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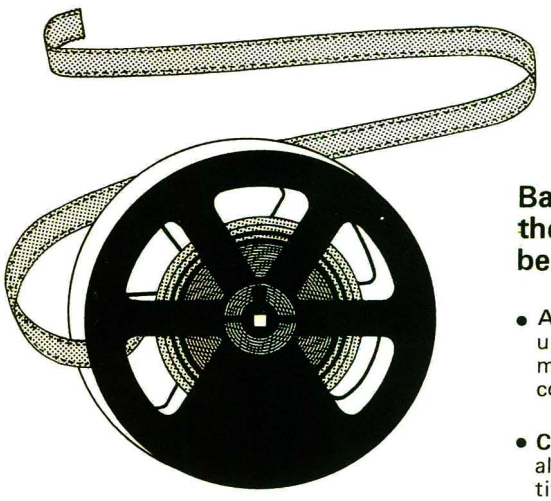

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SIMULTANEOUS ANALYSIS OF PHENYLGLYCOLS AND PHENYLETHANOLS IN HUMAN URINE BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY

DAVID J. EDWARDS*, MARGUERITE RIZK and JOHN NEIL

Department of Psychiatry, Western Psychiatric Institute and Clinic, University of Pittsburgh School of Medicine, Pittsburgh, Pa. 15261 (U.S.A.)

(Received April 10th, 1979)

SUMMARY

A method is described for the determination of the neutral metabolites formed from catecholamines and various other structurally related phenylethylamines by using gas chromatography—chemical ionization—mass spectrometry. These metabolites (phenylglycols and phenylethanols) were extracted from urine specimens and converted to pentafluoropropionyl derivatives which were separated on either 3% OV-1, 3% SP-2250, or 3% QF-1 packed columns. Our results demonstrate the presence in human urine of *p*-hydroxyphenylglycol, a metabolite of octopamine. One patient excreted 13 and 91 μg/day of free and total (free + conjugated) *p*-hydroxyphenylglycol, respectively. Treatment with a monoamine oxidase inhibitor reduced the excretion of total *p*-hydroxyphenylglycol to 30% of baseline level.

INTRODUCTION

The principal catabolic pathway of catecholamines, their O-methylated metabolites, and related phenylethylamines in neural tissues is via the oxidative deamination reaction catalyzed by monoamine oxidase. The aldehyde intermediates thus formed are metabolized further either by oxidation to a carboxylic acid or by reduction to an alcohol. Schanberg et al. [1] showed that 3-methoxy-4-hydroxyphenylethylene glycol (MHPG) is the major metabolite of norepinephrine in brain. Since norepinephrine is primarily converted in peripheral tissues to the acidic metabolite, vanilylmandelic acid (VMA), the urinary excretion of MHPG is often used as an index of norepinephrine metabolism in the central nervous system (CNS). The amount of MHPG excreted

*To whom correspondence should be addressed. Present address: Department of Pharmacology-Physiology, School of Dental Medicine, 541 Salk Hall, University of Pittsburgh, Pittsburgh, Pa. 15261, U.S.A.

per day as the free glycol plus its conjugates is relatively large (about 1—2 mg in man) and can be readily determined by gas chromatography with electron-capture detection of the trifluoroacetyl [2,3] or pentafluoropropionyl (PFP) [4] derivatives. Other neutral metabolites are generally present in biological fluids in much lower concentrations, but their measurement may nevertheless be an important step in studying the metabolism of their parent amines. Several investigators [4—7] have used gas chromatographic methods combined with electron-capture detection or mass spectrometry to determine the levels of 3-methoxy-4-hydroxyphenylethanol (MHPE), a neutral metabolite of dopamine, in brain, cerebrospinal fluid and urine. Although there are some discrepancies among the levels reported by different laboratories, it is clear that MHPE is a minor metabolite of dopamine and the significance of measuring this metabolite is not yet known. On the other hand, we have shown that phenylethylene glycol (PEG) is the major deaminated metabolite of phenylethanolamine [8]. By using gas chromatography—mass spectrometry (GC—MS) we have been able to measure PEG in rat and human urine and we have found that its excretion is elevated in untreated phenylketonuria [9]. In this paper we describe a method using GC—MS which permits the simultaneous determination of PEG, *p*-hydroxyphenylethanol (pHPE), *p*-hydroxyphenylglycol (pHPG), and MHPE, which are neutral metabolites of phenylethanolamine, *p*-tyramine, octopamine and dopamine, respectively, and MHPG and dihydroxyphenylethylene glycol (DHPG), both metabolites of norepinephrine.

EXPERIMENTAL

Urine specimens were obtained from patients on the Clinical Research Unit at the Western Psychiatric Institute and Clinic. All patients were drug-free for at least two weeks. The urines were collected in containers to which sodium metabisulfite had been added as a preservative. The urines were kept cold during the 24-h collection period, after which they were frozen until assayed.

Urine samples (3 ml) were added to 1 ml of 1 *M* sodium acetate (pH 6.0) and 0.5 ml of 2% EDTA and treated with 0.1 ml glusulase (Endo Labs., Garden City, N.Y., U.S.A.) at 37° for 20 h as described by Dekirmenjian and Maas [3]. The samples were then extracted according to the detailed procedures given elsewhere [9]. Briefly, the neutral metabolites were extracted with 3 × 10 ml of ethyl acetate. The combined organic phase was washed with 1 ml of 1 *M* $KHCO_3$ and evaporated just to dryness at 43° in a rotary evaporator. The residue was dissolved in 2 ml of ethyl acetate and stored at 4°. Calibration curves were prepared by the addition of 0—120 μl of a standard mixture (containing 2.5 μg/ml of PEG, pHPE, pHPG and 100 μg/ml of MHPG) to separate aliquots of a pooled urine sample and carrying them through the extraction procedure, without the addition of glusulase.

Aliquots of 200 μl of the ethyl acetate extracts obtained from samples and standards were reacted for at least 15 min at room temperature with 25 μl of pentafluoropropionic anhydride (PFPA; Pierce, Rockford, Ill., U.S.A.). In order to completely derivatize *o*-hydroxyphenylethanol (oHPE), a somewhat longer reaction time (30 min) was required, presumably due to steric hindrance. Two microliters of the reaction mixture were then injected into a

Finnigan Model 3200F quadrupole gas chromatograph—mass spectrometer which was operated in the chemical ionization (CI) mode (electron energy 90 eV). The chromatographic separations were carried out on a 5 ft. × 2 mm I.D. silanized U-column packed with either 3% OV-1, 3% SP-2250, or 3% QF-1 on 80—100 mesh Supelcoport (Supelco, Bellefonte, Pa., U.S.A.) using temperature programming. The injection port was maintained at 235°. Methane, which served as both the carrier gas and the reagent gas, was adjusted to a flow-rate (about 10 ml/min) which gave an ion source pressure of 1.0 torr. At the same time the sample was injected, a vacuum diverter was set to 40 sec with an automatic timer in order to prevent the solvent peak from entering the ion source. A six-channel programmable multiple-ion monitor (PROMIM) was used for selected ion monitoring by focusing on the most prominent ions.

The following standards were obtained from commercial sources and were used without further purification: phenylethylene glycol (PEG), Pfaltz and Bauer, Stamford, Conn., U.S.A.; *p*-hydroxyphenylethanol (pHPE), *m*-hydroxyphenylethanol (mHPE), *o*-hydroxyphenylethanol (oHPE) and *p*-hydroxyphenylpropanol (pHPP), Aldrich, Milwaukee, Wisc., U.S.A.; 3-methoxy-4-hydroxyphenylethanol (MHPE) and 3,4-dihydroxyphenylethanol (DHPE), Regis, Morton Grove, Ill., U.S.A.; and 3,4-dihydroxyphenylethylene glycol (DHPG), Sigma, St. Louis, Mo., U.S.A. *p*-Hydroxyphenylethylene glycol (pHPG) was synthesized by Dr. B.L. Goodwin.

RESULTS

Table I shows the CI mass spectra obtained with methane as the reagent gas for the PFP derivatives of several representative neutral alcoholic metabolites. The derivatives of all the metabolites except DHPE had their base peaks corresponding to the loss of pentafluoropropionic acid (*m/e* 164) from the M+1 ions. These ions appeared at *m/e* 267 for PEG and pHPE, *m/e* 281 for pHPP, *m/e* 429 for pHPG, *m/e* 591 for DHPG, *m/e* 297 for MHPE and *m/e* 459 for MHPG. The base peak of the derivative of DHPE resulted from the loss of a second PFP group in addition to PFPOH. The striking feature of these CI mass spectra is the appearance in most cases of only a single prominent ion in the mass spectra. Thus, for PEG, pHPE, DHPE and MHPE, no peak exceeded 4% of the abundance of the base peak. The M+1 ions were detectable in the spectra of only three of the derivatives, and then as peaks with a relatively low abundance: pHPP (1%), MHPE (2%), and MHPG (9%).

In order to reduce the fragmentation of the M+1 ions, we obtained CI spectra using isobutane as the reagent gas. Although in this case the relative abundance of the M+1 ions of MHPE and MHPG increased to 14% and 35%, respectively, still no M+1 ion was detected for PEG. However, because of practical considerations [10], we chose to use methane as the reagent gas for all further studies.

Fig. 1 shows the selected ion-current profiles obtained for a mixture of eight derivatives separated on three different columns. The retention times observed are summarized in Table II. The order by which the compounds eluted was the same on each of the three columns, except that MHPG eluted before MHPE on 3% SP-2250 and that DHPG eluted after both MHPE and MHPG on 3% QF-1.

TABLE I

CI MASS SPECTRA OF THE PFP DERIVATIVES OF SOME PHENYLGLYCOLS AND PHENYLETHANOLS

Mass spectra were obtained at 90 eV by scanning over the range from *m/e* 180 to *m/e* 800. Minor peaks (usually < 1% abundance) and isotope peaks are not shown. Abbreviations are defined in the Experimental section. The mass spectra for mHPE and oHPE were virtually identical to that of the isomer, pHPE (shown below) with base peaks at *m/e* 267 and peaks at *m/e* 295 and 307 equal to 3% abundance. mHPE, but not oHPE, had a peak at *m/e* 247 of 3% abundance.

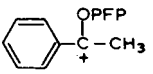
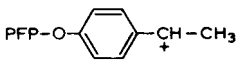
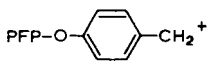
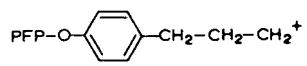
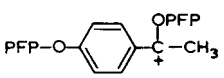
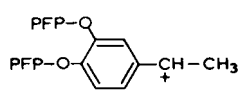
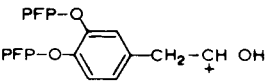
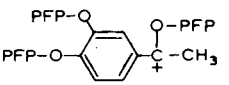
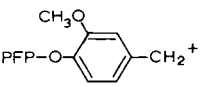
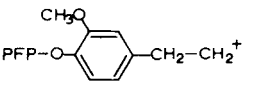
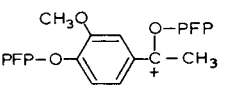
Compound	*m/e*	Relative abundance	Probable structure
PEG	267	100	
	295	3	$(M - PFPOH) + C_2H_5^+$
	307	2	$(M - PFPOH) + C_3H_5^+$
pHPE	247	4	*m/e* 267 — HF
	267	100	
	295	4	$(M - PFPOH) + C_2H_5^+$
	307	4	$(M - PFPOH) + C_3H_5^+$
pHPP	253	24	
	281	100	
	309	4	$(M - PFPOH) + C_2H_5^+$
	321	4	$(M - PFPOH) + C_3H_5^+$
	425	1	(M+1) — HF
	445	1	M+1
pHPG	267	15	*m/e* 429 — (OPFP) + 1
	429	100	
DHPE	263	2	*m/e* 283 — HF
	283	100	*m/e* 429 — (PFP) + 1
	311	4	*m/e* 429 — (PFP) + $C_2H_5^+$
	323	3	*m/e* 429 — (PFP) + $C_3H_5^+$
	429	2	

TABLE I (*continued*)

Compound	*m/e*	Relative abundance	Probable structure
	445	1	
DHPG	267	2	*m/e* 429 — (OPFP) + 1
	283	5	*m/e* 429 — (PFP) + 1
	429	10	*m/e* 591 — (OPFP) + 1
	445	2	*m/e* 591 — (PFP) + 1
	542	8	—
	591	100	
MHPE	277	3	*m/e* 297 — HF
	283	2	
	297	100	
	325	4	(*m/e* 297 — 1) + $C_2H_5^+$
	337	1	(*m/e* 297 — 1) + $C_3H_5^+$
	441	1	(M+1) — HF
	461	2	M+1
MHPG	297	14	*m/e* 459 — (OPFP) + 1
	411	9	—
	459	100	
	623	9	M+1

Urine specimens were analyzed on both 3% QF-1 and 3% OV-1. Equivalent results were obtained on either column. Since pHPG also produced a fragment at *m/e* 267 with an abundance 15% of the base peak (Table I), the appearance of this peak confirmed the identity of pHPG in these samples.

The results of analyses of five separate 24-h urine collections from the same patient are given in Table III. The average excretion of free + conjugated PEG, pHPE, pHPG and MHPG during the drug-free period was 22, 11, 91 and 1399 μg/24 h, respectively. DHPG and MHPE were also detected in these samples, but they were not quantitated. Treatment with a monoamine oxidase inhibitor resulted in a diminished excretion of pHPG and MHPG (to 30% and 14%, respectively, of the drug-free excretion level) but not of PEG and pHPE. The selected ion-current profiles for two of these samples, one obtained during the drug-free period and one during treatment with the monoamine oxidase inhibitor, are shown in Fig. 2.

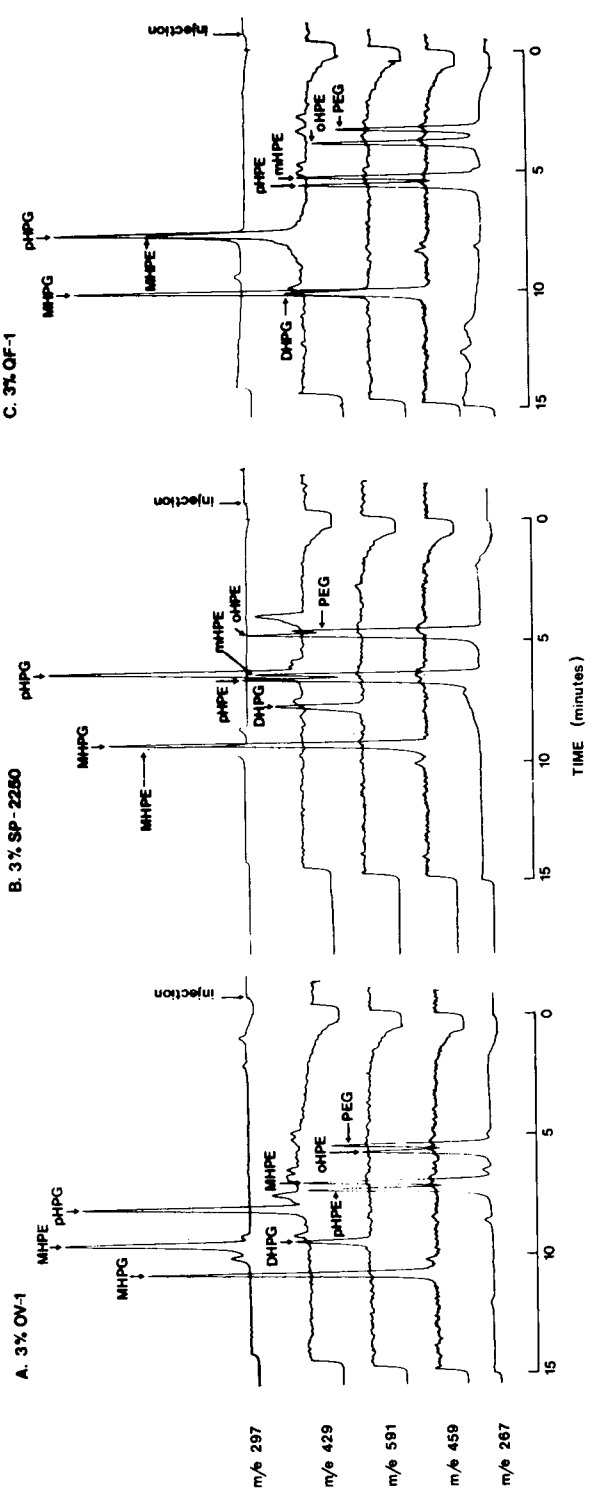


Fig. 1. Selected ion profiles for a standard mixture separated on: A, 3% OV-1 (programmed from 90° at 6°/min); B, 3% SP-2250 (from 90° at 6°/min); and C, 3% QF-1 (from 120° at 6°/min). The recorder was set at 2 V for the top channel (except for panel A, 1 V), 200 mV for each of the second, third and fourth channels, and 2 V for the bottom channel.

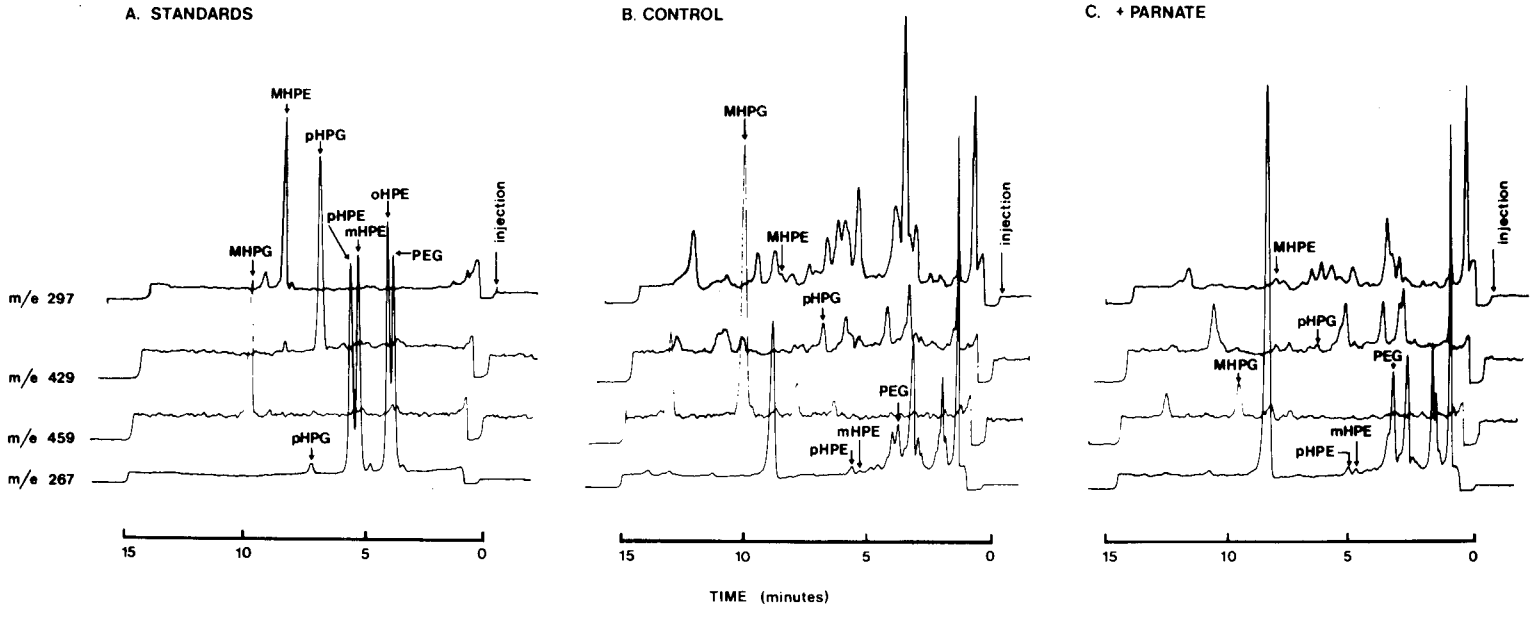


Fig. 2. Selected ion profiles for the analysis of phenylglycols and phenylethanols in urine specimens obtained from a patient drug-free and during treatment with a monoamine oxidase inhibitor (40 mg/day Parnate). A, Mixture containing 0.44 ng/μl of each standard (unextracted). Injection volume = 2 μl. B, Urine specimen obtained on day 1, drug-free (see Table III). C, Urine specimen obtained on day 48, during Parnate treatment. A 3% OV-1 column was used and programmed from 90° at a rate of 4°/min. The recorder was set for the channels from top to bottom at 500 mV, 200 mV, 200 mV and 1 V.

In separate aliquots of these urine specimens, the concentrations of the free metabolites were determined without treatment with glusulase. The average excretion of free pHPG and MHPG was 13 and 19 μg/24 h, respectively, or 14% and 1% of the total excretion of these metabolites. No free pHPE could be

TABLE II

RETENTION TIMES OF THE PFP DERIVATIVES OF PHENYLGLYCOLS AND PHENYLETHANOLS ON 3% OV-1, 3% SP-2250 AND 3% QF-1

All retention times are given in minutes.

Compound	Column		
	3% OV-1 (90°+ 6°/min)	3% SP-2250 90° + 6°/min)	3% QF-1 (120° + 6°/min)
PEG	5.41	4.56	3.30
oHPE	5.70	4.78	3.88
mHPE	6.96	6.38	5.33
pHPE	7.26	6.58	5.67
pHPG	8.60	6.93	8.28
DHPG	9.73	8.01	10.55
MHPE	10.39	10.32	8.62
MHPG	10.94	9.48	10.32

TABLE III

URINARY EXCRETION OF TOTAL NEUTRAL METABOLITES IN A SUBJECT BEFORE AND DURING TREATMENT WITH PARNATE (40 mg/day)

Parnate (®, Smith, Kline and French; tranylcypromine) was administered beginning on day 33.

Day	Parnate dose (mg/day)		PEG	pHPE	pHPG	MHPG
μg/24 h						
1	—		15	14	108	1680
4	—		28	13	145	1396
5	—		22	6	21	1121
		Mean	22	11	91	1399
48	40		32	10	18	144
49	40		22	17	35	254
		Mean	27	14	27	199
			(125%)	(127%)	(30%)	(14%)
μg/g creatinine						
1	—		8	7.4	57	893
4	—		15	7.0	78	755
5	—		12	3.3	12	623
		Mean	12	5.9	49	757
48	40		30	9.4	17	135
49	40		10	7.9	16	117
		Mean	20	8.7	17	126
			(171%)	(147%)	(34%)	(17%)

detected in these urine specimens, nor could either free pHPG or MHPG be detected in urines obtained during treatment with the monoamine oxidase inhibitor.

DISCUSSION

The alcoholic metabolites formed from biogenic amines are present in urine in significant amounts. Measurement of the concentrations of these metabolites in urine or tissues may be necessary in order to gain a complete picture of how these amines are metabolized and what are the effects of drugs. This may be particularly true for the β-hydroxylated phenylethylamines, since these compounds appear, at least in brain tissue, to be converted predominately to the corresponding neutral metabolite (i.e., the phenylglycol) rather than to an acidic one [11].

The present report provides a highly specific and sensitive method for the determination of alcoholic metabolites of phenylethylamines. The only limitation to the number of metabolites which can be analyzed simultaneously is the number of PROMIM channels available. The use of at least two different columns or in some cases monitoring two ion fragments insures that the peaks are correctly identified.

Quantitation was achieved by measuring peak heights and comparing them to standards carried through the extraction scheme. Plotting the peak heights against the amount of standard added resulted in linear relationships. In some cases, we added pHPP as an internal standard. Results obtained by measuring peak height ratios using the internal standard were virtually identical to those obtained by simply measuring the peak heights. The calibration curves using the internal standard were not improved, and in fact were slightly worse than those obtained without the internal standard. For this reason and because the internal standard required an additional PROMIM channel, we have routinely carried out our analyses without an internal standard.

The identification of pHPG in human urine to our knowledge represents the first demonstration of this metabolite in biological samples. The excretion of this metabolite, which is the β-hydroxylated analogue of pHPE, not surprisingly was 8-fold greater than pHPE. Nevertheless, compared to MHPG, the excretion of pHPG was only 7% as much. On the other hand, the concentration of octopamine, the amine precursor of pHPG, is exceedingly low in most tissues [12]. The concentration in brain, for example, is about 5 ng/g [12], or only 1% of the precursor of MHPG, norepinephrine. Taken together, these results support the suggestion based on the 5- to 10-fold increase in octopamine concentrations following treatment with a monoamine oxidase inhibitor that although the concentration of octopamine in tissues is very low, the turnover rate of this amine is nevertheless relatively large [12]. Our results suggest that the determination of pHPG excretion may provide a means of measuring the turnover of octopamine in vivo.

Treatment with 40 mg per day of the monoamine oxidase inhibitor, Parnate, resulted in a 70% reduction in the excretion of pHPG. In comparison, the excretion of MHPG was reduced by 86%, but no reduction was observed in the excretion of either PEG or pHPE. The cause of each of the deaminated neutral

metabolites not being reduced to the same extent is not known. One possible explanation is based on the fact that there are two forms of monoamine oxidase which differ in their substrate specificities and inhibitor sensitivities [13]. Norepinephrine is a specific substrate for type A monoamine oxidase [13], and octopamine is deaminated primarily by type A monoamine oxidase but is deaminated to some extent by type B monoamine oxidase [14]. Tyramine is deaminated by both forms of the enzyme, whereas phenylethanolamine is a specific substrate for type B monoamine oxidase [8]. Thus, the more specific a substrate is for the A form of monoamine oxidase, the greater is the reduction produced by Parnate in the excretion of the corresponding deaminated metabolite. The effect of Parnate on these metabolites would be explained if this drug were selectively blocking type A monoamine oxidase in vivo. However, there is no direct evidence for this, and in fact in vitro Parnate is nonspecific, inhibiting each form of the enzyme with about an equal potency [13]. These results could be explained alternatively if the drug produces a different degree of inhibition at different cellular locations. Thus, since norepinephrine and octopamine are presumed to be deaminated mainly within noradrenergic neurons, an accumulation of the drug by these neurons could account for the larger decrease in MHPG and pHPG formation.

Since this report was written, we have identified and quantitated pHPG in both rat brain and urine. Sprague—Dawley rats (300—350 g) were found to excrete (mean ± S.E.) 0.12±0.03 and 4.8±0.9 μg/24 h of free and total pHPG, respectively. The whole brain concentration of total pHPG was 3.1±0.6 ng/g [15].

ACKNOWLEDGEMENTS

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CHROMBIO. 417

NEW PROCEDURE FOR ISOLATION OF AMINO ACIDS BASED ON SELECTIVE HYDROLYSIS OF TRIMETHYLSILYL DERIVATIVES

KEITH L. CLAY and ROBERT C. MURPHY*

Department of Pharmacology, University of Colorado Medical School, Denver, Colo. 80262 (U.S.A.)

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SUMMARY

A rapid procedure for the isolation of amino acids from physiological fluids by class separation suitable for gas chromatographic and gas chromatographic—mass spectrometric analysis is described. A physiological fluid such as plasma is adjusted to pH 2 and extracted with diethyl ether to remove organic acids and neutrals. After precipitation of proteins with trichloroacetic acid, the aqueous plasma is dried and derivatized by trimethylsilylation. Organic compounds like sugars and amino acids are rendered soluble in petroleum ether leaving inorganic salts when the soluble layer is transferred. Separation of sugars from amino acids is achieved by taking advantage of the different rates of aqueous hydrolysis of the trimethylsilyl (TMS) derivatives. Mixing the petroleum ether extract with a small volume of water results in two phases. The petroleum ether layer contains TMS-sugar constituents of plasma and the aqueous layer contains free amino acids and amines. This procedure was used to isolate L-dopa, 3-O-methyldopa and tyrosine from human plasma in a quantitation assay using ^{18}O-labelled amino acids and gas chromatography—mass spectrometry.

INTRODUCTION

There has been a multitude of methods developed for the separation and analysis of amino acids present in physiological fluids involving ion-exchange, thin-layer, high-performance liquid, paper, and gas chromatographic techniques. Because of some unique advantages, amino acid analysis by gas chromatography has become a widely used procedure and has been recently reviewed [1]. The gas chromatographic (GC) procedures offer advantages of speed, sensitivity, and applicability to most naturally occurring amino acids and complete resolution of the twenty protein amino acids can be achieved with a single column in a total chromatographic time of less than one hour [2]. The necessity of making a derivative of the amino acid to increase thermal stability and to improve chromatographic behavior is often

*To whom correspondence should be addressed.

considered to be a disadvantage of the GC techniques; however one can use this to advantage. For example, derivatives suitable for electron-capture GC detection can be made, which profoundly increase the analytical sensitivity of amino acid quantitation [3]. Recent advances in combined gas chromatography—mass spectrometry (GC—MS) have made GC increasingly attractive as a chromatographic procedure for amino acid separation. Selected ion monitoring (SIM) of the GC effluent for ions specific to amino acids offers sensitivity comparable to that achieved with the electron-capture detector [4] and the combination of SIM analysis with stable isotopically labelled amino acids as internal standards gives a method with sensitivity and precision unmatched by other chromatographic methods [5].

Perhaps the major disadvantage in the analysis of amino acids by GC involves the effort required for isolation of these compounds from biological fluids in order to make them amenable to gas phase analysis. A large number of derivatives have been used to make amino acids sufficiently volatile and thermally stable for GC [1]. All of the derivatization procedures require prior removal of water; and considering the complexity of biological fluids with significant contributions of proteins and inorganic salts, some degree of purification is required. Ion-exchange procedures which yield the compounds of interest dissolved in a relatively large volume of aqueous solution, have been most widely used for isolation of amino acids. In studies involving many samples, the time spent in removal of water, following ion-exchange purification of the biological samples can become very inconvenient. In addition, amino acids labelled with isotopes in structural positions labile to exchange, present the possibility of loss of the isotopic label under conditions of prolonged exposure to aqueous solutions of low pH encountered in many ion-exchange procedures.

We report in this communication the development of a procedure for isolation of amino acids from biological sources which avoids the problems associated with ion-exchange purification of amino acids. The procedure is rapid, suited to the processing of large numbers of samples and delivers the amino acids in a form eminently suited for GC and GC—MS analysis. The procedure was developed primarily to facilitate isolation of amino acids under conditions which would avoid problems associated with the chemical instability of the catechol nucleus and with the possibility of loss of deuterium atoms in the aromatic ring of phenol- and catechol-amino acids.

EXPERIMENTAL

Reagents

Reagents and solvents were obtained from commercial suppliers and used without further purification. $H_2{}^{18}O$, 99 atom% ^{18}O was obtained from Mound Laboratories (Miamisburg, Ohio, U.S.A.) and used for synthesis of ^{18}O-carboxyl-labelled amino acids [$^{18}O_2$] L-dopa, [$^{18}O_2$] 3-O-methyldopa, and [$^{18}O_2$]-tyrosine by a procedure published elsewhere [6].

Blood samples from patients receiving L-dopa were collected from an indwelling catheter with a heparin lock after sufficient blood had been withdrawn to insure that dilution by the heparin solution was avoided. The blood

was transferred to a heparinized tube and immediately placed on ice. Within 30 min, the blood was centrifuged and the plasma collected. Plasma (0.2 ml) was then pipetted into a polypropylene centrifuge tube (Eppendorf) containing internal standards and 0.05 ml 3 *N* HCl; then frozen for subsequent analysis or analyzed immediately according to the procedure diagramed in Fig. 1.

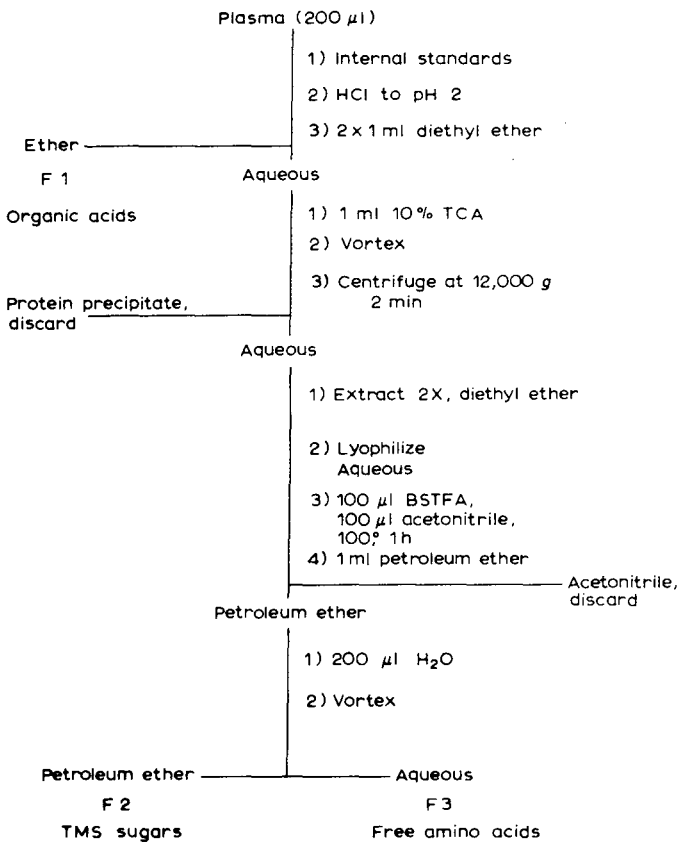

Fig. 1. Flow diagram for the sequential isolation of organic acids and non-polar neutrals (F1), trimethylsilyl ethers of polyhydroxy compounds (F2), and bases and amphoteric compounds (F3) from physiological fluids.

Procedure

The procedure for the isolation of amino acids is outlined in Fig. 1 and for the purpose of illustration, plasma is the physiological fluid. A small, precise volume of plasma (200 µl) which already contains internal standards and enough mineral acid to reduce the pH to below 2 is extracted twice with 1 ml of diethyl ether. The diethyl ether layers are removed and contain extractable organic acids (F1). The aqueous layer is then treated with 1 ml of 10% trichloroacetic acid (TCA) which precipitates plasma proteins after vigorous shaking. The capped tube is centrifuged at high speed (12,000 *g*) for 2 min in an Eppendorf Microfuge to pellet the proteins. The supernatant is separated and

extracted twice more with diethyl ether to remove TCA. It is essential to remove all the TCA. The clear, aqueous solution which is in a glass culture tube is lyophilized to complete dryness, and treated with 100 μl of bis(trimethylsilyl)trifluoroacetamide (BSTFA) and 100 μl of acetonitrile. The test-tube is sealed with a PTFE-lined cap and heated to 100° for 1 h in a heating block. The derivatization medium is briefly cooled and treated with 1 ml petroleum ether (b.p. 35—60°). Two layers result and the upper layer is transferred to a clean culture tube. Distilled water (200 μl) is added to this layer and the two-phase system vortexed for 30 sec. The petroleum ether (F2) is immediately removed from the small volume of water. This water layer contains underivatized amines and amino acids (F3) and can be further lyophilized to complete dryness and rederivatized with 25 μl BSTFA and 25 μl acetonitrile at 100° for 1 h. This solution or aliquots thereof can be directly injected into the gas chromatograph.

The procedure in Fig. 1 yields three fractions — organic acids and nonpolar neutrals (F1), polar neutrals (F2), and amines and amino acids (F3). For some applications, complete removal of sugar contaminants in the amine-containing fraction may be desirable and can be effected by a slight modification of this procedure (Fig. 1). After lyophilization of the water backwash (F3), rederivatization with BSTFA is carried out for 5 min at 100°, but without acetonitrile. Petroleum ether extraction will then remove any sugars not completely removed previously. Amines and amino acids are not efficiently derivatized in BSTFA alone and are not soluble in petroleum ether.

Apparatus

GC—MS analysis of the fractions produced in the procedure in Fig. 1 were carried out with a Finnigan 9500 gas chromatograph (Sunnyvale, Calif., U.S.A.) interfaced via an all-glass jet separator and transfer line to a Finnigan 3200 quadrupole mass spectrometer. Mass spectral data generated by monitoring the GC effluent were acquired and stored for later analysis by a Finnigan Model 6000 data system which also controlled the mass spectrometer operating parameters. GC was carried out on 3% SE-30 on Supelcoport 100—120 mesh. Helium carrier gas flow-rate was 30 ml/min. Injector, separator oven and transfer line temperature was maintained at 250°. All mass spectra were obtained under electron impact conditions at 70 eV.

Quantitation of tyrosine, dopa and 3-O-methyldopa was done isothermally at 210° by SIM analysis. With [$^{18}O_2$] L-dopa, [$^{18}O_2$] 3-O-methyldopa, and [$^{18}O_2$] tyrosine as internal standards, the mass spectrometer was focused on ions at *m/e* 218 for unlabelled dopa, 3-O-methyldopa, and tyrosine (this is a common ion for all three amino acids which retains the carboxyl ^{18}O atoms) [7] and at *m/e* 222 for [$^{18}O_2$] tyrosine, [$^{18}O_2$] dopa and [$^{18}O_2$] 3-O-methyldopa. Under computer control, the mass spectrometer was rapidly switched to acquire those three ions and generate an ion profile of abundance versus time as the compounds eluted from the gas chromatograph. When the analysis was terminated, the computer was used to integrate the areas under each of the ion profile curves whose identity had been established by known retention time. The ratios of the ion profiles of interest (e.g. *m/e* 218/222) were then compared to a standard curve which had been generated using known amounts of

each amino acid in distilled water and treated along with the plasma samples. The MS analysis of each plasma sample was complete in approximately 5 min. While data are not presented, the same procedure was also followed for quantitation of plasma aromatic amino acids, only using deuterium-labelled [$^2H_3$]-L-dopa, [$^2H_2$] tyrosine, and [$^2H_3$] 3-O-methyldopa. Since the deuterium atoms were in the aromatic ring of the internal standard amino acids, the mass spectrometer was set to record ion abundances at m/e 179, 181, and 182 to measure the labelled and unlabelled amino acids [7].

RESULTS AND DISCUSSION

Trimethylsilylation with a strong TMS donor such as BSTFA results in substitution of a TMS group for the active proton from such moieties as alcohols, carboxylic acids, amines and some amides [8]. The derivatives formed are non-polar compounds soluble in non-polar organic solvents, often in sharp contrast to the parent compounds. This non-polarity and increased volatility have made them useful derivatives for GC analysis, but their instability to hydrolytic conditions has imposed restrictions of anhydrous conditions upon preparation and storage. The hydrolytic instability, however, is different for each kind of TMS function, and it is this differential hydrolytic stability which forms the basis for the amino acid isolation scheme presented in Fig. 1. The basis for the purification procedure is that TMS ethers are much more stable to water hydrolysis than are TMS amines or TMS esters of carboxylic acids. Polyhydroxy TMS ethers such as sugars will not be hydrolyzed back to the polar sugar by brief exposure to water and will remain soluble in a non-polar solvent such as petroleum ether. TMS amines and TMS carboxylic acids will, however, be quickly hydrolyzed to free amines and acids and will no longer be soluble in such a non-polar solvent.

Fully silylated serine has been examined in some detail with respect to its solvolytic stability [9] and serves as an illustration of differential stability of TMS derivatives. Hydrochloric acid in diethyl ether cleaved only the N—TMS bond, while water or alcohol caused rapid solvolysis of both amino and carboxyl TMS groups. The same study also reported that alcoholysis of fully silylated tyrosine and hydroxyproline caused loss of carboxyl and amine TMS groups only, but did not cleave the TMS ethers.

The relative stability of TMS derivatives to water hydrolysis in the purification of amino acids is illustrated in Fig. 2A and B. Fig. 2A is a computer reconstructed total ionization plot of a mixture of amines, amino acids and sugars which was lyophilized, derivatized with BSTFA and acetonitrile, and subjected to temperature-programmed GC—MS. The components were identified by their mass spectra. Major components seen in the chromatogram are the sugars and citric acid. Only by utilizing the MS information was it possible to determine that tyrosine co-eluted with a hexose component. After water extraction it was apparent that the only components of the mixture to remain in the petroleum ether were the sugars and citric acid. There were no amino acids apparent even when the mass spectral data files were searched for specific ions. The amines were also removed from the petroleum ether by water hydrolysis and extraction. The amino acids and amines were efficiently extracted into

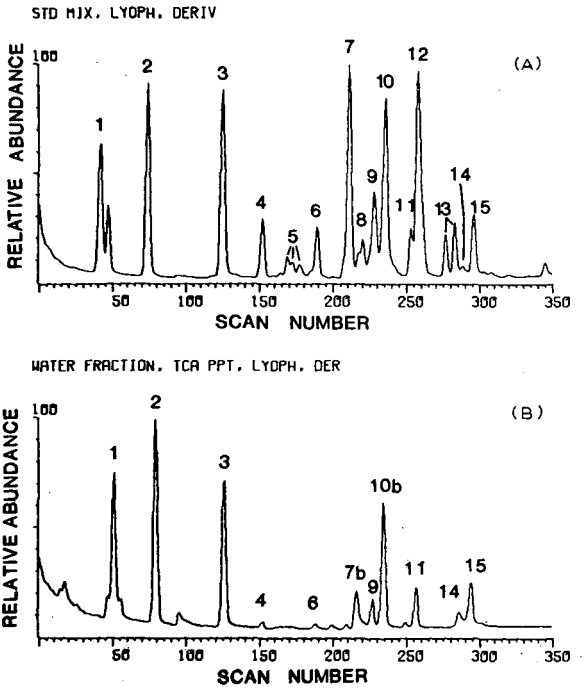

Fig. 2. Total ionization current (TIC) plots of a standard mixture of amines, amino acids and sugars. (A) TIC plot of the mixture lyophilized, derivatized and subjected to GC—MS analysis directly. (B) TIC plot of the water back-wash fraction (F3) lyophilized and derivatized. Individual components were identified by their mass spectra as: 1 = leucine; 2 = serine; 3 = α-aminobutyric acid + aspartic acid; 4 = tyramine-diTMS; 5 = ribose; 6 = 3-methoxytyramine-diTMS; 7 = citric acid; 8 = hexose; 9 = tyramine-triTMS; 10 = tyrosine + hexose; 10b = tyrosine; 11 = 3-methoxytyramine-triTMS; 12 = hexose; 13 = hexoses; 14 = tryptamine-diTMS; 15 = tryptophan + tryptamine-triTMS.

the water, as shown by Fig. 2B, which is the water back-wash (F3) after lyophilization and re-derivatization. There are no sugars apparent in the water fraction and the amino acids and amines are now free of these contaminants. Efficient mixing of the water with the petroleum ether fraction is essential for good extraction of amino acids and amines into the water. Recovery experiments with radiolabelled tyrosine and galactose demonstrated that the 30-sec period of vortex mixing recommended in Fig. 1 was sufficient to extract over 90% of the tyrosine from a derivatized plasma sample, while leaving the derivatized galactose exclusively in the petroleum ether phase. Shorter periods of vortex mixing resulted in proportionately less recovery of tyrosine. With more efficient mixing procedures, such as vigorous shaking of capped tubes, the mixing time can be reduced, but vortexing was found to be more convenient for handling large numbers of samples.

After protein precipitation, acid extraction, lyophilization and trimethylsilylation, major constituents of any plasma sample are hexoses. Fig. 3A is the reconstructed chromatogram of the petroleum ether-soluble TMS derivatives of components in a human plasma. Since amino acids are present, typical-

ly at 1—10 μg/ml in plasma, the specificity of the mass spectrometer is such that one could conceivably perform quantitative analysis of some amino acids by carefully selecting ions characteristic of each amino acid which are not present in the sugars. GC analysis alone, however, would not be possible for any amino acids which eluted in the region of the sugars. For our studies, it was desirable to use ^{18}O-labelled analogs of tyrosine, dopa and 3-O-methyl-dopa to monitor an ion common to all three amino acids which contained the oxygen-18 atoms, that being *m/e* 218 and *m/e* 222 for the ^{18}O-internal standards. As demonstrated in Fig. 3B and C, however, *m/e* 218 was a major ion in the contaminating sugars, arising as a natural isotope peak from an ion at *m/e* 217, which is an abundant ion characteristic of TMS-carbohydrates [10]. After removal of the sugars by the procedure of Fig. 1, the amino acid fraction was clean enough for both qualitative and quantitative analysis, mon-

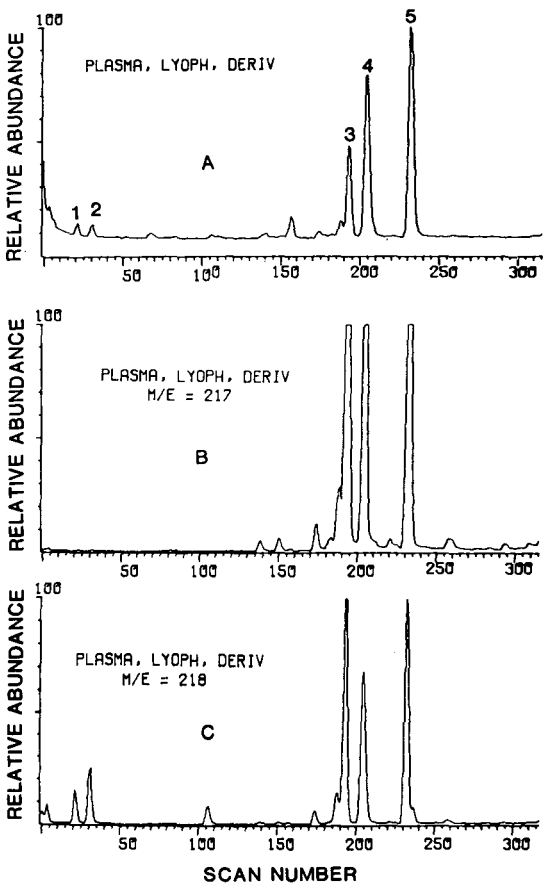

Fig. 3. Computer-reconstructed TIC plot (A) and mass chromatograms (B and C) for ions characteristic of TMS carbohydrates (*m/e* = 217, B) and TMS amino acids (*m/e* = 218, C). 200 μl of a human plasma sample were acidified with hydrochloric acid and deproteinized with TCA; the TCA was removed by ether extraction; the aqueous fraction was lyophilized to dryness, derivatized with acetonitrile and BSTFA and analyzed by GC—MS. Peaks were identified by their mass spectra as trimethylsilyl derivatives of: 1 = serine; 2 = threonine and 3—5 = hexoses.

itoring ions of *m/e* 218 and 222. Fig. 4 is the chromatogram of the water back-wash (F3) of the same sample of plasma shown in Fig. 3A. The major constituents of this fraction are now amino acids. If necessary, the remaining sugars can be removed by the slight modification described in Procedure. Since many amino acids are present in plasma in relatively high concentrations, it is obvious that any of these amino acid components of plasma can be measured using a small (200 μl) volume of plasma when the sugar contaminants are removed.

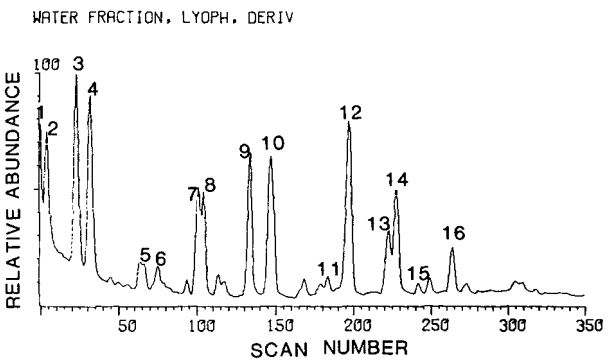

Fig. 4. Computer-reconstructed TIC plot of the water back-wash fraction (F3) of the plasma sample illustrated in Fig. 3, derivatized with BSTFA and acetonitrile and analyzed by GC—MS. Components were identified by their mass spectra as trimethylsilyl derivatives of 1 = Ile, 2 = Leu, 3 = Ser, 4 = Thr, 5 = Met, 6 = Asp, 7 = Phe, 8 = Glu, 9 = Gln, 10 = Lys-3-TMS, 11 = Hexose, 12 = Tyr and Lys-4TMS, 13 = Hexose, 14 = 3-O-methyldopa, 15 = dopa and 16 = Trp.

Fig. 5 illustrates application of this procedure to the analysis of 31 consecutive plasma samples from a Parkinsonian patient treated with L-dopa over the course of one day. Plasma samples were drawn at half-hourly intervals and then treated as described in Procedure. The total time required to prepare these samples by the described procedure for GC—MS analysis was less than one man-day.

Main contributions of this method to amino acid isolation are related to the speed, specificity, and to the mild conditions of the procedure. The method is essentially a class separation and produces three distinct fractions which are available for further analysis: acids, neutrals, and bases and amphoterics. The thrust of our work has been concerned with amino acid analysis, but the method also provides a possible route to convenient analysis of carbohydrates in biological fluids. The avoidance of ion-exchange procedures or strongly basic or acidic conditions should ensure that loss of labile isotopes would be minimized. The amino acids isolated as fraction F3 can be re-derivatized with any reagent desired, so that the method is applicable to studies with electron-capture detectors or for other specialized techniques. The method appears to be suited to a wide variety of biological fluids and we have successfully used the procedure to examine amines and amino acids in urine, human vitreous body and cerebrospinal fluid, in addition to plasma.

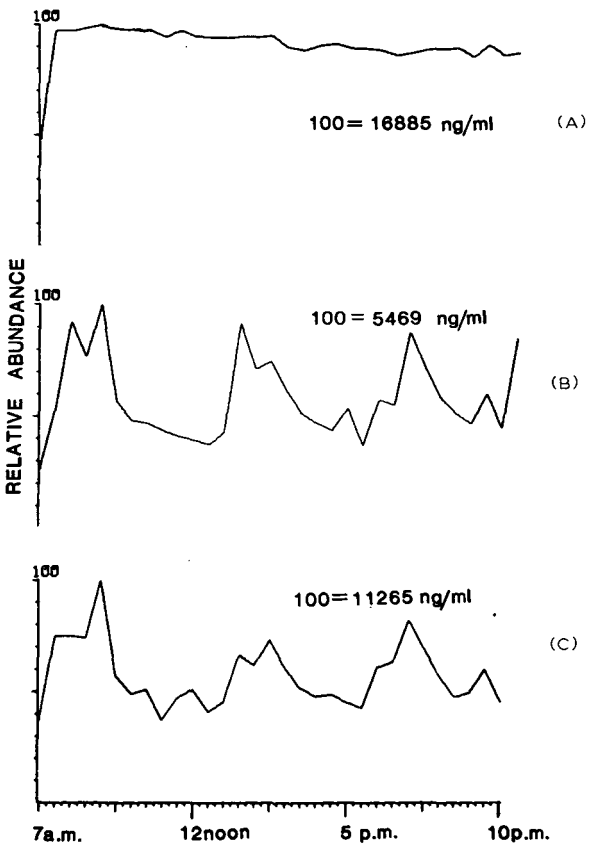

Fig. 5. Normalized plots of plasma concentration of (A) 3-O-methyldopa, (B) dopa and (C) tryptophan in a patient receiving chronic L-dopa therapy. L-Dopa was given at 7.00 a.m., 12 noon, 5.00 p.m. and 10.00 p.m. Blood samples were obtained at half-hourly intervals and the plasma amino acids were isolated as described in Fig. 1. Quantitations were performed by SIM analysis of the GC effluent, with the mass spectrometer focused to record ions of m/e = 218 and m/e 222 for the endogeneous and $^{18}O_2$-carboxyl-labelled internal standards, respectively. Ratios of the area of the ion profile curves for m/e = 218 and m/e = 222 were calculated by the computer and compared to a standard curve which was generated during the course of the analysis for each of the amino acids. Normalization was performed relative to the plasma sample of highest concentration for each amino acid, and the normalization value is shown for each compound.

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DETERMINATION OF ERYTHROCYTE AMINO ACIDS BY GAS CHROMATOGRAPHY

WILLIAM P. LEIGHTON, SEYMOUR ROSENBLATT and J.D. CHANLEY

Departments of Psychiatry and Biochemistry, The Mount Sinai School of Medicine of the City University of New York, Fifth Avenue at 100th Street, New York, N.Y. 10029 (U.S.A.)

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SUMMARY

Erythrocyte amino acid levels were determined, by gas chromatography, in a group of 34 normal human adults. No significant sex or age correlations were noted.

A method for the quantitative gas chromatographic analysis of free amino acids in erythrocytes is described. Following hemolysis and deproteinization the amino acids were isolated on a cation-exchange resin. Glutathione was removed from the amino acid mixture by adsorption on an anion-exchange resin. Following conversion to their N-acetyl-*n*-propyl esters, 19 amino acids were separated and quantitated by gas chromatography on a single column in 18 min. Typical reproducibility data indicate that a coefficient of variation of 2—5% is attainable.

INTRODUCTION

Although there is much information regarding the quantities of free amino acids in human plasma or serum in normal and abnormal individuals, little attention has been paid to such concentrations in erythrocytes. Recent studies have documented the importance of the red blood cell (RBC) in the inter-organ transport of free amino acids [1—4] many of which show a significant concentration differential across the erythrocyte cell membrane. The clinical importance of RBC amino acid levels has only been suggested; we have recently reported on a group of manic-depressive patients whose erythrocyte glycine concentration was significantly elevated in comparison to a group of control subjects [5].

The earliest published studies of RBC amino acids in normal adults [6,7] were done without the benefit of advanced analytical techniques, while more recent studies [3,8—11] have reported on small numbers of subjects. There has also been a general absence of specific information as to the sex and age

of the subjects studied, as well as to the time of specimen collection in relation to possible diurnal and nutritional effects, factors which are known to influence plasma amino acid levels [12—15]. It is therefore not surprising to find a wide range of reported values for many of the erythrocyte amino acids (see Table I).

In the past, amino acid analysis has depended on microbiological assay procedures [6] and various paper chromatographic techniques [7]. More

TABLE I

ERYTHROCYTE AMINO ACID LEVELS: SUMMARY OF AVERAGE VALUES REPORTED IN THE LITERATURE

For abbreviations of the amino acids see Table II. Their concentrations are given in μmoles/l.

Reference	6	7	8	3*	9	10	11	This report
Year	1951	1960	1971	1973	1976	1978	1978	1979
n	24	14	6	7	7	6	10	34
Sex: M/F	All M	?	?	All M	?	?	All F	11 M/23 F
Mean age	24	?	?	Adult	Adult	Adult	40	35/33
a.m.—fasting	Yes	Yes	?	Yes	?	Yes	Yes	Yes
Method††	MB	PC	IEC	IEC	IEC	IEC	IEC	GC
ALA		278	250	364	260	259	357	331
2-ABA		16	14	25		34		24
VAL	174	145	137	268	65	155	191	188
GLY		**	264	485	292	350	374	362
ILE	65	***	43	66	14	53	62	58
LEU	118	***	100	134	25	107	115	110
PRO		124		219	86	126	216	175
THR	135	102	154	167	82	113	122	124
4-ABA								12
SER		**	265	217	113	143	198	164
ASN				†	102	102	120	124
ASP			264	388	353		544	
MET	34	20	11	13	1	16	0	24
CYSH	32		7	0	0		0	
PHE	60	38	61	55	10	47	71	58
HYP								11
GLN		391	201	†	315	622	830	468
GLU		243	220		160	294	350	
TYR	58	47	78	69	27	57	75	70
ORN		120	149		105	113	153	179
HIS	70	85	115		55	82	131	
LYS	92	151	143		99	125	179	174
ARG	16	<8	25		5	42	0	29
TRP	14	14			1			

*Calculated from whole blood and plasma values.

**GLY + SER = 616.

***ILE + LEU = 137.

†GLN + ASN = 612.

††MB, microbiological assay; PC, paper chromatography; IEC, ion-exchange chromatography; GC, gas chromatography.

recently, ion-exchange chromatography has been most extensively utilized [3,8—11]. In addition, there have been significant advances in the techniques of gas chromatography (GC), some of which have been applied to amino acid analysis [16]. Advantages of GC include the low cost and greater versatility of the instrument, as well as greater sensitivity and reduced operating time. Of the many techniques which have been developed, the most useful are based on a two-step procedure: esterification of the carboxyl group, followed by acylation of the α-amino and other functional groups.

One example of this technique uses the N-trifluoroacetyl-*n*-butyl esters and requires two GC columns for complete separation [17,18]. Also used are the N-heptafluorobutyryl isobutyl esters which can be separated on a single column in 60 min [19—21]. A third procedure, based on the formation of the N-acetyl-*n*-propyl amino acid esters, has particular advantages: derivative preparation is simple and rapid, only a single GC column is required, and complete separation is achieved in less than 20 min [22—25]. Many successful applications of this technique have been reported [26—31], including the analysis of plasma and urine, but not of erythrocytes.

When considering the use of this technique for the analysis of erythrocyte amino acids, a potential source of serious error was apparent. Erythrocytes contain a large quantity of the tripeptide glutathione (γ-glutamyl-cysteinyl-glycine), which would be partially hydrolyzed during the esterification procedure in hot 8 *M* propanolic HCl. This would, in turn, lead to falsely elevated levels of glutamic acid, cysteine and glycine. Several methods were explored by which the glutathione could be separated from the amino acid mixture [32—36]; these were found to be too complex and unreliable. The procedure adopted for routine use was based on the adsorption of glutathione on an anion-exchange resin (AG 1-X8, 200—400 mesh, acetate form), and elution of the amino acids (except for dicarboxylic acids) with 0.05 *N* acetic acid [37]. This was shown to be a very dependable procedure as well as being simple and rapid.

The analytical procedure herein described was used to study the erythrocyte free amino acids in a group of normal adults of both sexes. Time of day and nutritional state were controlled by obtaining all blood specimens between 8 and 10 a.m., from subjects who had been fasting since the previous midnight. Informed consent was obtained from all subjects.

MATERIALS

Reagents

The following reagents were used: cation-exchange resin AG 50W-X8 (H^+), 100—200 mesh, and anion-exchange resin AG 1-X8 (CH_3COO^-), 200—400 mesh (Bio-Rad Labs., Richmond, Calif., U.S.A.); ammonium hydroxide and glacial acetic acid (reagent grade), trichloroacetic acid and sodium chloride (Fisher, Springfield, N.J., U.S.A.); acetic anhydride 99+ % (Aldrich, Milwaukee, Wisc., U.S.A.), ethyl acetate, triethylamine, *n*-propyl alcohol and acetone, each of highest available purity (Pierce, Rockford, Ill., U.S.A.); dry air, high-purity helium, prepurified hydrogen, electronic grade hydrogen chloride and prepurified nitrogen (Linde, South Plainfield, N.J., U.S.A.); amino acids, A grade

(Calbiochem, San Diego, Calif., U.S.A.); L-norleucine, grade I (for use as internal standard) (Sigma, St. Louis, Mo., U.S.A.); (additional high purity amino acids from Aldrich, and Alltech Assoc., Arlington Heights, Ill., U.S.A.); N-acetyl-*n*-propyl amino acids (Alltech).

Propylation reagent. The propylation reagent, propanolic HCl, was made by passing 27 g of anhydrous electronic grade HCl gas into 100 ml of cooled *n*-propanol. Stored at 4° in a glass-stoppered bottle the reagent lasts several months.

Acylation reagent. A mixture of acetone, triethylamine, and acetic anhydride (5:2:1) was prepared daily.

Resin columns

The columns were made by inserting a small quantity of Pyrex glass wool (Packard, Downer's Grove, Ill., U.S.A.) into a large-volume pasteur pipet (Fisher). The glass wool was gently pushed into the upper portion of the constricted pipet stem, and compacted just enough to prevent loss of resin, while avoiding a significant reduction of flow-rate. A 25-g quantity of each resin was washed with 4 × 100 ml of distilled water, and finally suspended in 100 ml of water for use as needed. A sufficient quantity of this washed resin suspension was applied to a column so that the resulting packed resin bed had a volume of 1.5 ml and measured 0.8 cm × 3.0 cm. The AG 50W-X8 (H^+) 100—200-mesh column contains 500 mg of dry resin and provides approximately 2.55 mequiv. of exchange capacity. The AG 1-X8 (CH_3COO^-) 200—400-mesh column contains 656 mg of dry resin and provides approximately 2.10 mequiv. of exchange capacity. A fresh bed of washed resin was used for each sample.

Standards

Stock solutions of selected individual amino acids, including norleucine (NLE), to be used as the primary internal standard, were made up in aqueous 0.1 *N* HCl at a level of 5 μmoles/ml. A solution of "secondary standards" was made up to contain 2-aminoadipic acid (2-AAA) and 2-aminoisobutyric acid (2-AIBA). A single "calibration standard" solution was likewise prepared which contained equimolar amounts of the following amino acids: 2-aminobutyric acid, alanine, valine, glycine, isoleucine, leucine, proline, threonine, 4-aminobutyric acid, serine, asparagine, methionine, cysteine, phenylalanine, hydroxyproline, tyrosine, ornithine, histidine, lysine, arginine and tryptophan. These solutions are stable for several months when stored in glass-stoppered bottles at 4°. Glutamine, however, is not stable in solution and must be freshly prepared at frequent intervals.

Glassware

Glassware included 13 mm × 100 mm and 16 mm × 125 mm disposable glass culture tubes (Fisher), 16 mm × 125 mm glass culture tubes with screw-cap (PTFE-lined) closures (Kimax No. 45066-A), 15-ml graduated Corex conical centrifuge tubes (Corning No. 8080-A), 40-ml graduated heavy-duty conical centrifuge tubes with screw-cap (PTFE-lined) closures (Corning No. 8142), 15 mm × 45 mm glass vials with screw-cap (PTFE-lined) closures

(Supelco, Bellefonte, Pa., U.S.A.), 145-mm and 230-mm disposable glass pasteur pipets and "large-volume" glass pasteur pipets (Fisher).

Equipment

The following equipment was used: "Temp-blok" module heaters (Scientific Products); Sorvall GLC-1 centrifuge; "Evapo-Mix" vortex evaporator (Buchler Instruments); "Vortex-Genie" mixer; Eppendorf pushbutton pipets.

Gas chromatograph

A Packard Model A7400 gas chromatograph with flame ionization detector and linear temperature programming was used together with a Honeywell Model 551 1-mV recorder. Injection was carried out using a Hamilton 701 RN syringe with a 26 S needle.

GC column

The glass column (76 cm × 6 mm O.D. × 2 mm I.D.) was terminated with a septum well at each end. This was packed with a mixed polar packing, consisting of 0.31% Carbowax 20 M, 0.28% Silar 5CP and 0.06% Lexan on Chromosorb W AW, 120—140 mesh (Alltech). The column ends were sealed with special high-temperature 1½-hole cylindrical septa (Supelco). Before connecting the outlet to the detector, the column was conditioned with a helium flow of 12 ml/min at 260° for 2 h, then at 220° for 16 h.

METHODS

Collection of blood

Blood specimens were obtained between 8 and 10 a.m. from subjects who had been fasting since midnight. A "vacutainer" apparatus was used to draw 20 ml, by antecubital venipuncture, into a rubber-stoppered tube containing 357 USP units of sodium heparin. Complete mixing of anticoagulant with blood was accomplished by repeated and gentle inversion of the tube. All specimens were promptly transported to the laboratory where immediate separation of erythrocytes from plasma was performed using 15-ml graduated "Corex" centrifuge tubes, which were spun for 30 min at 5000 rpm (3200 *g*) at room temperature.

Initial preparation of erythrocytes

The plasma was removed from the tube and a large-size pasteur pipet was used to carefully aspirate the buffy coat along with a portion of the packed red cells so that exactly 3 ml of the latter remained in the tube. To this were added 6 ml (2 parts) of distilled water, followed by thorough mixing. Four milliliters of this diluted RBC solution were transferred to a graduated, heavy-duty, 40-ml screw-top centrifuge tube, and a further dilution was made by adding 19 ml of distilled water. A PTFE-lined screw-cap was used to seal the tube, which was then subjected to quite vigorous agitation on a Vortex mixer for about 30 sec. Complete hemolysis was indicated by a change in appearance of the RBC solution: from cloudy and dark maroon in color to quite clear and bright cherry red. To this dilute hemolysate was added 1 ml of 50%

trichloroacetic acid solution, followed by 30 sec more of vigorous agitation. The precipitated hemoglobin and other proteins were removed by centrifugation at 2500 rpm (1200 *g*) for 30 min. The clear and colorless supernatant was passed through a folded cone of dry Whatman No. 2 filter paper to remove occasional floating clumps of precipitate. Approximately 20 ml of protein-free filtrate were recovered; this was placed in a tightly sealed plastic vial and stored at −70° for future analysis. Nine milliliters of this dilute preparation represent 0.5 ml of packed erythrocytes.

Pretreatment by ion-exchange chromatography

Nine milliliters of the dilute lysed protein-free erythrocyte solution were transferred to a 16 mm × 125 mm tube. To this were added 25 μl of 5 μmoles/ml norleucine internal standard solution. Using a pasteur pipet, the mixture was transferred to the first resin column (AG 50W-X8) and allowed to pass through the column at about 1 drop per 3 sec. Care was taken not to allow the column to run dry. The eluate was discarded. The sample tube was washed with 2 ml of distilled water and the washings transferred to the column. This was immediately followed with another 2 ml of distilled water and the eluate was discarded. The resin was eluted using 4 ml of 2 *N* NH_4OH followed by 2 ml of distilled water. After collecting the eluate in a 16 mm × 125 mm screw-top culture tube, it was concentrated to a small volume (about 0.5 ml) using the Evapo-Mix vortex evaporator with the water-bath at 40° (a short length of Tygon tubing was used for connecting the tube to the vortex evaporator). One milliliter of 0.05 *N* acetic acid was added to the sample, which was mixed well and transferred to the second resin column (AG 1-X8) [37]. The mixture was allowed to pass through the column at about 1 drop per sec, and the eluate was collected in a 16 mm × 125 mm screw-top culture tube. The sample tube was washed with 3 ml of 0.05 *N* acetic acid and the washings were transferred to the column. This was followed immediately with another 3 ml of 0.05 *N* acetic acid. Twenty-five microliters of the "secondary standards" solution, containing 2-AAA and 2-AIBA (5 μmoles/ml) were added to the combined column eluates. This solution, which was clear and colorless, was then evaporated to dryness on the vortex evaporator.

Derivatization

To insure complete dryness of the eluate residue, a stream of dry nitrogen was passed through the tube for 10 min. One milliliter of propylating reagent was added and the tube was flushed with nitrogen and capped firmly (using a PTFE-lined screw-cap). The tube was placed in a heating block at 110° for 20 min. (Note: Care was taken to use a tube which was free of chips or nicks around the tube lip — this prevented loss of pressure and evaporation of reagent during the propylation step.) The tube was cooled for 10 min before opening, and was then evaporated to dryness at 110° using a current of dry nitrogen. After cooling the tube briefly, 1 ml of freshly made acylating reagent was added, the tube was flushed with nitrogen and sealed tightly using a clean PTFE-lined screw-cap. The tube was placed in a heating block at 60° for 20 min. The cap was removed and the solution was carefully evaporated just

to dryness, using a gentle stream of dry nitrogen. (Care was taken not to exceed 60°, or 100 ml/min of gas flow per tube. Derivatized material at this point was relatively volatile and care was taken to prevent losses, particularly of alanine and valine.) The residue was redissolved in 2 ml of ethyl acetate. To this was added 1 ml of saturated sodium chloride solution. The tube was then capped and shaken vigorously. After centrifugation for 5 min at 700 *g*, the ethyl acetate layer was carefully transferred to a 15 mm × 45 mm glass vial. Again using gentle heat and nitrogen gas flow (as above) the sample was evaporated just to dryness. The residue was redissolved in 50 μl of ethyl acetate, mixed well, and the vial was sealed with a PTFE-lined screw-cap. The derivatized material, except for methionine and histidine, was stable for several weeks when stored at 4°. GC analysis was done using a 2-μl aliquot of this solution.

Gas chromatography

The following instrument settings were used: the helium carrier gas flow-rate was 25 ml/min at an inlet pressure of 30 p.s.i. The hydrogen and the air for the flame ionization detector were set at flow-rates of 30 and 300 ml/min, respectively. The inlet temperature was 250° and the detector temperature was 275°. The column oven was temperature programmed as follows: held at 100° for 1 min following injection, then increased at 10°/min to 215°, then ballasted to 250° and held for 10 min. Attenuation was set at 32×10^{-11} with a suppression current ranging from 0.5×10^{-10} to 0.2×10^{-10}, depending on the age of the column. A needle guide was used to prevent bending of the syringe needle and to prolong septum life by allowing multiple injections through a single hole; the depth of penetration by the needle was also thereby controlled and limited to a consistent 8 mm beyond the septum.

The GC performance was tested for resolution and sensitivity by injecting 2 μl of a mixture of pure derivatized amino acids in ethyl acetate containing 2 nmoles/μl of each derivative. The relative molar response factor (RMR_{NLE}) of each amino acid was calculated with respect to NLE by peak height measurement. Although peak area measurements are commonly used in GC analysis, they require the use of an additional (expensive) integrating device or, alternatively, more tedious manual methods. In the interest of simplicity, the direct manual measurement of peak height was used as the basis of all quantitative calculations. This technique did not involve any sacrifice in accuracy [38—40] since the internal standard method was used, all operating parameters were strictly controlled and carefully reproduced, and a standard amino acid mixture was chromatographed with every series of unknown samples so that RMR values were determined under identical GC conditions.

RESULTS AND DISCUSSION

Fig. 1 shows the separation of the N-acetyl-*n*-propyl esters of a mixture of 23 pure amino acids which were taken through the entire procedure, as described above. The secondary standards, 2-AAA and 2-AIBA, were added following resin column pretreatment. Each peak represents 5 nmoles of amino acid in the original mixture. Table II summarizes the retention temperatures,

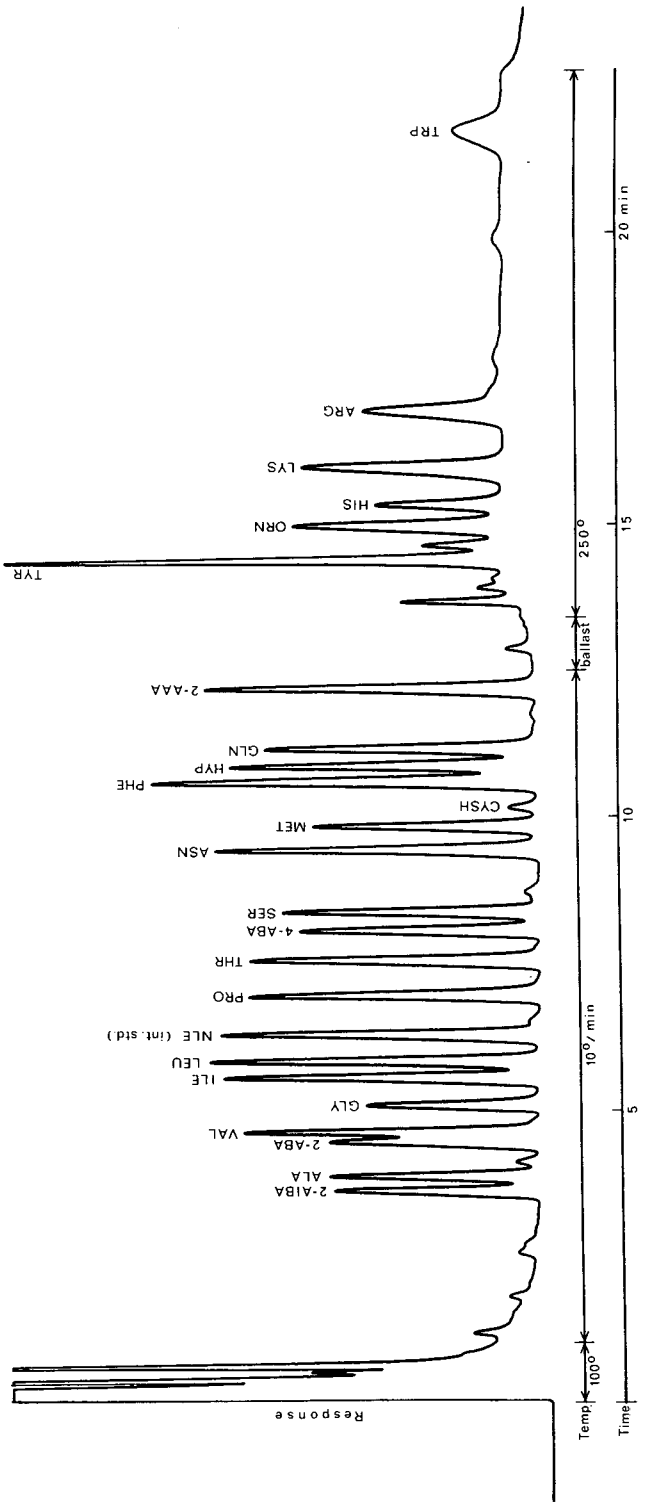

Fig. 1. GC separation of the N-acetyl-*n*-propyl esters of a mixture of 23 pure amino acids (for abbreviations see Table II) subjected to the complete procedure as described in Methods. The secondary standards 2-AAA and 2-AIBA were added after the resin columns. Temperature program: 1 min isothermal at 100°, followed by 10°/min to 215°, then ballasted to 250° and held for 10 min. Each peak represents 5 nmoles of amino acid derivative.

TABLE II

RETENTION TIME, RETENTION TEMPERATURE AND RELATIVE MOLAR RESPONSE OF N-ACETYL-*n*-PROPYL ESTERS OF AMINO ACIDS

Standard solution of 21 amino acids, plus GLN and NLE (as internal standard, I.S.) taken through complete procedure; 2-AAA and 2-AIBA added after resin columns. Data obtained from chromatogram shown in Fig. 1.

Amino acid	Abbreviation	Time (min)	Temperature (°C)	RMR_{NLE}
2-Aminoisobutyric acid	2-AIBA	3.6	125	0.66
Alanine	ALA	3.9	130	0.68
2-Aminobutyric acid	2-ABA	4.4	133	0.67
Valine	VAL	4.6	135	0.94
Glycine	GLY	5.0	139	0.55
Isoleucine	ILE	5.5	144	1.00
Leucine	LEU	5.9	148	1.04
Norleucine	NLE	6.2	151	1.00 (I.S.)
Proline	PRO	6.9	158	0.91
Threonine	THR	7.5	164	0.90
4-Aminobutyric acid	4-ABA	8.0	169	0.74
Serine	SER	8.3	172	0.79
Asparagine	ASN	9.4	183	1.01
Methionine	MET	9.8	187	0.69
Cysteine	CYSH	10.1	190	0.08*
Phenylalanine	PHE	10.6	195	1.19
Hydroxyproline	HYP	10.8	197	0.95
Glutamine	GLN	11.1	200	0.84
2-Aminoadipic acid	2-AAA	12.1	210	1.04
Tyrosine	TYR	14.4	250	1.64
Ornithine	ORN	14.9	250	0.69
Histidine	HIS	15.3	250	0.43*
Lysine	LYS	15.9	250	0.67
Arginine	ARG	16.8	250	0.44
Tryptophan	TRP	21.6	250	0.16*

*When resin columns are omitted, RMR_{NLE} value for cysteine is 0.38, for histidine is 0.58 and for tryptophan is 0.36.

retention times and relative molar response factors (RMR_{NLE}) which were obtained from the chromatogram in Fig. 1. The RMR_{NLE} value of an amino acid is defined as its peak height relative to the peak height of an equimolar amount of the primary standard NLE.

The secondary standard, 2-AAA, added after resin column clean-up and glutathione removal, served as a convenient monitor of recovery from the two resin columns. Comparison of the $RMR_{2\text{-}AAA}$ values for each amino acid in the "calibration standard" solution, as obtained with and without resin column treatment, showed that recovery was virtually quantitative (92—107%) for 19 of the amino acids as well as for the primary standard NLE. (During analysis of an unknown, the NLE peak height, relative to that of 2-AAA, was a clear indication of column elution efficiency.)

Significant and variable losses following the resin column procedure were

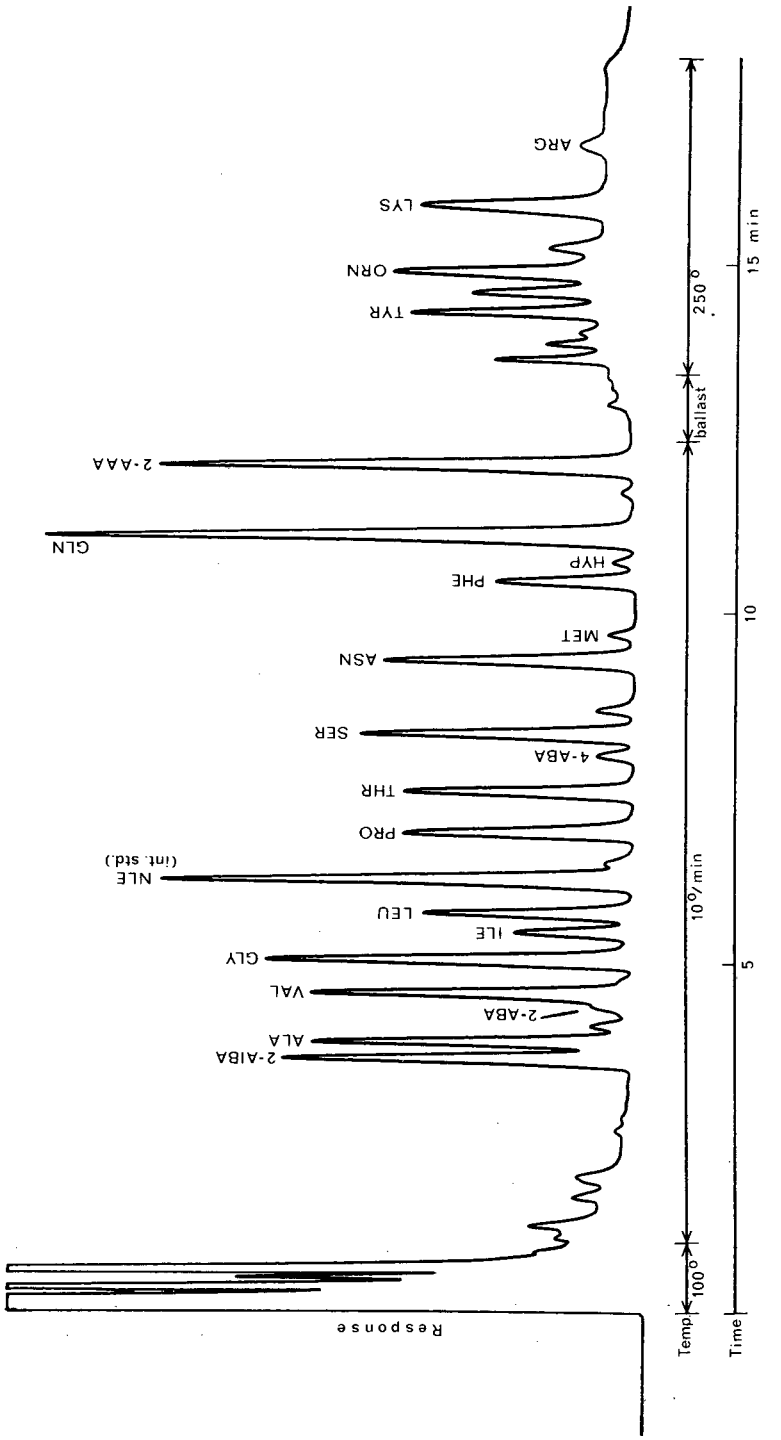

Fig. 2. Gas chromatogram of normal erythrocyte specimen subjected to the complete procedure. Temperature program as in Fig. 1. The three standard peaks represent 5 nmoles each of NLE, 2-AAA and 2-AIBA.

noted only for cysteine, histidine and tryptophan; their quantitation was therefore not reliable. It should be noted here that determination of erythrocyte cysteine and tryptophan is also not feasible by ion-exchange chromatography [10]. The dicarboxylic acids, aspartic and glutamic, were adsorbed on the anion-exchange column and were therefore not determined. This did, however, allow for the accurate quantitation of asparagine and glutamine which, under the conditions for esterification, were converted to the respective di-*n*-propyl esters of the parent acids. Reproducibility data for the 19 amino acids taken through the complete procedure show a coefficient of variation (C.V.) not exceeding 6%.

The secondary standard 2-AIBA forms the most volatile of all the derivatives studied here, and is therefore the first peak to elute from the GC column. A significant diminution in the size of this peak, relative to 2-AAA, was a warning that solvent evaporation, following acylation, had been excessive, and that some losses of alanine, 2-ABA, valine and possibly glycine had probably occurred. This problem, however, was seldom encountered in the course of many routine analyses.

Results of the application of this GC method of analysis to an erythrocyte sample from a normal adult is shown in Fig. 2. Clear and well-defined peaks can be identified for 19 amino acids, in addition to the NLE internal standard peak and the two secondary standard peaks, 2-AAA and 2-AIBA. A few minor unidentified peaks are present which do not interfere with the analysis.

The C.V. for simultaneous quadruplicate analysis of a single specimen was 8% for arginine, 6% for asparagine, and 1—4% for the other amino acids. When repeat analyses of a single specimen were done on different days, the C.V. values were slightly higher. Analysis of duplicate aliquots of a typical specimen of lysed erythrocytes, with and without a known amount of added glycine, indicated 100% recovery. When the glycine was added to the protein-free filtrate (following treatment with trichloroacetic acid) the recovery was 97%. Aliquots of selected erythrocyte specimens were sent to another laboratory for analysis by conventional automated ion-exchange chromatography. Values thereby obtained were in substantial agreement with the corresponding data from the GC analysis.

Table III summarizes the erythrocyte free amino acid concentrations in morning, fasting blood specimens obtained from 34 healthy adult volunteers. There were 23 female subjects, ranging in age from 21 to 60 years, and 11 male subjects whose age range was comparable (19—63). The mean, standard deviation, and range are shown for each of the 19 amino acids in both sex groups. (The mean concentration values for the combined group of 34 subjects are shown in Table I, for comparison with values published elsewhere.) Analysis of the data showed the absence of a significant correlation (r values in Table III) between subject age and the level of any amino acid in either sex group. The "two-tailed t test" was used to determine if there was a significant difference (P values in Table III) in the mean concentrations of any of the amino acids between the male and female groups. No sex group differences (NS) were seen in 17 of 19 erythrocyte amino acids. Differences of possible significance were indicated only for methionine ($P < 0.05$) and arginine ($P < 0.01$), both of which were found in very small amounts.

TABLE III

FREE AMINO ACID CONCENTRATIONS IN THE ERYTHROCYTES OF NORMAL ADULTS

For abbreviations, see Table II. Concentrations given in μmoles/l.

Amino acid	Males (n = 11)			Females (n = 23)			P* (M vs. F)
	Mean ± S.D.	Range	r (age)	Mean ± S.D.	Range	r (age)	
ALA	342 ± 76	213–441	+0.46	326 ± 67	228–438	+0.33	NS
2-ABA	26 ± 7	19– 39	+0.23	23 ± 7	13– 41	+0.03	NS
VAL	200 ± 30	166–275	+0.29	182 ± 31	123–232	+0.05	NS
GLY	351 ± 76	258–549	+0.75	368 ± 87	267–580	+0.06	NS
ILE	63 ± 13	44– 84	+0.03	56 ± 13	40–104	−0.05	NS
LEU	116 ± 17	89–137	−0.20	107 ± 19	78–172	−0.09	NS
PRO	190 ± 84	97–403	+0.31	168 ± 73	93–400	−0.24	NS
THR	123 ± 18	102–162	+0.28	124 ± 33	67–196	−0.12	NS
4-ABA	11 ± 5	6– 19	+0.06	12 ± 6	4– 27	+0.27	NS
SER	157 ± 14	135–179	+0.09	167 ± 26	123–219	−0.26	NS
ASN	120 ± 13	99–141	+0.16	126 ± 19	94–158	−0.18	NS
MET	26 ± 5	20– 35	−0.28	23 ± 3	18– 29	−0.18	<0.05
PHE	59 ± 12	48– 86	+0.21	58 ± 11	40– 85	+0.15	NS
HYP	14 ± 13	6– 51	+0.47	10 ± 6	4– 26	+0.19	NS
GLN	481 ± 31	420–519	+0.18	461 ± 78	310–589	−0.02	NS
TYR	72 ± 24	46–132	+0.14	69 ± 15	35–110	−0.07	NS
ORN	181 ± 28	152–230	+0.83	177 ± 37	112–249	+0.03	NS
LYS	181 ± 28	149–239	+0.68	170 ± 21	121–216	−0.02	NS
ARG	36 ± 13	17– 60	+0.76	26 ± 8	14– 45	−0.42	<0.01
Subject age (years)	35 ± 13	19– 63	1.00	33 ± 10	21– 60	1.00	

*Probability of the difference being due to chance, based on the "two-tailed test". NS, not significant.

Studies in progress have indicated the importance of controlling specimen collection time in regard to time of day and to food intake. These factors are related to quite significant changes in the levels of many erythrocyte amino acids.

CONCLUSION

We have described in this report a simple, rapid and reliable technique for the determination of free amino acids in erythrocytes. Interfering substances, principally glutathione, were removed by cation- and anion-exchange resins, and the amino acids were converted to their N-acetyl-*n*-propyl esters, separated and quantitated by gas chromatography. The comparable accuracy and precision, as well as reduced analysis time and equipment cost of the GC method make it a practical alternative to classical ion-exchange chromatography. As such, it seems well suited to facilitate further research in the field of amino acid metabolism in health and disease.

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DETERMINATION OF TRYPTOPHAN AND SEVERAL OF ITS METABOLITES IN PHYSIOLOGICAL SAMPLES BY REVERSED-PHASE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

DAVID D. KOCH and PETER T. KISSINGER

Department of Chemistry, Purdue University, West Lafayette, Ind. 47907 (U.S.A.)

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SUMMARY

A new method for the concurrent assay of three tryptophan metabolites at the picomole level is described. The method has been developed for blood, urine, cerebrospinal fluid, and tissue samples such as whole brain, brain parts, and endocrine glands. Tryptophan itself, serotonin, and 5-hydroxyindoleacetic acid are isolated initially on extraction columns, eluted with a suitable solvent, and injected onto a liquid chromatograph with an amperometric detector. This general approach may be applicable to a variety of other tryptophan metabolites and should be useful in both research and clinical investigations.

INTRODUCTION

Many analytical procedures have been devised for tryptophan (TRP), serotonin (5-HT), and serotonin's major metabolite, 5-hydroxyindole-3-acetic acid (5-HIAA). The most popular methods employ ultraviolet absorption, colorimetric, or fluorometric determinations, and these have been thoroughly reviewed [1]. More modern techniques such as radioenzymes, mass spectrometry, and gas chromatography—mass spectrometry have all been used with varying degrees of success [2, 3]. The need for chromatographic separation has led several investigators to choose liquid chromatography (LC). Many of these methods employ fluorescence detection [4—6] but generally require a derivatization step for greater sensitivity. Other LC methods using UV detection suffer from a lack of sensitivity [7, 8].

The present method allows concurrent assay of all three compounds from the same biological sample. Alternately, each can be measured alone, if so desired. Applicability to urine, serum, plasma, cerebrospinal fluid (CSF), and tissue homogenates arising from whole brain, brain parts, and endocrine glands is demonstrated. The method is based on the use of reversed-phase liquid chromatography with electrochemical detection (LCEC). Briefly, the selectivi-

ty arises from three processes: (a) an initial isolation step on small extraction columns, (b) the separation effected by the liquid chromatography, and (c) the electrode used as the detector. The basic isolation procedure has been adapted from the scheme of Hery et al. [9]. LCEC has been successfully applied to the determination of many drugs and metabolites [10—13], most notable being the measurement of tyrosine metabolites in brain tissue [14, 15]. Amperometric detection provides excellent sensitivity, more than adequate for most samples of interest. This method also has the advantages of relative simplicity and low cost. A recent report reviews the principles and methodology of liquid chromatography with electrochemical detection [16].

EXPERIMENTAL

Reagents

Hydrochloric acid, 6 *M*, 3 *M*, and 0.1 *M*. Sodium hydroxide, 3 *M* and 0.1 *M*. Perchloric acid, 4 *M* and 0.1 *M*. Acetate buffer, 0.1 *M* and pH 4.75: Dilute 82.04 g anhydrous sodium acetate and 58.0 ml of glacial acetic acid to 2 l for 1 *M* buffer; then dilute tenfold. Acetic acid, 1 *M*. Ammonium acetate, 1 *M*: Dilute 135 ml concentrated NH_4OH and 115 ml glacial acetic acid to 2 l. Disodium phosphate, 0.2 *M*: Dissolve 56.8 g anhydrous disodium phosphate in 2 l. Citric acid, 0.1 *M*: Dissolve 42.0 g citric acid in 2 l water plus 2 drops of toluene. Phosphate buffer A, 0.2 *M* and pH 6.50: Dissolve 4.46 g KH_2PO_4 and 2.44 g Na_2HPO_4 in 250 ml. Phosphate buffer B, 0.1 *M* and pH 6.80: Dissolve 1.86 g KH_2PO_4 and 1.98 g K_2HPO_4 in 250 ml. Ammonium acetate, 3 *M*: Dilute 50.7 ml concentrated NH_4OH and 43.1 ml glacial acetic acid to 250 ml. Ammonium hydroxide, 0.1 *M*: Dilute 3.4 ml concentrated NH_4OH to 500 ml. Phosphoric acid, 1.0 *N*: Dilute 4.51 ml concentrated H_3PO_4 to 200 ml. Acetate buffer, 0.1 *M* and pH 5.0: Dissolve 1.50 ml 6 *M* acetic acid and 1.31 g sodium acetate in 250 ml.

Authentic standards

5-Hydroxyindole-3-acetic acid (Sigma, St. Louis, Mo., U.S.A.), stock solution, 50 ng/μl, in 0.1 *M* $HClO_4$; store refrigerated and prepare fresh every 4 weeks. Serotonin (creatinine sulfate complex; Aldrich, Milwaukee, Wisc., U.S.A.) and tryptophan (Sigma) stock solutions, 50 ng/μl, in acetate buffer; store refrigerated and prepare fresh every 4 weeks. All concentrations of serotonin are reported as the free base.

Biological controls

Urine pool: Collect approximately 1 l of urine from healthy humans, acidify to pH 2 with 6 *M* HCl, and store 15-ml aliquots at —35° in glass scintillation vials. Serum (plasma) pool: Collect serum (plasma) from blood taken by venipuncture from healthy humans, mix, and store frozen at —35° in 3-dram vials. Plasma samples are drawn into Vacutainer tubes containing citrate to prevent clotting. Brain homogenate pool: Homogenize at least 3 rat brains in 10 ml 0.1 *M* $HClO_4$, collect homogenate and store at —35° in 3-dram vials.

Apparatus

Tissue samples are homogenized with a Brinkmann polytron homogenizer and centrifuged at 12,000 *g* for 20 min in a centrifuge refrigerated at 4° (Sorvall Model RC2-B). Small plasma and serum samples were centrifuged using a Brinkmann Eppendorf centrifuge. The isolation columns (Bio-Rad) are filled with the appropriate resin using a slurry of a weighed amount of dried resin and 0.1 *M* HCl. Amberlite CG-50 II cation-exchange resin (200—400 mesh, p.a. grade, Atomergic Chemetals Co., Plainview, N.Y., U.S.A.) was used for isolation of serotonin, Dowex AG-50 W-X2 cation-exchange resin (200—400 mesh, BioRad Labs., Richmond, Calif., U.S.A.) was used for tryptophan, and Sephadex G-10 gel filtration resin (40—120-μm particle size, Pharmacia, Uppsala, Sweden) extracted 5-HIAA. Dry-packing these columns is not recommended due to the air pockets and inhomogenous beds which sometimes result. Two Model LC-50 (Bioanalytical Systems) liquid chromatographs with carbon paste amperometric detectors (model TL-3) were equipped with stainless-steel columns slurry-packed with microparticulate reversed-phase packing material (μBondapak C_{18}, particle size 10 μm; Waters Assoc., Milford, Mass., U.S.A.). For each system two 15 cm × 4.0 mm I.D. columns were joined together with a short length of 1/16 in. stainless-steel tubing. Two identical chromatographs were used in order to minimize the time necessary to determine all three compounds and to utilize the considerable advantage gained by being able to use a lower electrode potential for some of the assays. The method is flexible enough that one chromatographic system would serve for many applications. The following mobile phases were used:

chromatograph 1: 0.5 *M* ammonium acetate, pH 5.1, 15% methanol. Mix 150 ml 1 *M* ammonium acetate, 150 ml water, 75 ml 1 *M* acetic acid, and 65 ml methanol. NOTE: Ammonium acetate is not stored below 1 *M* concentrations due to noticeable bacteria growth.

chromatograph 2: McIlvaine buffer, pH 4.0, 20% methanol. Mix 200 ml 0.2 *M* Na_2HPO_4, 350 ml 0.1 *M* citric acid, and 140 ml methanol.

The mobile phases were filtered through 0.22-μm (average pore size) filters (Millipore, Bedford, Mass., U.S.A.) and then thoroughly mixed with the appropriate amounts of methanol. Both instruments were used with a flow-rate of 1 ml/min and a rotary injection valve fitted with a 20-μl sample loop (Model 70-10, Rheodyne). The detector potential for chromatograph 1 was set at +500 mV vs. a Ag/AgCl reference electrode, while that for chromatograph 2 was +1.00 V. All glassware used in the procedure was silanized with trimethylchlorosilane [17].

Procedure

Column preparation. The isolation columns were packed as indicated above with the following amounts of resin: Amberlite, 0.15 g; Dowex, 0.30 g; Sephadex, 0.55 g. Once prepared, these columns could be recycled and used for at least 25 samples. Their performance was slightly better when wet, therefore, on the day the samples are to be run, the columns are washed with 12 ml of the appropriate initial solution. For Amberlite, this is phosphate buffer A; for Dowex, phosphate buffer B; and Sephadex, 0.1 *M* HCl. When the liquid stops flowing, the columns are ready for use. After eluting the

sample, a recycling sequence for each column of 3 *M* HCl, 3 *M* NaOH, 0.1 *M* HCl, 0.1 *M* NaOH, and 0.1 *M* HCl (10 ml of each) prepares them for the next sample.

Sample preparation. 1. Biological fluids. Urine, which can be analyzed fresh or from frozen samples, is adjusted to a pH of 5.0 before application to the first isolation column. This is usually possible by titration with a few drops of 1 *M* HCl and/or 3 *M* NaOH per 10 ml of urine. Serum and plasma are deproteinized by the addition of 100 μl of 4 *M* $HClO_4$ to every 1 ml of sample. After shaking the acidified sample and centrifuging at 15,000 *g* for 5 min, the clear supernatant is poured into a beaker and 1 ml of the pH 5, 0.1 *M* acetate buffer is added, along with 250 μl of NaOH to bring the pH to 5.0. Thus, the dilution factor used in calculating the original concentration of the sample is 40/99.

2. Tissue. Rats (Sprague-Dawley) were sacrificed ca. 10:00 a.m. each day by cervical dislocation; their brains were removed as rapidly as possible and frozen on dry ice, weighed to the nearest mg, and either stored frozen, dissected according to Konig and Klippel [18], or homogenized in 3 ml 0.1 *M* $HClO_4$. After centrifugation, the supernatant from the brain homogenates is adjusted to a pH of 5.0 by a method similar to that for plasma: 1 ml of the acetate buffer and 0.5 ml NaOH are added to every 2 ml of homogenate, with a resulting dilution factor of 4/7.

Isolation and determination of the compounds. A flow chart of the basic

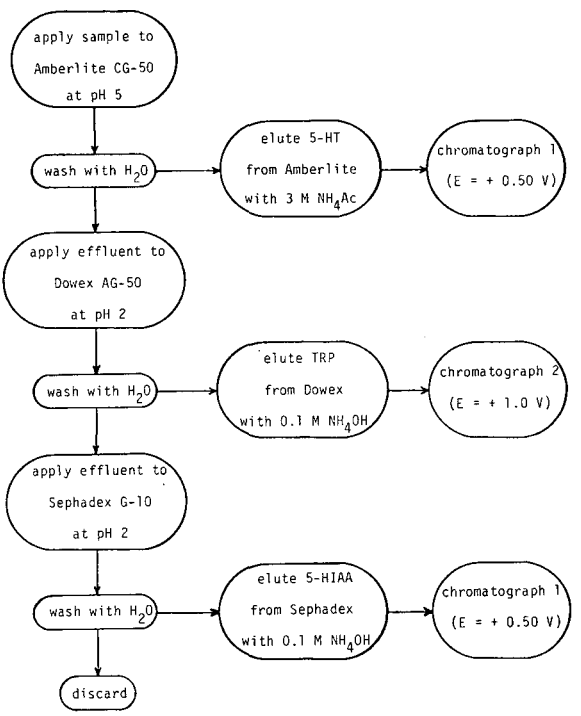

Fig. 1. Flow chart of the steps in the isolation procedure for the tryptophan metabolites. Each of the three compounds is injected individually.

steps in the isolation scheme is shown in Fig. 1. Two milliliters of the sample, now properly adjusted to pH 5.0, are passed through a column of Amberlite CG-50 to isolate the 5-HT. Within each group of 5 to 10 samples one from the standard pool should also be used. Wash the columns with water (5 ml) and elute with 3 *M* ammonium acetate (1.5 ml). Filter the eluent through a Millipore Swinnex assembly and inject a 20-μl portion onto chromatographic system 1 for quantitation of serotonin.

The effluents from each Amberlite column are adjusted to pH 2.0 and applied to a column of Dowex AG-50 resin, on which tryptophan is bound. A consistent method of adjusting the pH at this stage (and before the Sephadex) is to use aliquots of 1.0 *N* phosphoric acid. The columns are washed with water (5 ml) and eluted with 0.1 *M* NH_4OH. Generally 3.0 ml is used to elute the tryptophan, but larger amounts may be necessary, as described in the discussion. As the eluent begins to collect from the column, a drop of 6 *M* HCl is added to lower the pH and minimize oxidative decomposition of the sample. A 20-μl portion of the NH_4OH eluent is injected onto chromatographic system 2 for the quantitation of tryptophan.

Isolation of 5-HIAA from the effluents of the Dowex column is accomplished by adsorption on Sephadex G-10. The effluents are passed through at a pH of 2.0 (adjusted as for the Dowex), the columns washed with water (5 ml), and eluted with 0.1 *M* NH_4OH, 3.0 ml in the case of urine, and 1.0 ml in the case of plasma or brain after washing first with 500 μl. Again, a drop of 6 *M* HCl is added as the eluent begins to collect. Quantitation of 5-HIAA is accomplished after injecting 20 μl of the filtered eluent onto chromatographic system 1.

Calculations. The concentration of each compound is determined by measuring their respective peak heights and comparing with the peak heights obtained for the standard pool for the same sample type. The pool is calibrated using a standard addition method, adding sufficient volume of a standard solution of the three metabolites to make the added concentration fall within the desired levels. It is advantageous, of course, to employ different

TABLE I

STANDARD ADDITIONS FOR METHOD CALIBRATION

Urine			
5-HT:	50 ng/ml	100 ng/ml	150 ng/ml
5-HIAA:	1.5 μg/ml	3.0 μg/ml	4.5 μg/ml
TRP:	3.0 μg/ml	6.0 μg/ml	9.0 μg/ml
Serum and plasma			
5-HT:	100 ng/ml	200 ng/ml	300 ng/ml
5-HIAA:	10 ng/ml	20 ng/ml	30 ng/ml
TRP:	3.0 μg/ml	6.0 μg/ml	9.0 μg/ml
Brain homogenate			
5-HT:	150 ng/ml	300 ng/ml	450 ng/ml
5-HIAA:	150 ng/ml	300 ng/ml	450 ng/ml
TRP:	1.0 μg/ml	2.0 μg/ml	3.0 μg/ml

spikes for each compound in each pool, due to the great variability in concentration expected. The standard additions used in this work are shown in Table I.

Determine the peak heights for each compound in the pool and pool standard additions using the procedure described above. Plot the peak heights vs. the concentration of metabolite added and extrapolate to zero peak height to obtain the concentration in the original pool.

RESULTS

The present method has been optimized for the study of tryptophan, serotonin, and 5-hydroxyindoleacetic acid in urine, plasma, serum, CSF, and various regions of the brain. The assay is based on the use of small gravity-fed extraction columns to isolate the compounds prior to injection onto a reversed-phase liquid chromatograph. Final detection depends upon the compound's oxidation at a carbon electrode. Serotonin, 5-hydroxyindole-3-acetic

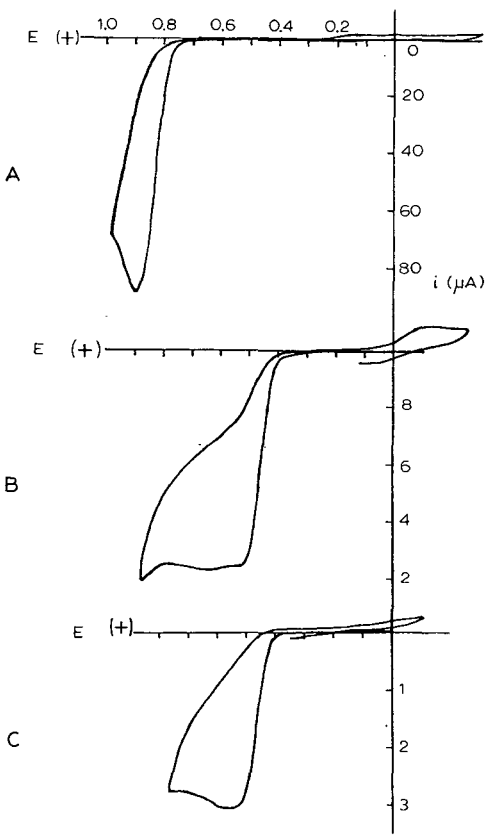

Fig. 2. Cyclic voltammograms of 50 μg/ml solutions of tryptophan (A), serotonin (B), and 5-HIAA (C). All solutions made from 0.1 *M* acetate buffer, pH 4.75. The working electrode was a 1.9 mm^2 disk packed with carbon paste, potentials are vs. a Ag/AgCl reference, and the scan rate was 250 mV/sec.

acid and tryptophan all can be oxidized at an accessible potential, as indicated by the cyclic voltammograms in Fig. 2. Typical chromatograms obtained for each compound are shown for human urine (Fig. 3) and plasma (Fig. 4), and rat brain homogenate (Fig. 5).

Our data from each of these matrices are reported in Tables II—IV, where a compilation of recent values is also presented. In each case, our data are in reasonable agreement with those obtained previously. The wide range in the levels may indicate methodological problems such as endogenous interferences or lack of sensitivity, difficulties which are surmounted in the present work by the isolation and detection scheme used.

Detection limits from an aqueous solution carried through the entire procedure were 1.0 ng/ml for 5-HT and 5-HIAA and 6.0 ng/ml for TRP. Practical detection limits for quantitation of each component in samples with a coefficient of variation of 5—8% were less than 2.0 ng/ml in the case of 5-HT and 5-HIAA, and 20 ng/ml for TRP. Absolute recovery, relative recovery, and reproducibility were adequate (see Table V) for each of the three compounds. Serotonin recovery can be improved by using larger volumes of Amberlite

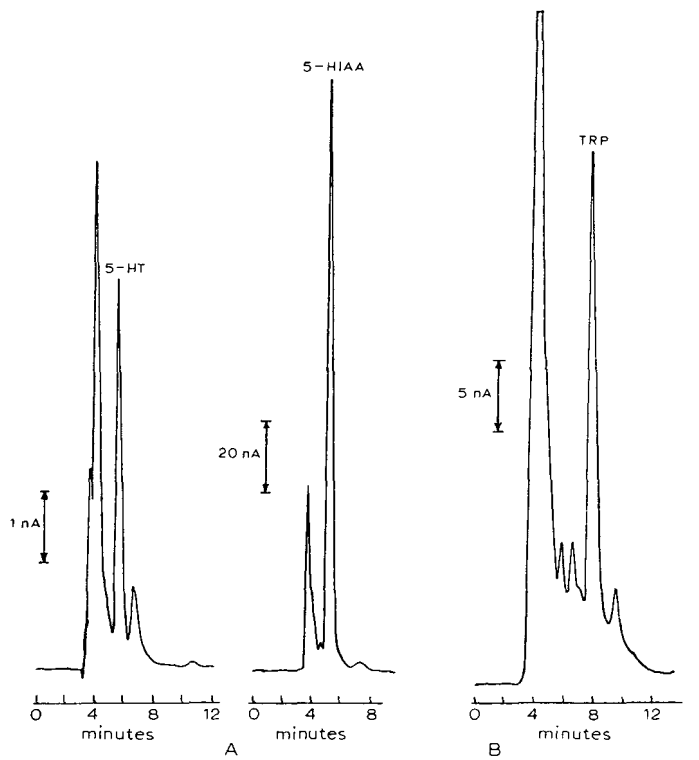

Fig. 3. Determination of urinary 5-HT, 5-HIAA, and TRP by LCEC. Chromatographic conditions: (A) 30 cm × 4 mm I.D. Waters μBondapak C_{18} mobile phase, system 1; flow-rate, 1 ml/min; 0.50 V detector potential vs. Ag/AgCl reference. (B) 30 cm × 4 mm I.D. Waters μBondapak C_{18}; mobile phase, system 2; flow-rate, 1 ml/min; 1.00 V potential. Typical concentrations and injected amounts: 5-HT: 103 ng/ml, 2.2 ng; 5-HIAA: 2.6 μg/ml, 33 ng; TRP: 12.3 μg/ml, 95 ng.

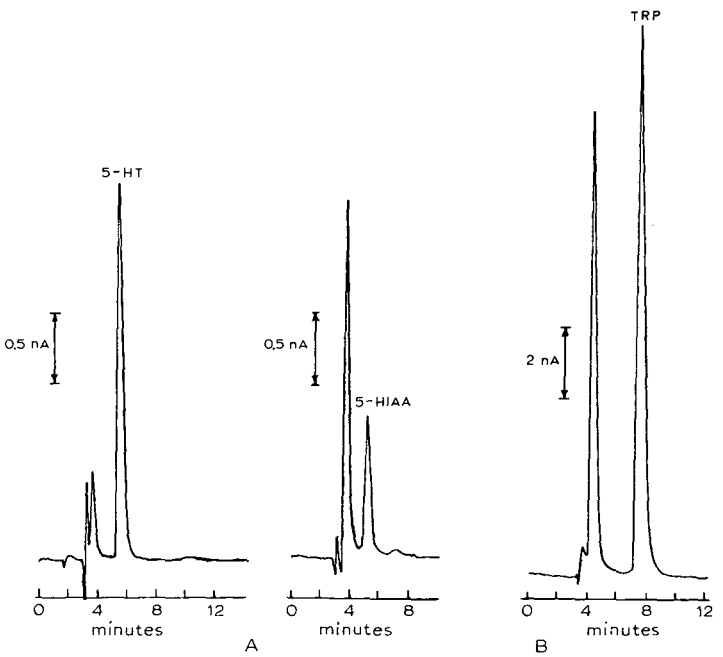

Fig. 4. Typical plasma sample. Chromatographic conditions as in Fig. 3. Typical concentrations and injected amounts: 5-HT: 28.3 ng/ml, 750 pg; 5-HIAA: 15.5 ng/ml, 620 pg; TRP: 12.1 μg/ml, 93 ng.

TABLE II

RAT WHOLE BRAIN VALUES

5-HT*	5-HIAA*	TRP**	Reference
710 ± 30	360 ± 10	3.31 ± 0.31	19
640 ± 30	530 ± 40	5.31 ± 0.06	20
450 ± 10	390 ± 10	4.10 ± 0.20	21
567 ± 25	446 ± 17		22
444 ± 79	349 ± 21	3.18 ± 0.47	23
521 ± 7	323 ± 11	7.91 ± 0.34	24
390 ± 70	580 ± 30	5.58 ± 0.24	25
510 ± 12	533 ± 21	3.75 ± 0.08	26
450 ± 50	870 ± 120	2.04 ± 0.19	27
510 ± 50	420 ± 50	2.0 ± 0.5	28
460 ± 30	503 ± 36		29
526 ± 81	442 ± 24	4.16 ± 0.23	3
238 ± 19	294 ± 7	6.92 ± 0.45	***

*ng/g wet weight, mean values ± S.D.
**μg/g wet weight, mean values ± S.D.
***This work, values ± S.D. One sample was repeated 4 times.

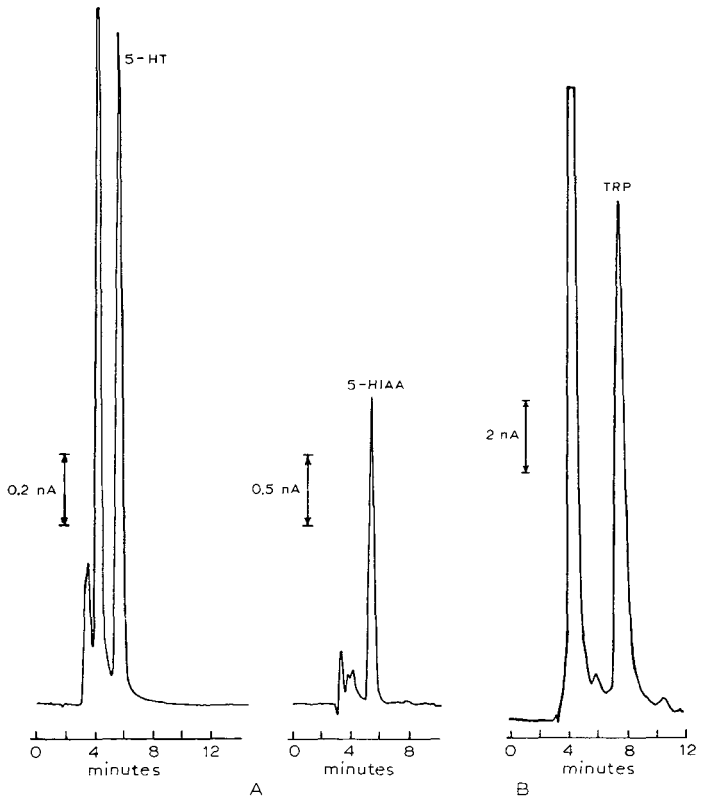

Fig. 5. Rat brain homogenate. Chromatographic conditions as in Fig. 3. Typical concentrations and injected amounts: 5-HT: 238 ng/g, 4.4 ng; 5-HIAA: 294 ng/g, 5.5 ng; TRP: 6.92 μg/g, 48 ng.

eluent, but the resulting dilution is undesirable. Both the recovery and precision of the method, for all three compounds but particularly for 5-HIAA, are aided by silanization of all glassware used in the procedure, presumably by avoiding the likelihood of losses due to adsorption onto the glass. The detector was found to be linear over the ranges of 0.010—80 ng of 5-HT or 5-HIAA injected and 0.10—100 ng of TRP injected. The linearity of the overall method was verified each time a standard addition calibration of a sample was performed. Demonstration of this linearity is given by the plot for 5-HT in urine (Fig. 6).

The identity of the peaks in each of the samples was confirmed by both their chromatographic and electrochemical behavior. Retention times for the three components in each sample were identical with aqueous standards, either injected directly or taken through the procedure. Also, retention times for all samples were compared with standards on LiChrosorb RP-18 (E. Merck, Darmstadt, G.F.R.) and Partisil PXS 5/25 ODS (Whatman, Clifton, N.J., U.S.A.) packing materials, both using two different mobile phases. In every case, co-chromatographic agreement resulted. Such consistency would not be expected unless the substances were in fact identical.

TABLE III

HUMAN PLASMA AND SERUM VALUES

5-HT*	5-HIAA*	TRP**	Reference
Plasma			
		9.8	30
	18 ± 5		31
		11.6 ± 4.7	32
8.5 ± 3			33
		1.57 ± 0.8	7
28.3 ± 2.1	15.5 ± 0.9	12.1 ± 0.4	†
Serum			
98 ± 8	<20***	10.5 ± 0.6	34
		11.1 ± 5	35
144 ± 46			36
84.1 ± 6.4	10.2 ± 0.5	9.82 ± 0.3	†

*ng/ml, mean values. In some cases the standard deviations are given, while in others, the range of data is indicated.

**μg/ml, total concentration, mean values. Standard deviations or ranges given. In one case, no standard deviation or range was included.

***Below detection limit.

†This work, values ± S.D. One sample was repeated 4 times. The range of data for 7 healthy males age 23—28 years was 13.3 ± 7.9, 16.5 ± 7.5, and 13.7 ± 5.5 in plasma and 39.5 ± 41.0, 10.2 ± 3.5, and 9.91 ± 3.1 in serum for serotonin, 5-hydroxyindole-3-acetic acid and tryptophan, respectively.

TABLE IV

HUMAN URINE VALUES

5-HT*	5-HIAA**	TRP**	Reference
		13.9	30
		1.7 ± 1.7	35
250 ± 20	1.1 ± 0.09		37
		4.2 ± 2.5	38
	10.0 ± 2.8		31
	4.3 ± 1.5		8
		27.9 ± 1	7
103 ± 8.2	2.6 ± 0.07	12.3 ± 0.80	***

*ng/ml, mean values ± S.D.

**μg/ml, mean values ± S.D. In a few cases, the range is shown. Where the data were given per 24 h or per g creatinine, the values reported here were calculated using 1200 ml urine per day and 1.5 g creatinine per day.

***This work, values ± S.D. One sample pool was repeated 7 times.

TABLE V

PRECISION OF THE ASSAY

	Absolute recovery (%) standards	Absolute recovery (%) samples *	Within-run coefficient of variation (%)
5-HT	77.8 ± 1.66	65.7 ± 6.7	6.6
5-HIAA	95.6 ± 7.5	82.8 ± 3.1	4.5
TRP	89.0 ± 2.6	58.7 ± 2.9	4.9

*These data are from 10 urine samples.

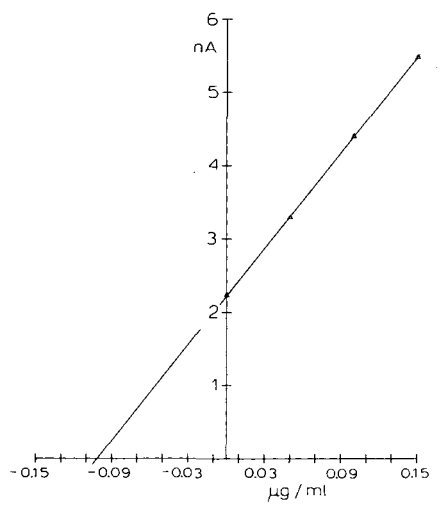

Fig. 6. Standard addition calibration for 5-HT in urine pool. Plot added concentration vs. peak height in nA.

Electrochemical confirmation was based on comparison of the hydrodynamic voltammograms of the actual sample eluent with aqueous standards for each compound. These curves are constructed by making repeated injections of a sample or standard at different electrode potentials, starting where the response is maximal and decreasing the potential, continuing until there is no response. The current (peak height) at each potential is divided by the current at the most positive potential to obtain the relative current ratio (ϕ) which is plotted vs. potential for both the sample and the standard. If the suspected compound and the sample are the same, their voltammetric curves will be identical at any potential. Hydrodynamic voltammograms for serotonin and the serotonin peak of a urinary Amberlite eluent are shown in Fig. 7. In this example, a slight amount of distortion does occur, but the agreement is evident. For every other possibility, the voltammograms coincide even more closely, except for the special case of 5-HIAA in urine, as will be explained below.

The hydrodynamic voltammograms point out not only the identity of the compound but also the specificity of the method for each compound. Inter-

ferences are virtually eliminated by the three criteria they must satisfy. First of all, an interfering molecule must behave in the same manner as the compound of interest on the isolation columns, then must co-chromatograph with that compound, and finally must be oxidized at the electrode potential used. The latter point can be of great advantage. For instance, the catecholamines behave most like serotonin and thus are eluted off the Amberlite, and may chromatograph close to serotonin, but they require a higher oxidation potential than 500 mV and thus are not detected. The accurate determination of serotonin in urine (and in other matrices, for that matter) is greatly improved by operating at 500 mV vs. e.g. 900 mV, as shown in Fig. 8.

Another way the detector potential offers selectivity is illustrated by the chromatograms of the Sephadex elution from a urine sample (Fig. 9). In the chromatograms, a large interference appears just after 5-HIAA which would completely mask the latter if it weren't for the fact that the interference doesn't begin to respond until about +550 mV. Thus, restricting the potential to 500 mV permits determination of 5-HIAA without the need to eliminate or resolve the interference.

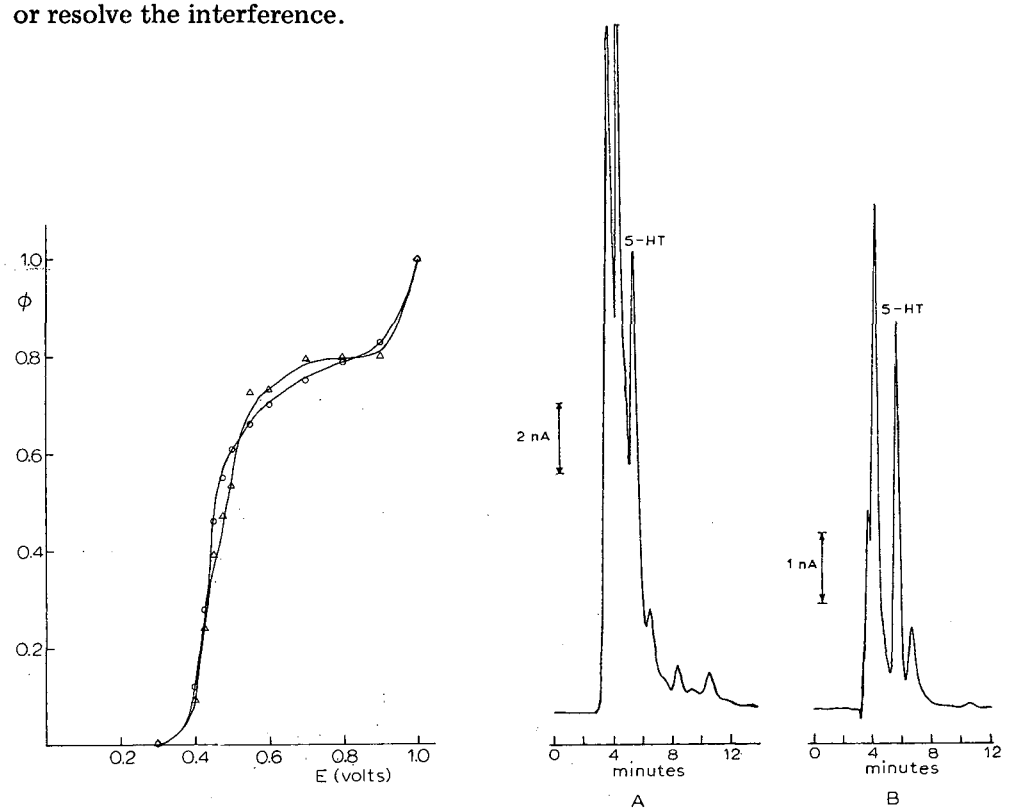

Fig. 7. Hydrodynamic voltammograms of seroronin standard (○) and the Amberlite eluate from a urine specimen (△).

Fig. 8. Selectivity based on the oxidation potential. Chromatograms of the same urine Amberlite eluate at two different potentials vs. the Ag/AgCl reference. (A) 900 mV; (B) 500 mV.

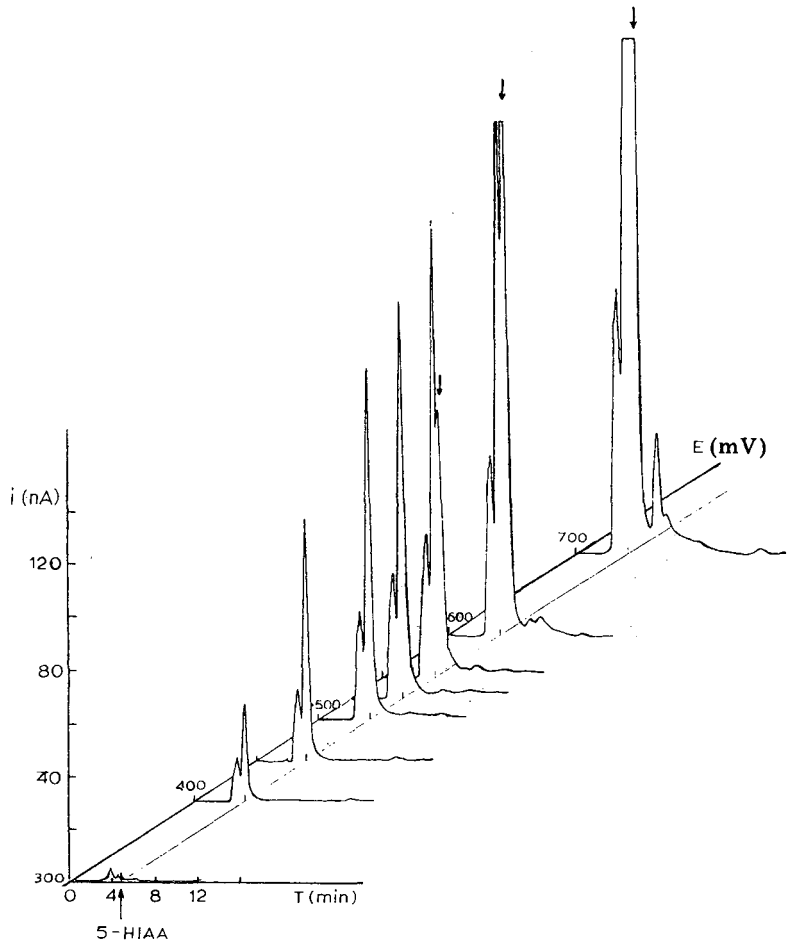

Fig. 9. Chromatograms of urinary 5-HIAA at different detector potentials, illustrating that judicious choice of the potential can eliminate interferences. Repeated injections of the sample were made at the following potentials vs. the Ag/AgCl reference: (mV) +700, +600, +550, +525, +500, +450, +400, +300. Each chromatogram is plotted vs. potential. Arrows indicate growth of the interfering peak.

DISCUSSION

At this point, the method has been used to determine tryptophan, serotonin, and 5-hydroxyindole-3-acetic acid levels in 25 rat brains, in 20 human plasma and serum samples, and in numerous urines from healthy humans and patients with disease states such as carcinoid tumor, melanoma, and neuroblastoma. The method also has been very successful when extended to other applications such as cerebrospinal fluid, free tryptophan in plasma, pineal gland, and other rat brain regions.

As the procedure has been described for quantitating all three compounds, it is possible for a single technician to complete 16 samples per day from

frozen specimens. However, the method is versatile and can be used when only one or two of the compounds are studied, increasing the sample rate considerably. For instance, 48 serotonin determinations per day are possible. If only 5-HT and 5-HIAA are desired, the Dowex step in the procedure may be omitted and the Amberlite eluates applied directly to the Sephadex. It is also possible to handle tryptophan alone, using only the Dowex columns. However, when performing the 5-HIAA individually, a slight addition to the procedure is helpful which cleans up every Sephadex chromatogram. This extra step is a wash with 5.0 ml of 0.02 *M* acetic acid which should follow the water wash. A large number of interferences are then eliminated, while 5-HIAA recovery is unchanged.

A study of the influence of the volume of sample applied to the isolation columns showed that 500 μl can be successfully used instead of 2 ml. This can be advantageous when the amount of sample is at a premium or the volume is low, e.g. homogenates of small brain regions. The elution volume also may be varied to suit individual purposes. As has been mentioned, 3.0 ml of 0.1 *M* NH_4OH is generally used to elute tryptophan, but when the amount of compound is high it is wise to increase the elution volume to enhance both the recovery and the linearity of the determination. Examples where this is most important include the determinations of tryptophan in urine, total tryptophan in plasma, and 5-HIAA in urine, since the unspiked concentrations are already large. Alternatively, and perhaps more satisfactorily, this problem is eliminated by replacing the TL-3 detector cell with one where the auxiliary electrode is positioned directly opposite the working electrode [39] (Bioanalytical Systems, Model TL-5A), which extends the linearity substantially. At the other extreme of elution volume, small amounts applied to the isolation columns demand elutions of minimal volume such as 500 μl. The first 500 μl contain little or none of the molecule desired, thus it is discarded in favor of collecting the second or third 500-μl fraction.

Another variable is the nature of the eluent. Different eluents can be employed to desorb the compounds which improve recoveries; however, other factors make them less desirable than those currently employed. For example, 3 *M* HCl elutes serotonin from the Amberlite with an 80% recovery, but the void volume response is much greater than that for 3 *M* ammonium acetate, which matches the mobile phase more closely. Improvement in the recoveries also results when more resin is packed into the columns, though at the expense of speed since the flow-rates will decrease.

The need for a fast, reliable procedure for tryptophan and its metabolites is apparent in view of the vast number of studies and the difficulties in interpreting results from the many different laboratories investigating similar problems. The LCEC method described here has several principal advantages which should make it useful in meeting this need. The three-way selectivity of liquid—solid extraction, liquid chromatography, and amperometric detection combine to provide very good specificity. The high sensitivity, simplicity, and good reproducibility with inexpensive reagents help to make it ideal for routine use. The versatility of the method, allowing analysis of one, two, or all three molecules, and applicability to every physiological sample commonly encountered are other desirable traits.

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Biomedical Applications

CHROMBIO. 407

DETERMINATION OF THERAPEUTIC AND TOXIC CONCENTRATIONS OF DOXEPIN AND LOXAPINE USING GAS—LIQUID CHROMATOGRAPHY WITH A NITROGEN-SENSITIVE DETECTOR, AND GAS CHROMATOGRAPHY—MASS SPECTROMETRY OF LOXAPINE

JOHN VASILIADES, TUOMAH M. SAHAWNEH and CARMEN OWENS

Department of Pathology, University of Alabama in Birmingham, Birmingham, Ala. 35233 (U.S.A.)

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SUMMARY

A gas—liquid chromatographic procedure is presented for the determination of therapeutic and toxic serum levels of doxepin and loxapine, using a nitrogen—phosphorus-sensitive detector. Amitriptyline is used as the internal standard. The method is accurate, sensitive and specific with no derivatization required prior to analysis. An advantage of the procedure is the small serum sample size needed for analysis and the selectivity and sensitivity of the detector, with the limit of detection being 3 and 2 μg/l for doxepin and loxapine, respectively. Nine cases of doxepin and loxapine misuse are presented. Serum doxepin concentrations ranged from 113 to 439 μg/l, with a loxapine concentration of 192 μg/l observed in one patient. The presence of the tricyclics was identified and confirmed by gas chromatography—mass spectrometry and the mass spectrum of loxapine is reported.

INTRODUCTION

Loxapine succinate (Loxitane), a dibenzoxazepine, and doxepin hydrochloride (Sinequan), a dibenzoxepine, are tricyclic derivatives (Fig. 1) which like other tricyclics such as amitriptyline, nortriptyline, imipramine, desimipramine and protriptyline, have effective antidepressant and antianxiety activity [1,2]. Loxapine is especially effective in the treatment of schizophrenia [3,4]. An apparent correlation between circulating blood levels and therapeutic effect has been reported for doxepin and other tricyclic antidepressants [5—7]. A definite correlation between administered daily dosage and therapeutic effect has also been established for loxapine [2—4]. Recently an attempt was made to correlate circulating blood levels and therapeutic effect for loxapine [8].

The more common use of these and other tricyclic drugs has brought about an increase in tricyclic abuse and self-inflicted poisonings [9]. Patients may take the drugs for therapeutic purposes or suicide gestures. Because of car-

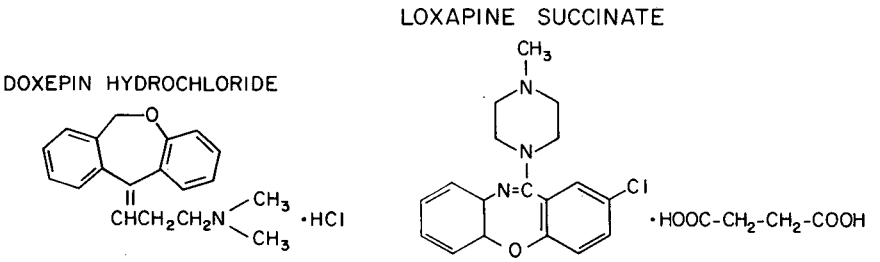

Fig. 1. Chemical structures of doxepin hydrochloride (Sinequan) and loxapine succinate (Loxitane).

diovascular and other complications, the identification and quantitation of doxepin and loxapine overdose is important.

A number of procedures have been reported for the determination of doxepin in serum [10—16]. Recently a procedure for the determination of loxapine and its metabolites has also been reported [8]. We report here a gas—liquid chromatographic (GLC) procedure using a nitrogen—phosphorus-sensitive detector for the simultaneous determination of doxepin and loxapine in serum. An advantage of this procedure is the small sample size used for analysis and the sensitivity and selectivity of the detector. No derivatization is required and quantitation is achieved from therapeutic to toxic levels. To illustrate the applicability of this procedure, eight representative patients involved in doxepin misuse and one patient involved in loxapine misuse as seen in the emergency room are presented.

EXPERIMENTAL

Reagents

All reagents used were spectral grade: heptane, chloroform, methanol, isobutanol. Anesthetic grade diethyl ether (J.T. Baker, Phillipsburg, N.J., U.S.A.) was used. Amitriptyline HCl (Merck, Sharp and Dohme, Rahway, N.J., U.S.A.), doxepin HCl (Sinequan; Pfizer, Brooklyn, N.Y., U.S.A.), and loxapine succinate (Loxitane; American Cyanamid Co., Pearl River, N.Y., U.S.A.) were used as the salts; however, all concentrations are expressed as the free base. A 1 g/l stock aqueous solution of doxepin, loxapine, and amitriptyline was prepared in deionized distilled water. A 10 mg/l working aqueous solution of the above was prepared in distilled deionized water. Serum standards were made up by the addition of small amounts of aqueous doxepin and loxapine 10 mg/l working standards to normal pooled human serum. An aqueous amitriptyline internal standard was added to the doxepin and loxapine standards and taken through the entire extraction procedure. A 0.5 *M* NaOH solution was prepared from solid sodium hydroxide, and a 0.1*M* HCl solution was prepared from concentrated hydrochloric acid.

Apparatus

Analyses were performed on a Perkin Elmer 3920 gas chromatograph equipped with a flame ionization and a nitrogen—phosphorus-sensitive detector

(Perkin Elmer, Norwalk, Conn., U.S.A.). A 1.8 m × 2 mm I.D. round glass column packed with 3% OV-17 on 100—120 mesh Gas-Chrom Q (Applied Science Labs., State College, Pa., U.S.A.) was used to accomplish separation with the following gas chromatographic (GC) conditions: helium carrier gas with a flow-rate of 40 ml/min, injector temperature 275°, interface temperature 275°. The column temperature was programmed as follows: initial temperature was maintained at 235° for 6 min and then programmed to 280° at 32°/min and maintained at 280° for 4 min. Air-flow to detector was 40 p.s.i., hydrogen flow to detector 12 p.s.i. The detector voltage was a 5.5—5.8 V setting on the variable power source; detector voltage was increased with bead aging for sensitivity. A Perkin Elmer 26 recorder set at a range of 1 mV and a chart speed of 10 mm/min was used to record all chromatograms.

The detector used in the analysis was the nitrogen—phosphorus-sensitive detector purchased from Perkin Elmer. The principle of its operation has been described [17].

Gas chromatographic—mass spectrometric (GC—MS) analyses were performed on a Hewlett-Packard 5985A quadrupole GC—MS system (Hewlett-Packard, Palo Alto, Calif., U.S.A.) using the electron impact (EI) mode. The system consists of an HP 5840A gas chromatograph interfaced with the mass spectrometer, an HP 7900 disc drive, an HP 2109 computer and a Tektronix 4012 graphic display. A 1.2 m × 2 mm I.D. column packed with 2% SP-2250 on Chromosorb W HP 100—120 mesh was used for the GC—MS analysis.

Procedure

The procedure used is a modification of our previous procedure [9]. To a 50-ml glass stoppered centrifuge tube add 2 ml of serum, 1 ml of 0.5 *M* NaOH and 30 ml of 4% isobutanol in *n*-heptane. Shake for 5 min, centrifuge for 5 min and filter through phase separation paper. To the organic filtrate add 5 ml of 0.1 *M* HCl, shake for 5 min, centrifuge for 5 min, and discard the organic layer. Wash the aqueous layer with 30 ml of *n*-heptane, back extract in 10 ml of ether, and evaporate to dryness.

The residue was dissolved in 25 μl of absolute ethanol or chloroform—methanol (1:1) and subjected to analysis by injecting 1 μl of the reconstituted sample onto the column.

In some cases where the internal standard was not carried through the extraction procedure, the internal standard was added with the absolute ethanol or chloroform—methanol mixture.

Standard curves were obtained by analyzing serum standards containing known amounts of doxepin and loxapine. Serum standards containing 50, 100, 200, 300, 400, and 500 μg/l of doxepin or loxapine were used for patients with therapeutic levels; serum standards containing 0.5, 1, 2, 3, 4 and 5 mg/l of doxepin or loxapine were used for toxic levels. The concentration of amitriptyline internal standard used for the therapeutic range was 100 μg/l, and the concentration for toxic levels was 1 mg/l. Following chromatography, the ratio of drug to internal standard peak area or peak height was calculated and plotted against its concentration. The doxepin or loxapine concentration in patients was obtained from the curve and calculated using the nearest serum standard. No significant difference between the two methods of calculation was observed.

RESULTS

Chromatograms of serum blanks, and a serum standard containing doxepin, loxapine and amitriptyline as internal standard, taken through the extraction procedure under the described conditions of study are shown in Fig. 2. The retention times at 235° were 3.6 and 4.1 min for amitriptyline and doxepin, respectively, and 9.5 min for loxapine after programming to 280° at 32°/min. Fig. 2 also indicates that the detector was most sensitive to loxapine, followed by amitriptyline and then doxepin. Sensitivity was governed by the ability of these drugs to form cyan-free radicals. Blank serum samples assayed in the same manner as standards and patients gave no significant peaks on the chromatogram that might interfere with the analysis (Fig. 2). However, Fig. 2 shows an impurity peak which appeared in some extractions that interferes with desmethyldoxepin and protriptyline; it was attempted to use the latter as an internal standard instead of amitriptyline. This interference could be overcome by decreasing the oven temperature or carrier gas flow-rate, but this would have increased the loxapine retention time resulting in increased analysis time. Under the conditions of this study, nortriptyline interferes with doxepin, which is in agreement with other literature findings [12]. In addition, desmethyldoxepin will interfere with protriptyline.

The absolute percentage recovery of doxepin and loxapine by our method averaged 65 ± 10% for serum standards of 50, 100, 200, 300, 400, and 500 μg/l. The percentage recovery relative to serum standards averaged 98 ± 10%. This compares well with previously described procedures for other tricyclic antidepressants [9].

The linearity of the entire assay was demonstrated by extracting 2 ml of serum samples in duplicate, containing 50, 100, 200, 300, 400, and 500 μg/l of doxepin and loxapine with 100 μg/l of amitriptyline as internal standard.

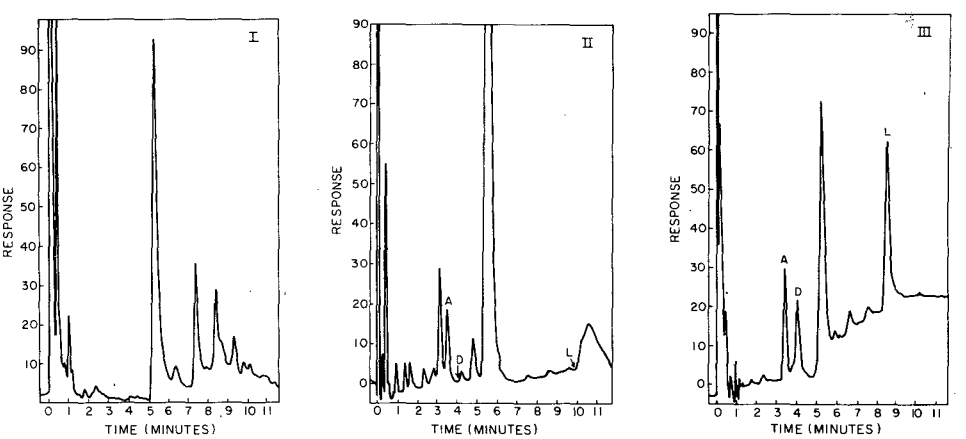

Fig. 2. Gas chromatograms of (I) serum blank, attenuation × 8; (II) serum blank with amitriptyline (A) as internal standard (100 μg/l), with D and L representing expected retention times for doxepin and loxapine, attenuation × 32; (III) serum standard containing 200 μg/l of doxepin (D), loxapine (L) and amitriptyline (A) as internal standard, attenuation × 4.

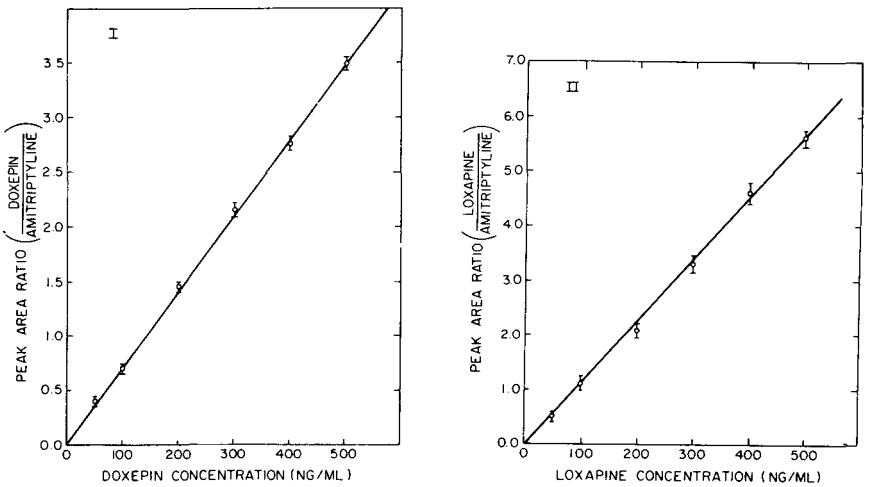

Fig. 3. Standard curves using serum standards containing 50, 100, 200, 300, 400 and 500 μg/l of doxepin (I) and loxapine (II). Plot of peak area ratio against concentration. Internal standard amitriptyline 100 μg/l. (I) Slope = 6.8×10^{-3}, y intercept = 5.3×10^{-2}, correlation (r) = 0.99, Syx = 0.03. (II) = 1.2×10^{-2}, y intercept = — 0.11, correlation (r) = 0.99, Syx = 0.06.

The extract was subjected to GC analysis under the described conditions, and the peak area ratio was plotted against concentration (Fig. 3). Linearity extended through the 500 μg/l standard for the lower concentration range of standards used and through the 5 mg/l standard for the higher concentration range. Aqueous standards of 10 μg/l of loxapine could easily be quantitated by this procedure.

The above procedure was used to determine serum doxepin and loxapine levels in patients seen in the emergency room and suspected of having ingested these drugs. A review of nine patients seen in the emergency room is summarized in Table I. Shown in Table I is the patient's alleged drug ingestion, clinical symptoms, and doxepin and loxapine serum levels. Some of the patients in Table I lack a serum concentration due to the unavailability of serum at the time of analysis. In all cases, doxepin and loxapine were identified and confirmed by three separate methods. Doxepin and loxapine were first identified in the urine and/or gastric samples of each patient by ultraviolet (UV) spectrophotometry, thin-layer chromatography (TLC), and GC as previously described [9]. Thin-layer plates were dried and sprayed with acidified iodoplatinate for visualization of the drugs and metabolites. Doxepin, loxapine, and possibly a doxepin metabolite in one of the samples (urine samples of patient No. 6) were observed. The TLC spots were scraped off and the drugs extracted for GLC analysis. The approximate concentrations of doxepin or loxapine in urine and/or gastric samples were calculated and are also given in Table I. The doxepin metabolite has a longer retention time than doxepin under the same GC conditions of study with a retention time of 1.37 relative to amitriptyline (Table II), and corresponds to that of desmethyldoxepin.

TABLE I

SUMMARY OF PATIENTS INVOLVED IN DOXEPIN AND LOXAPINE POISONING

Patient*	Drugs allegedly ingested	Clinical symptoms	UV spectra for		TLC**		Serum level (μg/l) of drug ingested (GLC)***
			Doxepin	Loxapine	Doxepin	Loxapine	
1. 27 years B-F	Sinequan unknown amount, 1/2 beer	Dizziness	+	—	+	—	Dox. 192
2. 25 years W-F	Sinequan or tricyclic subgroup, Triavil	Comatose, obtunded	+	—	+ G(811)	—	Dox. 431
3. 25 years W-F	Sinequan or tricyclic subgroup, Triavil	Semicomatose, pale and drousy but easily arousable	Unable to identify		—	+	Lox. 192
4. 31 years W-F	Sinequan 12/50 mg tablets	Lethargic but easily arousable	+	—	+ U(9.5)	—	Dox. Serum sample not available
5. 35 years W-M	Stelazine, Valium, and Sinequan		+	—	+	—	Dox. 112
6. 45 years W-M	Sinequan, suicide attempt		Unable to identify		+ U(14.2)	—	Serum sample not available
7. 59 years W-M	Librium, Sinequan and Valium	Depression, dizziness, slow in speech and thought	+	—	+ G(97.2) U(10.3)	—	Dox. 182
8.	Sinequan		+	—	+ U(5.2)	—	Dox. Serum sample not available
9. 38 years W-F	Sinequan, Omnipen, and Naprosyn		+	—	+ G(1.1)	—	Dox. Serum sample not available

*B = Black; w = white; f = female; m = male.
**G = Gastric sample; U = urine sample. Concentration in parentheses in mg/l; + = positive; — = negative.
***Values using peak area ratios for calculation. Single determinations.

TABLE II

RETENTION TIMES OF TRICYCLICS RELATIVE TO AMITRIPTYLINE ON 3% OV-17

Conditions: isothermal at 235°or programmed as in procedure.

Substance	Relative retention times			
	Ref. 9	Ref. 12	Ref.21	This work
Amitriptyline	1.00	1.00	1.00	1.00
Nortriptyline	1.18	1.12	1.16	1.14
Protriptyline	1.38	—	1.35	1.33
Imipramine	—	1.07	—	0.94
Desimipramine	—	1.24	—	1.14
Doxepin	—	1.13	—	1.17
Desmethyldoxepin	—	1.27	—	1.37
Loxapine	—	—		2.71*
SKF 525-A	—	—		1.32
Trihexyphenidyl	—	—		1.17

*Programmed to 280°.

Gas–liquid chromatographic analysis was performed on the serum to identify the drugs qualitatively and to quantitate them. Fig. 4 shows the chromatograms of two patients, one with a serum concentration of 192 μg/l of doxepin, and one with a serum concentration of 192 μg/l of loxapine. Good agreement was observed in the use of area or peak height ratios for calculating results. The within-run precision (C.V.) for n = 5, using a 200 μg/l serum control and using peak height, averaged 6% for doxepin and 5% for loxapine with a 97 ± 12% and 108 ± 10% recovery for doxepin and loxapine, respectively. The within-run precision at 1 mg/l averaged 3% and 5% for doxepin and loxapine, respectively.

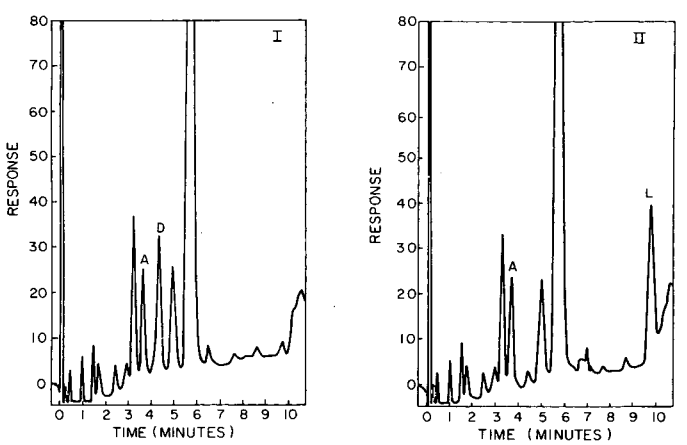

Fig. 4. (I) Gas chromatogram of a patient with a serum concentration of 192 μg/l of doxepin. Attenuation 1 × 32. A = Amitriptyline (100 μg/l), D = doxepin. (II) Gas chromatogram of a patient with a serum concentration of 192 μg/l of loxapine. Attenuation 1 × 32 for amitriptyline (A, 100 μg/l) and 1 × 64 for loxapine (L).

Serum levels quantitated by this method correlated well with the physical and clinical state of the patient, the higher the level the more obtunded and comatose the patient.

Gas chromatography—mass spectrometry

GC—MS was performed on the serum extracts to identify and confirm the presence of amitriptyline, doxepin, loxapine, and other possible substances present. A peak at *m/e* 58 with the same retention time as amitriptyline and doxepin confirmed the presence of these two compounds. The mass spectrum of loxapine is given in Fig. 5. Major peaks at *m/e* 83.2, 70.1, 257.1 and 193.0 were observed at the same retention times as loxapine which are indicative of the spectrum of loxapine (Fig. 5). During the mass spectral analysis, the presence of possible impurities present in the serum extracts was investigated. A number of impurities were looked for including tris(2-butoxyethyl) phosphate (B-D vacutainer plasticizer), and other plasticizers. The presence of a number of phthalates (*m/e* 149) at different retention times was observed, indicating contamination by various phthalate plasticizers.

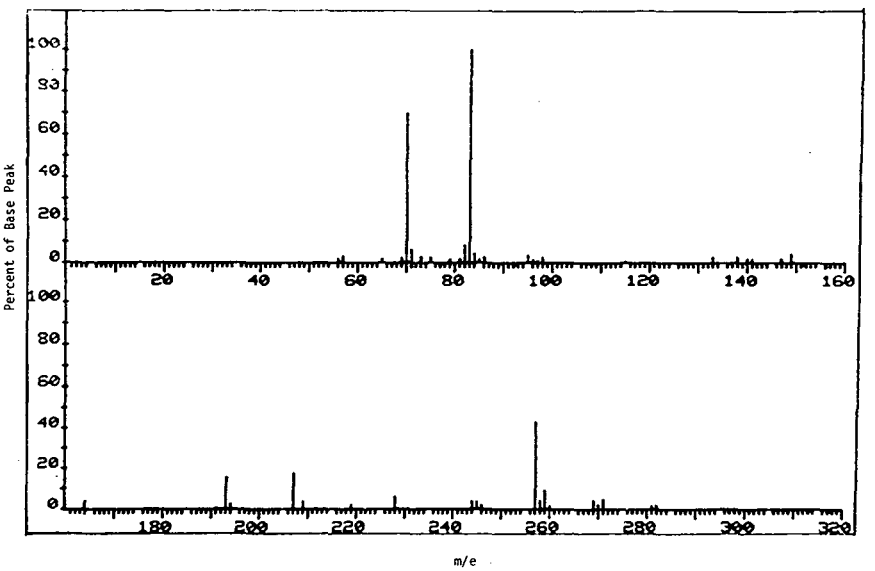

Fig. 5. GC—MS of loxapine using EI mode. Major peaks at *m/e* 83.2, 70.1, 257.1 and 193.0.

GC and GC—MS were also used for the identification and confirmation of loxapine and other tricyclics which were suspected in emergency room overdoses. Fig. 6 is the gas chromatogram of a urine extract of a patient seen in the emergency room who was suspected of having taken amitriptyline and loxapine. Fig. 7 gives the GC—MS total ion chromatogram of the same extract using *m/e* 70, 83, and 257 to monitor the presence of loxapine. The presence of amitriptyline was also confirmed by GC—MS by looking at the *m/e* 58 peak.

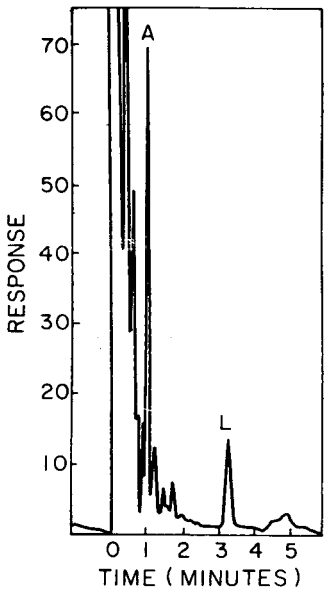

Fig. 6. Gas chromatogram of urine extract of a patient suspected of amitriptyline and loxapine misuse. Conditions: 280° isothermal. Attenuation 1 × 16. A = Amitriptyline, L = loxapine.

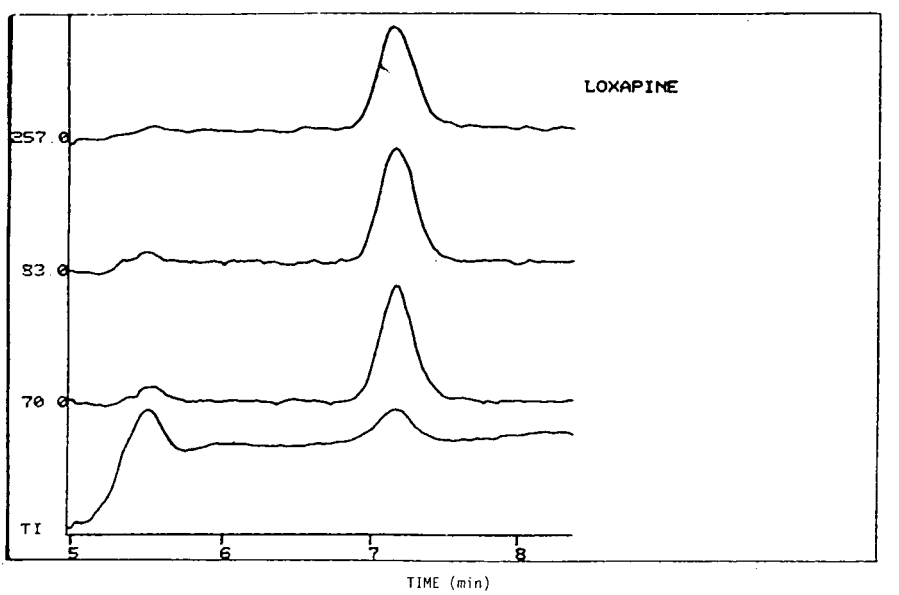

Fig. 7. Total ionization chromatogram of urine extract of patient (Fig. 6), using masses 70, 83, and 257 to monitor the presence of loxapine.

Solvent effects

The use of chloroform instead of ether in back-extracting the drugs from the 0.1 *N* HCl acidic solution and the use of ethanol instead of chloroform—methanol for redissolving the residue prior to GC were investigated. Serum blanks as well as sera containing 200 μg/l of doxepin and loxapine were extracted in the four different ways and chromatographed. Ether and ethanol were the preferred solvents to use for back-extracting and dissolving the final residue.

Internal standard

The use of different internal standards, SKF 525-A, trihexyphenidyl (Artane) and protriptyline, was investigated. Trihexyphenidyl could not be used since it has a retention time similar to that of doxepin under the conditions used in this study. SKF 525-A and protriptyline would interfere with the doxepin metabolite desmethyldoxepin which has a retention time similar to that of SKF 525-A and protriptyline [12]. Table II gives the GC retention times of all the tricyclics relative to amitriptyline and can be used to indicate the possible combinations of tricyclics that can be determined using the present procedure.

The effect of sample volume injected into the gas chromatograph on peak height ratio was also investigated. A definite decrease of the peak height ratio was observed with increased sample used for GC analysis. Doxepin with amitriptyline as the internal standard (n = 4) gave a 13% decrease in peak height ratio in going from 1 to 3 μl of sample injected onto the column. This is in agreement with the recently reported findings of decreased peak area ratios, in spite of the presence of an internal standard, with increasing sample volume in GC analysis [18]. Although a definite decrease in peak area ratio was reported between the ratio of analyte and internal standard with increasing sample

TABLE III

THIN-LAYER CHROMATOGRAPHIC DATA OF TRICYCLICS

Developing solvent: ethyl acetate—absolute ethanol—*n*-butanol—concentrated ammonium hydroxide (56:28:4:0.8). Positive acidic iodoplatinate reaction. R_F × 100 of amitriptyline = 62.

Drug	R_F relative to amitriptyline
Amitriptyline	1.0
10-Hydroxyamitriptyline	0.83
Nortriptyline	0.61
10-Hydroxynortriptyline	0.35
Protriptyline	0.44
Imipramine	0.98
Desimipramine	0.50
Doxepin	1.04
Desmethyldoxepin	0.35
Loxitane	1.10

volume, the authors [18] did not investigate the same effect on peak height ratio.

DISCUSSION

In a limited study of aged patients given 50—300 mg of doxepin in a single daily dose and plasma analyzed 10—12 h after the bedtime dose, levels of doxepin and desmethyldoxepin of 9—131 μg/l were obtained. Therapeutic response was observed in patients with minimum plasma levels of 110 μg/l [5]. In another study, patients given 75—200 mg over a 3—37-week period attained plasma levels of doxepin and desmethyldoxepin of 57—252 μg/l. Desmethyldoxepin was not detected in 5 out of 7 of these patient's blood samples [11]. Fatal cases of doxepin overdose have been reported with blood levels of doxepin ranging from 0.7 to 29 mg/l [10, 19].

Very little information is available in the literature regarding serum levels of loxapine for optimum therapeutic effect [8]. However, this drug is being seen more and more in emergency room patients, and laboratories should be aware of its presence and be able to identify and determine its concentration.

Loxapine succinate has absorption maxima in 2 M H_2SO_4 at 253 nm (ϵ = 1.25×10^4) and 293 nm (ϵ = 1.08×10^4) with a slight shift of the absorption maxima to 250 and 296 nm in basic solution.

Using TLC, loxapine has an R_F value of 0.69 while doxepin has an R_F of 0.65 (Table III). The R_F values of the various tricyclics and their metabolites using TLC under the present conditions of study are given in Table III. Doxepin and loxapine are not well separated by TLC. However, using GC with a 3% OV-17 column, both drugs are very well separated for identification, quantitation, and confirmation by GC—MS in serum, urine and gastric contents (Figs. 2 and 4). Fig. 6 gives the gas chromatogram of a patient's urine analyzed for amitriptyline and loxapine isothermally at 280°. If only loxapine is to be determined, the analysis can be carried out at 280° using prazepam as internal standard. Prazepam has a retention time of 1.33 relative to loxapine under the present conditions of study and can readily be determined at a concentration of less than 1 μg/ml. At 280°, diazepam (Valium) has a retention time relative to loxapine (6.0 min) of 0.78 while chlordiazepoxide (Librium) has a relative retention time of 1.10. At 280° chlordiazepoxide is not well separated from loxapine and would be expected to interfere. However, both drugs can be analyzed by using a column temperature of 260°. The present procedure can be modified to determine diazepam and other benzodiazepines, which are commonly misused with tricyclics.

With the present procedure, serum concentrations of the parent drug can be determined over a wide range with a precision of 5—10% with close to 100% relative recovery using serum standards. The five patients who ingested doxepin had levels of 113—439 μg/l, which indicates that although some patients were toxic, none of the patients had concentrations in the lethal range of 10 mg/l [10, 19]. In some of the cases other drugs or alcohol were present, making the effect of doxepin or loxapine difficult to interpret in view of the multiple drug ingestion. Only doxepin was determined in the present cases, since the active metabolite desmethyldoxepin was not detected in our initial screen-

ing. An interference peak with the same retention time as desmethyldoxepin (Fig. 2) was also observed in some extractions, making the use of this procedure for the quantitative determination of desmethyldoxepin impossible at low concentrations. The desmethyl metabolite has a longer retention time than doxepin (Table II) and would be seen at high concentrations under the present conditions of study. A number of procedures, which have recently been reported, can be used for the direct determination of desmethyldoxepin [11, 12].

Of seven patients on long-term doxepin therapy only two had a measurable concentration of desmethyldoxepin [11]. In four fatal cases of doxepin poisoning of patients who were known to be on doxepin, the concentration of doxepin ranged from 0.7 to 29 mg/l with the desmethyldoxepin being 0.1–6.2 mg/l [19]. The desmethyl metabolite accounted for only 2—22% of the total drug found in blood. In a study with tricyclic overdose patients, no correlation was found between the state of unconsciousness and total tricyclic concentration present [16]. However, if one calculates the percentage of parent drug present, the cases with a higher percentage of the unmetabolized drug appear to have a higher degree of unconsciousness.

Desmethyldoxepin's pharmacologic activity differs from that of the parent drug, the half-lives of doxepin and desmethyldoxepin being 16.8 and 51.3 h, respectively [20]. The present procedure can be used to study acute drug ingestions where metabolites have not accumulated to a large extent. For therapeutic monitoring of doxepin and loxapine, the present procedure could not be used to monitor the presence of metabolites. However, the determination of the parent drug alone may be of clinical usefulness.

GC—MS was used to confirm the presence of doxepin and loxapine in serum, gastric contents or urine, and to look at some of the possible interferences present. Looking at some of the extractions using this procedure (Figs. 2 and 4) by GC—MS, it became obvious that a contamination problem had occurred. After looking at these contaminants by GC—MS, it was realized that this was a phthalate contaminant as evidenced by the mass at *m/e* 149. After making new standards and changing solvents, many of the phthalate impurities disappeared. Although the phthalates did not interfere with the present analysis, possible interference could result from the use of this procedure for other tricyclics, especially in the early part of the chromatogram. GC—MS analysis of the various serum extracts indicated that substances were still present in the sera with an *m/e* of 149 mass units even after careful decontamination precautions. These residual peaks are due to phthalate plasticizers such as dioctylphthalate, dibutylphthalate and butylbutoxyphthalate. The large interfering peak after doxepin appears to be dioctylphthalate and does not appear to be tris(2-butoxyethyl) phosphate. To prove this, a red stoppered B-D vacutainer was placed in methanol and the dissolved plasticizers were analyzed by GC—MS. The spectrum of the serum impurity after doxepin (Fig. 4) was not consistent with the spectrum of tris(2-butoxyethyl) phosphate, but is similar to that of dioctylphthalate [21—23]. The stopper extract showed a number of plasticizers containing phthalates (*m/e* 149) including the tris(2-butoxyethyl) phosphate impurity (*m/e* 57, 85.1, 125, 100, 101) which did not appear in high concentration in the serum extracts.

Studies using chloroform or ether for back-extracting the drugs from the HCl aqueous layer indicate that ether is a better solvent to use. Chloroform is a less desirable solvent to use since it extracts a higher concentration of interfering serum components which are not seen in ether extracts. The use of ethanol for redissolving the evaporated extract prior to analysis is also preferred over chloroform because of the interference by chloride with the rubidium bead. Using chloroform, an abnormal chromatogram is observed due to the binding of chloride with the rubidium, resulting in a non-equilibrium state of rubidium atoms around the bead [17]. Using ethanol or some nonhalogenated solvent seems to correct the whole problem of baseline shifts.

A column packed with 3% SE-52 for analysis was also investigated. Although the column was appropriate for single drug analysis, it could not separate amitriptyline from doxepin and could not, therefore, be used for this analysis.

The use of different internal standards confirmed our initial belief, and that of others [12], that amitriptyline should be used as the internal standard. Although the possibility exists that amitriptyline ingestion may interfere with the amitriptyline internal standard used, it seems unlikely since in most cases it will be identified during the initial screen. If amitriptyline is suspected, then another internal standard (such as prazepam) will have to be used, or the analysis may have to be run without an internal standard, being especially careful that the injected volume is kept constant. Peak areas would be used for quantitation.

Using an internal standard to correct for sampling errors due to volume changes has recently been reported to be inaccurate [18]. Thus, one must maintain a constant volume of injection and a constant concentration of internal standard for accurate results. In addition, the concentration of the standard must be close to the concentration of the unknown. Using an internal standard and calculating peak height ratio, we also confirmed the above observation that a decrease in peak height ratio of standard to internal standard occurs with an increase in volume injected. We observed this decrease in peak height ratio, using a nitrogen-selective detector. In the previous study [18], a thermal conductivity detector and a flame ionization detector were used. Thus, for accurate results the volume injected must be kept constant, and the concentration of standard used must be close to the unknown concentration. Using peak areas with an internal standard, an error of 15% was observed in going from 1 to 2 μl of sample injected. We observed an error of 13% using peak height ratios upon changing the volume by 1 μl, which is in agreement with the peak area studies [18].

The procedure presented is a sensitive and selective direct method for the determination of doxepin and loxapine in serum. For best results, a constant volume of extract must be injected, and the use of nonchlorinated solvents is recommended for greater baseline stability using a nitrogen-sensitive detector. Low levels can be quantitated with the lower limit of detection of doxepin and loxapine being 3 and 2 μg/l, respectively. No derivatization is necessary, only a simple extraction of the pure drug. In addition, doxepin, loxapine and amitriptyline are eluted from the column after a steady baseline has been obtained. No solvent front of significant length is observed. Quantitation of drug levels can be easily accomplished by using peak height ratios instead of

peak area ratios. Other drugs in biological materials may be determined by this procedure or by a minor modification of it.

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MEASUREMENT OF ADRIAMYCIN (DOXORUBICIN) AND ITS METABOLITES IN HUMAN PLASMA USING REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND FLUORESCENCE DETECTION

RICHARD N. PIERCE and PETER I. JATLOW

Department of Laboratory Medicine, Yale University School of Medicine, New Haven, Conn. 06510 (U.S.A.)

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SUMMARY

We describe a method for measuring adriamycin and its major metabolite, adriamycinol, in plasma, using reversed-phase high-performance liquid chromatography and fluorescence detection. The lower limit of detection is approximately 1 ng/ml for both compounds; within-day coefficients of variation are 3.6% and 4.4% for adriamycin and adriamycinol, respectively. A slight modification of this procedure also allows measurement of aglycone metabolites.

INTRODUCTION

Adriamycin (Ad, doxorubicin) is an anthracycline antibiotic which has been used in the treatment of a variety of neoplastic diseases in man [1, 2]. In the study of the pharmacokinetics of this drug, several methods for measurement of adriamycin and its metabolites in biological samples have been used, including total extractable plasma fluorescence [3, 4], thin-layer chromatography (TLC) followed by elution and fluorescent measurement [5], TLC with quantitative fluorescent scanning of the thin-layer plate [6], and radioimmunoassay (RIA) [7]. High-performance liquid chromatography (HPLC) with a diphenyl bonded column has been used by Langone et al. [8] as a preparatory step for separating fractions prior to RIA. Israel et al. [9] have applied adsorptive and reversed-phase (diphenyl bonded) chromatography to the measurement of adriamycin and its metabolites in rabbit bile using both ultraviolet and fluorescence detection. Using ultraviolet and visible light detection, Eksborg [10] has studied the separation of adriamycin, daunomycin, and their hydroxyl metabolites under various conditions, but he did not evaluate biological fluids. We

describe a sensitive reversed-phase (ODS column) HPLC procedure, employing fluorescence detection for the separation and quantitation of adriamycin, adriamycinol, and their respective aglycones, in human plasma. The closely related compound daunomycin (Da) and its aglycone derivative (daunomycinone) are used as internal standards.

MATERIALS AND METHODS

Apparatus

We initially used a Perkin-Elmer dual pump high-pressure liquid chromatograph (Model 601) equipped with a Rheodyne injector valve (Model 7120) with a 20-μl loop and a Model 204-S Perkin-Elmer fluorescent detector. For later work a Perkin-Elmer (Series 2) high-performance liquid chromatograph and a Perkin-Elmer Model 650-10LC fluorescent detector were used, with comparable results. The column ultimately used (25 × 0.26 cm) had a C_{18} (5-μm) reversed-phase packing, ODS "Hi-Eff." (Applied Science Labs., State College, Pa., U.S.A.). In preliminary work other columns evaluated were C_{18} (10 μm): Partisil 10/25 ODS and Partisil 10/25 ODS-2 (Whatman, Clifton, N.J., U.S.A.).

Reagents and standards

Adriamycin HCl was a gift of Adria Laboratories (Columbus, Ohio, U.S.A.) and adriamycinol HCl was a gift of Farmitalia (Milan, Italy). Daunomycin HCl was obtained from Sigma (St. Louis, Mo., U.S.A.) and acetonitrile "Distilled in Glass" from Burdick and Jackson Labs. (Muskegon, Mich., U.S.A.). Other reagents and drugs used for interference studies were obtained from usual commercial sources.

Stock solutions of adriamycin, adriamycinol, and daunomycin were made in methanol (concentration 100 μg/ml) and stored at −20°. Aglycones of all of these drugs were made as previously described [2] by mild acid hydrolysis (0.1 *M* HCl at 55° for 45 min) followed by neutralization, extraction of the aglycone into chloroform, evaporation and redissolution of the residue in methanol. Purity was checked by TLC [2] and by HPLC. The concentrations of the aglycone solutions were calculated, based on the molar extinction of daunomycinone [11] and the equivalent molar extinction for the aglycones of adriamycin and adriamycinol [2, 12]. Concentrations for daunomycin and daunomycinone internal standard solutions were 2 μg/ml, in methanol.

Plasma standards were made by supplementing blood bank plasma with stock solutions of each of the drugs to final concentrations of 2—200 ng/ml. Aliquots were protected from light and stored frozen at −20°.

Plasma samples from patients receiving adriamycin were collected in heparin. The plasma was separated and stored in the dark at −20° until assayed within one week. Samples for interference studies were obtained from patients not receiving adriamycin. These were chosen to include samples from patients with renal failure and hepatic disease, as well as samples from patients receiving cancer chemotherapy other than adriamycin, and samples with in vitro hemolysis.

Extraction procedure

Daunomycin, the internal standard (50 μl, 100 ng) was added to each 2.00-

ml sample and mixed in a 15-ml PTFE-lined screw-top glass test-tube. Following alkalinization with 0.1 ml of 0.2 *M* sodium hydroxide the sample was extracted with 10 ml chloroform—isopropanol (2 : 1) for 10 min. After centrifugation (1000 *g* for 10 min) the upper aqueous layer was removed by aspiration to facilitate transfer of the lower organic layer to a standard test tube. After drying with anhydrous sodium sulfate the organic layer was transferred to a tapered 15-ml centrifuge tube and evaporated in a water-bath at 40° under an air stream. During evaporation the sides of the tube were washed with chloroform or chloroform—methanol (1 : 1) in order to increase recovery. The residue was dissolved in 50—100 μl of methanol, and 10—20 μl were submitted to chromatography.

The extraction procedure for the aglycones was identical except that the internal standard used was 50 μl (100 ng) of daunomycinone.

Chromatographic procedure

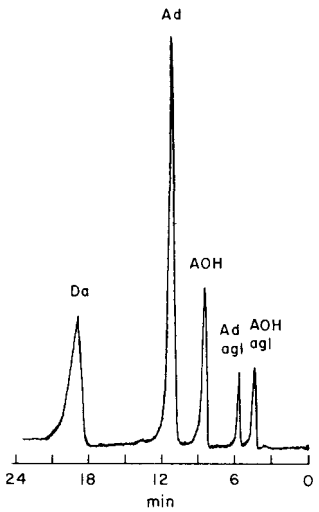
The mobile phase consisted of 50% acetonitrile (by volume) in 50% 0.01 *M* phosphoric acid (pH 2.3). The flow-rate was 1 ml/min, and column temperature was maintained at 25°. The fluorometer was set at 465 nm excitation wavelength (slit width 10 nm) and 580 nm emission (slit width 20 nm). Quantitation was based on peak height ratios.

For measurement of aglycones, the percentage of acetonitrile in the mobile phase was decreased to 36—40%; otherwise, the procedure was identical.

To exclude drug interferences, methanolic solutions of drugs were injected in quantities greater than would be expected in 2 ml of plasma.

RESULTS

Standard curves based on peak height ratios for adriamycin and adriamycinol were both linear (r = 0.99) in the ranges 0—100 ng/ml and 0—50 ng/ml, respectively. The analysis of ten samples, each containing 10 ng/ml adriamycin and 5 ng/ml adriamycinol, showed within-day coefficients of variation of 3.6% and 4.4%, respectively. Retention times were: daunomycin, 18 min (k = 4.7); adriamycin, 11.4 min (k = 2.44); adriamycinol, 8.7 min (k = 1.45); adriamycinone, 5.4 min (k = 0.72); adriamycinol aglycone, 4.2 min (k = 0.36) (see Fig. 1). Extraction of many plasma and serum blank samples resulted in peaks with retention times of 2.7—5.2 min (Fig. 2); therefore, to obtain better resolution of early eluting peaks, the procedure was modified for aglycones by reducing the proportion of acetonitrile to 36—40%. Using 40% acetonitrile, retention times were: daunomycinone, 19 min (k = 4.8); adriamycinone, 9 min (k = 1.6); adriamycinol aglycone, 6.4 min (k = 0.67); and all peaks in drug-free plasma eluted between 3 and 6 min (Fig. 3). Using these modified conditions and daunomycinone as an internal standard, linear standard curves were obtained for adriamycinone (range 0—20 ng/ml, r = 0.99) and adriamycinol aglycone (range 0—40 ng/ml, r = 0.99). Analysis of ten replicate samples revealed coefficients of variation of 5.1% (adriamycinone) and 4.0% (adriamycinol aglycone). Recoveries for the parent drug, the metabolites, and the two internal standards were in the range 65—75%. These were corrected by the use of plasma standards and the use of an internal standard carried through the entire extraction



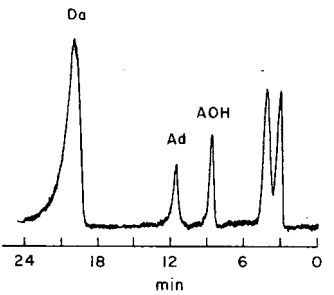


Fig. 1. Mixture of 5 drugs: daunomycin (Da), adriamycin (Ad), adriamycinol (AOH), adriamycinone (Ad agl), and adriamycinol aglycone (AOH agl). Chromatographic conditions: 50% acetonitrile, 50% 0.01 *M* phosphoric acid, flow-rate 1 ml/min, temperature 25°. Fluorescence detection: excitation wavelength 465 nm, emission 580 nm.

Fig. 2. Plasma sample supplemented with 5 ng/ml adriamycin (Ad) and 5 ng/ml adriamycinol (AOH); internal standard 50 ng/ml daunomycin (Da). Chromatographic conditions as in Fig. 1. Unidentified peaks (3—5 min) were present in chromatograms from all plasma or serum samples tested.

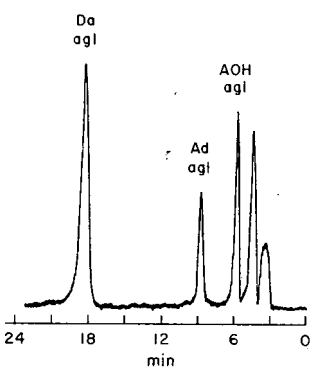


Fig. 3. Plasma sample supplemented with 7.8 ng/ml adriamycinone (Ad agl) and 7.6 ng/ml adriamycinol aglycone (AOH agl); internal standard 50 ng/ml daunomycinone (Da agl). Chromatographic conditions: 40% acetonitrile, 60% 0.01 *M* phosphoric acid, flow-rate 1 ml/min, temperature 25°.

procedure. Careful temperature control during the solvent evaporation step of the extraction procedure was critical; if the temperature exceeded 45° these drugs, including adriamycin, its metabolites, and both internal standards, might decompose.

Analysis of forty commonly used drugs, including naturally fluorescent compounds, analgesics, tranquilizers, and sedatives, revealed no interfering peaks

(see Table I). However, methyldopa and oxycodone eluted at 6 min (using 50% acetonitrile), which was slightly later than adriamycinone, 5.4 min.

TABLE I

DRUGS TESTED FOR INTERFERENCE WITH MEASUREMENT OF ADRIAMYCIN AND ITS METABOLITES

Acetaminophen	Meperidine, normeperidine
Allylisobutylbarbital	Methadone
Amitriptyline	Methaqualone
Amobarbital	Methyldopa
Aspirin	Morphine
Caffeine	Nitroprusside
Chlordiazepoxide	Nortriptyline
Chlorpromazine	Pentazocine
Codeine	Pentobarbital
Desipramine	Phenobarbital
Diazepam, nordiazepam	Procainamide, N-acetylprocainamide
Diphenhydramine	Propoxyphene
Dopamine	Propranolol
Furosemide	Protriptyline
Hydroxyzine	Quinidine
Imipramine	Secobarbital
	Theophylline

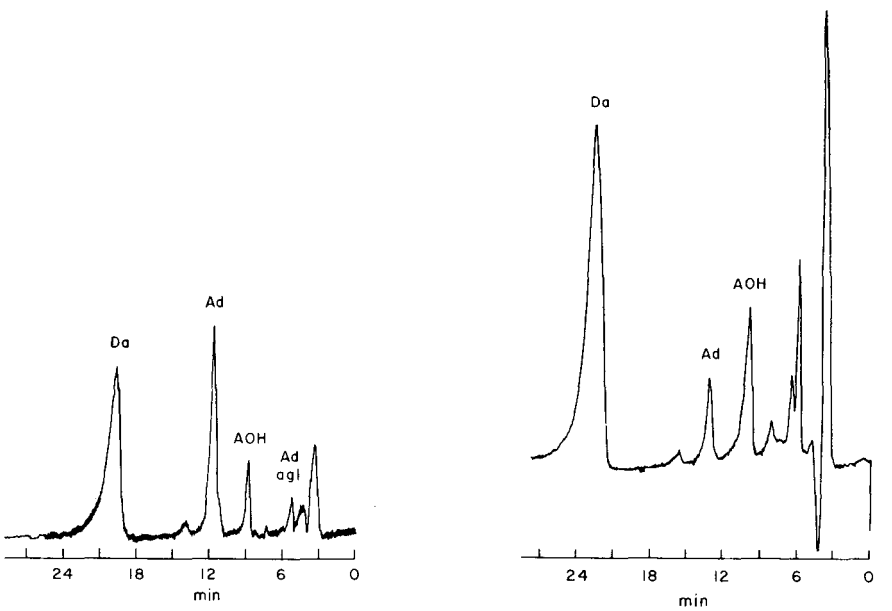


Fig. 4. Sample from patient who received 45 mg adriamycin 2 h previously. Chromatographic conditions as in Fig. 1. Peaks represent: Ad, adriamycin (25 ng/ml); AOH, adriamycinol (5.5 ng/ml); Ad agl, adriamycinone (1 ng/ml). Internal standard is Da, daunomycin (50 ng/ml).

Fig. 5. Sample from patient who received 30 mg adriamycin 15 h previously. Chromatographic conditions as in Fig. 1. Peaks represent: Ad, adriamycin (4.0 ng/ml); AOH, adriamycinol (4.8 ng/ml). Peak corresponding to adriamycinone coelutes with plasma peaks. Internal standard is Da, daunomycin (50 ng/ml).

Samples from patients not receiving adriamycin were analyzed by the same procedure and showed no interferences for adriamycin, adriamycinol, daunomycin, or daunomycinone. When analyzed by the modified procedure for aglycones, samples from two patients (both patients receiving multiple drugs including methyldopa) resulted in several minute peaks coeluting with adriamycinone and adriamycinol aglycone; these peaks corresponded to less than 2 ng/ml of either aglycone equivalent.

A chromatogram from a patient receiving adriamycin is illustrated in Fig. 4. The major peaks correspond to adriamycin, adriamycinol, and adriamycinone. Fluorescent scanning of each of these three peaks was performed on a subsequent sample by stopped flow technique. The resultant activation and emission scans were all identical with those obtained from the authentic compounds under the same conditions. Figs. 5 and 6 show chromatograms from the plasma of a second patient who had received 30 mg of adriamycin 15 h previously. Using 50% acetonitrile (Fig. 5), peaks correspond to 4.0 ng/ml adriamycin and 4.8 ng/ml adriamycinol; but peaks which would correspond to aglycones are not resolved from unidentified plasma components. Reduction of the acetonitrile concentration to 40% (Fig. 6) improves the resolution of these early peaks, with adriamycinone 7 ng/ml, and adriamycinol aglycone less than the limit of detection for this assay (<1 ng/ml). Fig. 7 shows a chromatogram from

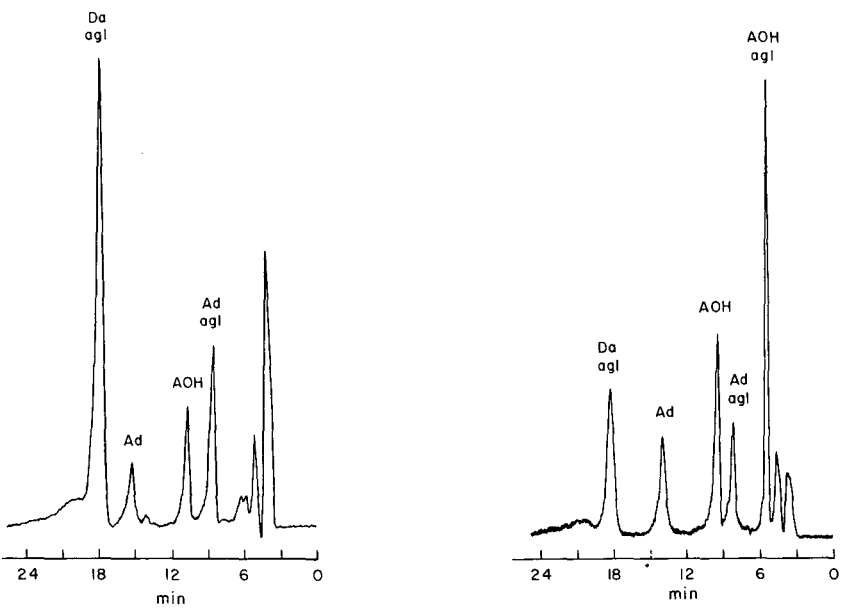


Fig. 6. Sample from the same patient as in Fig. 5. Chromatographic conditions modified; 40% acetonitrile, 60% 0.01 *M* phosphoric acid, flow-rate 1 ml/min, temperature 25°. Peaks: Ad, adriamycin; AOH, adriamycinol; and Ad agl, adriamycinone (7 ng/ml). Internal standard is Da agl, daunomycinone (50 ng/ml).

Fig. 7. Sample from patient who received 96 mg of adriamycin 17 h previously. Chromatographic conditions as in Fig. 3 (modified method). Major peaks: Ad, adriamycin (20 ng/ml); AOH, adriamycinol (21 ng/ml); Ad agl, adriamycinone (12 ng/ml) and AOH agl, adriamycinol aglycone (29 ng/ml). Internal standard is Da agl, daunomycinone (50 ng/ml).

another subject who had received 96 mg of adriamycin 17 h previously. In this instance adriamycin and relatively high concentrations of adriamycinol and aglycone metabolites are present. Since this chromatogram was run under modified conditions (40% acetonitrile) daunomycinone is the internal standard; daunomycin is not present.

DISCUSSION

Pharmacokinetics of adriamycin and its metabolites have been studied in man [4, 5, 13, 14], rabbits [15], mice and rats [9, 16], monkeys [7], as well as in enzyme systems [17]. The major metabolite in humans and in other species studied is adriamycinol, though eight metabolites in the urine [13] and eleven metabolites in bile [18] have been reported. Since both adriamycin and adriamycinol have cytotoxic effects [19], it would be desirable to measure both of these compounds. Assays based on total extractable fluorescence measure the parent drug and fluorescent metabolites without discrimination. Additionally, it is reported that total plasma extractable fluorescence may be elevated due to the presence of certain steroids or bile acids [14] causing interference, particularly at low drug levels. For the separate quantitation of metabolites the fluorescent assays have been used in conjunction with chromatographic separation, most commonly using TLC. RIA measures adriamycin and adriamycinol equally, and cross reacts with the aglycones to a lesser degree [7]. The use of HPLC for separation, followed by RIA for quantitation, has been described [8].

Eksborg [10] has studied systematically the use of reversed-phase HPLC for separation of adriamycin and daunomycin, and their hydroxyl metabolites, with ultraviolet or visible light absorbance spectrophotometric detection. However, because fluorescence detection has the potential for both increased sensitivity and specificity, we have selected this mode. Our results indicate sensitivity to drug levels less than 2 ng/ml, and interferences from common drugs tested are negligible. The sensitivity and linearity range are appropriate for measurement of the levels of adriamycin and adriamycinol expected following the usual therapeutic dose of this drug.

In preliminary work several hydrocarbon reversed-phase columns were tried. Columns with 10-μm particle size packing did not provide sufficient separation and yielded broad peaks. The use of 5-μm packing resulted in substantial improvement in resolution and peak configuration.

For measurement of adriamycin and adriamycinol, daunomycin was used as an internal standard; it has both structural similarity and an identical fluorescent spectrum with these two drugs. For the smaller but less water soluble aglycones, daunomycinone was used as an internal standard. These two internal standards were not compatible with use in a single extraction. Using chromatographic conditions optimized for adriamycin (50% acetonitrile), daunomycinone would coelute with adriamycinol; conversely, if conditions were optimized for measurement of the aglycones (36—40% acetonitrile), retention of daunomycin was 30 min.

We evaluated this procedure on several patients who had received adriamycin at various times before blood sampling. Adriamycin, adriamycinol, and ap-

parent levels of adriamycinone were found in all patients. The third patient (Fig. 7), from whom the sample was obtained 17 h after medication, showed peaks corresponding to 12 ng/ml of adriamycinone and 29 ng/ml of adriamycinol aglycone, in addition to the parent compounds. All these peaks showed fluorescent emission and activation scans identical to the authentic material. We cannot of course verify that in vitro degradation did not contribute to the presence of these aglycones. However, supplemented plasma standards showed no detectable in vitro degradation under identical conditions; although, if kept at room temperature for 36 h, adriamycin and adriamycinol showed degradation corresponding to 34% and 11%, respectively. Although very little of the unconjugated aglycones has been thought to occur in plasma [4, 6], Riggs et al. [18] and Watson and Chan [6] have detected the presence of aglycone metabolites in human plasma using TLC.

ACKNOWLEDGEMENT

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REVERSED-PHASE LIQUID CHROMATOGRAPHIC DETERMINATION OF PLASMA LEVELS OF ADRIAMYCIN AND ADRIAMYCINOL

STAFFAN EKSBORG*, HANS EHRSSON and INGRID ANDERSSON

Karolinska Pharmacy, Fack, S-104 01 Stockholm 60 (Sweden)

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SUMMARY

A method is given for the determination of adriamycin and its main metabolite, adriamycinol in plasma from cancer patients after administration of adriamycin as the free drug or as a complex with DNA.

Adriamycin and adriamycinol are extracted in a column from 1 ml of plasma (pH 8.6) using a mixture of chloroform—1-heptanol (8:2). After re-extraction into phosphate buffer pH 2.2, the separation is performed as reversed-phase liquid chromatography on a LiChrosorb RP-2 (5 μm) column with a mobile phase of acetonitrile—water, acidified with phosphoric acid.

The precision by quantitation with photometric detection was better than 5% within the range 50—300 ng/ml. Plasma levels of adriamycin and adriamycinol in a cancer patient are presented in this paper.

INTRODUCTION

Adriamycin (Fig. 1) is one of the most promising new antineoplastic drugs [1,2]. The therapy with adriamycin, like with other anthraquinone glycosides, is restricted by drug-induced myocardial infarction [3,4]. The cumulative dose of adriamycin should not exceed 550 mg/m^2 [3]. Administration of adriamycin as a complex with a macromolecular carrier, herring sperm DNA, has recently been used to overcome the serious heart toxicity of the drug [5—7].

Pharmacokinetic studies of adriamycin in man after administration as free drug or as DNA complex require a reliable method for determination of plasma levels. Such a method should permit simultaneous determination of adriamycinol (Fig. 1), a metabolite of adriamycin, with similar cytotoxic activity as the intact drug [8,9]. The pharmacokinetics of adriamycin has previously been

*To whom correspondence should be addressed.

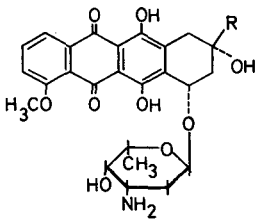


Fig. 1. Structural formulae. Adriamycin, R = $COCH_2OH$; adriamycinol, R = $CH(OH)CH_2OH$.

studied with unselective methods such as the radioimmunoassay (RIA) technique and measurements of total fluorescence of plasma extracts [10—12]. Analytical methods including a chromatographic step offer a higher selectivity, and have been used recently [13—16].

The present paper gives an analytical method for simultaneous determination of adriamycin and adriamycinol in plasma after administration of adriamycin as the free drug or as the DNA complex. The proposed analytical method comprises of column extraction from the buffered plasma sample into an organic phase, re-extraction into a small acidic aqueous phase and separation by reversed-phase liquid chromatography. A high detection sensitivity as well as a high detection selectivity of the method is obtained by the use of a photometric or a fluorimetric chromatographic detector. The construction of the method has been based upon previously reported studies of the extraction properties and reversed-phase liquid chromatographic separation of antraquinone glycosides [17,18], cf. ref. 19.

EXPERIMENTAL

Apparatus

The spectrofluorimeter used was an Aminco-Bowman 4-8202 B, and the pH meter an Orion Research Model 701/digital pH meter equipped with an Ingold combined electrode type 401.

Glass equipment. All glass equipment, with the exception of micro-pipettes, were silanized before use by treatment with dichlorodimethylsilane (5% by volume) in toluene, followed by washing with dry methanol.

Chromatographic system. The detectors were a LDC Spectromonitor II (500 nm, 10-mm path length, 8-μl volume) and a Schoeffel Instrument FS 970 Fluorimetric Detector (435/550 nm; 5-μl cell volume). A LDC 711 Solvent Delivery System pump was used. The columns were stainless steel (length 150 mm, 4 mm I.D., 1/4 in. O.D. Column end fittings were modified Swagelok connectors. A Rheodyne (Model 70-10) injection valve with a sample loop of 300 μl was used. The support employed was LiChrosorb RP-2 (Merck, Darmstadt, G.F.R.) with a mean particle diameter of 5 μm. The chromatographic system was thermostated to 25.0 ± 0.1°.

Chemicals

Adriamycin and adriamycinol were kindly supplied by Farmitalia (Milan, Italy), and desipramine chloride by A.B. Hässle (Mölndal, Sweden). The mobile

phases were prepared from acetonitrile (Merck, Uvasol), phosphoric acid (Merck, p.a.) and distilled water. All other chemicals were of analytical grade and used without further purification.

Chromatographic technique

The columns were packed by the balanced density slurry technique previously described [20] with tetrachloroethylene as suspending medium. The columns were washed with *n*-hexane and acetone (100 ml of each) before use. The mobile phase was passed through the chromatographic system until constant retention of the solutes was obtained. Less than 50 ml was usually required.

Incubation of whole blood samples

To 4-ml whole blood samples (heparinized) were added 100 μl of 10^{-2} *M* phosphoric acid containing adriamycin (40 μg/ml). After addition of 100 μl of 10^{-2} *M* NaOH the samples were incubated at 25° for between 5 min and 22 h. At the end of the incubation time the samples were centrifuged at 4080 *g* for 10 min. The plasma fractions were analysed for adriamycin and adriamycinol according to the proposed analytical method.

Plasma samples from cancer patients

Blood samples (5—7 ml) were collected in 10-ml glass test-tubes (Vacutainer) containing 250 I.U. heparin (freeze dried) immediately before and at appropriate times after the commencement of drug administration. The samples were immediately centrifuged at 4080 *g* for 10 min. The plasma fraction was carefully aspirated and frozen at —20° until assay.

Spiking of plasma samples

The precision and accuracy of the proposed analytical method were tested by analysis of blank plasma samples, spiked with adriamycin and adriamycinol [19].

Analytical method

Extraction procedure. The plasma sample (1.0 ml) is carefully mixed with 0.10 ml phosphate buffer pH 8.6, μ = 1.0 and 1.5 g of diatomaceous earth (acid washed Celite 545), and quantitatively transferred into an empty extraction column (6 mm I.D.). The column is eluted with chloroform—1-heptanol (8:2, v/v), the first 7 ml being collected in a centrifuge tube. The organic phase is extracted for 10 min with 0.300 ml phosphate buffer, pH 2.2, μ = 0.1, containing 10 μg/ml of desipramine. The aqueous (upper) phase from the extraction procedure is transferred into a centrifuge tube with tapered bottom (0.2 ml) containing 2 ml of hexane and centrifuged. (This step is included to facilitate the transfer of the aqueous phase into the chromatographic column without contamination with organic phase.)

Liquid chromatographic isolation and quantitation. Part of the aqueous (lower) phase (0.050—0.300 ml) is injected into the chromatographic column (support: LiChrosorb RP-2, 5 μm mean particle diameter; mobile phase: acetonitrile—water—0.1 *M* phosphoric acid (20:70:10); mobile phase flow-rate

0.8—1.0 ml/min). The concentration of the solutes in the eluate is measured by photometric (500 nm) or fluorimetric (435/550 nm) detection. Quantitation is based on peak area measurements.

RESULTS AND DISCUSSION

Metabolic activity in whole blood samples

Adriamycin has been reported not to be convertable into adriamycinol by human blood cells [21]. No traces of adriamycinol were found in plasma after incubation of whole blood samples with adriamycin for 0—2 h (Table I). Low amounts of adriamycinol were, however, found after incubation for 4—22 h, but the conversion of adriamycin to adriamycinol was found to be considerably slower than the corresponding formation of daunorubicinol from daunorubicin, a cytostatic drug with a structure similar to adriamycin [19]. No formation of adriamycinol, when handling the blood samples according to the procedure described above, is likely to occur.

TABLE I

FORMATION OF ADRIAMYCINOL FROM ADRIAMYCIN IN WHOLE BLOOD SAMPLES

Whole blood samples from a healthy volunteer incubated with 1 μg of adriamycin per ml at 25°.

Time (h)	Relative amounts adriamycinol/adriamycin
0	<0.01
1	<0.01
2	<0.01
4	0.04
6	0.08
22	0.24

Extraction procedure

Optimum degree of extraction is obtained at pH 8.4 for adriamycin and 8.6 for adriamycinol [17]. Under these conditions 94% and 62% of the drug and its metabolite, respectively, are extracted into the organic phase using equal phase volumes. Quantitative extraction can be obtained by an increased phase volume ratio. For the quantitative extraction (>99%) of adriamycinol from 1 ml of an aqueous phase, the volume of the organic phase must exceed 60 ml. Such a large volume is very inconvenient in the re-extraction step preceeding the injection of the sample into the liquid chromatograph.

In the present method, column extraction is used for quantitative transfer of adriamycin and adriamycinol from buffered plasma (pH 8.6) into an organic phase [chloroform—1-heptanol (8:2, v/v)]. The drug and the metabolite are completely eluted within the first 7 ml of the organic extractant passing through the column.

The extraction degree of adriamycin and adriamycinol from a buffer solution (pH 8.6, μ = 0.1) were found to be strongly influenced by the presence of herring sperm DNA, most likely as a result of complex formation [19]. A

25-fold excess (by weight) of DNA reduced the extraction degree of adriamycin and adriamycinol (200 ng/ml) by 20 and 35%, respectively, in batch experiments. When using the column extraction technique as in the proposed method, the influence of DNA on the extraction yield is negligible within therapeutic plasma levels of adriamycin and adriamycinol.

Adriamycin and adriamycinol are separated from aglycones, formed as metabolites [8], by re-extraction into a small volume of acidic phase. Conditions for quantitative re-extraction can be calculated from constants given in ref. 17. A phase volume ratio organic phase:aqueous phase of 25 makes it necessary to use an aqueous phase of pH < 2.6 for quantitative transfer of adriamycin and adriamycinol from the organic into the aqueous phase.

To avoid the effect of adsorption phenomena by the extraction, a secondary amine, desipramine (10 μg/ml) was added to the acidic aqueous phase [19], used in the re-extraction step.

The stability of adriamycin and adriamycinol at 25° was studied by liquid chromatography. At pH 2.2 no degradation was observed within 24 h. Storage for 2 h at pH 8.6 gave recoveries of 94 and 97% of adriamycin and adriamycinol, respectively.

Chromatographic isolation

Separation of anthraquinone glycosides by reversed-phase liquid chromatography has previously been studied in respect to selectivity and retention [18]. The highest performance of the chromatographic system was obtained using LiChrosorb RP-2 as the support with a mobile phase containing 20—30% (v/v) of acetonitrile in water, acidified with phosphoric acid. The retention of adriamycin and adriamycinol was strongly affected by the concentration of acetonitrile, Fig. 2 [18]. In the method 20% of acetonitrile is used giving a complete separation of the drug and the metabolite within 10 min.

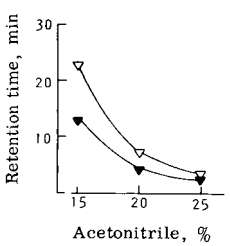


Fig. 2. Retention time and concentration of acetonitrile in mobile phase. Support: LiChrosorb RP-2 (5-μm mean particle diameter). Sample: adriamycin (▽) and adriamycinol (▼), 2.5 nmoles of each in 100 μl of mobile phase. Mobile phase, phosphoric acid (10^{-2} *M*) in acetonitrile—water; mobile phase flow-rate, 1.0 ml/min.

Detection selectivity and sensitivity

Liquid chromatographic determination of adriamycin and adriamycinol is possible with a very high detection selectivity. A high detector response is obtained by photometric detection at 500 nm as well as by fluorimetric detection at 435/550 nm, i.e. under conditions where almost no endogenous compounds

and other drugs may interfere. Highest detection sensitivity was given by the fluorimetric detector. A sample containing 2 ng of each adriamycin and adriamycinol gave a signal-to-noise ratio of 3 under the chromatographic conditions used in the analytical method. Photometric detection at 500 nm gave about 3—5 times lower sensitivity. Besides the high detection selectivity and sensitivity a further advantage of the fluorimetric detector is given by the fact that, compared to photometric detectors considerably larger sample volumes can be injected. Only minor disturbances of the baseline are caused by the non-retarded solvent peak. Band broadening of the solute peaks is minimized by a proper choice of sample solvent [22]. In this method, phosphate buffer pH 2.2 is used by which the solutes are almost completely retarded.

Quantitative determination

Fluorimetric detection (435/550 nm). Evaluation of the amount of adriamycin and adriamycinol in an unknown sample is based on peak area measurement and the use of a calibration graph, obtained by running known amounts of adriamycin and adriamycinol through the chromatographic system. Identical standard curves [Y (mm^2) = 8.58 + 6.51 X (ng); r = 0.9999, n = 20] were obtained for the two compounds within the range 10—300 ng. By increasing the sensitivity setting of the detector it was possible to quantitate as little as 2 ng of each compound.

Photometric detection (500 ml). Quantitation is based on peak area measurement. The amount of sample in the chromatographic peak can be calculated by means of eqn. 1:

$$M = Y \times u \times b \times \epsilon^{-1} \quad (1)$$

where M = amount of sample in mmoles, Y = peak area in mm^2, u = ml/mm chart paper, b = absorbance/mm chart paper and ϵ = molar absorptivity of the migrating compound [22]. The molar absorptivity of adriamycin and adriamycinol were found to be identical (7.89×10^3).

TABLE II

RECOVERY AND PRECISION

Photometric detection at 500 nm.

Plasma level (ng/ml)	Recovery (%)		Added as**
	Adriamycin	Adriamycinol	
50	85.9 ± 2.24*	93.3 ± 1.64	Free drug
100	89.7 ± 4.66	90.4 ± 3.89	Free drug
280	90.5 ± 3.43	93.2 ± 2.04	Free drug
50	95.4 ± 3.83	95.7 ± 4.11	DNA complex
100	95.1 ± 3.00	96.3 ± 3.19	DNA complex
225	99.7 ± 1.07	96.4 ± 2.40	DNA complex

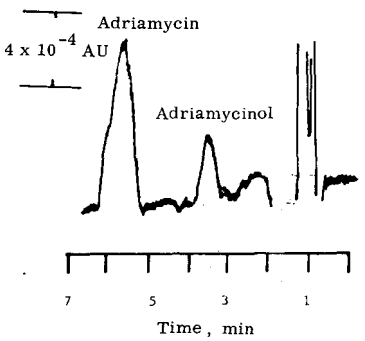
*Relative standard deviation (n=9).
**For details see ref. 19.

Recovery and precision

The recovery and precision of the method at various drug levels are presented in Table II. Adriamycin and adriamycinol can be determined with a precision better than 5% at plasma levels above 50 ng/ml (photometric detection).

Plasma samples from patients

A chromatogram of a plasma sample from a cancer patient treated with adriamycin is shown in Fig. 3. Plasma levels of adriamycin and adriamycinol in a cancer patient treated with adriamycin are shown in Fig. 4. Further pharmacokinetic studies are in progress.



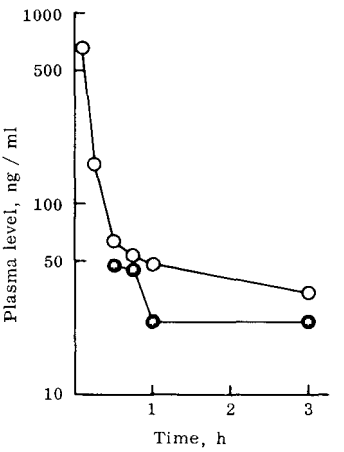


Fig. 3. Chromatogram from cancer patient plasma containing adriamycin and adriamycinol. Chromatographic conditions as given by the analytical method (photometric detection). Plasma concentrations: adriamycin, 44 ng/ml; adriamycinol, 8 ng/ml.

Fig. 4. Plasma levels of adriamycin and adriamycinol after intravenous infusion of adriamycin. ○ = Adriamycin, ● = adriamycinol. Administered dose of adriamycin, 1.0 mg/kg.

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SPECTROFLUORIMETRIC DETERMINATION OF DIPYRIDAMOLE IN SERUM — A COMPARISON OF TWO METHODS

J.M. STEYN

Department of Pharmacology, Medical School, University of the Orange Free State, P.O. Box 339, Bloemfontein 9300 (Republic of South Africa)

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SUMMARY

Two spectrofluorimetric methods for the determination of dipyridamole in plasma are described. The thin-layer chromatographic—fluoridensitometric method utilizes 1 ml of plasma which is extracted at pH 10 with diethyl ether—dichloromethane (80:20). The organic phase is evaporated to dryness, reconstituted in 250 μl dichloromethane and 5 μl are spotted on a silica gel 60 plate. The plate is developed in ethyl acetate—methanol—ammonia (85:10:5), dried, dipped in a paraffin wax solution, dried, and scanned using 380 nm as excitation wavelength, a 430 nm cut-off filter, and collecting all emitted light on the photomultiplier. Quantitation was done by the external standard method, peak heights being measured and a calibration graph constructed. For the spectrofluorimetric method 1 ml of plasma is extracted at pH 10 with 8 ml of hexane—isoamyl alcohol (95:5) and the organic phase used directly for the measurement of the fluorescence intensity (excitation 405 nm, emission 495 nm). Quantitation was done by measuring the fluorescence of standards that were treated as above and constructing a calibration graph of concentration versus fluorescence intensity. Concentrations of unknowns were found by interpolation from this graph. The two methods were found to exhibit good correlation but the spectrofluorimetric method proved to be more amenable to the analysis of a large number of samples.

INTRODUCTION

When asked to perform a bioavailability study on tablets containing dipyridamole (Fig. 1) a review of the literature revealed that only a few analytical procedures for this drug had been published [1—4]. None of these methods suited our particular needs and an analytical method for the determination of dipyridamole in plasma had to be developed. Due to the highly fluorescent nature of this drug, it was decided to utilize this property as a means of determination.

This paper describes two fluorimetric procedures for the determination of dipyridamole in plasma; namely, a thin-layer chromatographic (TLC)—fluoridensitometric method, and a spectrofluorimetric method where the fluores-

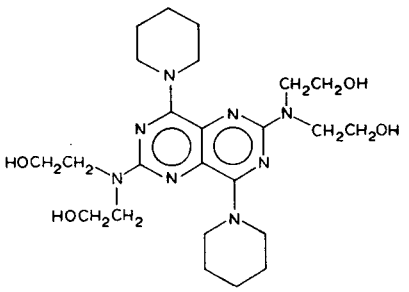

Fig. 1. Chemical structure of dipyridamole [2,6-bis(diethylamino)-4,8-dipiperidinopyrimido-(5,4-*d*)-pyrimidine].

cence is measured in cuvettes after extraction into a suitable solvent. The two methods are compared as to their reproducibility and accuracy. Both methods are easy to perform and sensitive enough to determine dipyridamole in concentrations that would be expected in the plasma of patients being treated with this drug.

MATERIALS AND METHODS

Reagents

All reagents and solvents used were of guaranteed reagent grade (E. Merck, Darmstadt, G.F.R.). All solvents used in the TLC procedure were distilled prior to use.

Extraction solvent

(a) Fluoridensitometric method: A mixture of diethyl ether—dichloromethane (80:20) was used.

(b) Spectrofluorimetric method: A mixture of hexane—isoamyl alcohol (95:5) was used.

TLC developing solvent

A mixture consisting of ethyl acetate—methanol—28% ammonia (85:10:5) was used; this solvent was prepared immediately before use.

Dipping solution

Paraffin wax (m.p. 42—44°) was dissolved in light petroleum (b.p. 40—60°), 140 g in 2 l.

Stock solutions

Stock solutions of dipyridamole were prepared by weighing about 10 mg accurately on a Mettler ME22 electronic microbalance and adding sufficient distilled methanol to result in a solution containing 2 μg of dipyridamole per μl of methanol from which a solution containing 200 ng of dipyridamole per μl was prepared. These solutions were prepared fresh before each series of determinations.

Buffer solution

Aqueous solutions of 0.2 *M* Na_2CO_3 and 0.2 *M* $NaHCO_3$ were mixed until a pH of 10 was reached.

Apparatus

A Perkin-Elmer MPF3 spectrofluorimeter equipped with a Xenon lamp and a thin-layer scanning attachment was used to measure the fluorescence of the spots for the thin-layer procedure. The excitation wavelength used was 380 nm while a cut-off filter of 430 nm was selected for the emitted light. The instrument was used in the reflectance mode.

Fluorescence of dipyridamole when determined by means of the spectrofluorimetric method was measured on a Perkin-Elmer Model 204 spectrofluorimeter equipped with a xenon lamp. The excitation wavelength used was 405 nm and the emission wavelength 495 nm. The intensity of the fluorescence was measured by a disc integrator coupled to the recorder. The peak intensity was measured for a period of 20 sec and the number of counts recorded.

Thin-layer chromatographic equipment

Pre-coated silica gel TLC plates (Merck) with a layer thickness of 0.25 mm and without a fluorescence indicator were used. The extract was applied to the plate by means of a 5-μl micro capillary. The TLC plates were developed in a Shandon Chromatank (Shandon Scientific, London, Great Britain).

Analytical procedures

TLC—fluoridensitometric method. To 1 ml of plasma was added 1 ml of carbonate buffer (pH 10) and the mixture was extracted with 5 ml of distilled diethyl ether—dichloromethane (80:20) by shaking for 15 min on a mechanical shaker. The phases were separated by centrifugation (800 *g*, 2000 rpm, swing rotor), the organic phase transferred to a conical evaporation tube and dried at 50° under a stream of nitrogen. The residue was dissolved in 250 μl of redistilled dichloromethane and 5 μl were applied to the thin-layer plate. The plate was developed in the developing solvent to a height of 6 cm in an unsaturated atmosphere, dried with a stream of air at room temperature, left in the dark for 10 min and then dipped in the paraffin wax solution. After drying as described above, the plate was again left in a dark place for 10 min to allow stabilisation of the fluorescence, after which time it was ready for scanning.

Quantitation was done by processing five standard concentrations and spotting the concentrated extracts in duplicate on two different positions on the plate. Peak heights were measured, averaged, and plotted on a graph against concentration. The equation for this line, which conformed to a power curve, was derived through regression analysis and the concentrations of unknown samples were calculated by substituting their peak heights into this equation.

Spectrofluorimetric method. To 1 ml of plasma was added 1 ml of buffer (pH 10) and the mixture extracted with 8 ml of hexane—isoamyl alcohol (95:5) by shaking for 15 min on a mechanical shaker. After separation of the phases by centrifugation, 3 ml of the organic phase were transferred to a

quartz cuvette and the fluorescence measured using the conditions and equipment described above. Quantitation was done by measuring the intensity of fluorescence of five standards spanning the expected range of concentrations and plotting the number of counts vs. concentration. An equation for the best straight line fit through these points was obtained by linear regression analysis and the unknown concentrations were calculated by substitution of their peak heights into this equation.

Interference from other drugs

The drugs listed in Table I, in concentrations higher than would be expected in the therapeutic situation, together with dipyridamole (1 μg/ml), were subjected to the procedures described for both the thin-layer and spectrofluorimetric procedures. The fluorescence of the contaminated samples was measured and compared with an unadulterated sample. No interference was found.

TABLE I

DRUGS TESTED FOR INTERFERENCE

Acebutolol*	Hydrochlorothiazide
Amitryptiline	Lorazepam
Bamipine	Norephedrine
Benzoctamine	Penbutolol*
Carbamazepine	Procainamide*
Chlormezanone	Procaine*
Chlorpromazine*	Propranolol*
Clothiapine	Quinidine*
Diazepam	Salicylamide
Diphenhydramine	Thioridazine
Ephedrine	Trifluoroperazine
Flurazepam	Trimipramine

*Compounds with intrinsic fluorescence.

RESULTS

TLC—fluoridensitometric method

This method proved to be very good in the concentration range exceeding 50 ng/ml. It was found that the calibration curve of peak height vs. concen-

TABLE II

REPRODUCIBILITY OF THE TLC—FLUORIDENSITOMETRIC METHOD

Concentration spiked (ng/ml)	Peak height (mean ± S.D.)	*n*	Coefficient of variation
200	13.3 ± 1.2	3	9
400	31.0 ± 3.6	3	11
800	83.3 ± 9.6	3	12
1200	143.7 ± 5.9	3	4.1
1600	102.7 ± 9.6	3	4.8

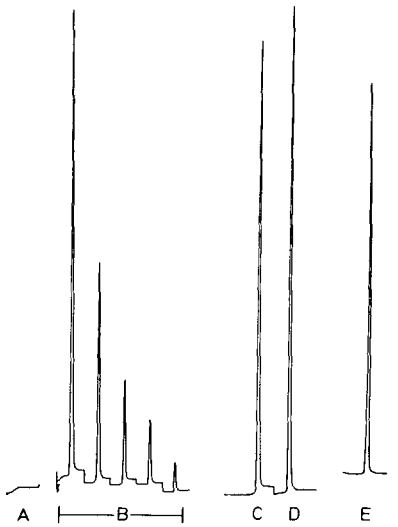

Fig. 2. Representative tracing of a TLC fluoridensitogram. (A) Plasma blank. (B) Standards from 400 ng/ml to 25 ng/ml. (C) 1200 ng dipyridamole per ml plasma extracted and compared with D, the expected amount. (E) Extract of a patient's plasma.

tration results in a line that fits a power curve very well (Table II). The reproducibility is also illustrated in Table II. A representative tracing of a TLC—fluoridensitogram of standards and a sample extract is illustrated in Fig. 2.

Spectrofluorimetric method

This method can be used for concentrations from 50 ng/ml upwards. The calibration graphs exhibit linear characteristics to a concentration of 1800 ng/ml. The particulars for the calibration graph as well as the reproducibility of the extraction procedure are illustrated in Table III.

TABLE III

REPRODUCIBILITY OF EXTRACTION OF SPECTROFLUORIMETRIC METHOD

Concentration spiked (ng/ml)	Counts (mean ± S.D.)	*n*	Coefficient of variation
100	21 ± 1	3	4.8
200	39 ± 2	3	5.1
400	75 ± 4	3	5.6
800	137 ± 4	3	3.1
1200	207 ± 10	3	4.7
1600	261 ± 4	3	1.4
1800	294 ± 5	3	1.7

COMPARISON OF SENSITIVITY OF THE TWO METHODS

The sensitivity limits of the two methods were compared by spiking serum with dipyridamole so as to obtain a range of concentrations from 10 ng/ml to 200 ng/ml. After extraction and evaporation of the extraction solvent 200 μl of dichloromethane were added to the residue and 10 μl portions of the different extracts were determined by means of the methods described above. In the case of the spectrofluorimetric method 10 μl of the reconstituted extract were added to 3 ml of hexane—isoamyl alcohol and the fluorescence was measured. When a comparison was made in this way the TLC—fluoridensitometric method proved to be more sensitive than the spectrofluorimetric method. It was found that as little as 15 ng/ml of dipyridamole would give a 20% of full-scale deflection when analyzed by means of the TLC—fluoridensitometric method, while giving virtually no response when using the spectrofluorimetric method. The minimum detectable quantity when using the latter method in the manner described above was 100 ng/ml.

EXTRACTION EFFICIENCY

To 1 ml of serum were added 1200 ng of dipyridamole, extracted with distilled diethyl ether—dichloromethane (80:20) and compared by means of the TLC—fluoridensitometric method with the expected theoretical amount. Extraction efficiency proved to be 92% (Fig. 2C and D). The extraction efficiency for the extraction used in the spectrofluorimetric method was 94%.

DISCUSSION

When the investigations into a method for determining dipyridamole were started it was found that certain solvents had a decomposing effect on the drug. It was found that in concentrations of 500 ng/ml the fluorescence disappeared when left overnight in chloroform and carbon tetrachloride, but remained unaffected when dissolved in dichloromethane, hexane, dichloromethane—diethyl ether (20:80), hexane—isoamyl alcohol (95:5) or methanol. In the light of these findings it was decided to prepare fresh standard solutions before each series of determinations. During the development of the TLC–fluoridensitometric method it was found that the quality of the plates had a marked effect on the fluorescence of the spots and that in some instances there was a significant difference in the intensity of the fluorescence of the same quantity of dipyridamole when applied on different positions on the plates. It was therefore necessary to spot each extract twice, at different positions on the plate. This, together with the observation that the calibration graph had to be constructed from five concentrations due to the non-linear characteristics thereof, had the effect that only five determinations could be done on one plate.

The effect of paraffin wax on the fluorescence of the spots [5] was quite dramatic and it was found that the intensity of dipyridamole fluorescence on the plate increased fifteen-fold. The mechanism of this effect is not clear but it may be due to a decrease of the surface scattering of the plate. The stability of the fluorescence of the spots was also investigated and it was found that

during the first 10 min after dipping of the plate there was a slight increase in the fluorescence but after this time the fluorescence remained stable for about 2 h, after which there was steady decline.

Since no internal standard is used, a crucial step in the procedure is the transfer of the organic phase, after initial extraction, to the evaporation tube. For the method to be accurate and reproducible it is essential that the amount of solvent transferred should be reproducible. Investigation of this procedure proved that the mean weight of solvent transferred was 4.4 g (5.2 ml) with a coefficient of variation of 2.7%, which is adequate for this kind of procedure.

With the number of samples that had to be assayed for the bioavailability study in mind it was decided to investigate the possibility of measuring the fluorescence of dipyridamole in cuvettes after a single extraction. This proved to be quite feasible and the method also proved to be amenable to a large number of samples since the procedure is very simple and the extracted dipyridamole was stable for several hours in the solvent employed.

To determine the correlation between the two procedures control samples containing two different concentrations of dipyridamole were made up and analysed without revealing the concentration to the analyst. Analyses were made on four different occasions and the results are displayed in Table IV. This result also serves to illustrate the accuracy and reproducibility of the two methods.

TABLE IV

DATA TO ILLUSTRATE THE PRECISION AND ACCURACY AND THE CORRELATION BETWEEN THE TLC—FLUORIDENSITOMETRIC AND SPECTROFLUORIMETRIC METHODS

Concentration spiked (ng/ml)	*n*	Amount recovered (ng/ml)	
		Spectrofluorimetric method (mean ± S.D.)	TLC method (mean ± S.D.)
270	4	259 ± 10.9	267.6 ± 14.3
680	4	665 ± 16.1	699.8 ± 28.7

TABLE V

CORRELATION BETWEEN SPECTROFLUORIMETRIC AND TLC—FLUORIDENSITOMETRIC METHODS WHEN A PATIENT'S PLASMA IS ANALYSED

Sample	Time after medication (h)	Concentration found (ng/ml)	
		Spectrofluorimetric method	TLC method
B1	1	1426, 1626	1380, 1503
B2	2	290, 320	316, 304
B3	4	154, 182	166, 177
B4	8	230, 270	251, 274
B5	12	106, 126	116, 150

A possible disadvantage of the spectrofluorimetric procedure is that it may be less selective than the TLC method due to the possible interference of metabolites. Since the extraction may not be selective it is possible that metabolites may contribute to the fluorescence and result in a falsely high concentration. To rule out this possibility a normal healthy volunteer was given 100 mg of dipyridamole. Blood was collected at 1, 2, 4, 8 and 12 h after medication. The samples were analysed by means of both the methods. The results, displayed in Table V, show a very good correlation between the two methods and it would appear as if metabolites do not significantly contribute to the value obtained by the spectrofluorimetric method when a patient receives a single dose of the drug.

The stability of dipyridamole in serum when stored at −20° was also determined; over a period of 22 days no deterioration could be found.

From the above it is therefore evident that the two methods are comparable in accuracy and precision but that the spectrofluorimetric method is more amenable to the analysis of a large number of samples.

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Biomedical Applications

CHROMBIO. 413

Note

Aromatic amino acids in amniotic fluid samples analyzed by reversed-phase liquid chromatography with spectrophotometric detection

ANTÉ M. KRSTULOVIC and SALVATORE CIRIELLO

Chemistry Department, Manhattanville College, Purchase, N.Y. 10577 (U.S.A.)

and

LAURA BERTANI-DZIEDZIC and STANLEY E. GITLOW

Catecholamine Research Laboratory, Department of Medicine, The Mount Sinai School of Medicine, City University of New York, New York, N.Y. 10029 (U.S.A.)

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The importance of prenatal diagnosis of diseases caused by aberrations in amino acid metabolism cannot be overestimated. Disorders such as tyrosinosis, phenylketonuria, and neonatal hypertyrosinemia are manifested in abnormal serum levels of tyrosine. Since the treatment requires a low amino acid diet in early infancy [1, 2], it is essential to monitor these compounds rapidly and sensitively.

In addition, in disorders in the metabolism of tryptophan, a precursor of serotonin, excretion of abnormal amounts of its metabolites has been observed in a number of conditions, such as bladder cancer [3], breast cancer [4, 5], Hodgkin's disease [6], vitamin B_6 deficiency [7], depression [8, 9] and migraines [10].

However, while current research has focused mainly on the analysis of catecholamine metabolites [11] in amniotic fluid, no efforts have been made to devise simple and routine analytical methods for the assessment of aromatic amino acid levels in normal subjects and patients with metabolic disorders.

Paper chromatography [12] does not possess the reproducibility necessary for quantitative analysis of trace levels of aromatic amino acids in amniotic fluid samples. Gas—liquid chromatography [13], although rapid and highly sensitive, requires elaborate derivatization procedures, which makes it unsuitable for routine clinical use.

Therefore, we have investigated the use of reversed-phase liquid chromatography in the analysis of tyrosine and tryptophan in amniotic fluid samples.

EXPERIMENTAL

Apparatus

A Model 6000A solvent delivery system, Model U6K universal liquid chromatograph injector, and a Model 660 solvent programmer (Waters Assoc. Milford, Mass., U.S.A.) were used in all determinations. The UV detection system consisted of two Model SF 770 Spectroflow monitors from Kratos Inc., Schoeffel Instrument Division (Westwood, N.J., U.S.A.).

The column was a stainless-steel μBondapak C_{18} (10-μm particle size), obtained from Waters Assoc.

Reagents

The reagents used were all of highest purity. Reference compounds were purchased from Sigma (St. Louis, Mo., U.S.A.), spectral grade acetonitrile from Burdick and Jackson Labs. (Muskegon, Mich., U.S.A.), and potassium dihydrogen phosphate from Mallinckrodt (St. Louis, Mo., U.S.A.). Reference solutions of tyrosine and tryptophan were prepared at 1.0 m*M* concentration and kept frozen when not in use.

Chromatographic conditions

The low-strength eluent was a 0.1 *M* solution of potassium dihydrogen phosphate, pH of 2.50, and the high-strength eluent acetonitrile—water (3:2, v/v). A linear gradient from 0% to 80% of high-strength eluent in 45 min was used. The low-strength eluent was always filtered through a Millipore membrane filter (Millipore, Bedford, Mass., U.S.A.), Type GS with a pore size of 0.22 μm, and the high-strength eluent was regularly degassed under vacuum. The flow-rate was 1.4 ml/min, and the temperature was ambient at all times.

Samples

Amniotic fluid samples were obtained by transabdominal amniocentesis from subjects who were tested for chromosomal abnormalities and fetal neural tube defects in a genetic testing center. Samples were obtained from the 16th to 24th week of gestation, and they were kept frozen at −10° until analyzed.

Prior to the chromatography, samples were filtered through Millipore membrane filters, Type HA, pore size 0.22 μm to remove the particulate matter.

Peak identification

Initial identification of the peaks of interest was based on retention behavior and co-chromatography with the reference compounds. Since the high-performance liquid chromatographic effluents were simultaneously monitored at two wavelengths, peak height ratios were computed for the peaks in amniotic fluid samples and compared with those of the reference compounds. In addition, stopped-flow UV spectra were also obtained. Although these spectra characteristically lack in fine structure, this method has nevertheless proven to be a powerful tool for peak identification [14].

RESULTS

Prior to the analysis of amniotic fluid samples, the chromatographic conditions were optimized for the separation of tryptophan and tyrosine. Fig. 1 illustrates the separation of the reference compounds, detected at 235 nm and 285 nm. The low wavelength of 235 nm was necessary for monitoring the creatinine content in amniotic fluid samples.

Twelve samples of amniotic fluid were then analyzed using the described analytical technique. A typical chromatogram of a sample of amniotic fluid, monitored at 235 nm and 285 nm, is shown in Fig. 2.

The identitiy of chromatographic peaks was deduced from evidence accumulated from the retention behavior, co-chromatography with the reference compounds, peak height ratios, and stopped-flow UV spectra. A comparison of the spectra of chromatographic peaks in the amniotic fluid with those of the reference compounds is shown in Fig. 3.

Since the concentration of amino acids in body fluids varies with water

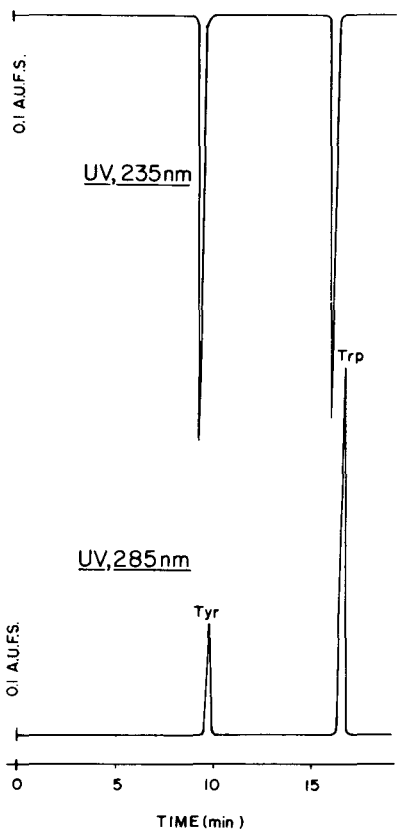

Fig. 1. A chromatogram of a synthetic mixture of tyrosine (Tyr) and tryptophan (Trp), detected at 235 nm and 285 nm. Amount injected: 3 nmoles each. Chromatographic conditions: column, μBondapak C_{18}; low-strength eluent 0.1 *M* KH_2PO_4, pH 2.50; high-strength eluent acetonitrile—water (3:2, v/v); gradient, linear, from 0% to 80% of high-strength eluent in 20 min; flow-rate, 1.4 ml/min; temperature, ambient.

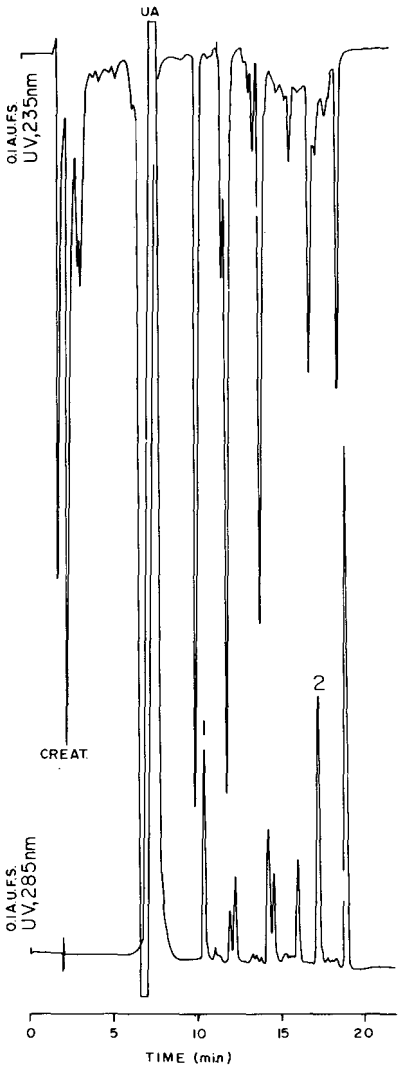

Fig. 2. Chromatogram of a sample of amniotic fluid. Volume injected: 100 μl. Chromatographic conditions as in Fig. 1.

intake, the levels of tryptophan and tyrosine were expressed in mg per mg of creatinine, which was measured simultaneously at the wavelength of 235 nm. The concentration range for tyrosine and tryptophan in 12 samples of amniotic fluid was 0.682—1.35 mg per mg of creatinine and 0.184—0.548 mg per mg of creatinine, respectively.

In conclusion, the described reversed-phase liquid chromatographic method for the rapid determination of tyrosine and tryptophan in amniotic fluid has great potential for the assessment of normal values of aromatic amino acids and early diagnosis of phenylketonuria, and other disorders involved in aberrations in the metabolism of aromatic amino acids.

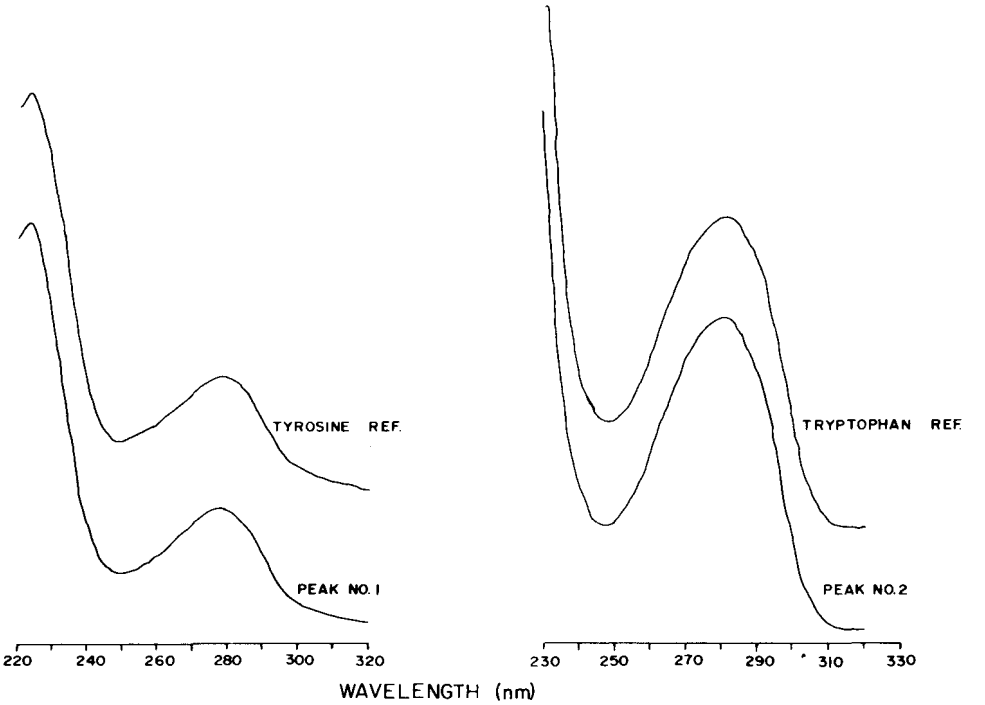

Fig. 3. Stopped-flow UV spectra of the reference compounds and peaks in amniotic fluid sample (scanning rate = 100 nm per min); absorbance, 0.1 a.u.f.s..

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Biomedical Applications

CHROMBIO. 420

Note

Rapid liquid chromatographic determination of tryptophan, tyrosine, 5-hydroxyindoleacetic acid and homovanillic acid in cerebrospinal fluid

GEORGE M. ANDERSON, J. GERALD YOUNG and DONALD J. COHEN

Yale Child Study Center, 333 Cedar Street, New Haven, Conn. 06510 (U.S.A.)

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Tryptophan (TRP) and tyrosine (TYR) are precursors for the brain neurotransmitters serotonin (5-HT) and dopamine (DA), respectively. The major brain metabolite of 5-HT is 5-hydroxyindoleacetic acid (5-HIAA), while DA is predominantly metabolized to homovanillic acid (HVA). The measurement of these precursors and metabolites in cerebrospinal fluid (CSF) gives an indication of brain turnover of the neurotransmitters and is the most direct method available for assessing human brain neurochemistry.

TRP and TYR have been determined in CSF with fluorometric methods [1—4] and by using amino acid analyzers [5—7]. In general, the methods are time-consuming and require relatively large sample volumes (0.25—2.0 ml). Recently TRP has been quickly determined in 1—20 μl of CSF using a liquid chromatographic—fluorometric technique [8—10]. The acid metabolites, 5-HIAA and HVA, have been determined in CSF using fluorometry [11—19], gas chromatography—mass spectroscopy [20—23], and liquid chromatography coupled with either flow-through fluorometric [8—10, 24] or amperometric detectors [25].

We have developed a combined liquid chromatographic—fluorometric/amperometric method capable of determining all four of the compounds of interest within 9 min.

EXPERIMENTAL

Apparatus

Liquid chromatography was performed using an Altex 110 A pump (Altex Scientific, Berkeley, Calif., U.S.A.), a Waters Assoc. U6K injector and a μ-Bondapak C_{18} reversed-phase column (300 mm × 3.9 mm I.D., average particle size 10 μm) (Waters Assoc., Milford, Mass., U.S.A.). An Aminco Fluoromonitor (American Instrument, Silver Spring, Md., U.S.A.) was modified as previously described [8—10]. A further modification entailed cutting 2.0 × 20 mm slits

in the cylindrical flow-cell holder opposite the entrance and exit slits. In addition, the entire holder was dipped in 6 *M* HCl for several minutes, resulting in a dull grey finish. These changes reduced the background light level three to four fold. The amperometric detector consisted of a Bioanalytical Systems electrochemical controller (LC-2A), a carbon paste (silicon oil) working electrode, a Ag/AgCl reference electrode, and a Plexiglas thin-layer detector cell and reference electrode compartment (Bioanalytical Systems, West Lafayette, Ind., U.S.A.). A 5l-μm spacer gasket was used, and the working electrode was set at + 0.8 V versus the reference electrode. The detector and reference cells were enclosed in a grounded faradaic cage. The fluorometric and amperometric detectors were connected in series, with the amperometric detector downstream.

Reagents

Standards were purchased from Sigma (St. Louis, Mo., U.S.A.). Stock solutions (10 mg/100 ml) were made up in distilled water with 0.1% ascorbate added. Diluted standards (0.1—5.0 ng/μl) were made up daily in 0.1 *M* HCl. The solvent system was prepared by mixing, for at least 1 h, 300 ml of glass-distilled methanol (Burdick and Jackson Labs., Muskegon, Mich., U.S.A.) with 1700 ml of pH 4.0, 0.01 *M* sodium acetate (adjusted with glacial acetic acid).

Methods

Usually 20 μl of unprocessed CSF, obtained by lumbar puncture, was directly injected into the system. Sample preparation consisted of centrifuging the CSF at 400 *g* for 10 min before injecting. The solvent system was delivered at a flow-rate of 1.5 ml/min. The compounds were quantitated by peak height measurements; single point standards were used as a linear response (peak height versus concentration) was observed over the working range.

RESULTS AND DISCUSSION

A chromatogram of TYR, TRP, 5-HIAA, and HVA standards is shown in Fig. 1. The retention times and absolute detection limits for the compounds are listed in Table I. Standards were determined at the 1—50 ng level with within-day coefficients of variation of from 2—11% (typically 5%). An unprocessed human lumbar CSF sample chromatogram is shown in Fig. 2. The column used

TABLE I

CHROMATOGRAPHIC AND DETECTABILITY DATA

Compound	Retention time (min)*	Detection limits (pg)**	
		Amperometric	Fluorometric
Tyrosine	2.4	60	350
Tryptophan	4.6	400	25
5-Hydroxyindole-acetic acid	6.2	10	45
Homovanillic acid	7.7	24	2000

*For chromatographic conditions see Experimental.

**Injected quantity giving a signal-to-noise ratio of 2.0.

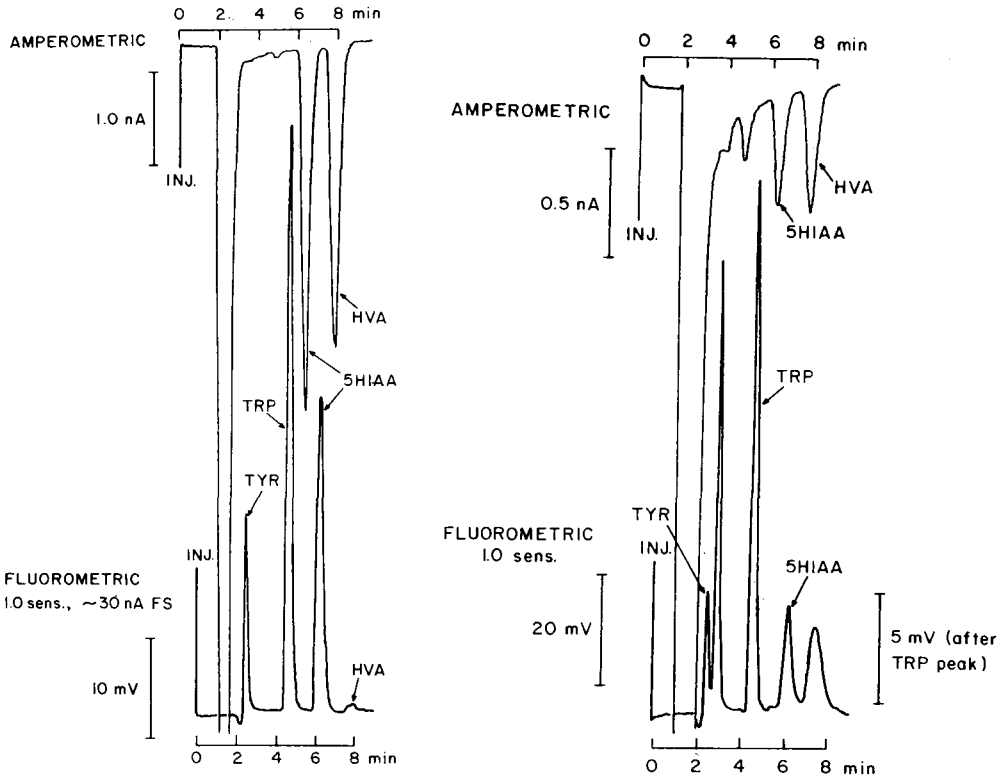

Fig. 1. Chromatogram of standards (25 ng TYR, 5 ng TRP, 5 ng 5-HIAA, 10 ng HVA) with amperometric and fluorometric detection. TYR was not usually separated from the amperometric solvent front. See Experimental for chromatographic conditions.

Fig. 2. Chromatogram of a 20-μl unprocessed lumbar CSF sample. Concentrations for the sample shown are: TYR, 1.04 μg/ml; TRP, 396 ng/ml; 5-HIAA, 32.6 ng/ml and HVA, 89.7 ng/ml. The fluorometric peaks immediately following the TYR and 5-HIAA peaks are unidentified. See Experimental for chromatographic conditions.

for the separations shown in Figs. 1 and 2 had been used to analyze more than 500 CSF and deproteinized plasma samples over a seven-month period. During that time the efficiency, or plate count (N), had decreased from ca. 2700 to ca. 1000. In a pooled human CSF sample the compounds were determined with the following coefficients of variation: TYR 6.7%; TRP 2.5%; 5-HIAA (fluorometric) 5.1%; 5-HIAA (amperometric) 2.9%, and HVA 10.7%. A standard addition study was performed by adding standards in amounts from 1—20 times the normal levels. The percent recoveries were: TYR 110 ± 5.9; TRP 93.7 ± 5.2; 5-HIAA 108 ± 6.1; and HVA 104 ± 4.0%.

The identities of the peaks observed in CSF were confirmed by chromatographing samples with solvent systems containing 10, 5 and 3% methanol or acetonitrile. The sample peaks always coeluted with the appropriate standard. The concentration ranges observed for the compounds have been similar to those reported in the literature.

A comparison between the fluorometric and amperometric determinations of 5-HIAA levels in 25 different human CSF samples is shown in Fig. 3. The methods were well correlated; usually 5-HIAA levels were taken to be the mean of the two methods.

A collaborative study is being undertaken comparing this method, a gas chromatographic—mass spectroscopic method, and a standard fluorometric procedure for determining 5-HIAA and HVA in CSF.

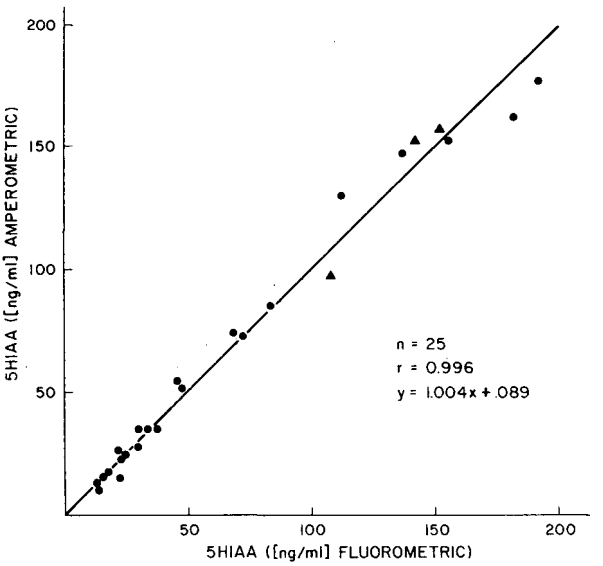

Fig. 3. The amperometric values for 5-HIAA plotted against the corresponding fluorometric values for 25 human lumbar CSF samples. Points plotted as ▲ are one-half the observed values.

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Biomedical Applications

CHROMBIO. 403

Note

High-performance liquid chromatography of hemoglobins

I. Determination of hemoglobin A_2

KAREN M. GOODING, KAI-CHUN LU and FRED E. REGNIER

Department of Biochemistry, Purdue University, West Lafayette, Ind. 47907 (U.S.A.)

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The separation of hemoglobin variants has traditionally been carried out by electrophoresis [1, 2] or ion-exchange chromatography on carbohydrate gels [3]. Ion-exchange chromatography gives good resolution and quantitation but is very slow. In an effort to decrease analysis time, microchromatography on small ion-exchange columns was implemented for the determination of specific hemoglobin variants [3]. These columns take only 30—200 min to develop but fractions must be collected manually and the procedure cannot be automated. Over the past few years, support materials for the high-performance liquid chromatography of proteins have been developed and refined [4—6]. The method described herein uses a high-performance anion-exchange support for the rapid determination of hemoglobins A_2 and A_0.

Hemoglobin A_2 levels are elevated in certain clinical states, most notably, in β-thalassemia [7]. A rapid method for the routine determination of Hb A_2 and A_0 is therefore valuable in the clinical recognition of patients with certain hemoglobinopathies.

EXPERIMENTAL

Chemicals

Tris(hydroxymethyl)aminomethane (Tris) was purchased from ICN Pharmaceuticals (Cleveland, Ohio, U.S.A.). Sodium acetate and glacial acetic acid were from Mallinckrodt (Paris, Ky., U.S.A.). The Hb A_2 Quik Column Control (2.47% Hb A_2) was from Helena Laboratories (Beaumont, Texas, U.S.A.). The blood was from anonymous samples which would have been discarded after other determinations had been made.

Apparatus

SynChropak AX 300 columns, 250 × 4.1 mm I.D., particle size 10 μm,

were obtained from SynChrom, Inc. (Linden, Ind., U.S.A.). A Micromeritics 7000 liquid chromatograph and a Model 785 variable wavelength detector (Micromeritics Instrument Corp., Norcross, Ga, U.S.A.) were used for the analyses with a Model CV-6-UHPa-N-60 injection valve (Valco, Houston, Texas, U.S.A.).

Methods

Solvent A (0.02 *M*, pH 8.0) was prepared by adding 2.42 g of Tris to 1 l of distilled water and adjusting the pH with acetic acid. Solvent B was the same as solvent A with the addition of 13.6 g of sodium acetate (0.1 *M*). Hemoglobin standards were dissolved in distilled water at a concentration of 15 mg/ml. Hemolyzed blood was diluted tenfold. No further sample preparation was necessary. Sample size was 10—20 μl. Full-scale absorbance of the detector was set at 0.05 at a wavelength of 410 nm. A 10-min linear gradient from 0 to 30% was used with a step to 100% after A_0 had eluted. The flow-rate was 2.5 ml/min.

RESULTS AND DISCUSSION

Fig. 1 shows the analysis of the hemoglobin standard under the above conditions. Slight variations in operating conditions can result in radical differences in resolution, as is seen in Fig. 2. Here Hb A_2 and A_0 are widely separated but Hb A_1 elutes with Hb A_0. Fig. 3 shows a sample of blood from a person with

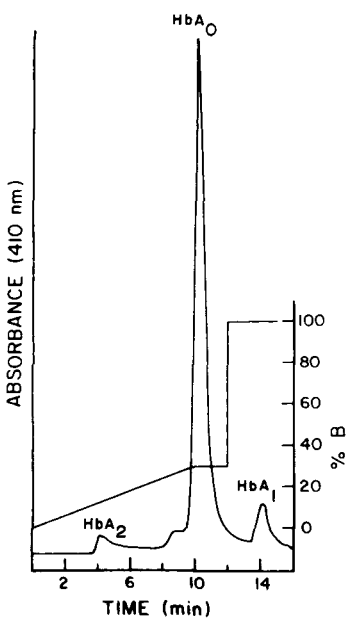

Fig. 1. Analysis of standard Hb A_2 sample. Column: SynChropak AX300, 250 × 4.1 mm I.D. Solvents A and B as in text, with a 10-min linear gradient from 0 to 30% then step to 100%. Flow-rate 2.5 ml/min; pressure 1200 p.s.i. Detection at 410 nm.

β-thalassemia trait. The Hb A_2 peak was approximately twice as large as that of normal blood.

Analysis of hemoglobins by high-performance ion-exchange chromatography allows rapid separations with the capabilities of automated injection and quantitation. With a 5-min recycle time, samples may be analyzed every 20 min with minimal preparation. A drop of blood is more than adequate for the procedure.

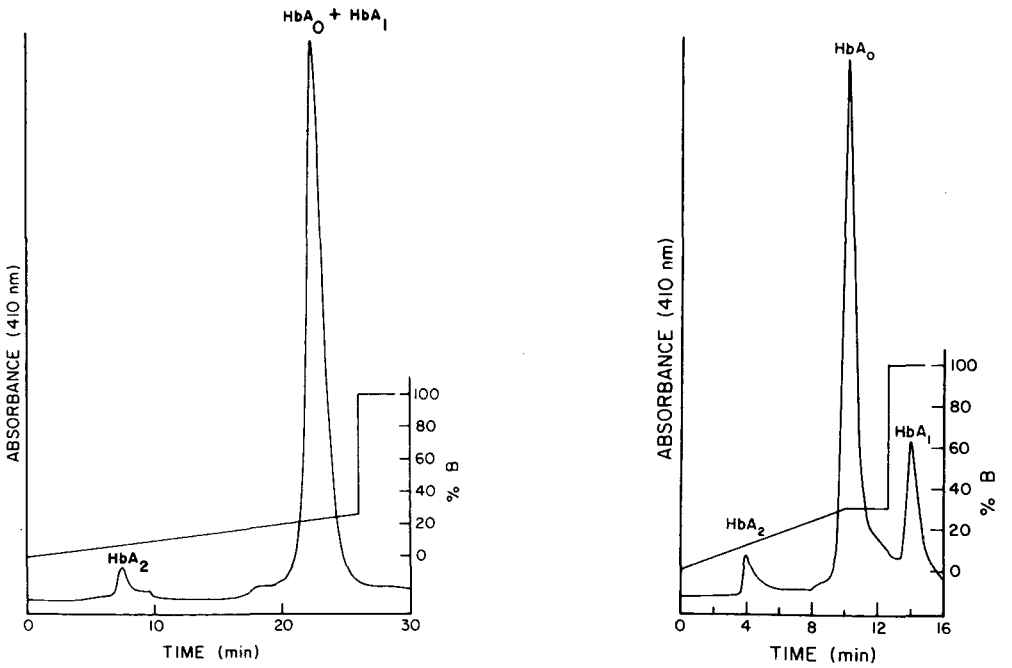

Fig. 2. Analysis of standard Hb A_2 sample. Column: SynChropak AX 300, 250 × 4.1 mm I.D. Solvents A and B as in text, with a 40-min linear gradient from 0 to 40% then step to 100%. Flow-rate 2.0 ml/min; pressure 1020 p.s.i. Dtection at 410 nm.

Fig. 3. Analysis of human blood showing β-thalassemia trait. Conditions as in Fig. 1.

ACKNOWLEDGEMENTS

The authors wish to thank Ms. Marsha Borders for her advice and assistance. They would also like to thank Dr. William Jacobsen at St. Elizabeth Hospital, Lafayette, Indiana, for blood samples. This work was supported by Grants HL20751 and GM25431 from the National Institutes of Health, U.S. Public Health Service. This is journal paper number 7549 from the Purdue University Agriculture Experiment Station.

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Biomedical Applications

CHROMBIO. 405

Note

Gas chromatographic determination of (*o*-methyl-α-phenylbenzyloxy)acetic acid levels in human serum following therapeutic doses of orphenadrin (Disipal®)

J. HUISMAN* and L.L. LIEBREGT

Department of Clinical Chemistry, Psychiatric Centre "Zon en Schild", Utrechtseweg 266, Amersfoort (The Netherlands)

and

J.H.H. THYSSEN

University Hospital, State University Utrecht, Department of Clinical Endocrinology, Utrecht (The Netherlands)

(First received January 15th, 1979; revised manuscript received August 6th, 1979)

Orphenadrin [N,N-dimethyl-2-(*o*-methyl-α-phenylbenzyloxy)ethylamine] is a centrally acting skeletal muscle relaxant initially used as a therapeutic agent for the symptomatic treatment of Parkinson's disease. Medication with anticholinergic anti-parkinsonian drugs is also required for psychiatric patients medicated with long-acting (depôt) neuroleptics [1].

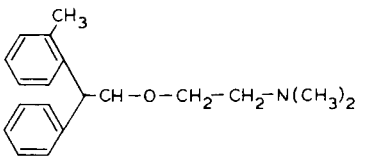
The metabolism, distribution and excretion of orphenadrin in man has been studied by several authors [2, 3]. Using [^{3}H]orphenadrin Ellison et al. [2] detected several metabolites by means of thin-layer chromatography. One of the major metabolites of orphenadrin, (*o*-methyl-α-phenylbenzyloxy)acetic acid (OMBOA), excreted in the urine as the glucuronide, accounted for about 13% of a 100-mg oral dose of [^{3}H]orphenadrin ingested by a volunteer. One or more of the metabolites of orphenadrin was shown to interfere in the determination of thyroxin in serum [4, 5] or possibly even affect thyroid function; for this reason we planned to investigate the influence of a prolonged use of orphenadrin on the thyroid function of psychiatric patients. As we planned to correlate serum levels of the most important metabolite of orphenadrin with thyroid function, the development of a method for the determination of OMBOA was necessary.

*To whom correspondence should be addressed.

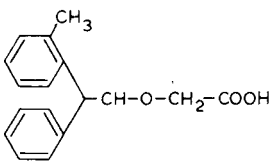
MATERIALS AND METHODS

Reagents

N,N-Dimethyl-2-(*o*-methyl-α-phenylbenzyloxy)ethylamine · HCl (orphenadrin) and (*o*-methyl-α-phenylbenzyloxy)acetic acid (OMBOA) were kindly supplied by Gist-Brocades, Haarlem, The Netherlands (Fig. 1). The internal standard, primidone, was obtained from ICI-Holland (Rotterdam, The Netherlands); a solution of 2.5 μg/ml in redistilled chloroform was prepared. All other reagents and solvents were obtained from Merck (Darmstadt, G.F.R.).



N, N-Dimethyl-2-(*o*-methyl-α-phenylbenzyloxy)ethylamine (Orphenadrin)



(*o*-methyl-α-phenylbenzyloxy)acetic acid (OMBOA)

Fig. 1. Chemical structures of orphenadrin and OMBOA.

Extraction procedure

A 1.0-ml volume of serum, acidified with 0.2 ml of 1.0 *M* HCl, was extracted for 1 min on a Vortex mixer with 10.0 ml of internal standard solution in chloroform. After centrifugation for 5 min at 2500 *g*, 9.0 ml of the chloroform layer were transferred and evaporated to dryness at 50° and under reduced pressure. The residue was dissolved in 100 μl of absolute ethanol.

Gas chromatography

A Pye series 104 gas chromatograph equipped with a flame-ionisation detector and connected with a SpectraPhysics SP-4000 chromatography data system was used. Silanised glass columns (1.8 m × 2 mm I.D.) were packed with 3% OV-17 on Chromosorb W AW DMCS HP, 80—100 mesh (Chrompack, Middelburg, The Netherlands).

The gas chromatographic conditions were: injector block at 260°; detector at 270°; column oven at 250°; nitrogen flow-rate 40 ml/min; hydrogen and air pressure 14.5 and 13.0 p.s.i., respectively; chart speed 0.5 cm/min.

A 2.0-μl aliquot of the ethanolic solution of the extraction residue (100 μl) was injected into the gas chromatograph. The retention times of OMBOA and primidone (internal standard) under the conditions described were about 325 and 195 sec, respectively (Fig. 2a).

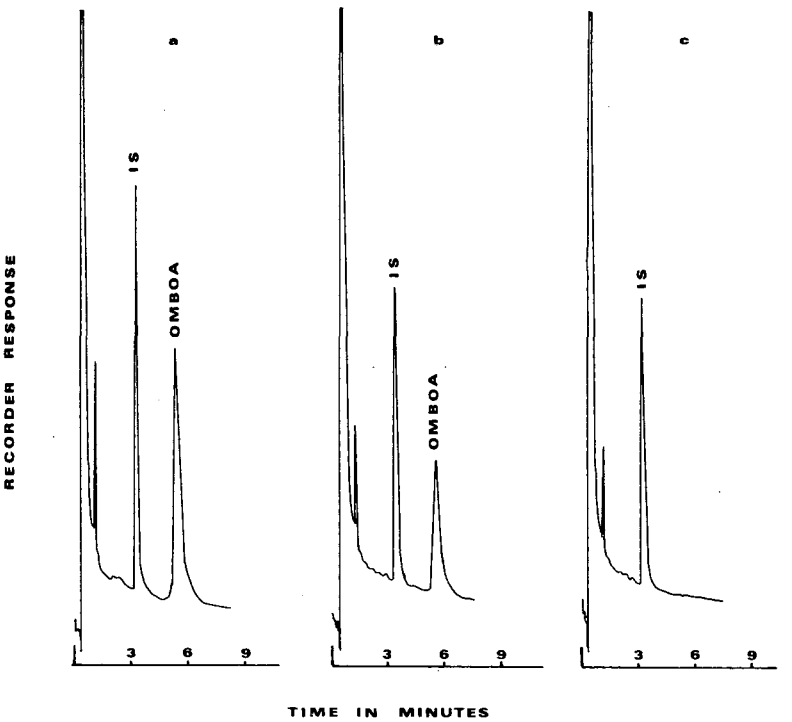


Fig. 2. Gas chromatographic analysis of OMBOA in human serum. (a) Gas chromatogram of a chloroform extract of a standard solution of OMBOA in serum (72 μg/ml). (b) Gas chromatogram of a chloroform extract of serum from a patient who ingested 300 mg orphenadrin daily for more than 1 year. (c) Gas chromatogram of human serum after addition of orphenadrin, four of its metabolites and primidone (internal standard, IS) showing the absence of interference from these compounds in the retention area of OMBOA.

RESULTS AND DISCUSSION

Accuracy and reproducibility

Calibration curves were constructed by analysing serum samples to which different amounts of OMBOA were added to yield concentrations of 12, 24, 36, 48, 60 and 72 μg/ml.

A linear relationship between the peak area response ratios of OMBOA and primidone versus concentration was observed; the slope of the linear regression line was 0.9989, thus validating the linearity of the calibration curve. This standard curve covers the concentration range of serum OMBOA in patients using therapeutic amounts of orphenadrin.

Reproducibility of the determination of OMBOA, verified by the repeated assay of the same sample of serum, yielded a mean concentration of 23.6 μg/ml with a coefficient of variation of 5.3% (n = 8).

The minimum detectable amount of OMBOA was 13 ng (signal-to-noise ratio = 3:1), thus the sensitivity limit of the assay is 0.8 μg/ml of serum. Inter-assay variability determined on consecutive days yielded a mean value of 27.3 ± 5.5% (n = 6).

Recovery

Overall recovery of OMBOA taken through the analytical procedure was determined by a comparison of the peak area ratios, obtained from a serum containing 30 μg/ml of OMBOA, with those of a standard of the same concentration injected directly. The single chloroform extraction yielded an effective recovery of 83,4% with a coefficient of variation of 2.5% (n = 6).

Specificity

Several metabolites of orphenadrin have been described by Ellison et al. [3]; besides OMBOA they identified N-methyl-2-(*o*-methyl-α-phenylbenzyloxy)ethylamine, 2-(*o*-methyl-α-phenylbenzyloxy)ethylamine, N,N-dimethyl-2-(*o*-methyl-α-phenylbenzyloxy)ethylamine-N-oxide and *o*-methylbenzhydrole.

Serum spiked with these metabolites was processed under the conditions described for the assay of OMBOA. No interference was detected, as can be seen from Fig. 2c.

Salicylic acid, the major metabolite of aspirin, was also processed under these conditions but the retention time proved to be only about 70 sec. The proposed method is therefore specific for the metabolite OMBOA.

So far, 28 serum samples of patients not receiving orphenadrin have been tested. In none of them has a peak been observed during gas chromatography which interferes with the primidone or OMBOA peaks.

Application in human subjects

Blood samples were obtained from 28 psychiatric patients treated with different amounts of orphenadrin during a period of at least 1 year. The serum was separated and stored at —25° prior to analysis.

The steady-state concentrations of OMBOA found are shown in Fig. 3 as a function of the daily dosage of the drug. The correlation between the OMBOA concentrations and the daily dose of orphenadrin proved to be poor, the coefficient of correlation being 0.6498. A representative chromatogram is shown in Fig. 2b.

From these results it can be seen that there is considerable individual variation in absorption, metabolism and/or elimination of orphenadrin in man.

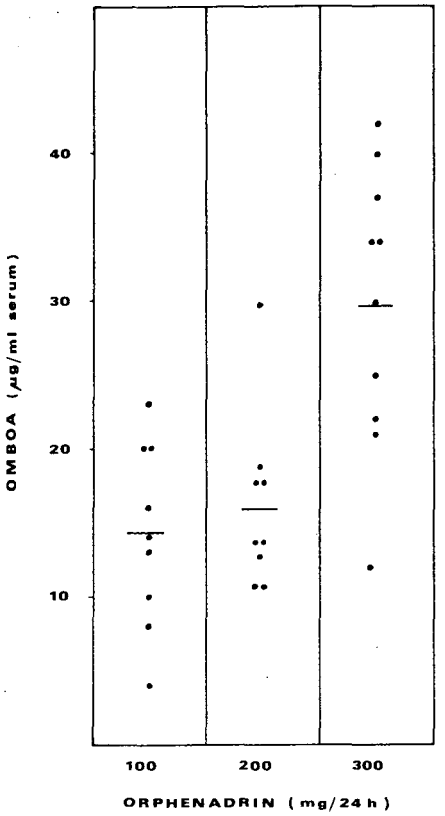


Fig. 3. Relationship between the daily dose of orphenadrin and the serum concentration of OMBOA in psychiatric patients.

CONCLUSIONS

The proposed technique permits the rapid assay of OMBOA in serum with an adequate degree of accuracy and specificity. The sensitivity of the assay (0.8 µg/ml) is sufficient to measure serum levels of OMBOA, the major metabolite, after thereapeutic doses of orphenadrin.

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Biomedical Applications

CHROMBIO. 406

Note

Simultaneous determination of disopyramide and its mono-N-dealkylated metabolite in plasma by gas—liquid chromatography

MIRJA-LIISA AITIO

Department of Pharmacology, University of Turku, Turku (Finland)

(First received March 13th, 1979; revised manuscript received August 6th, 1979)

Disopyramide is a new anti-arrhythmic agent with a broad spectrum of efficiency. It has been comparatively well tolerated [1]. Studies correlating the plasma levels of disopyramide with clinical effects are few, and different therapeutic ranges have been reported: 1.5—6 μg/ml [2], 3—5 (6) μg/ml [1], 2—4 μg/ml [3], and even 3—8 μg/ml [4].

The major metabolic pathway of disopyramide in man is N-dealkylation; the resulting mono-N-dealkylated disopyramide (MND) represents 15—25% of the dose given [5]. At present there is no information in the literature about the plasma levels of the metabolite during long-term therapy with disopyramide. In animal studies the metabolite has been shown to be active and possibly to have a positive inotropic effect [6]. The early fluorimetric assay [7] did not differentiate between the parent drug and the metabolite. Thereafter, several gas—liquid chromatographic methods have been described [2, 8—11]. Only one of these, the method of Hutsell and Stachelski [8], includes an assay for N-dealkylated disopyramide. The latter usually breaks down and is eluted as three poorly separated peaks under gas chromatographic conditions suitable for disopyramide. This can be avoided by acetylation [8]. A high-performance liquid chromatographic assay of disopyramide [12, 13] measures MND without derivatization, but the equipment is not yet available everywhere.

The method described in this paper was developed for the routine laboratory use for the simultaneous analysis of disopyramide and MND from the same sample with a single injection.

EXPERIMENTAL

Materials

Disopyramide [4-diisopropylamine-2-phenyl-2-(2-pyridyl)butyramide] phosphate was supplied by Leiras, Turku, Finland, and was used as an aqueous solution of 0.1 mg/ml in 0.1 *M* hydrochloric acid. Mono-N-dealkyldisopyramide [MND, 4-isopropylamino-2-phenyl-2-(2-pyridyl)butyramide] and the internal standard, *p*-chlorodisopyramide [4-diisopropylamino-2-*p*-chlorophenyl-2-(2-pyridyl)butyramide] were gifts from Roussel Laboratories, Wembley, Great Britain. Both of these were used as 0.05 mg/ml solutions in 0.1 *M* hydrochloric acid. Diethyl ether, chloroform, sodium hydroxide and sulphuric acid were of analytical reagent grade. Glass microfibre paper GF/A was purchased from Whatman, Maidstone, Great Britain, cut to 7 × 7 cm pieces, turned into rolls and heated at 570° for 20 min, to remove impurities, before use.

Gas—liquid chromatography

A Varian Model 2100 gas chromatograph equipped with an alkaline flame ionization detector and linked to W+W 1200 recorder was used. Integration of peak areas was performed using a Spectra-Physics integrator. The column, detector and injector temperatures were 250, 285 and 275°, respectively. The nitrogen (carrier gas) flow-rate was 28 ml/min and those of air and hydrogen approximately 300 and 50 ml/min, respectively. A glass column 3 ft. long was silanized by dichloromethylsilane overnight, rinsed in toluene and ethanol and dried. The column was packed with 3% OV-17, 100—120 mesh, on Chromosorb W.

Extraction procedures

Sample, or standard (1 ml of EDTA plasma), with 100 μl (5 μg) of the internal standard solution, was pipetted into 20-ml glass tubes. The mixture was made alkaline by the addition of 100 μl of 2 *M* sodium hydroxide. A glass microfibre paper roll, cut in two halves, was dropped into the tube containing the sample. Within 1 min the plasma was totally absorbed into the paper; thereby, clotting of the plasma was completely avoided★. Diethyl ether (3 ml) was added and the tube contents were mixed. After pouring the ether into another tube, the extraction was repeated. The combined ether phases were acidified (500 μl of 0.05 *M* sulphuric acid) in tapered, stoppered tubes. After mixing, the phases were separated by centrifugation. The organic layer was removed very carefully. The remaining aqueous phase was again made alkaline (300 μl of 2 *M* NaOH), and 3 ml of chloroform were added. The tube contents were mixed, and the layers separated. The aqueous layer was discarded, and the chloroform layer was poured into a stoppered tube containing 25 μl of acetic anhydride, in order to make an acetate derivative of MND. After evaporation to dryness under a stream of nitrogen, the residue was redissolved in 25 μl of ethanol, and 2-μl aliquots were injected onto the column.

★Use of glass microfibre paper in the extraction was a suggestion by Torsti Yrjänä, Ph.D., from the Central Laboratory of Turku University Hospital, which is gratefully acknowledged.

All pipetting was performed manually, and all mixing was done in a vortex-type mixer (30 sec.).

Calibration

Calibration graphs were obtained by adding known amounts of disopyramide and MND to human plasma samples. They were straight lines in the range of 0.5—12.5 μg/ml for disopyramide and 0.5—7.5 μg/ml for MND. The regression lines of the calibration graphs were $y = 0.257x - 0.005$ and $y = 0.179x + 0.023$, for disopyramide and MND, respectively. The correlation coefficient was 0.999 in both cases.

RESULTS AND DISCUSSION

Disopyramide, *p*-chlorodisopyramide, and the acetyl derivative of MND gave sharp well-separated peaks with retention times of 1.5, 2.4, and 4 min, respectively (Fig. 1). Both disopyramide and acetyl-MND could be determined from a single chromatographic injection. The short retention times allow analysis of several samples in a short period of time, making this method suitable for routine laboratory analysis. The back-extraction of Hayler and Flanagan [11] removed an endogenous interfering peak which is probably cholesterol — authentic cholesterol appears as a coincident peak that has a retention time very close or similar to that of acetyl-MND (Fig. 1). Although a nitrogen-sensitive detector was used, this peak might constitute a problem. Therefore, the ether layer must be aspirated very carefully to remove any residual ether which contains the interference peak.

The extraction efficiency of the method for disopyramide was 71.6 ± 7.8% (S.D.) (*n* 22), at disopyramide levels of 1.5, 5.0 and 10.0 μg/ml. The efficiency is somewhat low compared to other methods [8, 9]. This is obviously due to the use of the glass-fibre paper: about 1 ml of ether remains in the paper after the extraction. The glass-fibre paper, however, has many advantages: all emulsification of the plasma is avoided; clotting of plasma that is occasionally seen upon addition of concentrated sodium hydroxide does not occur; no centrifugation after extraction is needed, and after extraction, the organic phase can simply be poured into another tube.

Both within-batch and between-batch precisions of the assay procedure were determined (Tables I and II); both were clearly better for disopyramide than MND. This difference most probably is not due to variability in acetylation, because in the very recent high-performance liquid chromatographic determination of disopyramide and MND, not using acetylation, the same difference was seen [13]. With disopyramide the precision was reasonable at drug plasma levels with clinical significance, whereas at low plasma levels a larger coefficient of variation was seen. When duplicate samples were assayed, at therapeutic plasma levels the coefficient of variation for disopyramide was 4—6%, reasonably good for a gas—liquid chromatographic assay procedure. On the other hand, the precision of the assay for MND was rather poor at the levels ordinarily encountered, that is at levels below 1 μg/ml. At these plasma levels, however, MND most probably has no clinical significance: in animal studies [6] MND was about four times less active than disopyramide. On the

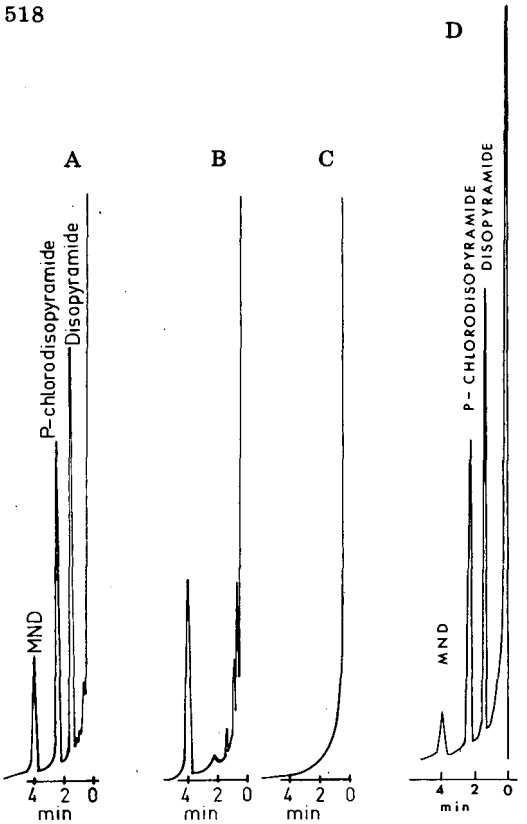

Fig. 1. Gas chromatographic tracings of plasma extracted as described in the methods. (A) Plasma spiked before the extraction process with disopyramide (3 μg/ml), mono-N-dealkyldisopyramide (3 μg/ml), and the internal standard (*p*-chlorodisopyramide). (B) Blank plasma extracted and analyzed without the back-extraction to sulphuric acid. (C) The same blank plasma extracted and analyzed as described in the methods. (D) Plasma sample of a patient undergoing disopyramide therapy, 600 mg daily (disopyramide 3.6 μg/ml, MND 1.4 μg/ml).

TABLE I

WITHIN-BATCH PRECISION OF THE ASSAY OF DISOPYRAMIDE AND MONO-N-DEALKYLDISOPYRAMIDE (MND)

Values were calculated from duplicates of all patient samples analyzed during three months. The analyses were performed by two persons.

Drug concentration (μg/ml)	Coefficient of variation (%)	No. of assays
Disopyramide		
<2.5	5.0	16
2.5—4.0	5.7	47
>4.0	4.1	27
MND		
All determinations	13.2	80
>1.5	10.9	17

TABLE II

BETWEEN-BATCH PRECISION OF THE ASSAY OF DISOPYRAMIDE AND MONO-N-DEALKYLDISOPYRAMIDE (MND)

The figures represent mean ± S.D. of ten successive analyses, during three months, performed by two persons, of plasma samples spiked with the indicated amounts of disopyramide and MND, and of a sample from a patient given disopyramide. The analyses were carried out in duplicate.

Expected value (μg/ml)	Analytical result (μg/ml ± S.D.)	Coefficient of variation (%)
Disopyramide		
1.0	1.10 ± 0.097	8.83
4.0	4.01 ± 0.220	5.53
8.0	8.60 ± 0.319	3.71
Unknown	3.38 ± 0.128	3.78
MND		
1.0	1.02 ± 0.177	17.3
4.0	3.89 ± 0.488	12.8
8.0	8.09 ± 0.711	8.8
Unknown	0.52 ± 0.149	28.7

other hand, I have seen patients with exceptionally high MND levels, with simultaneous, relatively low levels of the parent drug [14]. It is in these cases, when it becomes important to know the plasma level of MND, and the present assay, although admittedly not as good for MND as for disopyramide, is reasonably good at MND levels exceeding 2 μg/ml (Tables I and II). The precision of a method using high-performance liquid chromatography was similar to that of the present study [13].

Hutsell and Stachelski [8] were the first to describe a gas chromatographic assay for both disopyramide and MND. Their extraction procedure was more tedious, and included a florisil separation. They also needed larger sample sizes and big volumes of organic solvents.

N-Acetylprocainamide and diazepam have been reported to interfere in this type of assay [15]. Procainamide and disopyramide are very seldom used in combination. N-Acetylprocainamide has, in this assay, a retention time similar to the internal standard, *p*-chlorodisopyramide. Approximate levels of disopyramide can be determined, however, by assaying samples with and without the internal standard. Diazepam has the same retention time as disopyramide, but even an unusually high plasma level of 500 ng/ml of diazepam appeared as a peak corresponding to an apparent level of disopyramide below 0.5 μg/ml; therefore, possible error due to diazepam levels usually encountered is no serious handicap in the present assay procedure.

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Note

Determination of 1-diethylcarbamoyl-4-methylpiperazine (diethylcarbamazine) in human plasma and urine

G.D. ALLEN, T.M. GOODCHILD and B.C. WEATHERLEY*

Department of Drug Metabolism, The Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS (Great Britain)

(First received April 12th, 1979; revised manuscript received August 6th, 1979)

Diethylcarbamazine (Banocide, Hetrazan) (DEC) has been used for many years for the treatment of human and animal filariases.

Spectrophotometric methods for measuring DEC through formation of ion-pairs have been utilized by Lubrum [1] and Ramachandran [2]. Procedures based on this principle, however, lack the specificity and sensitivity of gas chromatographic methods.

Bogan [3] has developed a gas chromatographic method for measuring diethylcarbamazine in animal plasma and tissues, and while the procedure is an improvement over the above methods, the sensitivity is somewhat lacking as the minimum detectable concentration is 400 ng/ml and there are operational difficulties with column priming and poor chromatography.

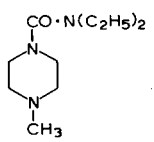
The present method can detect concentrations of 10 ng/ml from 0.5 ml human plasma, and therefore can be applied to the clinical situation where levels below 500 ng/ml are obtained later than 12 h after oral dosing of 200 mg of DEC citrate.

EXPERIMENTAL

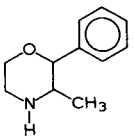
Reagents and materials

Diethylcarbamazine (I) is a basic compound, soluble in most organic solvents and in aqueous solutions of organic acids, and stable in the presence of 2 *M* aqueous sodium hydroxide.

*To whom correspondence should be addressed.



I



II

Phenmetrazine (3-methyl-2-phenylmorpholine hydrochloride) (II) was chosen as internal standard because of its similarity to DEC in extraction properties, its appropriate retention time and its ready availability; analogues of DEC are not commercially available. Other chemicals used were ethyl acetate (redistilled), *n*-hexane and sodium hydroxide (all AnalaR grade; BDH, Poole, Great Britain). The sodium hydroxide was employed as a 2 *M* solution in water.

Standard plasma solutions of DEC citrate were prepared from an aqueous solution of concentration 2 mg/100 ml, serially diluted using plasma as appropriate. Urine standards were prepared from a solution in human urine of concentration 10 mg/100 ml, serially diluted using urine as appropriate. The internal standard was prepared by dissolving phenmetrazine hydrochloride in distilled water to a concentration of 20 μg/ml.

Glassware

Screw-capped 10-ml Sovirel tubes (V.A. Howe, London, Great Britain) were used in the extraction and BC24/C14T tapered test-tubes (Quickfit and Quartz, J.A. Jobling, Staffordshire, Great Britain) were used for solvent evaporation.

Gas—liquid chromatographs

A Perkin-Elmer F30 instrument, modified to allow sample injection from a Hewlett-Packard 7670A autosampler, was used with a nitrogen flame ionization detector. This was operated under standard conditions with hydrogen and air flow-rates of 3 and 50 ml/min, respectively, and the standing current adjusted to 10 pA.

A Hewlett-Packard 5735A gas chromatograph, equipped with 7671A autosampler was also used with a nitrogen flame ionization detector. Again this was operated under standard conditions with hydrogen and air flow-rates of 3 and 50 ml/min, respectively, and the standing current adjusted to 16 pA.

Operation of the autosamplers and processing of data were carried out using a Hewlett-Packard 3352B minicomputer-based laboratory automation system.

Extraction procedure for plasma and urine

Preliminary experiments having shown that extraction of DEC and phenmetrazine was quantitative into ethyl acetate using aqueous sodium hydroxide, and that this solvent produced a clean gas chromatogram in the region of the measured compounds when blank fluids were extracted, the following procedure was devised. To each plasma (0.5 ml) or urine (1 ml) sample (standard as prepared above, or unknown) in a Sovirel tube were added 100 μl of phen-

metrazine standard solution (2 μg as hydrochloride). The mixture was made alkaline with 500 μl of 2 *M* sodium hydroxide solution for plasma, or 1 ml for urine, and ethyl acetate (5 ml) was added. The phases were mixed by mechanical tumbling end-over-end at 15/min for 10 min, separated by centrifugation at 3000 *g* for 5 min and the organic layer transferred as completely as possible to a tapered test-tube. Five ml more ethyl acetate were added to the aqueous

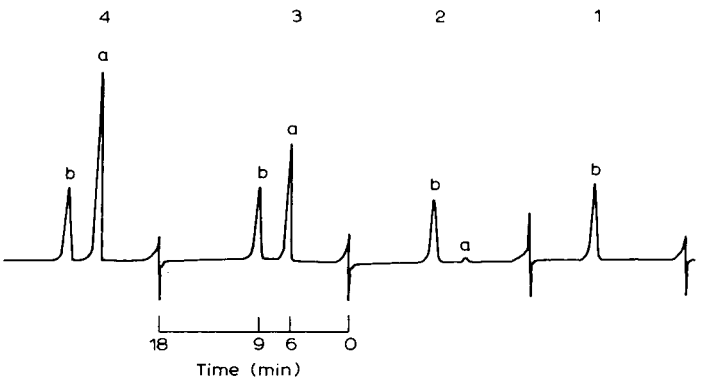


Fig. 1. Gas—liquid chromatography traces of plasma samples extracted and analysed for DEC citrate. Peaks: a = DEC; b = phenmetrazine. 1, Pre-dose patient plasma sample; 2 and 3, patient plasma samples containing 0.27 and 5.16 μg/ml, respectively; 4, plasma standard of concentration 8.26 μg/ml.

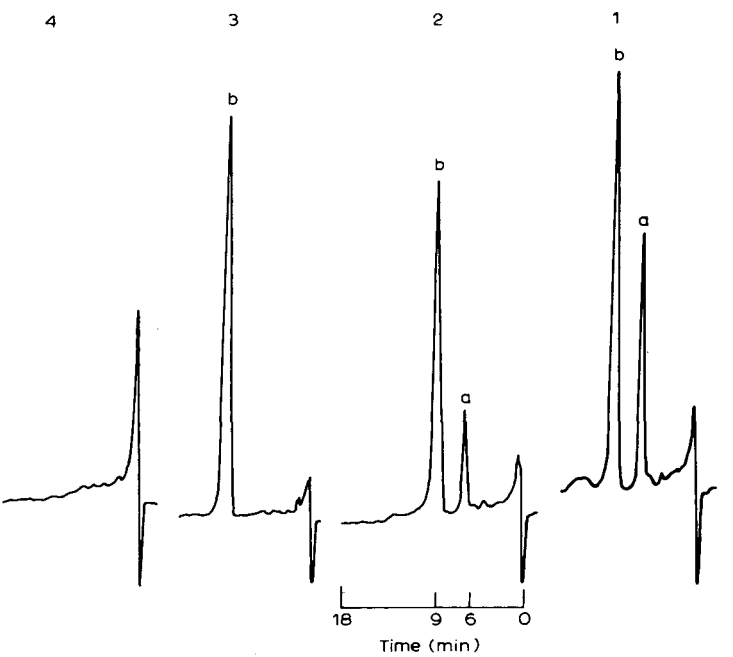


Fig. 2. Gas—liquid chromatography traces of urine samples extracted and analysed for DEC citrate. Peaks: a = DEC; b = phenmetrazine. 1, Urine standard of concentration 13.00 μg/ml; 2, urine sample containing 5.01 μg/ml; 3, pre-dose urine sample containing phenmetrazine; 4, pre-dose patient urine sample.

phase, the extraction repeated, and the organic phase added to the first extract. The ethyl acetate was removed using dry nitrogen at room temperature and the residue redissolved in 200 μl of hexane using a Vortex mixer to wash the sides of the tube. Samples were transferred to Hewlett-Packard microvials for analysis by gas chromatography, sample injection being 5 μl.

Gas—liquid chromatography

Several stationary phases were investigated, and it was concluded that 2% Carbowax 20M, 5% KOH, on Chromosorb G AW DMCS (100—120 mesh) was the optimum in terms of separating the drug and internal standard from endogenous biological material, and of eliminating adsorption and tailing seen on silicone phases [3]. The column was maintained at 160°, the injection port at 180° and the detector at 240°. Nitrogen was used as carrier gas at a flow-rate of 40 ml/min. Under these conditions the retention time of DEC was 6 min and that of phenmetrazine 9 min.

The peaks from DEC and phenmetrazine were identified by retention times, and the 3352B data system was programmed to calculate the concentrations of DEC by peak area ratios referred to standards extracted and run with the samples.

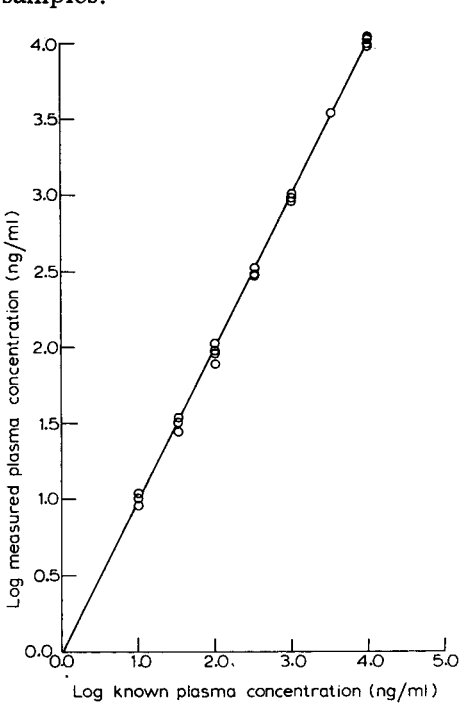


Fig. 3. Validation graph for analysis of DEC citrate in human plasma. Each concentration was measured in quadruplicate; the line is a linear regression of the logarithms of the measured concentrations on the spiked concentrations with a correlation coefficient of 0.9996.

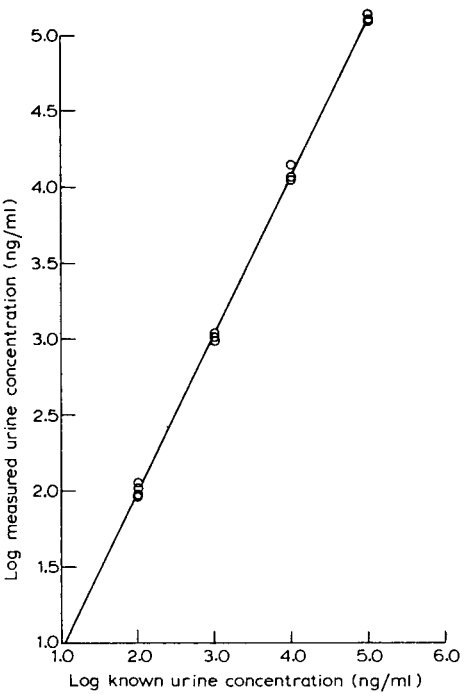
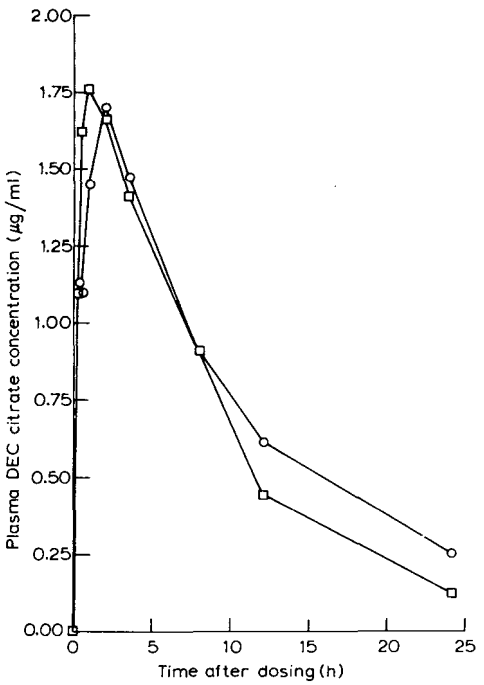


Fig. 4. Validation graph for analysis of DEC citrate in human urine. Each concentration was measured in quadruplicate; the line is a linear regression of the logarithms of the measured concentrations on the spiked concentrations with a correlation coefficient of 0.9995.



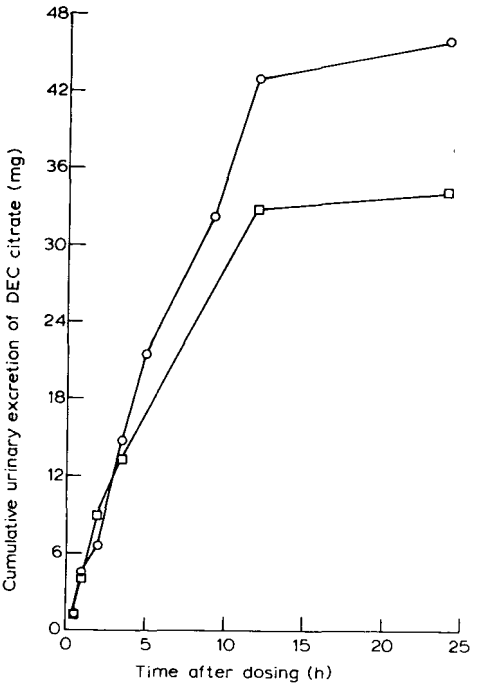


Fig. 5. Plasma concentrations of DEC citrate in two volunteers dosed orally with 200 mg Banocide.

Fig. 6. Accumulated urinary excretion of unchanged DEC citrate in the same volunteers as in Fig. 5.

RESULTS

Typical chromatograms of plasma (Fig. 1) and urine (Fig. 2) standards and samples from patients are shown, together with validation graphs showing the linearity and range (Figs. 3 and 4). Standard samples were re-analysed after storage at −20°, including freshly prepared standards; plasma samples are stable for at least six weeks, and urine samples stable for at least three months at this temperature.

Recoveries of DEC were measured by comparing gas chromatogram peak areas from DEC citrate standards, carried through the procedure, with peak areas from DEC base in methanolic solution. Each determination was carried out in triplicate, and the mean recoveries (± standard deviation) from plasma and urine were 95 ± 8% and 33 ± 7%, respectively.

Clinical studies

This analytical procedure was applied to samples from informed and consenting volunteers and from patients dosed with Banocide (DEC citrate); preliminary results have been reported by Rée et al. [4], and data on the concentration—time course are shown in the following graphs (Figs. 5 and 6). Urine

samples which were beyond the calibration range of 100 μg/ml were diluted with control urine and re-analysed.

Results of a field study in patients receiving a topical application of DEC citrate, and detailed pharmacokinetic calculations on the oral data presented here will be published at a later date.

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Note

Determination of the diuretic triamterene in the plasma and urine of humans by high-performance liquid chromatography

R.R. BRODIE, L.F. CHASSEAUD, T. TAYLOR and L.M. WALMSLEY

Department of Metabolism and Pharmacokinetics, Huntingdon Research Centre, Huntingdon (Great Britain)

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Triamterene is a potassium-sparing diuretic which increases sodium, chloride, and to a lesser extent bicarbonate, excretion [1].

Earlier studies of triamterene concentrations in the plasma and urine of humans were carried out using somewhat non-specific fluorimetric methods [2—4] which determined the parent drug and its metabolites together. A more specific assay, involving fluorimetry has also been described [5].

Because the reported procedures were not considered sufficiently sensitive for bioavailability studies of triamterene-containing formulations, a high-performance liquid chromatographic (HPLC) method utilising fluorimetric detection has been developed and is described in this paper. Since these studies were completed, a dissimilar high-performance liquid chromatography—fluorescence detection assay has been reported [6] which requires extraction of triamterene from plasma as the perchlorate ion-pair, a silica column and does not include an internal standard. Its sensitivity appears to be marginally less than the procedure described below.

EXPERIMENTAL

Materials

Triamterene, 6-*p*-hydroxytriamterene and 6-*p*-methoxytriamterene (Fig. 1) were kindly provided by Pharma Schwarz (Monheim, G.F.R.). Standard solutions of triamterene and the internal standard *p*-methoxytriamterene were prepared at a concentration of 1 μg/ml in methanol and stored at 4°. Reagents were of analytical grade and inorganic reagents were prepared in freshly glass-distilled water.

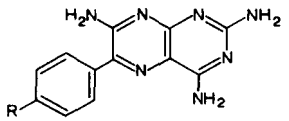

Fig. 1. Chemical structure of triamterene (R = H), 6-*p*-hydroxytriamterene (R = OH) and 6-*p*-methoxytriamterene (R = OCH_3).

Extraction procedure

Plasma samples (1 ml) were transferred into 10-ml pointed centrifuge tubes and spiked with a solution of the internal standard (40 μl, containing 40 ng of *p*-methoxytriamterene). Sodium bicarbonate (200 mg) was added and the mixture extracted by shaking it for 1 min with ethyl acetate (5 ml). After centrifugation for 10 min, the organic layer was carefully transferred into a 10-ml pointed centrifuge tube and evaporated to dryness under a stream of nitrogen at 37°. The sides of the tube were washed with ethyl acetate to ensure that all the residue was washed to the bottom of the tube, and the ethyl acetate evaporated to dryness. The residue was dissolved in methanol (20—50 μl) and an aliquot (5—12 μl) injected into the chromatograph.

Urine samples (100 μl) were diluted with distilled water (1 ml), spiked with internal standard (40 ng) and taken through the same extraction procedure as described for plasma.

High-performance liquid chromatography

The chromatograph consisted of a Waters M6000A pump (Waters Assoc., Northwich, Great Britain) coupled to a Perkin-Elmer LC 1000 fluorescence detector (Perkin-Elmer, Beaconsfield, Great Britain) operated at an excitation wavelength of 365 nm and an emission wavelength of 440 nm. Peak area measurements were obtained using a Hewlett-Packard 3380A Advanced Reporting Integrator (Hewlett-Packard, Hitchin, Great Britain). Injection was performed by syringe (25 μl, Precision Sampling, Baton Rouge, La., U.S.A.) via a U6K universal injector (Waters Assoc.). The column was stainless steel (300 × 4 mm I.D.), prepacked with μBondapak C_{18} (mean particle diameter, 10 μm) (Waters Assoc.).

Chromatography was performed in a reversed-phase mode using a solvent system of 45% (v/v) methanol in aqueous 0.1% (w/v) potassium dihydrogen phosphate with the final pH adjusted to 3.8 with phosphoric acid. The mobile phase flow-rate was 2 ml/min. The retention times (t_R) of triamterene and internal standard (*p*-methoxytriamterene) were 2.6 min and 3.1 min, respectively (Fig. 2), and the metabolite *p*-hydroxytriamterene (t_R 1.9 min) did not interfere with the assay.

Calibration curves

Calibration curves were constructed of peak area ratio measurements of triamterene to internal standard against concentration over concentration ranges of up to 40 ng/ml in plasma and 800 ng/ml in urine. Samples of blank plasma (1 ml) were spiked with triamterene at concentrations of 1, 10, 20, 30

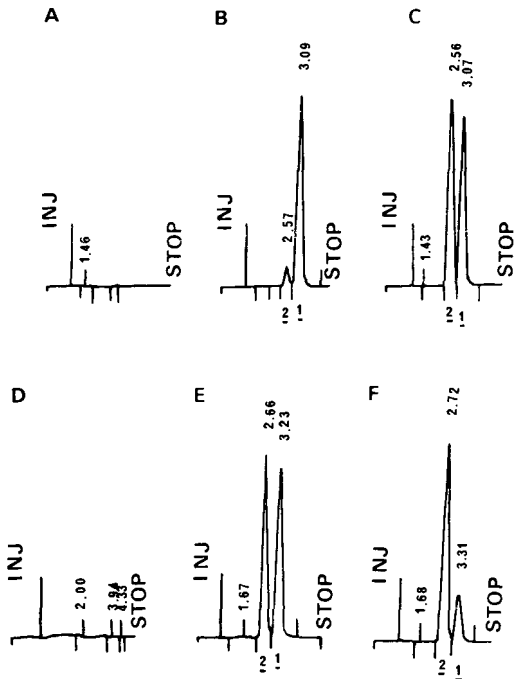

Fig. 2. Chromatograms of (A) predose control plasma; (B) and (C) plasma samples containing 1 and 10 ng/ml of triamterene, respectively; (D) predose control urine; and (E) and (F) urine samples containing 100 and 400 ng/ml of triamterene, respectively. Column (300 × 4 mm I.D.) prepacked with μBondapak C_{18}; flow-rate, 2 ml/min; solvent system, 45% (v/v) methanol—aqueous 0.1% (w/v) potassium dihydrogen orthophosphate; excitation and emission wavelengths, 365 and 440 nm, respectively; scale expansion, ×50; integrator attenuation, 32. Peaks: 1 = internal standard (*p*-methoxytriamterene, 40 ng/ml); 2 = triamterene.

and 40 ng/ml and with internal standard at 40 ng/ml. Samples of predose urine (100 μl) were spiked with 10, 20, 30, 40 and 80 ng of triamterene and 40 ng of internal standard. The samples were taken through the extraction procedure described previously.

Studies in humans

Plasma and urine samples obtained from human volunteer subjects dosed with a commercially available triamterene formulation (50-mg capsule) were analysed by the foregoing procedures. The studies in volunteers were carried out under conditions similar to those described by Brodie et al. [7].

RESULTS AND DISCUSSION

Precision

Extraction and measurement at each concentration were repeated on fourteen occasions from plasma and on six occasions from urine. The precision of the method for the measurement of triamterene was indicated by the coefficient of variation of peak area ratios which was ± 11%, ± 3% and ± 2% at

1 ng/ml, 20 ng/ml and 40 ng/ml, respectively, when extracted from plasma (1 ml) and ± 3%, ± 2% and ± 2% at 100 ng/ml, 300 ng/ml and 800 ng/ml, respectively, when extracted from urine (100 μl).

The coefficient of variation of peak area ratio measurements of a non-extracted mixture of triamterene and internal standard was ± 1% throughout the analysis of all plasma and urine samples.

Accuracy

The calibration curve for the measurement of triamterene in plasma was constructed from fourteen replicates at five concentrations over the range, and plots of peak area ratios against concentration were linear ($y = a + bx$, where $a = 0.0119$, $b = 0.0864$, correlation coefficient $r = 0.9991$) and the value of the intercept was not significantly different from zero ($P > 0.05$). The equation for the line forced through the origin was $y = 0.0868$ (± 0.0003 S.D.)x, where y is the peak area ratio and x is the concentration of triamterene (ng/ml). The accuracy of the method as defined by the 95% confidence limits of the least squares regression line forced through the origin, i.e. taking the calibration line as an estimate of the concentration of triamterene in plasma, was ± 122%, ± 6% and ± 3% at 1 ng/ml, 20 ng/ml and 40 ng/ml, respectively.

The calibration curve for the measurement of triamterene in urine was constructed from six replicates at five concentrations over the range, and plots of peak area ratios against concentration were linear ($y = a + bx$, where $a = 0.1214$, $b = 0.0802$, correlation coefficient $r = 0.9984$, and y is the peak area ratio and x is the amount of triamterene in 100 μl of urine). The value of the intercept was shown to be significantly different from zero. The 95% confidence limits for the curve were ± 25%, ± 7% and ± 4% at 100 ng/ml, 400 ng/ml and 800 ng/ml, respectively.

Recovery

The recovery of internal standard (40 ng/ml) from plasma (1 ml) was determined by comparison of peak area ratio measurements of internal standard to triamterene of standards taken through the extraction procedure to those injected into the chromatograph without extraction. The mean recovery of internal standard was 80.3% ± 2.8 S.D. ($n = 6$).

TABLE I

RECOVERY OF TRIAMTERENE FROM PLASMA AND URINE

Concentration added to plasma (ng/ml)	Recovery* (%)	Concentration added to urine (ng/ml)	Recovery* (%)
1	90.3	100	79.4
10	69.9	200	88.3
20	78.9	300	87.0
30	78.5	400	85.9
40	76.4	800	86.2

*Means of 14 determinations (plasma) and 6 determinations (urine) at each concentration.

The mean recovery of internal standard from urine (100 μl) at a concentration of 40 ng/100 μl was determined similarly and was 90.4% ± 1.3 S.D. (n = 6).

The mean recovery of triamterene from plasma was determined by comparison of peak area ratio measurements of non-extracted standards to those of extracted standards corrected for 100% recovery of internal standard, and was 78.8% ± 7.4 S.D. (Table I).

The mean recovery of triamterene from urine was determined similarly and was 85.4% ± 3.5 S.D. (Table I).

Stability of triamterene

The stability of triamterene in plasma and urine under the storage conditions used was tested by storing spiked plasma and urine samples for one week at −20° before analysis and then comparing the results obtained with freshly spiked standards. No decomposition was detected in either plasma or urine. Similarly results of samples re-analysed after three weeks storage at −20° were in good agreement with those attained initially and showed no decomposition.

Limit of detection

No interfering peaks were present in predose (blank) plasma samples taken from each subject prior to the start of the study. The limit of detection based on instrumental noise was set at 1 ng/ml (Fig. 2), but levels in the higher pg/ml range could be detected. The limit of detection using an ultraviolet absorption detector was poorer (λ_{max} 230 nm) and was about 10 ng/ml.

Concentrations of triamterene in plasma

After single oral doses of triamterene, a peak of mean concentrations of

TABLE II

CONCENTRATIONS OF TRIAMTERENE (ng/ml) IN THE PLASMA OF SIX HUMAN SUBJECTS AFTER SINGLE ORAL DOSES OF 50 mg

Time (h)	Subject No.						Mean ± S.D.
	1	2	3	4	5	6	
0.25	4	1	1	1	<1	<1	–
0.5	8	3	6	20	1	5	7.2 ± 6.7
0.75	13	7	8	20	2	10	10.0 ± 6.1
1	14	13	10	23	5	16	13.5 ± 6.0
1.5	13	14	15	17	8	26	15.5 ± 6.0
2	12	11	15	16	10	20	14.0 ± 3.7
3	8	10	8	12	14	16	11.3 ± 3.3
4	6	10	11	9	19	15	11.7 ± 4.6
6	4	6	10	8	10	13	8.5 ± 3.2
8	3	4	6	6	5	14	6.3 ± 3.9
12	2	6	6	3	2	3	3.7 ± 1.9
16	1	5	6	2	1	3	3.0 ± 2.1
24	1	3	3	1	2	2	2.0 ± 0.9
30	1	1	2	2	3	2	1.8 ± 0.8
36	1	3	1	2	2	2	1.8 ± 0.8

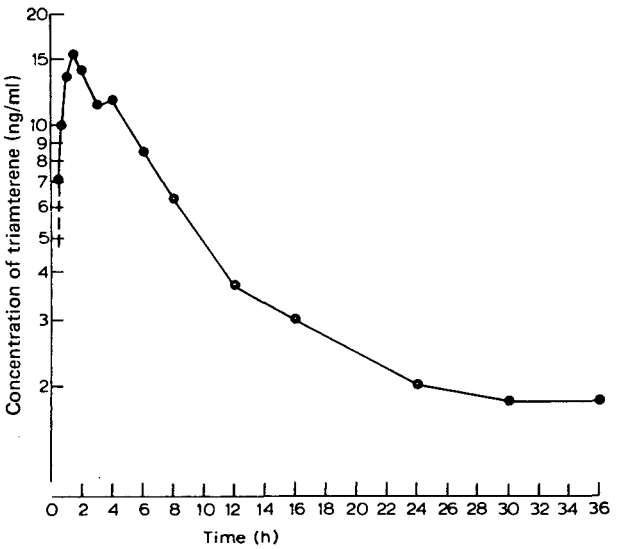

Fig. 3. Mean plasma concentrations of triamterene during 36 h after an oral dose of 50 mg of drug in a capsule. Semilogarithmic scale.

parent drug in plasma of 15.5 ng/ml was reached at 1.5 h after dosing (Table II) and was disproportionately lower than peak levels reported after oral doses of 100 mg of [^{14}C]-triamterene [8]. Thereafter mean concentrations declined to 8.5 ng/ml at 6 h, to 3.7 ng/ml at 12 h and to low but maintained levels of about 2 ng/ml between 24 and 36 h. Between 1.5 and 12 h, mean plasma concentrations declined with a half-life of about 5 h (Fig. 3), a value about twice that reported by Pruitt et al. [8] but the data indicate that this may not be the terminal elimination phase (Fig. 3).

Concentrations of triamterene in urine

During 24 h after single oral doses of triamterene, a mean of 1.6% dose was excreted in the urine (Table III) as parent drug, which is somewhat lower than

TABLE III

EXCRETION OF UNCHANGED TRIAMTERENE IN URINE (0—24 h) AFTER SINGLE ORAL DOSES OF 50 mg

Subject No.	Concentration (ng/ml)	Amount excreted (mg)	Dose excreted (%)
1	530	0.76	1.52
2	410	0.71	1.42
3	720	1.37	2.74
4	390	0.36	0.72
5	180	0.26	0.52
6	1300	1.43	2.86
Mean ± S.D.	588 ± 391	0.82 ± 0.49	1.63 ± 0.99

was reported after higher doses of the drug [8]. There was a six-fold variation in the proportion of triamterene excreted unchanged in 0—24 h urine.

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Journal of Chromatography, 164 (1979) 534—540
Biomedical Applications

CHROMBIO. 414

Note

Determination of naftidrofuryl in the plasma of humans by high-performance liquid chromatography

R.R. BRODIE, L.F. CHASSEAUD and T. TAYLOR

Department of Metabolism and Pharmacokinetics, Huntingdon Research Centre, Huntingdon (Great Britain)

and

J.O. HUNTER and P.J. CICLITIRA*

Addenbrookes Hospital, Cambridge (Great Britain)

(Received May 16th, 1979)

Naftidrofuryl is a new drug [1—4] recently introduced for the treatment of cerebral and peripheral vascular disorders [5—7]. The metabolism of the drug has been studied in animals using radioisotopic techniques [8] and a fluorimetric assay for measurement of the parent drug has also been used [9] with a limit of detection in plasma of about 0.1 μg/ml set by background interference. The sensitivity and specificity of the assay for the measurement of the drug in plasma can be improved by using a procedure based on reversed-phase high-performance liquid chromatography. The results obtained are described in this paper.

EXPERIMENTAL

Materials

All reagents were of analytical grade and all inorganic reagents were prepared in freshly glass-distilled water. Diethyl ether was freshly redistilled prior to use and acetonitrile was HPLC, far U.V. grade (Fisons Scientific Apparatus, Loughborough, Great Britain).

Standard solutions of naftidrofuryl [N-diethylaminoethyl-2-tetrahydrofurfuryl-3-(1′-naphthylpropionate) as the oxalate salt, Fig. 1] and LS 140 [the internal standard, oxalate salt of N-dimethylaminoethyl-2-tetrahydrofurfuryl-3-(1′-naphthylpropionate)] were prepared at a concentration of 10 μg/ml in acetonitrile and stored at 4°. Samples of naftidrofuryl and LS 140 were supplied by Lipha (Lyon, France).

*Drummond Research Fellow, 1977—1979.

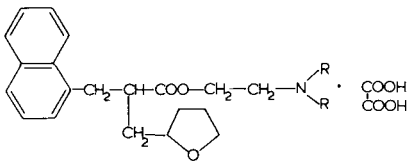

Fig. 1. Chemical structure of naftidrofuryl (R = C_2H_5) and LS 140 (internal standard, R = CH_3) oxalate salt.

Extraction procedure

Plasma samples (1 ml) were transferred into conical centrifuge tubes (10 ml), spiked with internal standard (10 μl, containing 100 ng LS 140) and made alkaline by the addition of ammonia solution (0.5 ml), 0.88 ammonia—water (1:10). The mixture was extracted with diethyl ether (5 ml) by vortexing it for 2 min on a Whirlymixer (Fisons Scientific Apparatus). The extracts were centrifuged at 2000 *g* for 10 min and the separated ether layer carefully transferred to another conical centrifuge tube. The ether extract was evaporated to dryness under nitrogen at 37° and the walls of the tube rinsed with more ether to ensure that all the residue was at the bottom of the tube. The ether was again evaporated and the residue dissolved in mobile phase (20 μl). After centrifugation at 2000 *g* for 10 min, as much as possible of the clear solution was injected into the chromatograph.

Calibration procedure

Samples of control (drug-free) plasma (1 ml) were spiked with naftidrofuryl oxalate at concentrations of 20, 50, 100, 200 and 300 ng/ml and with internal standard at a fixed concentration of 100 ng/ml. The samples are taken through the extraction procedure described previously.

Instrumentation

The liquid chromatograph consisted of a Waters Model 6000A pump (Waters Assoc., Cheshire, Great Britain) fitted to an LC3 variable wavelength UV detector (Pye Unicam, Cambridge, Great Britain) operated at 222 nm at λ_{max} for naftidrofuryl dissolved in the mobile phase. Injection was by syringe (25 μl, Precision Sampling, Baton Rouge, La., U.S.A.) via a U6K universal injector (Waters Assoc.). Peak area ratio measurements were quantified using a 3380A computing integrator (Hewlett-Packard, Slough, Great Britain). Mass spectra were obtained using a Micromass 16F mass spectrometer (V.G. Organic, Cheshire, Great Britain) operated in the electron impact mode of ionisation using an electron beam energy of 70 eV and a trap current of 100 μA. The ion source was operated at a temperature of 210° and samples were introduced by direct insertion probe.

Chromatography

Chromatography was performed in a reversed-phase mode. The column was constructed of stainless steel (30 cm × 0.4 cm I.D.) and prepacked with μBondapak C_{18} (mean particle diameter 10 μm) (Waters Assoc.). A precolumn constructed of stainless steel (7 cm × 0.2 cm I.D.) and dry-packed with

pellicular Co:Pell®ODS (particle diameter 25—37 μm) (Whatman, Maidstone, Great Britain) was installed in series in front of the main analytical column to protect it from contamination and was changed routinely if the back pressure in the system increased. The precolumn appeared to have no effect on the chromatographic separation or resolution and was used merely to remove endogenous material from the extracted samples. The mobile phase consisted of 50% acetonitrile in aqueous potassium dihydrogen orthophosphate (0.5%, w/v) with the final pH adjusted to pH 4 with phosphoric acid. A flow-rate of 2 ml/min was maintained.

Fig. 2 illustrates the separation of naftidrofuryl from the internal standard with retention times of 3.5 min and 2.8 min, respectively, and a total analysis time of 10 min.

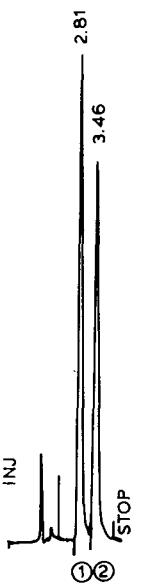

Fig. 2. Chromatogram of reference standards. Peaks: 1 = internal standard (LS 140); 2 = naftidrofuryl. Conditions: column, μBondapak C_{18} (30 cm × 4 mm I.D.); mobile phase, 50% acetonitrile in aqueous potassium dihydrogen orthophosphate (0.5%, w/v) pH 4; flow-rate, 2 ml/min; UV detection at 222 nm with 1 V output to 3380A integrator.

Plasma samples

The method of analysis was applied to plasma samples obtained from two male volunteer subjects after each had received an oral dose of 100 mg of naftidrofuryl oxalate contained in a capsule (batch No. 22.267; Lipha). The conditions of the volunteer studies were similar to those described by Brodie et al. [10].

RESULTS AND DISCUSSION

Concentrations of naftidrofuryl (as oxalate salt) were calculated from calibration lines constructed by plotting peak area ratios of drug to internal standard over the concentration range 20—300 ng/ml naftidrofuryl in plasma. Extraction and measurement at each concentration were repeated on five occasions and peak area ratios showed a coefficient of variation of ± 5% at 20 ng/ml and ± 3% at 300 ng/ml, indicating a good precision for the measurement of naftidrofuryl in plasma.

The calibration line was linear ($y = -0.0187 + 0.0095\ x$, correlation coefficient $r = 0.9987$) and where the value of the intercept was not significantly different from zero ($P > 0.05$). The equation of the line forced through the origin was $y = 0.0094$ (± 0.0001 S.D.)$\cdot x$, where y is the peak area ratio and x is the concentration of naftidrofuryl (ng/ml) present. The accuracy of the method defined by 95% confidence limits of the least squares regression line forced through the origin, i.e. taking the calibration line as an estimate of the concentration of naftidrofuryl in plasma, was ± 57.5% at 20 ng/ml, ± 8.7% at 134 ng/ml and ± 4.1% at 300 ng/ml. The recovery of internal standard from plasma was 92% ± 4 S.D. ($n = 5$). The mean overall recovery of naftidrofuryl from plasma (95% ± 5 S.D., $n = 25$) over the concentration range 20—300 ng/ml was calculated by comparing peak area ratio measurements of non-extracted standards to those of extracted standards corrected for recovery of internal standard (Table I).

No interfering peaks were present in the predose (control) plasma (Fig. 3). The limit of detection based on the instrumental parameters used was 5 ng naftidrofuryl per ml with an integrator slope sensitivity setting of 3 mV/min. Since naftidrofuryl is fluorescent (excitation λ_{max} 286 nm and emission λ_{max} 326 nm), it should be possible to improve the sensitivity of the assay using the appropriate fluorescence detector.

Concentrations of naftidrofuryl are reported as the oxalate salt, concentrations of the free base can be determined by allowing for molecular weight differences (conc. of free base = 0.81 × conc. of oxalate salt). When applied

TABLE I

PRECISION OF THE METHOD AND RECOVERIES OF NAFTIDROFURYL FROM PLASMA

Concentration of naftidrofuryl added to plasma (ng/ml)	Coefficient of variation (%) ($n = 5$)	Recovery (%)
20	5	96
50	5	94
100	3	92
200	3	94
300	3	97
mean		95 ± 5 S.D.

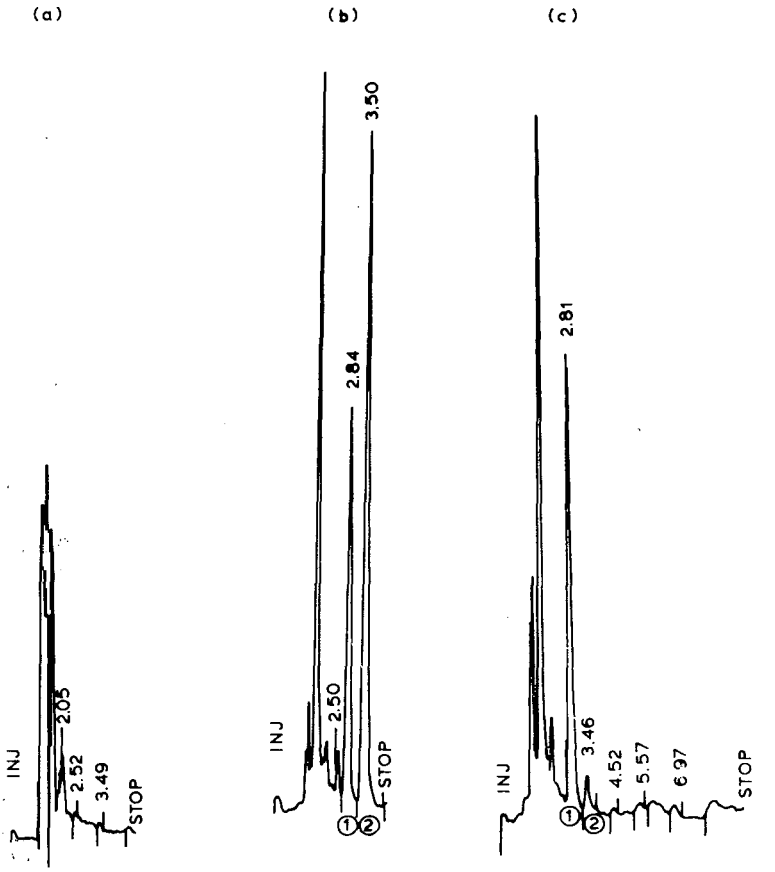

Fig. 3. (a) Predose (control) plasma extract; (b) 0.5 h postdose plasma extract containing 194 ng/ml naftidrofuryl; (c) 5 h postdose plasma extract containing 11 ng/ml naftidrofuryl. Conditions as for Fig. 2; peaks: 1 = internal standard; 2 = naftidrofuryl.

to the collected samples, the method showed that peak concentrations of naftidrofuryl occurred at 0.75 and 0.5 h after dosing (215 ng/ml and 212 ng/ml in Subjects 1 and 2, respectively) and were below the limit of detection (5 ng/ml) at 7 h after dosing (Table II). The descending portion of the concentration—time curves appeared to be composed of at least two linear sections, presumably associated with distribution and elimination phases, respectively. The half-life of the terminal linear section was approximately 1.2 h (Fig. 4).

The specificity of the method was tested by collection of the effluent corresponding to the naftidrofuryl peak which was then extracted and subjected to mass spectrometry. The mass spectrum obtained by this procedure was identical to that obtained with authentic reference material showing a molecular ion at *m/e* 383 and fragments at *m/e* values of 368, 267, 141, 99 and 86 present in similar proportions in both spectra.

TABLE II

CONCENTRATIONS OF NAFTIDROFURYL (AS THE OXALATE SALT) IN THE PLASMA OF HUMAN SUBJECTS AFTER SINGLE ORAL DOSES OF 100 mg NAFTIDROFURYL

Time after dosing (h)	Concentrations of naftidrofuryl (ng/ml)		
	Subject 1	Subject 2	Mean
0.5	194	212	203
0.75	215	135	175
1.0	190	79	135
1.5	94	56	75
2.0	68	33	51
2.5	40	25	33
3.0	35	16	26
4.0	19	10	15
5.0	11	5	8
7.0	<5	<5	<5

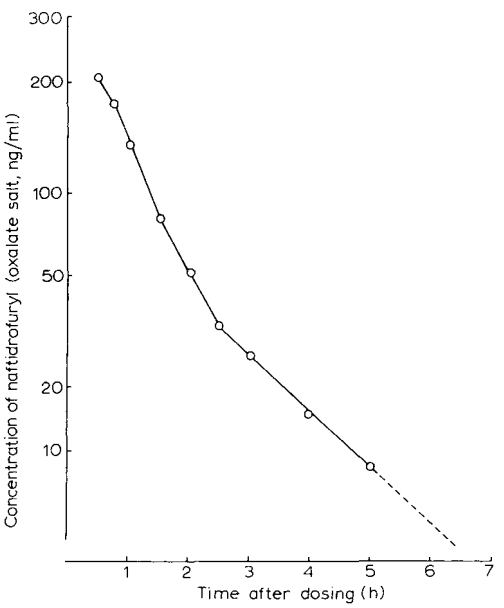

Fig. 4. Semi-logarithmic plot of mean plasma concentrations of naftidrofuryl with time after an oral dose of 100 mg to two human subjects.

ACKNOWLEDGEMENTS

We are grateful to Dr. A. Meynaud, Lipha, Lyon, France, for his help, advice and encouragement. We also thank Miss A. Roberts for her technical assistance and Mr. S.R. Biggs for performing the mass spectrometry.

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CHROMBIO. 415

Note

High-performance liquid chromatographic determination of glipizide and some other sulfonylurea drugs in serum

ELISABETH WÅHLIN-BOLL and ARNE MELANDER

Department of Clinical Pharmacology (Malmö General Hospital), University of Lund, and Department of Community Care Sciences, Dalby; Malmö and Dalby (Sweden)

(Received May 16th, 1979)

Sulfonylurea drugs are widely used in the treatment of diabetes mellitus of the maturity-onset character. The first sulfonylurea generation, comprising compounds such as tolbutamide and chlorpropamide, is being gradually replaced by second-generation sulfonylureas, such as glibenclamide and glipizide, which offer a markedly enhanced potency without a corresponding increase in toxicity. Sensitive and selective chemical methods for the determination of blood concentrations of tolbutamide and chlorpropamide are available and have been applied in clinical studies [1—3]. For the second-generation drugs, however, no such methods have been developed so far.

The present report describes a high-performance liquid-chromatographic (HPLC) technique for the measurement of glipizide concentrations in blood. The method also permits the determination of the blood concentrations of tolbutamide, chlorpropamide and glibenclamide. Glibornuride is used as internal standard in the assay of glipizide and glibenclamide. Chlorpropamide serves as internal standard for tolbutamide and vice versa.

MATERIALS AND METHODS

The technique described below is the finally adopted method for the measurement of glipizide concentrations in serum. The influence of variations in extraction media, pH, internal standards, and UV absorption wavelengths are described under Results and discussion, and so are the techniques for measurement of glibenclamide, tolbutamide and chlorpropamide.

Apparatus

A Waters Model 6000 pump, equipped with a U6K injector and a Varian Vari-Chrom UV—Vis detector were used.

Column

μBondapak C_{18} columns (0.3 m × 3.9 mm I.D.; 10-μm particles) were obtained prepacked from Waters Associates, Göteborg, Sweden.

Chemicals

Mixtures of methanol and phosphate buffers were used for elution. Degassing was carried out by sonication for 15 min. All reagents employed were of analytical grade and were used without further purification. Glipizide (Mindiab®) and two glipizide metabolites, the 3-cishydroxycyclohexyl and the 4-transhydroxycyclohexyl derivatives were kindly supplied by Dr. Tosolini, Istituto Carlo Erba per Ricerche Terapeutiche, Milan, Italy. Glibenclamide (Euglucon®) and tolbutamide (Artosin®) were gifts from Dr. V. Hrtska, Boehringer-Mannheim, Mannheim, G.F.R. Glibornuride (Glutril®, Hoffman-LaRoche & Co., Basle, Switzerland) was used as internal standard. Chlorpropamide (Diabinese®) was obtained from Pfizer Corp., Groton, Conn., U.S.A. Standard solutions were made in methanol and were found to be stable for at least 3 months when kept refrigerated.

Extraction procedure

Eight hundred nanograms of glibornuride (internal standard) and 1 ml of HCl (0.05 mol/l) were added to 0.5 ml of serum, resulting in a pH of about 3. Extraction was made with 3 ml of benzene, during gentle automated shaking for 10 min. After centrifugation, the organic phase was transferred to a conical tube and evaporated to dryness in a water-bath at 45° under a stream of air. The extract was re-dissolved in 50 μl of methanol. An aliquot of 20 μl was injected into the chromatograph.

Blood samples

Venous blood samples were obtained from patients on medication with glipizide, glibenclamide, tolbutamide or chlorpropamide. Serum was prepared by centrifugation, and was stored at −20° until assayed. For the initial method development, and for making serum standards, known amounts of the different drugs were added to drug-free serum.

RESULTS AND DISCUSSION

Extraction media

The following extraction media were tried: hexane, diethyl ether, toluene, chloroform, benzene, methylene chloride, ethylene dichloride, butanol, and hexane—diethyl ether (1:1). Of these, only benzene was found suitable, as the others either extracted glipizide badly or yielded interfering peaks from their dried residues.

Influence of pH on glipizide yield

Glipizide is a weak acid with a pK_a of 5.94 [4]. As it was the aim to keep the drug undissociated to the largest possible degree, a pH of 3.0—3.5 seemed appropriate for the extraction. It was found that the yield was maintained at about 84% up to pH 5. At pH 6 and 7, the yield had fallen to 4%.

Influence of phosphate buffer on glipizide capacity factor

Changes in the amount of phosphate buffer in the mobile phase altered the capacity factor for glipizide as shown in Fig. 1. It appeared that a content of 40% phosphate buffer of 0.01 mol/l and a pH of 3.5 would be optimal.

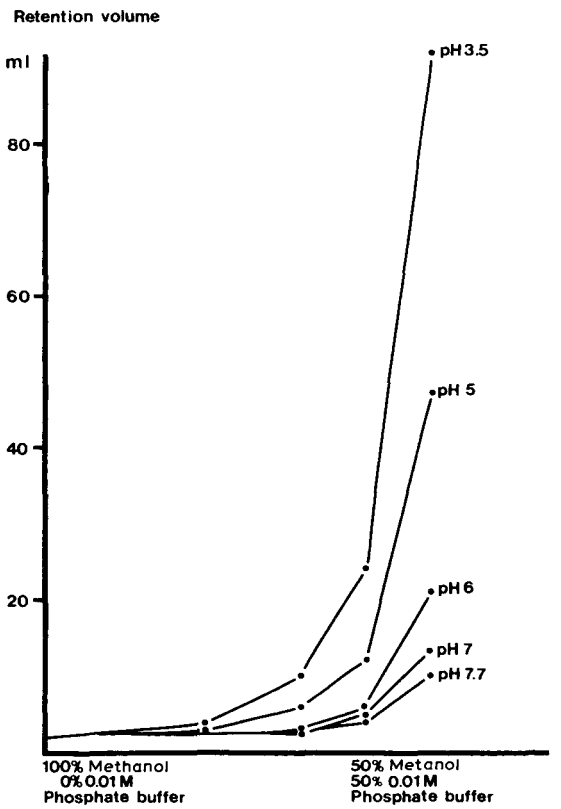

Fig. 1. Influence on the capacity factor for glipizide of amount of phosphate buffer and of pH in the mobile phase.

Detection wavelength and limit

Absorption maxima were 225 nm for glipizide and 275 nm for glibornuride. The molar absorption at the chosen detection wavelength, 225 nm, was found to be 24,000 for glipizide and 16,000 for glibornuride. The detection limit for glipizide was 10 ng/ml, using an injection volume of 20 μl. The sensitivity of the spectrophotometer was kept on 0.02 a.u.f.s. throughout. A typical chromatogram is shown in Fig. 2.

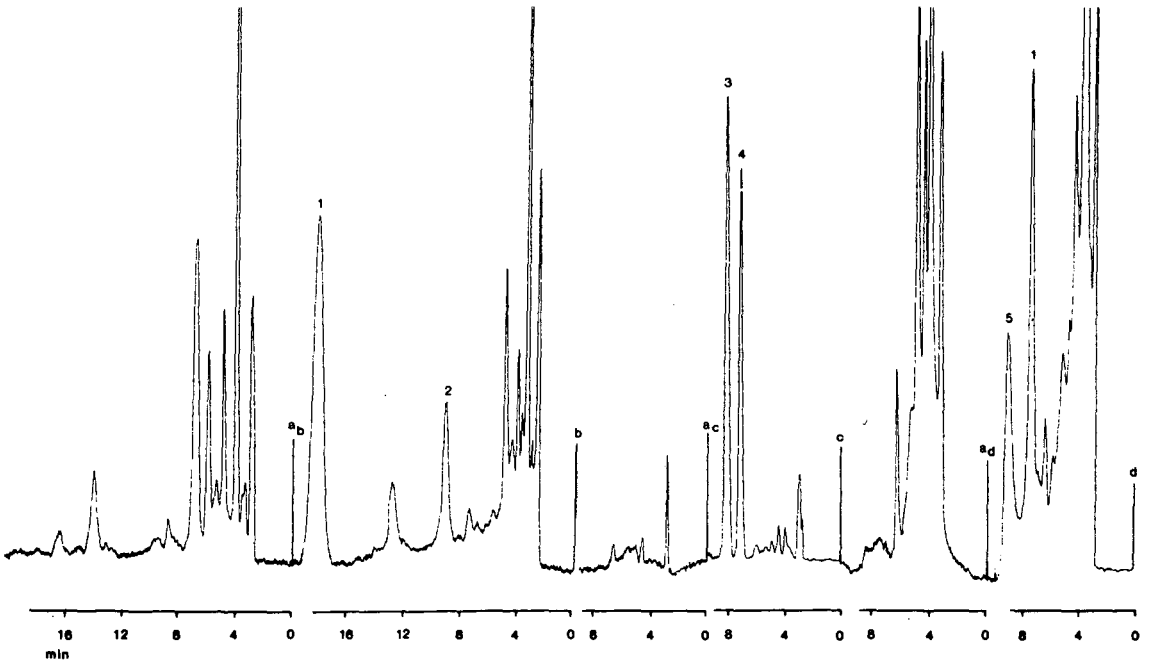

Fig. 2. Chromatograms of serum blanks (a_b, a_c, a_d), and serum from patients being treated with glipizide (b), chlorpropamide (c) and glibenclamide (d). Peaks: 1 = glibornuride, 2 = glipizide, 3 = tolbutamide, 4 = chlorpropamide, 5 = glibenclamide.

Quantitation

The standard curve was linear over a range of 20—1500 ng of glipizide. Analyses of the same sample ten times in the same run yielded a standard deviation of 1.8%. Inter-assay was found to be 6.2% (S.D.) as judged from eleven consecutive assays.

Internal standards

The following compounds were tested as internal standards: tolbutamide, chlorpropamide, glibenclamide, methylglipizide and glibornuride. Of these, only glibornuride and glibenclamide had a retention volume that was not equal to that of any endogenous compound. Efforts to clean up serum were unsuccessful. Under conditions optimal for glipizide detection, both glibornuride and glibenclamide eluted later than glipizide. Glibornuride eluted closer to glipizide than did glibenclamide and was hence chosen as internal standard. The total retention volume was 18 ml.

Metabolites

Neither of the two glipizide metabolites found in human plasma — the 3-*cis*-hydroxycyclohexyl derivative and the 4-*trans*-hydroxycyclohexyl de-

rivative — interfered with the parent drug in the chromatogram; both eluted with the serum front.

Glibenclamide

Glibenclamide was extracted with benzene after acidification to pH 3. The mobile phase was 30% 0.01 *M* phosphate buffer (pH 3.5) in 70% methanol. Glibornuride served as internal standard.

Tolbutamide and chlorpropamide

The therapeutic serum concentrations of these two sulfonylureas are in the range of several μg/ml, while those of glipizide apparently are in the ng/ml range, i.e. 1000-fold less. This made it possible to use the glipizide extraction procedure also in the determination of tolbutamide and chlorpropamide in serum, even though the two compounds could not be used as internal standards. Indeed, this method was found to be very sensitive and rapid and to employ as little as 50 μl of serum. Toluene could be used as extraction medium instead of benzene; however, more serum was then needed, as the dried residue of toluene yielded some slowly eluting peaks that interfered significantly when detector sensitivity was high. Tolbutamide was used as internal standard for chlorpropamide and vice versa.

Interference of biguanides

Sulfonylureas are often used in combination with biguanides, such as metformin or phenformin. It was of particular importance, therefore, to assess whether biguanides would interfere with the determination of glipizide, glibenclamide, tolbutamide or chlorpropamide. No such interference was seen with either metformin or phenformin.

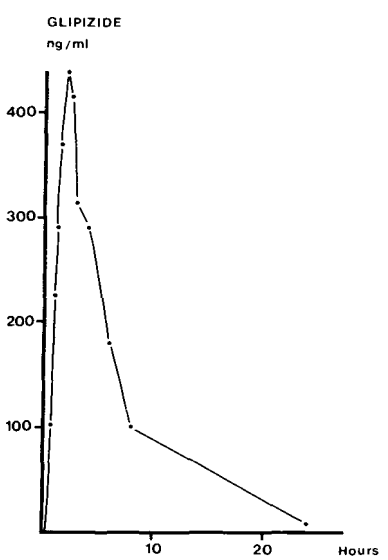

Fig. 3. Serum concentrations of glipizide during a 24-h period after ingestion of a single dose of 5 mg by a subject previously unexposed to the drug.

Glipizide in serum

Fig. 3. shows a glipizide concentration curve in serum from a subject who ingested 5 mg of glipizide. It is seen that the method allows detection of the drug over the whole 24-h period examined.

CONCLUSION

The presented method appears to be sufficiently selective, sensitive and rapid to allow accurate and precise measurements of the serum concentrations of glipizide and some other sulfonylureas during therapeutic conditions.

ACKNOWLEDGEMENTS

The skillful technical assistance of Mrs. Hannelore Claesson is gratefully acknowledged. The study was supported by grants from the Swedish Board of Health and Social Welfare (the Dalby project), from the Swedish Medical Research Council (No. 04X-05657), and from Istituto Carlo Erba per Ricerche Terapeutiche, Milan, Italy.

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Biomedical Applications

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Book Review

Biological and biomedical applications of isoelectric focusing, edited by N. Catsimpoolas and J. Drysdale, Plenum Press, New York, XV + 351 pp., Price US$ 39.00, ISBN 0-306-34603-6.

This is a third volume of a series entitled Biological Separations, edited under the general supervision of Dr. Catsimpoolas. The high standard that has been established in the previous volumes is preserved also in this one. The book offers a well-balanced survey of the status quo of the applications of isoelectric focusing methods to various fields of biomedical separations. Both the editors and the authors of individual contributions have succeeded remarkably well in compiling a book which, without looking at the chapter headings, gives the impression that it has been written by a single author. Individual chapters are devoted to saliva, cerebrospinal fluid and urinary proteins, to the analysis of haemoglobins, allergens, muscle proteins, immunoglobulins, nuclear non-histone proteins, components of the cell membranes, vegetable and microbial proteins and, inevitably, serum proteins.

At first glance one might object that some classes of proteins are not included; but, in fact, those omitted are those which have not yet been subjected to isoelectric focusing, although it is feasible to expect successful results with them. Connective tissue proteins and crystallins are such examples. In any case, this is not a drawback to the book. On the other hand, a more expanded index to the book would be very handy.

The general concept of the volume is similar to others in the Biological Separations series — an overview. Thus, those readers looking for a laboratory handbook are not likely to select this one. For those looking for well and didactically presented information about biomedical applications of isoelectric focusing this volume is a recommended buy.

Prague (Czechoslovakia) ZDENĚK DEYL

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Book Review

Affinity chromatography, edited by O. Hoffmann-Ostenhof, M. Breitenbach, F. Koller, D. Kraft and O. Scheiner, Pergamon, Oxford, New York, 1978, XVI + 374 pp., price US$ 40.00, ISBN 0-08-022632-9.

This book records the proceedings of an International Symposium on Affinity Chromatography held in Vienna, Austria, on September 20—24, 1977.

The texts of the main lectures and the abstracts of short communications (4—6 pages long) are classified into seven parts. The first part, devoted to gel matrices, coupling techniques and charge transfer chromatography, contains the text of the lecture presented by Egly and Porath. Part II, devoted to affinity chromatography of enzymes, is the most extensive one. Very valuable discussions on affinity chromatography of enzymes are presented in terms of the operational capacity of immobilized ligands in the article by Dean and Watson. In the same part of the book, Lowe discusses the use of hydrophobic and hydrophilic spacers in connection with the method of immobilization of coenzymes, while the contribution of Mosbach describes the preparation and the use of immobilized adenine coenzymes both in general ligand affinity chromatography and as active coenzymes. Numerous examples of the successful use of affinity chromatography in enzymology are given in the following sixteen abstracts.

Hydrophobic interaction chromatography is the topic of Part III. The titles of the two main lectures included in this part are: Homologous series of hydrocarbon-coated agarose in hydrophobic chromatography (Shaltiel et al.) and Adsorption of proteins on hydrophobic matrices at high salt concentrations (Påhlman). The abstracts of three short communications complete the abovementioned lectures.

Part IV is devoted to matrix-bound antigens and antibodies. The introductory lecture by Kristiansen is presented under the same title. The wide application of affinity chromatography in immunology is evident from the fourteen abstracts that follow this lecture.

Part V, entitled Matrix-bound lectins and affinity chromatography of cells, commences with an article on fractionation of lymphocytes on insolubilized Helix pomatia A hemagglutinin and wheat germ agglutinin (by Hammarström et al.) and is completed by a number of examples in seven abstracts.

One of the most important applications of affinity chromatography is undoubtedly in the isolation of hormone receptors. This is dealt with in Part VI. The isolation of the receptors of insulin and estrogens, together with

a number of further examples, is the subject of the contribution by Parikh. This lecture is completed with one abstract on the purification of estrogen receptor, based on the use of soluble biospecific macromolecules.

The last part is introduced with an article on Affinity techniques in the isolation of specific polysomes and mRNA: purification of rat albumin mRNA by Taylor et al. It is followed by three abstracts from the field of isolation of DNA, RNA and polysomes. A subject index terminates the book.

The book under review complements other monographs on affinity chromatography, for example Immobilized biochemicals and affinity chromatography (edited by R.B. Dunlap, Plenum, New York, 1974), Affinity chromatography (by C.R. Lowe and P.D.G. Dean, Wiley-Interscience, London, 1974), Affinity techniques (edited by W.B. Jakoby and M. Wilchek, Academic Press, New York, 1974), Affinity chromatography (by J. Turková, Elsevier, Amsterdam, 1978) with some new ideas and also partly overlapping with the compendium of lectures on Chromatography of synthetic and biological polymers, Vol. 2, Hydrophobic, ion exchange and affinity methods (edited by R. Epton, Ellis Horwood, 1978).

Since the aim of the majority of the lecturing authors was principally the presentation of the latest results, the book mainly reviews and summarizes the papers published in 1977. The lectures included in the book are expertly written by the most prominent scientists who develop affinity methods, but they comprize predominantly of data already published since then.

Prague (Czechoslavakia) JAROSLAVA TURKOVÁ

Author Index

Subject Index

GENERAL INFORMATION

(A leaflet *Instructions to Authors* can be obtained by application to the publisher.)

Types of Contributions. The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications*: Regular research papers (Full-length papers), Short communications and Notes. Short communications are preliminary announcements of important new developments and will, whenever possible, be published with maximum speed. Notes are usually descriptions of short investigations and reflect the same quality of research as Full-length papers, but should preferably not exceed four printed pages. For reviews, see page 2 of cover under Submission of Papers.

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1 A. T. James and A. J. P. Martin, *Biochem. J.*, 50 (1952) 679.
2 L. R. Snyder, *Principles of Adsorption Chromatography*, Marcel Dekker, New York, 1968, p. 201.
3 H. C. S. Wood and R. Wrigglesworth, in S. Coffey (Editor), *Rodd's Chemistry of Carbon Compounds, Vol. IV, Heterocyclic Compounds*, Part B, Elsevier, Amsterdam, Oxford. New York, 2nd ed., 1977, Ch. 11, p. 201.
4 E. C. Horning, J.-P. Thenot and M. G. Horning, in A. P. De Leenheer and R. R. Roncucci (Editors), *Proc. 1st Int. Symp. Quantitative Mass Spectrometry in Life Sciences, Ghent, June 16–18, 1976*, Elsevier, Amsterdam, Oxford, New York, 1977, p. 1.

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