEF.

NEW BOOKS

The serum concentration of drugs (Int. Congress Series, No. 501), edited by F.W.H.M. Merkus, Excerpta Medica, Amsterdam, New York, 1979, 300 pp., price Dfl. 150.00, US$ 73.25, ISBN 0-444-90126-4.

Genetic metabolic diseases: Early diagnosis and prenatal analysis, by H. Galjaard, Elsevier/North-Holland Biomedical Press, Amsterdam, New York, 1980, XVI + 880 pp., price Dfl. 285.00, US$ 139.00, ISBN 0-444-80143-X.

The role of non-specific immunity in the prevention and treatment of cancer, edited by M. Sela, Elsevier/North-Holland Biomedical Press, Amsterdam, New York, 1980, XVI + 590 pp., price Dfl. 195.00, US$ 95.00, ISBN 0-444-80156-1.

Side effects of drugs, Annual 4, 1980, edited by M.N.G. Dukes, Excerpta Medica, Amsterdam, New York, 1980, 396 pp., price Dfl. 130.00, US$ 63.50, ISBN 0-444-90130-2.

Mathematics and statistics for the bio-sciences, by G. Eason, C.W. Coles and G. Gettinby, Ellis Horwood (Wiley), Chichester, 1980, ca. 450 pp., price ca. US$ 49.50, £ 20.00, ISBN 0-85312-175-3.

Gel chromatography: Theory, methodology and applications, by T. Kremmer and L. Boross, Wiley, Chichester, New York, Brisbane, Toronto, 1979, 299 pp., price £ 16.50, ISBN 0-471-99548-7.

Modern size-exclusion liquid chromatography: Practice of gel permeation and gel filtration chromatography, by W.W. Yau, J.J. Kirkland and D.D. Bly, Wiley-Interscience, New York, Chichester, Brisbane, Toronto, 1979, XVII + 476 pp., price £ 15.00, ISBN 0-471-03387-1.

Drug level monitoring – Analytical techniques, metabolism, and pharmacokinetics, by W. Sadée and G.C.M. Beelen, Wiley-Interscience, New York, Chichester, Brisbane, Toronto, 1980, XIII + 495 pp., price £ 22.20, ISBN 0-471-04881-X.

NEW JOURNAL

Chemical, Biomedical and Environmental Instrumentation (formerly Chemical Instrumentation), edited by E.M. Chait, Marcel Dekker, New York, Vol. 10 (4 issues), 1980, subscription price per volume US$ 55.00, individual subscription price per volume US$ 27.50, ISSN 0190-4094.

MEETING

4th INTERNATIONAL SYMPOSIUM ON AFFINITY CHROMATOGRAPHY AND RELATED TECHNIQUES

The 4th International Symposium on Affinity Chromatography and Related Techniques – Theoretical Aspects, Industrial and Biomedical Applications will be held from June 22–26, 1981 in the University of Nijmegen, The Netherlands. The scope of the meeting will cover the following topics:

Theoretical Aspects – Ligand/ligate interaction in homogeneous and heterogeneous systems. General theory of electrostatic, hydrophobic and charge-transfer interaction. Theoretical analyses of affinity separations. Matrix structure. Column/batch procedures.

Polymeric Matrices and Ligand Immobilization – Natural synthetic polymers. Immobilization of ligand molecules.

Applications – Isolation and purification.

The proceedings of the symposium will be published by Elsevier Scientific Publishing Company in the Analytical Chemistry Symposia Series.

Plenary lectures will be presented by invited speakers. Participants wishing to present a paper and/or poster should address themselves for detailed information to the organizing committee at the following address: Secretariat, Department of Organic Chemistry/Faculty of Sciences, Katholieke Universiteit, Toernooiveld, 6525 ED Nijmegen, The Netherlands.

PUBLICATION SCHEDULE FOR 1980

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

MONTH	D 1979	J	F	M	A	M	J	J	A	S	O	N	D
Journal of Chromatography	185 186	187/1 187/2 188/1	188/2 189/1 189/2	189/3 190/1	190/2 191 192/1	192/2 193/1 193/2 193/3	194/1 194/2 194/3	195/1 195/2 195/3	196/1 196/2 196/3	197/1 197/2 198/1	The publication schedule for further issues will be published later.		
Chromatographic Reviews			184/1	184/2					184/3				
Biomedical Applications		181/1	181/2	181/ 3-4	182/1	182/2	182/ 3-4	183/1	183/2	183/3	183/4		

INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 193, No. 3, pp. 529–532. A free reprint can be obtained by application to the publisher)

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