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JOURNAL OF CHROMATOGRAPHY BIOMEDICAL APPLICATIONS

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IDENTIFICATION OF METABOLITES DIAGNOSTIC FOR ORGANIC ACIDURIAS BY SIMULTANEOUS DUAL-COLUMN CAPILLARY GAS CHROMATOGRAPHY

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SUMMARY

To offer a procedure with increased resolution compared to packed-column gas chromatography, we developed a dual-capillary method of gas chromatography for diagnosing organic acidurias. We derivatized and injected organic acids repeatedly on two different bonded phase columns (DB-1, DB-1701) to establish a table of methylene units. Compounds in urine specimens were identified by their characteristic pair of methylene units. With this method, we are able to identify 120 metabolites in urine. Accordingly, the procedure provides a cost-effective alternative to routine gas chromatography—mass spectrometry.

INTRODUCTION

Organic acidurias are genetic disorders generally caused by the diminished activity or complete absence of specific enzymes involved in the metabolism of amino acids. These inborn errors of metabolism result in the excretion of specific organic acids in the urine at abnormal concentrations. Some examples of common organic acidurias are isovaleric acidemia, methylmalonic aciduria, maple syrup urine disease, and propionic acidemia [1]. Although clinical symptoms may be present, a definitive diagnosis of an organic aciduria has usually required identification of specific metabolites by gas chromatography—mass spectrometry (GC—MS). In recent years, however, gains in diagnosing organic acidurias have been made through advances in GC alone [2—5].

In the late 1960s, Horning et al. [2] investigated the separation of urinary acids and other compounds of biological interest on OV-1 and OV-17. They found that the thermostability of these packing materials increased the range of

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compounds that could be studied by GC. Methylene unit (MU) values for trimethylsilyl (TMS) derivatives of many biochemically important organic acids were later reported for OV-1 and OV-17 by Butts [3]. The two columns gave a characteristic pair of MU values for a particular compound. Tanaka and coworkers [4, 5] employed the difference in the polarity of these columns to diagnose organic acidurias without the use of GC—MS. The identity of an organic acid was determined by comparing its MU value on OV-1 and OV-17 with the MU values of known organic acids.

Since urine contains as many as several hundred compounds, it is not always possible to adequately resolve and identify the important constituents without GC—MS. Capillary column GC offers an advantage over packed-column GC. The increased number of theoretical plates available with capillary GC greatly increases the resolution of complex matrices, thus increasing the reliability of identification based on MU values. Recently, capillary columns have been employed to separate organic acids. In a limited study, De Jong [6] compared the MU values of 30 normally occurring organic acids separated on a capillary column of OV-1701 to the MU values reported earlier for a packed column of OV-17 [4].

We report here a dual-column GC method employing bonded-phase capillary columns of different polarities to screen for organic acidurias. From repeated injections of TMS derivatives, we prepared a table of reference MU values to identify 120 organic acids of biological interest.

MATERIALS AND METHODS

Reagents

All organic acids and hydrocarbon standards were purchased as free acids or stable salts from commercial vendors. Organic acids, not available commercially, were detected in selected urine samples and identified by GC—MS. Hydroxylamine hydrochloride was purchased from Fisher Scientific (Pittsburgh, PA, U.S.A.). TriSil BSA Formula P was obtained from Pierce (Rockford, IL, U.S.A.). All gases were commercial grade and passed through moisture and oxygen traps (for helium carrier gas only), while all solvents were pesticide grade.

Sample preparation

Samples were prepared by a procedure essentially the same as the procedure of Tanaka et al. [5]. TMS derivatives were formed by the addition of 100 μl of TriSil BSA Formula P to the dried sample and heating at 60°C for 30 min.

Instrumentation

Fused-silica capillary columns, Durabond-1 (DB-1) and Durabond-1701 (DB-1701) (J & W Scientific, Rancho Cordova, CA, U.S.A.), were of 0.25 μm film thickness and 30 m × 0.25 mm I.D. A Hewlett-Packard 5880A gas chromatograph (Hewlett-Packard, Avondale, PA, U.S.A.) was equipped with dual flame ionization detectors, electrometers, and data terminals. An HP 7671A autosampler injected 1 μl of sample. The inlet liner was packed with 10% OV-1 on Chromosorb W HP 80—100 mesh (Alltech Assoc., Deerfield, IL, U.S.A.) and

split the injected sample 1:50 (column flow/split inlet flow). Carrier gas flow-rate (helium) at 50°C was set at 1 ml/min. Make-up gas (helium) and hydrogen flow-rates were each set at 30 ml/min. Air flow-rate at the flame-ionization detector was set at 300 ml/min. After a 1-min delay, the oven temperature was programmed from 50°C to 270°C at 8°C/min. The final temperature was held for 5 min followed by a post-run oven temperature of 280°C for 10 min. The injector and detectors were maintained at 250°C and 330°C, respectively. This configuration permitted automated simultaneous analysis of a series of samples on two capillary columns.

The packed columns were 10% OV-1 on 100—120 mesh Gas-Chrom Q (Alltech Assoc.) and 10% OV-17 (10% SP-2250 on 100—120 mesh Supelcoport, Supelco, Bellefonte, PA, U.S.A.). Both silane-treated glass columns (1.88 m × 2 mm I.D.) were installed in a Model 3700 gas chromatograph (Varian, Palo Alto, CA, U.S.A.) equipped with dual flame ionization detectors and a single electrometer. After a 1-min delay, the oven temperature was programmed at 8°C/min from 70°C to 290°C, while the injector and detector were maintained at 250°C and 300°C, respectively. The carrier gas (helium) was set at a flow-rate of 30 ml/min. A 1-μl aliquot of the derivatized sample was injected manually.

Formulation of the methylene unit table

Oximes of the keto acid standards were formed as described [5]. The internal standard solution (pentadecanoic and capric acid 1 mg/ml in ethyl acetate) was added to each organic acid standard prior to derivatization. A sufficient amount of each organic acid was used to enable 1/4 to full scale deflection upon derivatization and injection. An on-line computer calculated methylene units by linear interpolation based on the retention times of even and odd hydrocarbon standards (C_{10} to C_{25}) for the DB-1 and DB-1701 columns, respectively.

Gas chromatography—mass spectrometry

Unknown peaks present in urine samples were identified with an HP 5985B gas chromatograph—mass spectrometer. The system consisted of an HP 5840 GC and HP 1000 minicomputer and disk drives containing a user-built library of organic acids. Methylene units were computed by a user-written program.

RESULTS

Methylene unit table

The MU values for 120 compounds chromatographed on DB-1 and DB-1701 are listed in Table I. These values represent the mean of three to ten injections on two columns of each bonded phase. There was no significant difference in MU values between the two different columns of the same bonded phase. The small standard deviation of the multiple injections permitted MU windows for identification of ± 0.03. In contrast, the packed-column method in our laboratory required MU windows of ± 0.06. For compounds not commercially available, patient urine specimens were prepared and the MU values of metabolites were computed after identification by GC—MS. These compounds are denoted by an asterisk.

TABLE I

METHYLENE UNITS OF VARIOUS COMPOUNDS ON DB-1 AND DB-1701 CAPILLARY COLUMNS

Compound	DB-1	DB-1701	Compound	DB-1	DB-1701
Lactic acid	10.61	11.16	2-Keto-3-methylvaleric acid (D-oxime)	12.88	13.41
Hexanoic acid	10.69	11.24	Glycerol	12.92	12.61
Glycolic acid	10.73	11.45	Maleic acid	12.96	14.16
Oxalic acid	11.19	12.38	Succinic acid	13.08	14.10
Glyoxylic acid (oxime)	11.20	11.91	Thymol	13.12	13.73
o-Cresol	11.27	11.97	Methylsuccinic acid	13.23	14.18
2-Hydroxybutyric acid	11.29	11.80	Propionylglycine* (I)	13.30	15.83
p-Cresol	11.42	12.16	2-Ketocaproic acid (oxime)	13.31	13.80
3-Hydroxypropionic acid	11.42	12.07	Glyceric acid	13.41	13.89
Pyruvic acid (oxime)	11.48	12.04	Fumaric acid*	13.44	14.38
Dipropylacetic acid	11.56	12.02	Acetylglycine (II)	13.59	15.17
3-Hydroxybutyric acid	11.64	12.15	Glutaric acid	13.91	15.02
3-Hydroxyisobutyric acid*	11.70	12.16	3-Methylglutaric acid	14.16	15.17
2-Hydroxyisovaleric acid	11.71	12.13	Capric acid	14.51	15.09
2-Ketobutyric acid (oxime)	11.88	12.40	Isovalerylglycine (II) (oxime)	14.58	16.26
2-Hydroxyvaleric acid	11.99	12.49	Mandelic acid	14.73	15.77
2-Methyl-3-hydroxy-butyric acid*	12.00	12.48	Adipic acid	14.97	16.12
Acetoacetic acid	12.00	13.10	Malic acid	14.98	15.79
2-Ketoisovaleric acid (oxime)	12.09	12.58	*trans*-3-Dihydro-muconic acid	15.03	16.31
Methylmalonic acid	12.09	13.02	Isovalerylglycine (I) oxime	15.05	17.03
3-Hydroxyisovaleric acid*	12.16	12.58	Salicylic acid	15.07	16.17
Urea	12.30	14.65	4-Phenylbutyric acid	15.10	16.40
2-Ethylhydracrylic acid	12.31	12.81	Pyroglutamic acid	15.10	17.05
Benzoic acid	12.33	13.45	2-Keto-4-methiolbutyric acid (oxime)	15.12	16.19
3-Hydroxyvaleric acid*	12.38	12.86	2-Phenylglycine	15.20	16.11
2-Hydroxyisocaproic acid	12.38	12.84	*trans*-Cinnamic acid	15.26	16.78
Acetoacetic acid	12.40	13.10	3-Methyladipic acid	15.28	16.40
2-Hydroxy-3-methyl-valeric acid	12.47	12.88	Tiglylglycine* (I)	15.41	17.93
2-Ketovaleric acid (oxime)	12.52	13.05	Undecanoic acid	15.49	16.10
Acetylglycine (II)	12.53	15.06	*cis*-Oxalacetic acid	15.53	16.21
Glyoxylic acid	12.59	13.09	*o*-Hydroxyphenyl-acetic acid	15.60	16.81
Octanoic acid	12.63	13.19	*m*-Hydroxybenzoic acid	15.60	16.70
2-Keto-3-methylvaleric acid (L-oxime)	12.70	13.13	2-Hydroxyglutaric acid	15.76	16.66
Phosphoric acid*	12.73	13.90	Phenyllactic acid	15.83	16.89
Ethylmalonic acid	12.77	13.66	Pimelic acid	15.92	17.12
2-Hydroxycaproic acid	12.82	13.30	*m*-Hydroxyphenyl-acetic acid	15.96	17.22
Phenylacetic acid	12.82	14.06	3-Hydroxy-3-methyl-glutaric acid	16.06	16.85
2-Ketoisocaproic acid (oxime)	12.86	13.31	*p*-Hydroxybenzoic acid	16.22	17.43
			2-Ketoglutaric acid (oxime)	16.27	17.20
			p-Hydroxyphenylacetic acid	16.27	17.57

TABLE I (continued)

Compound	DB-1	DB-1701	Compound	DB-1	DB-1701
Phenylpyruvic acid (oxime)	16.30	17.43	*p*-Hydroxyphenylglycine	18.75	19.73
Dodecanoic acid	16.47	17.06	3-Indoleacetic acid	18.80	22.42
Tartaric acid	16.55	17.21	Decanedioic acid	18.84	20.09
Octanedioic acid	16.87	18.10	*p*-Hydroxyphenyllactic acid	19.06	20.10
2-Ketoadipic acid (oxime)	17.07	18.08	*p*-Hydroxyphenylpyruvic acid (oxime)	19.34	20.44
trans-Aconitic acid	17.44	18.72	3,4-Dihydroxymandelic acid	19.36	20.19
cis-Aconitic acid	17.44	18.70	Pentadecanoic acid	19.43	20.04
Orotic acid	17.44	18.47	*o*-Hydroxyhuppuric acid (III)	19.52	21.03
Tridecanoic acid	17.48	18.09	Palmitic acid	20.40	21.02
4-Hydroxy-3-methoxybenzoic acid	17.54	18.94	*o*-Hydroxyhippuric acid (II)	20.50	23.26
4-Hydroxy-3-methoxyphenylacetic acid	17.59	19.12	*trans*-2-Dodecenedioic acid	21.23	22.79
Gentisic acid	17.80	18.82	5-Hydroxyindole-3-acetic acid	21.99	24.01
Nonanedioic acid	17.82	19.08	Linoleic acid	22.01	22.95
Hippuric acid (II)	17.92	19.80	Linolenic acid	22.05	23.09
Hippuric acid (I)	18.05	21.34	Palmitoleic acid	20.16	20.92
2,4-Dihydroxybenzoic acid	18.18	19.21	Oleic acid	22.08	22.85
Homogentisic acid	18.34	19.47	Stearic acid	22.39	23.07
Citric acid	18.35	19.17	Tetradecanedioic acid	22.69	23.99
Isocitric acid*	18.35	19.27	Arachidonic acid	23.59	24.70
Tetradecanoic acid	18.40	19.03	Hexadecanedioic acid	24.77	26.09
Methylcitric acid* (2*R*,3*S*)	18.58	19.25			
Methylcitric acid* (2*S*,3*S*)	18.66	19.34			

*Compounds identified by GC—MS in patient specimens of known organic acidurias.

Column efficiency

To determine if capillary columns provided greater resolution, we calculated several chromatographic parameters for the capillary and packed columns (Table II). The separation number (*TZ*, Trennzahl value) is an approximation of the number of compounds that may be resolved between two consecutive *n*-alkanes of known peak width at one-half peak height. Four to five compounds can be resolved on DB-1 or DB-1701 for each compound resolved on OV-1 or OV-17. Capillary columns had significantly more theoretical plates per unit length and total theoretical plates than the packed columns.

Analysis of selected urine specimens from patients with organic acidurias

We further demonstrated the increased resolution of capillary GC by analyzing some representative patient samples. To verify the value of the MU method for identification, we compared the identities of compounds provided by this procedure with those provided by GC—MS. In the organic acidurias studied, the GC—MS confirmed the identities provided by the MU method.

Propionic acidemia

In propionic acidemia, the absence of propionyl coenzyme A (CoA)

TABLE II

COMPARISON OF COLUMN EFFICIENCY

$$TZ = \frac{t_{R(x+1)} - t_{R(x)}}{W_{h(x+1)} + W_{h(x)}} - 1 \qquad N = 5.54(t_R/W_h)^2$$

$t_{R(x+1)}$ = Retention time of the hydrocarbon peak (C_{13} or C_{18}); $t_{R(x)}$ = retention time of hydrocarbon peak (C_{12} or C_{17}); $W_{h(x+1)}$ = width of hydrocarbon peak at half height (C_{13} or C_{18}); $W_{h(x)}$ = width of hydrocarbon peak at half height (C_{12} or C_{17}); N = the number of plates.

	OV-1	OV-17	DB-1	DB-1701
TZ (C_{12}—C_{13})	6.4	5.9	31.6	30.3
TZ (C_{17}—C_{18})	4.5	4.5	20.5	20.8
Total plates (C_{15})	4330	3670	109,600	110,700
Plates per m (C_{15})	2405	2040	3650	3690

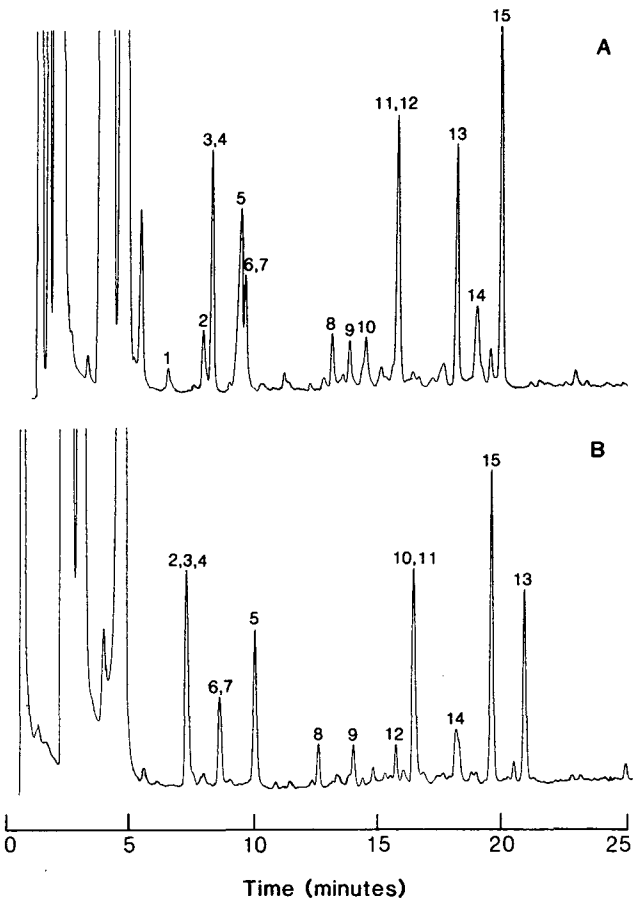

Fig. 1. OV-1 (A) and OV-17 (B) urinary organic acid profiles of a patient with propionic acidemia. Compounds identified are: 1 = lactic acid, 2 = 3-hydroxypropionic acid, 3 = 3-hydroxybutyric acid, 4 = 3-hydroxyisobutyric acid, 5 = urea, 6 = 3-hydroxyvaleric acid, 7 = 2-ethylhydracrylic acid, 8 = capric acid, 9 = adipic acid, 10 = 3-methylcrotonylglycine (I), 11 = *p*-hydroxyphenylacetic acid, 12 = 2-ketoglutaric acid (oxime), 13 = hippuric acid, 14 = methylcitric acid, 15 = pentadecanoic acid. The MU table from Tanaka et al. [4] was used for compound identification.

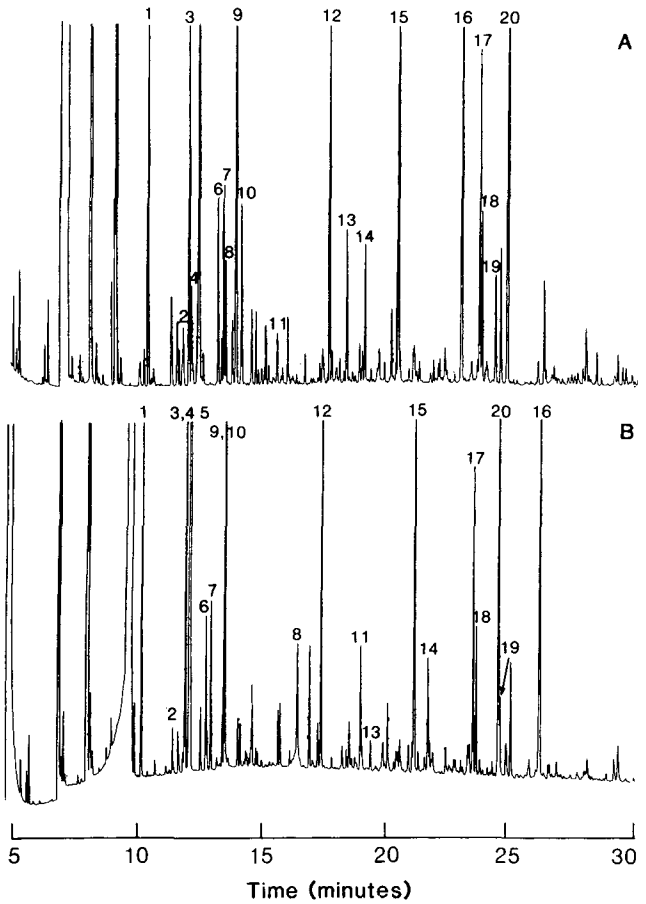

Fig. 2. DB-1 (A) and DB -1701 (B) urinary organic acid profiles of a patient with propionic acidemia. Compounds identified are: 1 = lactic acid, 2 = 2-hydroxybutyric acid, 3 = 3-hydroxypropionic acid, 4 = pyruvic acid (oxime), 5 = 3-hydroxybutyric acid, 6 = 2-hydroxyvaleric acid, 7 = 2-ketoisovaleric acid (oxime), 8 = urea, 9 = 2-hydroxyisocaproic acid, 10 = 2-hydroxy-3-methylvaleric acid, 11 = propionylglycine (I), 12 = capric acid, 13 = adipic acid, 14 = tiglylglycine (I), 15 = *p*-hydroxyphenylacetic acid, 16 = hippuric acid (I), 17 = methylcitric acid (2*R*,3*S*), 18 = methylcitric acid (2*S*,3*S*), 19 = *p*-hydroxyphenyllactic acid, 20 = pentadecanoic acid.

carboxylase prevents the conversion of propionyl CoA to methylmalonyl CoA in leucine and isoleucine metabolism. As a result, several metabolites accumulate in the urine which are useful for diagnosing the disorder. A patient with propionic acidemia was analyzed by the packed-column (Fig. 1) and capillary column (Fig. 2) procedures. As seen in the packed-column chromatograms, several peaks had shoulders indicating the presence of additional compounds. These compounds could not be identified by GC alone because of the peak overlap. Using capillary columns, however, these peaks were well resolved, enabling compound identification.

For example, methylcitrate (the most important metabolite diagnostic for propionic acidemia), was not readily identified by packed column GC and con-

firmation by GC—MS was necessary. The poorly resolved peak on OV-17 did not permit identification of methylcitrate. Identification was not a problem with capillary GC. In fact, the diastereomers of methylcitrate were resolved. In addition, secondary metabolites [propionylglycine (I), tiglyglycine] were identified by capillary GC but not by packed GC.

Maple syrup urine disease

Maple syrup urine disease, also known as branched-chain keto aciduria, results from an enzymatic defect in the degradation of the branched-chain amino acids: leucine, isoleucine, and valine. Fig. 3 shows the capillary chromatograms of a patient with maple syrup urine disease; the presence of lactic, pyruvic, and

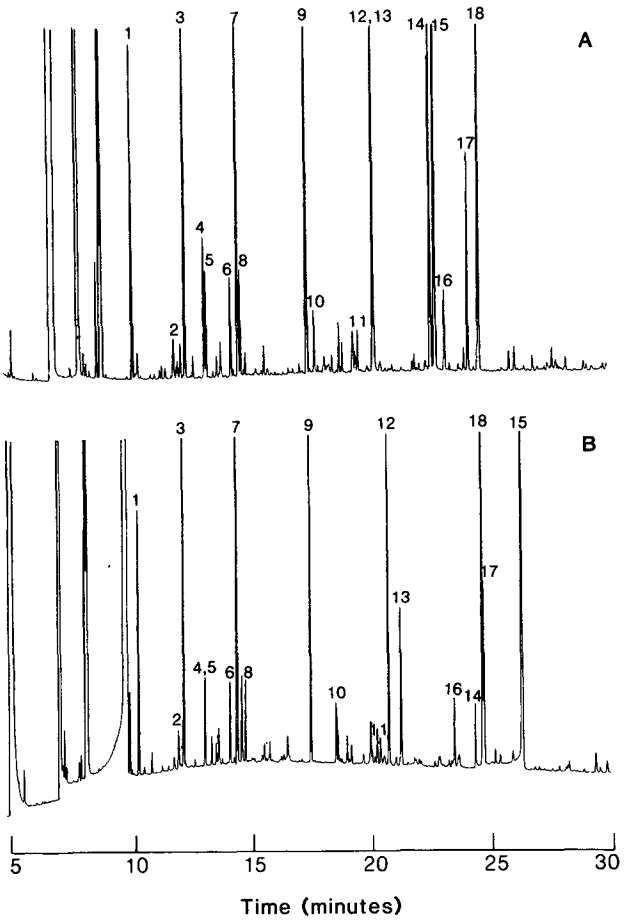

Fig. 3. DB-1 (A) and DB-1701 (B) urinary organic acid profiles of a patient with maple syrup urine disease. Compounds identified are: 1 = lactic acid, 2 = pyruvic acid (oxime), 3 = 2-hydroxyisovaleric acid, 3-hydroxybutyric acid, 4 = 2-ketoisovaleric acid (oxime), 5 = 3-hydroxyisovaleric acid, 6 = 2-keto-3-methylvaleric acid (L-oxime), 7 = 2-ketoisocaproic acid (oxime), 8 = 2-keto-3-methylvaleric acid (D-oxime), 9 = capric acid, 10 = mandelic acid, 11 = phenyllactic acid, 12 = 2-ketoglutaric acid (oxime), 13 = *p*-hydroxyphenylacetic acid, 14 = hippuric acid (II), 15 = hippuric acid (I), 16 = citric acid, 17 = *p*-hydroxyphenyllactic acid, 18 = pentadecanoic acid.

3-hydroxyisovaleric acids also indicates ketoacidosis. Of particular interest is the resolution of 2-ketoisovaleric acid (oxime) and 3-hydroxyisovaleric acid on the DB-1 and the resolution of 2-ketoisocaproic acid (oxime) and 2-keto-3-methylvaleric acid (oxime) on DB-1701. Neither pair of compounds were resolved on the similar packed column phases (not shown).

Isovaleric acidemia

In isovaleric acidemia, a deficiency of isovaleryl-CoA dehydrogenase prevents the conversion of isovaleryl-CoA to methylcrotonyl-CoA. In Fig. 4, the chromatograms from an isovaleric acidemia patient exhibit baseline resolution of the mono-, and di-TMS isovalerylglycines from capric acid (an internal standard). When large amounts of isovalerylglycine were present in the specimens we were unable to resolve isovalerylglycine from the capric acid on packed columns but we were able to on the capillary columns.

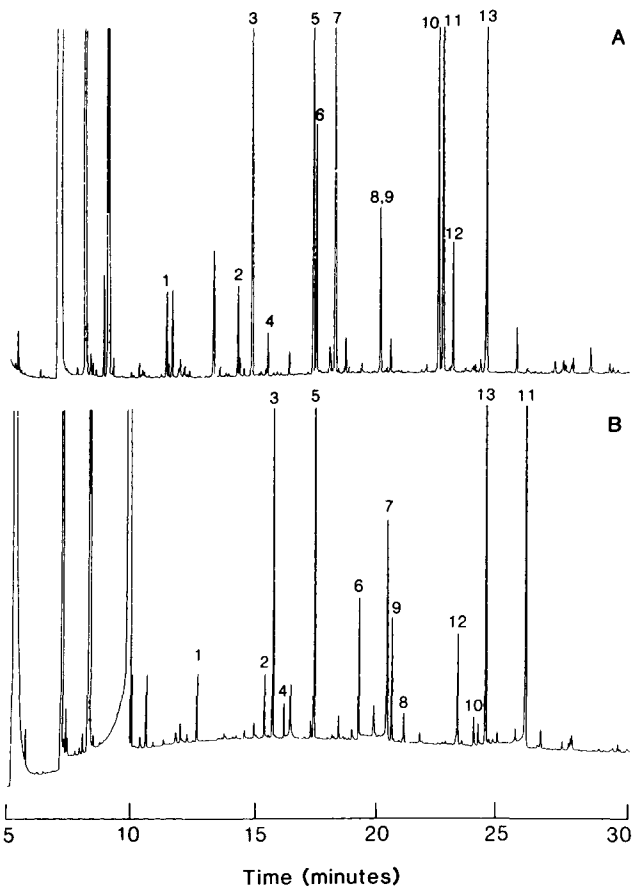

Fig. 4. DB-1 (A) and DB-1701 (B) urinary organic acid profiles of a patient with isovaleric acidemia. Compounds identified are: 1 = oxalic acid, 2 = phosphoric acid, 3 = succinic acid, 4 = fumaric acid, 5 = capric acid, 6 = isovalerylglycine (II), 7 = isovalerylglycine (I), 8 = *p*-hydroxyphenylacetic acid, 9 = 2-ketoglutaric acid (oxime), 10 = hippuric acid (II), 11 = hippuric acid (I), 12 = citric acid, 13 = pentadecanoic acid.

DISCUSSION

In recent years, GC has gained increasing acceptance as an analytical tool for diagnosing organic acidurias [1]. In these inherited disorders, abnormal amounts of specific metabolites accumulate in the urine. Identification of these compounds has routinely required that the gas chromatograph be coupled to a mass spectrometer. In a recent study, Tanaka and coworkers [4, 5] were able to diagnose 23 inherited disorders using GC alone. In their procedure, specimens were chromatographed on two packed columns of different stationary phases. Organic acids were identified according to a specific pair of MU values from the two columns.

We have successfully employed the procedure of Tanaka and coworkers [4, 5] in our laboratory. It has been necessary on a portion of the specimens, however, to confirm identities of poorly resolved compounds by GC—MS. Since publication of this procedure in 1980, bonded-phase fused-silica capillary columns with extraordinary numbers of theoretical plates have been introduced. Bonded phases have also shown a great degree of thermal stability. Considering the problems of resolution encountered with packed columns, we developed a dual-column GC method based on bonded-phase capillary columns.

Using this dual-column capillary method, we established an MU table and found less variability in the MU values of standards such that better identifications were possible. The increased resolution and sensitivity of the capillary columns permitted excellent identification of primary as well as secondary metabolites important in organic acidurias.

These results indicate a significant improvement, compared to packed-column methods, in the separation of constituents from urine when subjected to GC on bonded-phase capillary columns. In our laboratory, this new method has now replaced the procedure using packed columns. We have developed a reliable and accurate method for the diagnosis of organic acidurias and for all practical purposes GC—MS confirmation is not needed for routine study of organic acidurias.

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REFERENCES

1 S.I. Goodman and S.P. Markey, Diagnosis of Organic Acidemias by Gas Chromatography—Mass Spectrometry, Alan R. Liss, New York, 1981.
2 M.G. Horning, E.A. Boucher and A.M. Moss, J. Gas Chromatogr., 6 (1967) 297.
3 W.C. Butts, Anal. Biochem., 46 (1972) 187.
4 K. Tanaka, D.G. Hine, A. West-Dull and T.B. Lynn, Clin. Chem., 26 (1980) 1839.
5 K. Tanaka, A. West-Dull, D.G. Hine, T.B. Lynn and T. Lowe, Clin. Chem., 26 (1980) 1847.
6 A.P.J.M. De Jong, J. Chromatogr., 233 (1982) 297.

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CHROMBIO. 2531

DETERMINATION OF AMINO ACIDS BY SEPARATION OF THEIR ION PAIRS WITH DODECYL SULPHATE

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SUMMARY

The ion pairs of amino acids with dodecyl sulphate were separated on a reversed-phase column (Beckman Ultrasphere™ I.P.) using a sequence of eluents that are prepared by mixing 0.2 *M* phosphoric acid (containing 10 m*M* sodium dodecyl sulphate), 0.2 *M* sodium acetate (pH 4.50; containing 10 m*M* sodium dodecyl sulphate) and methanol. Mixtures of the amino acids commonly occurring in tissues, except taurine and related weakly basic amino acids, can be analysed at a rate of 95 min per sample at a sensitivity of less than 50 pmol per amino acid. Elution modes for specific amino acids (α-difluoromethylornithine, γ-vinyl-4-aminobutyric acid, 4-aminobutyric acid, putreanine) and non-essential amino acids that allow higher separation rates are reported.

The method is suitable for fully automated routine amino acid analysis.

INTRODUCTION

Among the various strategies of amino acid determinations by high-performance liquid chromatography (HPLC), separation of ion pairs of the non-derivatized amino acids seems to be not in much use. In a recent review [1] this possibility was barely mentioned, although the high quality of reversed-phase columns and the successful application of this type of method for the separation of biogenic amines [2—4] is in favour of it. In a study of literature we found only four pertinent publications [5—8] of which only one [8] seemed to give satisfactory separations. In our attempt to adopt this method, the low buffering capacity of the eluent proved disadvantageous.

Therefore, an alternative elution system was established. The method described in this paper has been applied to the determination of the amino acids in tissue extracts.

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EXPERIMENTAL

Chemicals

Common laboratory chemicals, including 2-mercaptoethanol and *o*-phthalaldehyde, were from Merck (Darmstadt, F.R.G.). L-Amino acids for the preparation of standard solutions (in 0.1 *M* perchloric acid) were from Sigma (St. Louis, MO, U.S.A.) and sodium dodecyl sulphate (electrophoresis purity reagent) was from Bio-Rad Labs. (Richmond, CA, U.S.A.).

Apparatus

A Varian Vista 5500 liquid chromatograph equipped with a loop injector valve with a 200-μl loop and a Model 8085 autosampler was used. Separations were performed on a thermostated (25°C) column (Beckman Ultrasphere^{TM} I.P.). This column (25 cm × 4.6 mm I.D.) was filled with 5-μm pellicular material with chemically bonded C_{18} groups. The separation column was protected by a guard column (7 cm × 2.1 mm I.D.) filled with Pellicular ODS (C_{18} groups bonded to 37—53 μm particles; Whatman, Clifton, NJ, U.S.A.). The column eluate (flow-rate 1 ml/min) was mixed in a 1:1 ratio with the *o*-phthalaldehyde—2-mercaptoethanol reagent, and after passing through a PTFE coil (1 m × 0.5 mm I.D.) fluorescence was continuously recorded using a Varian Fluorichrome filter fluorimeter (excitation, 345 nm; emission, 455 nm). This fluorescence detector was equipped with a 12.5-μl flow cell. Signals were usually recorded at two sensitivities (Omniscribe, two-channel pen recorder, Houston Instruments, Gistel, Belgium).

Solvents

Aqueous solutions were prepared using tap water distilled over phosphoric acid. Gradients were prepared by mixing the following three solvents. Solvent A: 0.2 *M* phosphoric acid containing 10 m*M* sodium dodecyl sulphate. Solvent B: 0.2 *M* sodium acetate (pH adjusted to 4.50 with acetic acid) containing 10 m*M* sodium dodecyl sulphate. Solvent C: methanol.

o-Phthalaldehyde—2-mercaptoethanol reagent

The *o*-phthalaldehyde—2-mercaptoethanol reagent was prepared by dissolving 50 g boric acid and 31.5 g sodium hydroxide in 1 l water; 3 ml of Brij-35 solution, 3 ml of 2-mercaptoethanol and a solution of 400 mg of *o*-phthalaldehyde dissolved in 5 ml of methanol were added to the borate buffer. Potassium hydroxide could not be used since potassium dodecyl sulphate is precipitated when mixed with the column eluent.

The reagent was stored in dark bottles and used without further precautions, however, not longer than two days.

Tissue preparation and sample application

Tissue extracts were prepared by homogenisation with 10 vols. of 0.2 *M* perchloric acid [10 ml/g of tissue; tissue amounts ranging from a few milligrams to several grams (whole organs)] and centrifugation. The supernatants were first diluted with the same volume of distilled water. Further dilutions were made with 0.1 *M* perchloric acid. Samples were applied on the column in 0.1 *M*

perchloric acid solution in a volume of up to 200 μl which volume did not significantly influence the separations.

RESULTS

The separation of a complex amino acid mixture (Table I) in the form of ion pairs with dodecyl sulphate, using a reversed-phase column, was achieved by a relatively complicated sequence of solvent mixtures which differed both in pH and methanol content (Table II). Owing to the detection reaction with *o*-phthalaldehyde only amino acids with primary amino groups were included in the standard amino acid mixture.

Fig. 1 shows the separation of a mixture of amino acids and related compounds. Taurine, hypotaurine, cysteic acid and related weakly basic amino acids seem not capable of forming ion pairs with sodium dodecyl sulphate and are therefore eluted close to the solvent front. All other common amino acids listed in Table I are sufficiently well separated, as to allow their quantitative determination. D,L-α-Difluoromethylornithine or norvaline can be used as internal standard.

TABLE I

CODE NUMBER AND CAPACITY FACTOR (k') OF THE AMINO ACIDS AND PEPTIDES SEPARATED UNDER STANDARD ELUTION CONDITIONS

Code No.	Amino acid	k'
1	Taurine, hypotaurine, cysteic acid, phosphoethanolamine	0.4
2	Glutamine	3.6
3	Serine	3.8
4	Aspartic acid	4.3
5	Glutamic acid	4.6
6	Glutathion	5.0
7	Threonine	5.4
8	Glycine	5.7
9	Cystine	6.1
10	α-Alanine	6.2
11	β-Alanine	7.1
12	α-Difluoromethylornithine (DFMO)	8.6
13	4-Aminobutyric acid (GABA)	9.4
14	Tyrosine	10.1
15	Valine	10.6
16	Methionine	11.1
17	Norvaline	12.0
18	Ornithine	13.3
19	Lysine	16.6
20	Histidine	17.9
21	Isoleucine	18.4
22	Phenylalanine	21.0
23	Leucine	21.6
24	γ-Vinyl-GABA (4-aminohex-5-enoic acid; GVG)	22.4
25	Homocarnosine	23.8
26	Carnosine	24.3
27	Tryptophan	24.9
28	Arginine	28.0
29	Putreanine [N^1-(2-carboxyethyl)-1,4-butanediamine]	28.8

TABLE II

COMPOSITION OF THE ELUENT FOR THE SEPARATION OF A COMPLEX AMINO ACID MIXTURE

End time: 80 min; equilibration time: 15 min; flow-rate: 1 ml/min; column temperature: 25° C. For solvent composition see Experimental.

Elution time (min)	Percentage solvent		
	A	B	C
0	63	27	10
10	63	27	10
11	40	60	0
20	40	60	0
21	41	56	3
30	41	56	3
31	35	60	5
57	35	60	5
58	0	75	25

Complete separation was achieved within 80 min. Before the next sample can be applied a 15-min equilibration period with the initial solvent mixture is required.

Samples containing amino acids in concentrations ranging between 200 pmol/ml and 10 nmol/ml were separated on three different days and the chromatograms were evaluated by peak height measurements. Even with this simple evaluation procedure, the standard deviation (S.D.) was $\leqslant \pm 5\%$ of the respective mean value for all samples containing 500 pmol or more per ml, although

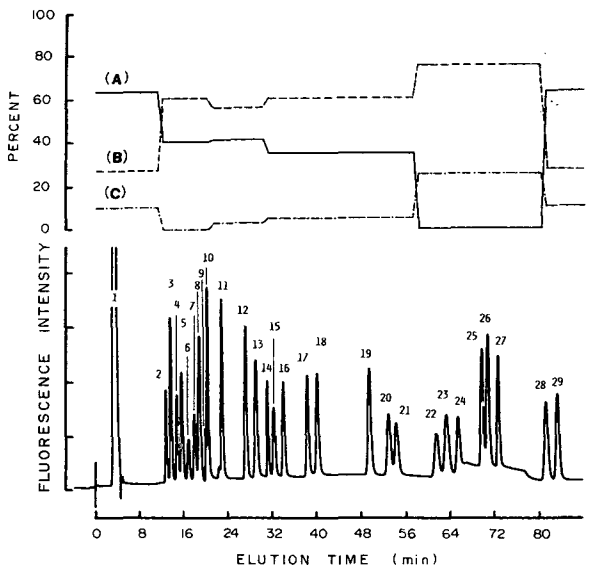

Fig. 1. Gradient composition and separation of the ion pairs with dodecyl sulphate of amino acids (1 nmol) by gradient elution of a reversed-phase column. For the code numbers see Table I; for details of the method see Experimental.

the fluorescence intensity observed after reaction with *o*-phthalaldehyde varies greatly, depending on the structure of the amino acids as can be seen from Fig. 1. Within the mentioned range the relationship between concentration and recorded peak height was linear ($r^2 < 0.99$) for all amino acids.

In Figs. 2 and 3 separations of perchloric acid extracts of mouse brain and liver are shown. They demonstrate the practical applicability of the method. Amino acid concentrations were determined in the brains of CD_1 male albino mice (weighing between 35 and 40 g). The values obtained from these measurements were in good agreement with published data [9, 10], which were obtained by conventional ion-exchange column chromatography. The average standard deviation (for all amino acids) was ± 14.5% of the mean values of a total of nineteen brains. Repeated separations of the same brain extract were performed with a reproducibility indentical to that obtained with standard amino acid mixtures (S.D. ⩽ ± 5% of the mean values; $n = 4$).

If the complete pattern of amino acids is not required, the gradient elution can be interrupted at any point, by changing the solvent composition to 65% B and 35% C so that all following amino acids are rapidly eluted. Shorter runs can

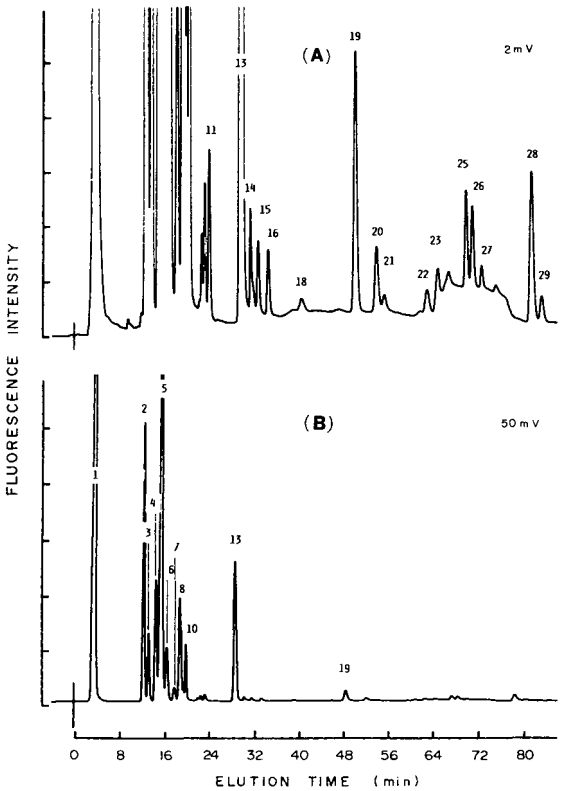

Fig. 2. Separation of the amino acids of a mouse brain extract. A whole brain was homogenised with 10 vols. of 0.2 *M* perchloric acid. The perchloric acid extract was diluted with the same volume of water and a 200-µl aliquot was separated under conditions identical with those shown in Fig. 1. (A) Recording of fluorescence intensity at a recorder sensitivity of 2 mV (full scale); (B) same run as in A, but recorded at 50 mV (full scale). For code numbers see Table I; for details of the method see Experimental.

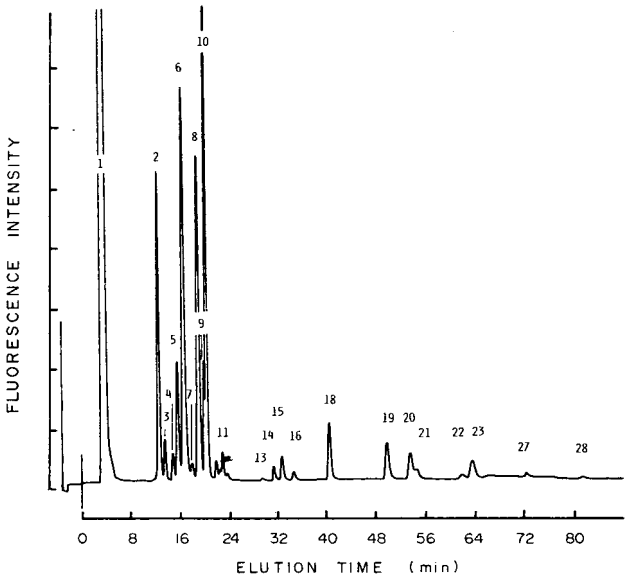

Fig. 3. Separation of the amino acids of a mouse liver extract. The perchloric acid extract was diluted 1:1 with water (see Fig. 2). Separation conditions are the same as those in Fig. 1, recorder sensitivity 50 mV (full scale). For code numbers see Table I.

be achieved by elution programmes which are designed for specific separations.

The determination of the non-essential amino acids is a very frequent requirement. They can be separated by isocratic elution with the following solvent mixture: A = 63%; B = 27%; C = 10%. Glutamine, serine, aspartate glutamate, glutathion, threonine, glycine and with somewhat lower sensitivity β-alanine, GABA and α-alanine can be determined in tissue extracts within 35 min with this elution mode (Fig. 4). Because of their low concentration in tissues amino acids with a capacity factor higher than that of alanine do normally not influence significantly the next separations.

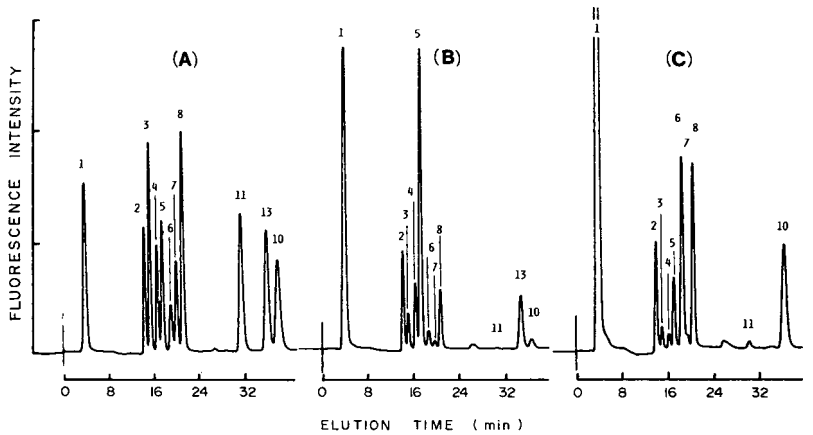

Fig. 4. Isocratic separation of the non-essential amino acids. (A) Standard amino acid mixture; (B) perchloric acid mouse brain extract (1:19 dilution); (C) perchloric acid mouse liver extract (1:19 dilution). Solvent: A = 63%; B = 27%; C = 10%; flow-rate 1 ml/min. For details of the method see Experimental and Results; for code numbers see Table I.

TABLE III

COMPOSITION OF THE ELUENT FOR SENSITIVE GABA DETERMINATION

End time: 25 min; equilibration time: 10 min; flow-rate: 1 ml/min; column temperature: 25°C. For solvent composition see Experimental.

Elution time (min)	Percentage solvent	
	B	C
0	97	3
8	97	3
9	65	35

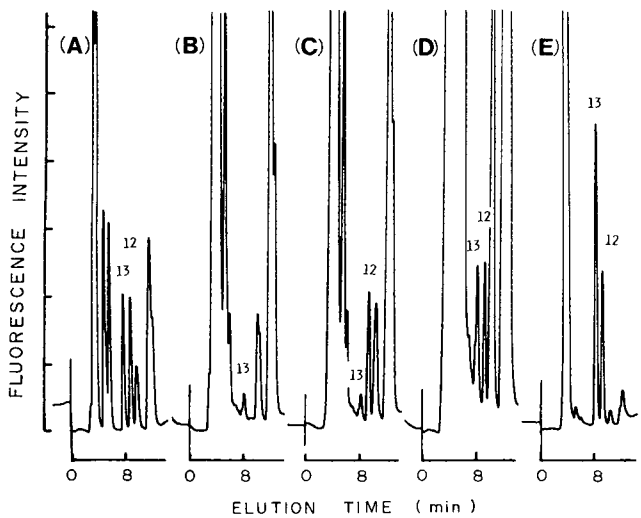

Fig. 5. Elution mode suited for the sensitve determination of GABA and α-difluoromethylornithine. Separation is achieved by elution for 8 min with a mixture of 97% B and 3% C. The more polar amino acids are eluted with 65% B and 35% C. (A) Complete standard amino acid mixture (see Table I); (B) perchloric acid mouse liver extract (1:9 dilution); (C) perchloric acid liver extract (1:9 dilution) with 1 nmol/ml α-difluoromethylornithine; (D) perchloric acid liver extract (1:1 dilution) with 1 nmol/ml α-difluoromethylornithine; (E) mouse brain extract (1:99 dilution) with 1 nmol/ml α-difluoromethylornithine. For details of the separation method see Experimental and Results; for code numbers see Table I.

Owing to peak broadening at isocratic elution mode GABA determinations are of restricted sensitivity with the above-mentioned system. The determination of GABA in amounts smaller than 50 pmol is possible using the binary system described in Table III. In Fig. 5, separations of a brain extract and liver extracts are shown. α-Difluoromethylornithine, the most widely used irreversible inhibitor of ornithine decarboxylase [11], can advantageously be used as internal standard, or, alternatively, the method can be used for the sensitive determination of this drug. The GABA peak in a 1:9 dilution of a mouse liver extract corresponds to about 40 pmol, the α-difluoromethylornithine peak to 200 pmol (Fig. 5). One separation including equilibration with the initial eluent requires 35 min.

TABLE IV

COMPOSITION OF THE ELUENT FOR SENSITIVE DETERMINATION OF PUTREANINE

End time: 40 min; equilibration time: 10 min; flow-rate: 1 ml/min; column temperature: 25°C. For solvent composition see Experimental.

Elution time (min)	Percentage solvent		
	A	B	C
0	33	58	9
18	33	58	9
19	0	65	35

Putreanine is an amino acid uniquely occurring in brain [12]. The gradient described in Table IV is suitable for its complete separation from all other components of extract from brains of mammals and birds. In adult mouse brain 22 ± 3 nmol/g (n = 19), in the brain of a one-day-old chicken 4.3 ± 0.7 nmol/g (n = 4) of putreanine was determined. Using an ion-exchange chromatographic method [13] 17.9 ± 1.9 nmol/g putreanine was found in mouse brain. The same elution mode can also be used for the determination of arginine, and in tissue extracts for ornithine and lysine (Fig. 6). In urine these latter two amino acids were co-migrating with other, not identified components.

γ-Vinyl-GABA is an irreversible inhibitor of 4-aminobutyrate: 2-oxoglutarat aminotransferase [14] with anticonvulsant properties [15]. It is presently in clinical trial as antiepileptic drug and has also great importance as a tool in the study of the GABA neuronal systems. Although it is a close analogue of GABA,

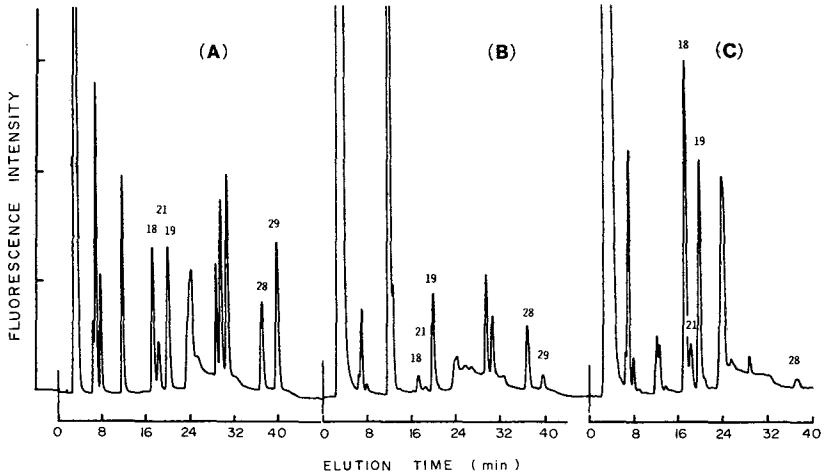

Fig. 6. Elution mode suited for the determination of some basic amino acids. Separation is achieved by a two-step gradient: 0—18 min: A = 33%; B = 56%; C = 11%; 19—35 min: B = 65%; C = 35%. (A) Standard amino acid mixture; (B) perchloric acid mouse brain extract; (C) perchloric acid mouse liver extract. In urine samples ornithine co-migrates with a not yet identified compound. For details of the separation method see Experimental and Results; for code numbers see Table I.

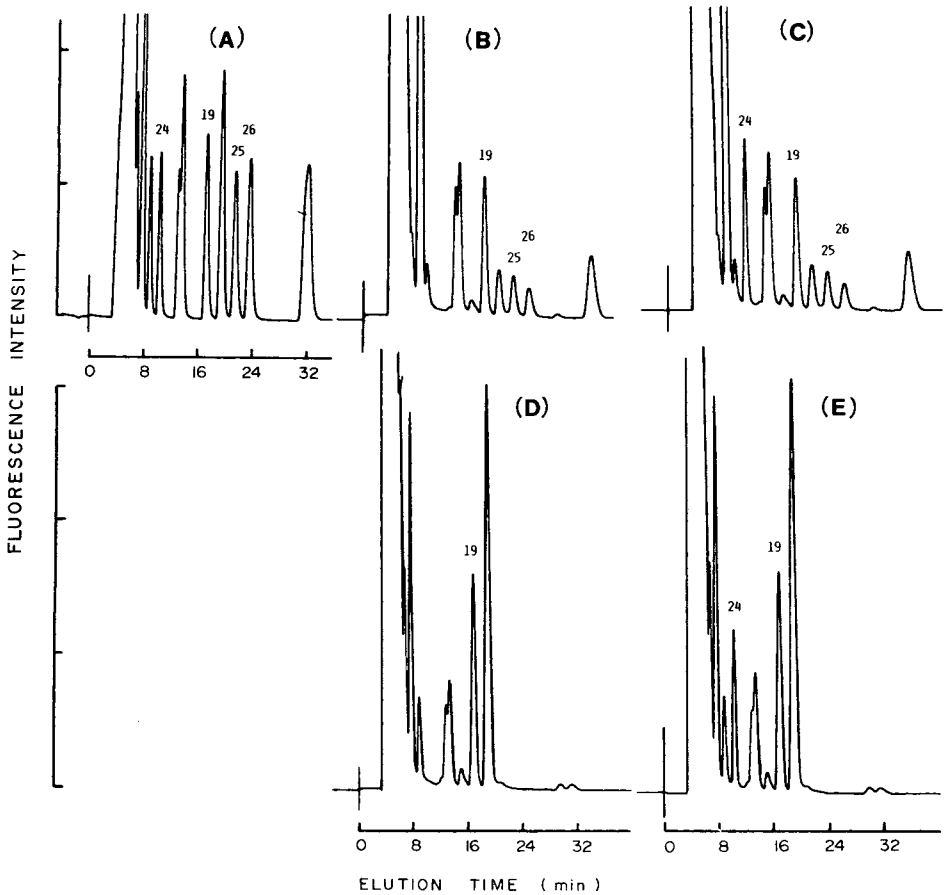

Fig. 7. Elution method suited for the determination of carnosine, homocarnosine, lysine and γ-vinyl GABA. Isocratic elution with a mixture of 70% B and 30% C. (A) Complete standard amino acid mixture (see Table I); (B) perchloric acid mouse brain extract (1:1 dilution with water); (C) same extract as in B with 1 nmol γ-vinyl GABA per 0.1 ml; (D) perchloric acid mouse liver extract (1:1 dilution with water); (E) same extract as in D with 1 nmol γ-vinyl GABA per 0.1 ml. For details of the method see Experimental and Results; for code numbers see Table I.

γ-vinyl-GABA has surprisingly a much higher capacity factor than GABA (Table I). It elutes at a position where, owing to a step in the gradient, the baseline is not flat, so that the standard system is not well suited for its sensitive determination. However, isocratic elution with a mixture of 70% B and 30% C separates γ-vinyl-GABA from all components of brain or liver extracts, as can be seen in Fig. 7. The method allows one to determine about 50 pmol of γ-vinyl-GABA. Lysine, homocarnosine and carnosine are also eluted as uniform peaks and can be determined at the same time, whereas ornithine and histidine and arginine and putreanine, respectively, co-migrate in this system.

DISCUSSION

Methods separating ion pairs of amino acids on reversed-phase columns

resemble in many respects methods employing ion-exchange resin filled columns, with the advantage that the reversed-phase columns do not show volume changes during elution with pH gradients. A practical advantage of both these methods, as compared with methods employing pre-chromatographic derivatization, is the minimal sample preparation and the stability of most amino acids in acidic extracts, which can be directly separated.

The separations described in this paper suggest that our method is a reasonably sensitive and rapid routine method for amino acid determinations in biological materials.

It appears that the retention times of certain amino acids are extremely sensitive to changes of pH and polarity. An example is the difference between the capacity factor of α-alanine in the standard gradient system (Fig. 1) and the isocratic system which is used for the determination of the non-essential amino acids (Fig. 4). While α-alanine elutes in the first system well ahead GABA (k' = 7.1), it appears only after GABA in the second system (k' = 12.1). In contrast, the capacity factor of GABA is similar in the two systems (k' = 9.4 and 11.5, respectively). It is evident from this observation that only devices with very precise pumps, which allow one to form highly reproducible gradients, are suitable for amino acid separations.

In a previous work [8], a simple gradient was pointed out as advantageous. For the separation of complex amino acid mixtures, we needed, however, a complicated sequence of eluents which were prepared by mixing three different components. Admittedly, it is a certain advantage, if a simple device can be used for the solution of a given analytical problem. However, the present state of technical development makes it easy to produce complex gradients in a highly reproducible fashion, so that the usefulness of a separation method is not hampered in principle by a complicated elution pattern. We consider our work as an example of the use of contemporary technical HPLC equipment in an important area. It demonstrates the great practical advantage of gradient formation of three components.

The versatility of the system is demonstrated by determinations of specific amino acids, or groups of amino acids, using simplified gradients and short runs. All examples described in Results have been repeatedly tested or routinely used. Owing to gradual changes of the column characteristics it is necessary to adapt the elution system from time to time by small corrections of the eluent composition.

The retention of the reversed-phase column was sufficiently high for nearly all pertinent amino acid separations. Samples of 200 µl (0.1 *M* perchloric acid) containing acid-soluble material of as much as 9 mg of tissue were routinely applied for the determination of putreanine, without deterioration of the separations.

The sensitivity of the method is limited by the background fluorescence of the *o*-phthalaldehyde—2-mercaptoethanol reagent; the presence of sodium dodecyl sulphate in the eluent prohibits the use of potassium hydroxide for the preparation of the reagent, because of precipitate formation. An improved reagent could increase the sensitivity to the level of methods using precolumn derivatisation with *o*-phthalaldehyde [1].

REFERENCES

1 R.F. Pfeifer and D.W. Hill, Adv. Chromatogr., 22 (1983) 37.
2 J.H. Knox and J. Jurand, J. Chromatogr., 125 (1976) 89.
3 J. Wagner, M. Palfreyman and M. Zraika, J. Chromatogr., 164 (1979) 41.
4 N. Seiler and B. Knödgen, J. Chromatogr., 221 (1980) 227.
5 M.T.W. Hearn and N.Z. Hancock, Med. J., 86 (1977) 352.
6 J.C. Kraak, K.M. Jonker and J.F.K. Huber, J. Chromatogr., 142 (1977) 671.
7 M.K. Radjai and R.T. Hatch, J. Chromatogr., 196 (1980) 319.
8 T. Hayashi, H. Tsuchiya and H. Naruse, J. Chromatogr., 274 (1983) 318.
9 G. Levi, J. Kandera and A. Lajtha, Arch. Biochem., 119 (1967) 303.
10 H.C. Agrawal, J.M. Davis and W.A. Himwich, J. Neurochem., 15 (1968) 917.
11 B.W. Metcalf, P. Bey, C. Danzin, M.J. Jung, P. Casara and J.P. Vevert, J. Amer. Chem. Soc., 100 (1978) 2551.
12 Y. Kakimoto, T. Nakajima, A. Kumon, Y. Matsuoka and I. Sano, J. Biol. Chem., 244 (1969) 6003.
13 N. Seiler, B. Knödgen, G. Bink, S. Sarhan and F. Bolkenius, Adv. Polyamine Res., 4 (1983) 135.
14 M.J. Jung, B. Lippert, B.W. Metcalf, P. Böhlen and P.J. Schechter, J. Neurochem., 29 (1977) 797.
15 P.J. Schechter and Y. Tranier, in N. Seiler, M.J. Jung and J. Koch-Weser (Editors), Enzyme-Activated Irreversible Inhibitors, Elsevier, Amsterdam, 1978, p. 149.

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CHROMBIO. 2533

HIGHLY SENSITIVE ASSAY FOR CHOLINE ACETYLTRANSFERASE ACTIVITY BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

A highly sensitive assay for choline acetyltransferase activity by high-performance liquid chromatography with electrochemical detection was devised. This assay method is based on the separation of acetylcholine and choline on a Develosil Ph-5 reversed-phase column (a phenyl column), followed by their enzymatic conversion to hydrogen peroxide through post-column reaction with acetylcholinesterase and choline oxidase. The sensitivity of the system is excellent and 5 pmol of acetylcholine enzymatically formed could be detected. The linearity between the peak height and the amount of acetylcholine was observed over the range of 5 pmol to 5 nmol. Some enzymatic properties were investigated by using a soluble fraction of bovine caudate nucleus as enyzme. The Michaelis constants of the enzyme for choline and acetyl coenzyme A were 0.3 m*M* and 0.03 m*M*, respectively. The enzyme exhibited the maximum activity over the pH range 7.4—9.5. The regional distribution of choline acetyltransferase activity in rat brain was examined. The order of the activity from the highest to the lowest agreed with the reported brain distribution of the enzyme: striatum, pons plus medulla oblongata, cerebral cortex, thalamus plus hypothalamus, olfactory bulb and cerebellum.

INTRODUCTION

Choline acetyltransferase (acetyl-CoA : choline O-acetyltransferase, E.C. 2.3.1.6; CAT) is the enzyme which catalyses the formation of neurotransmitter acetylcholine (ACh) from acetyl-CoA and choline (Ch). CAT is recognized as a specific marker for cholinergic neurons and is thought to play an important role together with the action of acetylcholinesterase (AChE) in regulating the amount of ACh in the brain. Recently, it was reported that the amount of ACh is decreased significantly in the Meynert nucleus of the patients with senile

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dementia [1]. It is therefore conceivable that CAT activity is much affected in some cerebroneuronal disorders as well as in dementia or ageing.

The measurement of CAT activity has been performed by several methods. These include radiometric assay [2—4], colorimetric assay [5, 6], fluorometric assay [7], and enzyme cycling technique [8]. Because of the absence of a suitable detection system, a simple and sensitive assay of ACh and Ch by high-performance liquid chromatography (HPLC) could not be attained. However, Potter et al. [9] have recently developed a highly sensitive and simple procedure using HPLC with electrochemical detection (ED). The principle of the technique is based on the separation of ACh and Ch on a reversed-phase column, followed by their enzymatic conversion through post-column reaction with AChE and choline oxidase to hydrogen peroxide which is detectable electrochemically by a platinum electrode. This method was found to be simple and reproducible, but ACh was eluted as a somewhat broad and tailed peak owing to the strong adsorption of ACh to the octadecylsilane (ODS) even in the presence of 1.2 m*M* tetramethylammonium (TMA). We found that this problem was solved by employing a phenyl column, which is less hydrophobic than ODS. This modification provided a sharp and symmetrical elution pattern of ACh. Using this improved method, we first describe a highly sensitive and simple HPLC assay for CAT activity in brain tissues.

EXPERIMENTAL

Materials

Acetylcholine chloride, choline chloride, acetyl-CoA, eserine sulphate, AChE type V-S (electric eel), and choline oxidase (*Alcaligenes*) were obtained from Sigma (St. Louis, MO, U.S.A.); TMA chloride and sodium 1-octanesulphonate (SOS) were from Nakarai (Kyoto, Japan); ethylhomocholine (EHC) was a gift from Tokai Irika (Tokyo, Japan). A Develosil Ph-5 packed column (spherical silica chemically bonded with phenyl groups, particle size 5 μm, 250 × 4.6 mm I.D.) for reversed-phase HPLC was obtained from Nomura Chemical (Seto, Japan). A guard column (10 × 4 mm I.D.) was dry-packed with Develosil Ph with particles size of 15—30 μm. All other chemicals used were of analytical grade.

Bovine caudate nucleus was dissected from a fresh brain and stored at −20°C. Rats were decapitated, and the whole brain was dissected on a glass plate over ice into six parts: striatum, pons plus medulla oblongata, cerebral cortex, thalamus plus hypothalamus, olfactory bulb, and cerebellum. The brain tissues were frozen and stored at −20°C.

The enzyme solution for the measurement of CAT activity was prepared from frozen brain tissues by homogenization in 12.5 ml of 25 m*M* sodium phosphate buffer, pH 7.4, per g of wet weight, using a Teflon homogenizer with twenty up and down strokes, followed by centrifugation at 20 000 *g* for 60 min at 4°C. The supernatant obtained was used as an enzyme solution.

A standard mixture of 19.2 μM ACh, 19.2 μM Ch and 23.1 μM EHC in 0.2 *M* perchloric acid was prepared daily from the stock solutions of 0.5 m*M* ACh, 0.5 m*M* Ch and 0.6 m*M* EHC in 0.01 *M* hydrochloric acid which were stored at 4°C.

Assay of CAT activity

The standard incubation mixture consisted of the following components [10] in a total volume of 200 μl (final concentrations in parentheses): 100 μl of substrate solution containing 10 m*M* choline chloride (5 m*M*), 0.4 m*M* acetyl-CoA (0.2 m*M*), 0.2 m*M* eserine sulphate (0.1 m*M*), 0.3 *M* sodium chloride (0.15 *M*), and 20 m*M* EDTA—2Na (10 m*M*) in 0.1 *M* sodium phosphate buffer, pH 7.4 (0.05 *M*), and 100 μl of enzyme solution in 25 m*M* sodium phosphate buffer, pH 7.4.

Incubation was done at 37°C for 20 min, and the reaction was stopped with 50 μl of 1 *M* perchloric acid in an ice-bath. After 10 min, 10 μl of 0.6 m*M* EHC in 0.01 *M* hydrochloric acid as an internal standard were added and the reaction mixture was centrifuged at 1600 *g* for 10 min at 4°C. A 150-μl aliquot of the clean supernatant was taken, and a 10-μl aliquot was injected into the HPLC system. For the control experiments, the enzyme solution was boiled at 95°C for 5 min, or either acetyl-CoA or Ch was omitted from the substrate solution.

The concentration of protein was determined using a Bio-Rad Protein Assay Kit (Bio-Rad Labs., Richmond, CA, U.S.A.) using γ-globulin as a standard.

Chromatographic conditions

Most of the chromatographic conditions used were the same as those reported by Potter et al. [9]. The HPLC system consisted of a PM-30A dual-piston pump, a LC-4B amperometric detector equipped with a TL-10A platinum electrode (Bioanalytical Systems, West Lafayette, IN, U.S.A.), a Rheodyne 7125 injector with a 200-μl sample loop (Berkeley, CA, U.S.A.), a Develosil Ph-15/30 guard column and a Develosil Ph-5 analytical column (250 × 4.6 mm I.D.). A 1-μm filter was placed between the injector and the guard column.

The mobile phase was 0.01 *M* sodium acetate—citrate buffer (pH 5.0) containing 0.4 m*M* TMA and 30 mg/l SOS, which was filtered through a 0.45-μm membrane filter (Toyo Roshi, Tokyo, Japan) and degassed with a water aspirator for a few minutes prior to use. The flow-rate was 0.8 ml/min.

AChE (2 U/ml) and choline oxidase (1 U/ml) in 0.2 *M* potassium phosphate buffer, pH 8.5, were pumped with a Minipuls 2 peristaltic pump (Gilson, Villiers Le Bel, France) at a rate of 0.5 ml/min. This enzyme reagent solution was mixed with the column effluent through the tee placed between the column and a reaction coil (Teflon tubing, 10 m × 0.5 mm I.D.). The enzymatic reaction was performed at room temperature in this coil, which was connected to the amperometric detector cell. The electrode potential was set to +0.5 V against an Ag/AgCl reference electrode for the detection of hydrogen peroxide. Under these conditions the retention times were: solvent front, 6.0 min; Ch, 12.6 min; EHC, 21.0 min; ACh, 25.8 min.

RESULTS

It was found that ACh, Ch and EHC could be measured with very high sensitivity by using the present HPLC—ED method. The linear response of

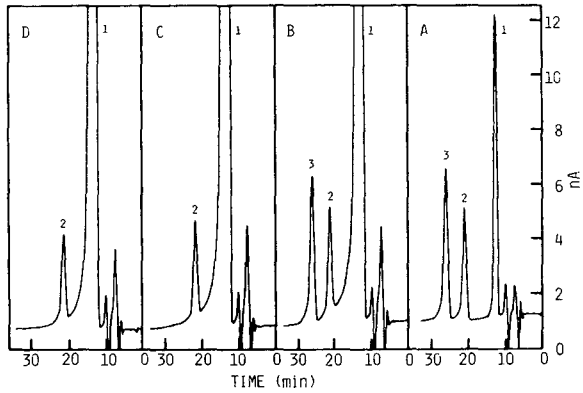

Fig. 1. HPLC elution pattern of the incubation mixtures for choline acetyltransferase with the soluble fraction of bovine caudate nucleus as enzyme. The conditions are described in the experimental section. The incubation mixture contained 40 μl (64 μg of protein) of the soluble fraction as enzyme and 5 m*M* choline and 0.2 m*M* acetyl-CoA as substrates. Internal standard, ethylhomocholine (6.0 nmol), was added in all cases after the enzyme reaction had been stopped by the addition of perchloric acid. (A) Standard samples in 0.2 *M* perchloric acid. Peaks: 1 = choline (192 pmol), 2 = ethylhomocholine (231 pmol), 3 = acetylcholine (192 pmol). (B) Experimental incubation with 40 μl of the soluble fraction of bovine caudate nucleus. (C) Control incubation with an inactivated enzyme. Soluble fraction of bovine caudate nucleus was boiled at 95° C for 5 min. (D) Control incubation without acetyl-CoA.

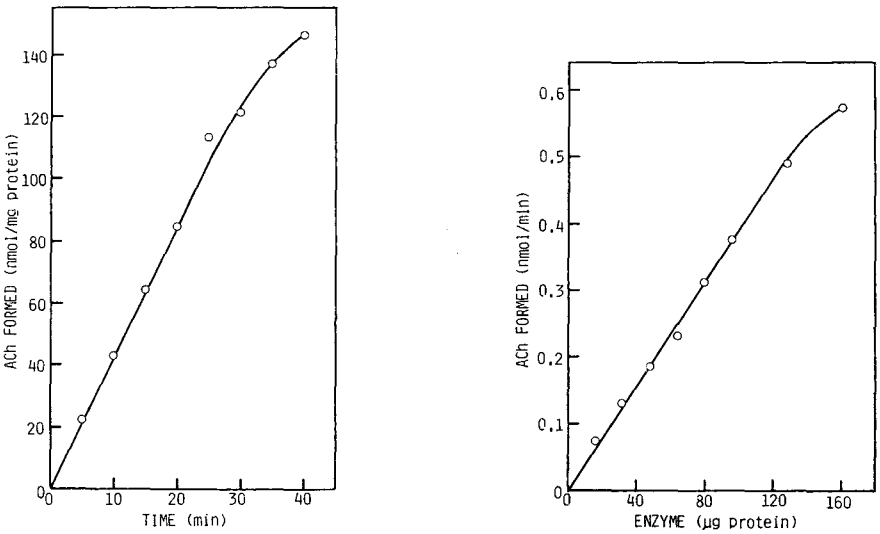

Fig. 2. The rate of acetylcholine formation by choline acetyltransferase using the soluble fraction of bovine caudate nucleus as the enzyme source at 37° C. Standard incubation mixture containing 40 μl (64 μg protein) of the enzyme solution was used as described under Experimental.

Fig. 3. Choline acetyltransferase activity in the soluble fraction of bovine caudate nucleus as a function of enzyme concentration. The standard incubation system was used and incubation was carried out for 20 min at 37° C.

the peak height of the electrochemical detector for the amounts of ACh injected was observed from 5 pmol to 5 nmol.

The chromatographic pattern of the CAT reaction with a soluble fraction of bovine caudate nucleus as enzyme is shown in Fig. 1. Fig. 1A shows the separation of standard samples of ACh, Ch and EHC. It should be noted that ACh is eluted as a sharp and symmetrical peak as those of Ch and EHC. The experimental incubation with 40 μl (64 μg of protein) of the enzyme solution from bovine caudate nucleus (Fig. 1B) shows the significant formation of ACh during the reaction at 37°C for 20 min. In the control incubation, in which the enzyme solution was boiled (Fig. 1C) or the substrate, acetyl-CoA was omitted from the reaction mixture (Fig. 1D), no formation of ACh was observed. These facts clearly indicate that the formation of ACh during the incubation can be ascribed to the enzymatic action of CAT present in the brain tissue.

The rate of ACh formation using the soluble fraction of bovine caudate nucleus as CAT proceeded linearly up to 30 min at 37°C as shown in Fig. 2. Accordingly, we selected 20 min as a standard assay condition. CAT activity as a function of enzyme concentration is shown in Fig. 3. Complete linearity was observed between 16 and 128 μg of protein of the soluble fraction.

The reproducibility of the assay with replicates of the same sample was 100 ± 2.6% (C.V. for seven determinations).

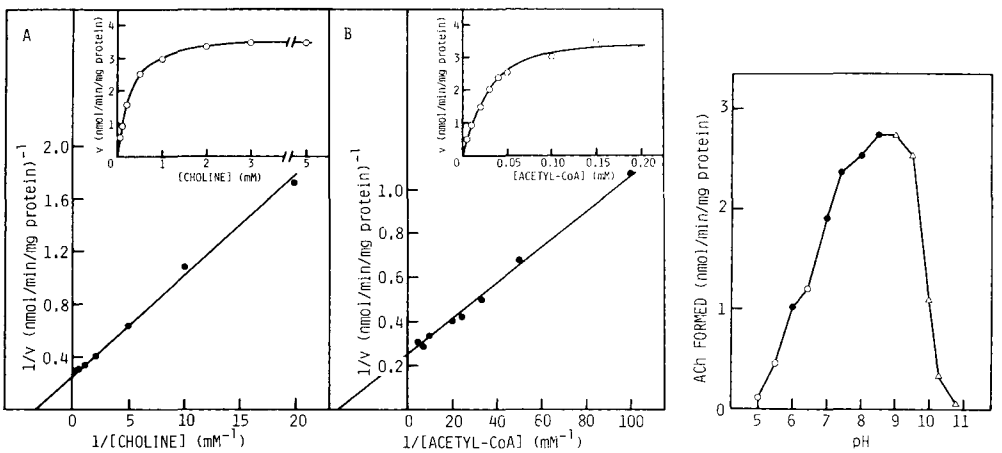

Fig. 4. Lineweaver—Burk and Michaelis—Menten plots for the evaluation of the kinetic parameters of choline acetyltransferase from the bovine caudate nucleus. The main panel shows Lineweaver—Burk plots and the inserted panel shows Michaelis—Menten plots. The standard incubation mixture containing 40 μl (64 μg of protein) of the soluble fraction was used, and incubation was carried out for 20 min at 37°C. (A) The concentration of choline was changed. (B) The concentration of acetyl-CoA was changed.

Fig. 5. Effect of pH on the formation of acetylcholine by choline acetyltransferase in the soluble fraction of bovine caudate nucleus. The incubation mixture consisted of 0.2 *M* buffer solution, 0.15 *M* sodium chloride, 5 m*M* choline chloride, 0.2 m*M* acetyl-CoA, 0.1 m*M* eserine sulphate, 10 m*M* EDTA—2Na, and 40 μl (64 μg of protein) of the soluble fraction as enzyme in a total volume of 200 μl. The incubation was carried out for 20 min at 37°C as described under Experimental. The following buffers were used; (○) sodium acetate buffer; (●) potassium phosphate buffer; (△) glycine buffer.

Fig. 4 shows the Lineweaver—Burk and Michaelis—Menten plots for Ch and acetyl-CoA. It was found that the CAT obeys simple Michaelis—Menten-type kinetics and the Michaelis constants (K_M) were 0.3 m*M* and 0.03 m*M* for Ch and acetyl-CoA, respectively. The same values for the maximum velocity (V_{max}) (230 pmol ACh formed per 20 min per injection; 0.0047 μmol per min per mg protein) were obtained from Fig. 4A and B, respectively.

The pH dependence of CAT activity in bovine caudate nucelus was measured by the present method. As shown in Fig. 5, a broad optimum was observed in the alkaline region and the enzyme exhibited maximum activity over the pH range 7.4—9.5. In the present method, we adopted pH 7.4 for the standard assay condition because it is near the physiological condition and has been used as the optimum condition by many investigators.

We have applied the present method to measure the CAT activity in different regions of rat brain. As shown in Table I, the highest activity was found in the striatum, followed by pons plus medulla oblongata, cerebral cortex, thalamus plus hypothalamus and olfactory bulb; the lowest activity was in the cerebellum.

TABLE I

CHOLINE ACETYLTRANSFERASE (CAT) ACTIVITY IN VARIOUS RAT BRAIN REGIONS

Brain samples were dissected out and processed as described under *Materials*. Results represent mean ± S.E. for a group of four animals. Activity is expressed in pmol of acetylcholine formed per min per mg protein.

Brain region	CAT activity
Striatum	4983 ± 487
Pons plus medulla oblongata	3245 ± 107
Cerebral cortex	2493 ± 134
Thalamus plus hypothalamus	2011 ± 90
Olfactory bulb	1085 ± 76
Cerebellum	249 ± 5

DISCUSSION

An assay method for CAT activity by HPLC—ED has been first reported in this paper. Potter et al. [9] first developed an assay method for ACh and Ch by HPLC—ED in brain tissues of rats sacrificed by microwave irradiation. However, in our experience on their original method using ODS as a column support, ACh was eluted as a broad and tailed peak compared with that of Ch. We examined different column supports, and found that Develosil Ph-5, a phenyl column, was most effective for the separation of ACh, Ch and EHC.

The present assay method for CAT activity has many advantages. First, it is highly sensitive. The limit of sensitivity was about 5 pmol of ACh formed enzymatically. The sensitivity was found to be even higher than that of various radiometric assays, in which the limit is about 100 pmol of ACh. The sensitivity is high enough to allow us to estimate the CAT activity in submilligram samples of brain tissue. Secondly, the procedure is simple and specific.

Deproteinized reaction mixture could be directly analysed by HPLC. Furthermore, there is no need to isolate ACh from the reaction mixture as in the case of radioassay. The specificity is excellent, because the method is based on HPLC—ED and two specific enzymatic reactions. Thirdly, it is very reproducible. Although the detector sensitivity decreases by about 50% after 8—10 h of successive operation of the system, due to possible contamination on the surface of the platinum electrode, it is easy to correct the decreased sensitivity by using the peak height of an internal standard, EHC, and to restore the sensitivity by washing the electrode with methanol. The coefficient of variation (C.V.) of 2.6% for the peak height of ACh was obtained with seven different incubations using the same enzyme solution.

In this study the K_M values of bovine caudate nucleus enzyme for Ch and acetyl-CoA were obtained as 0.3 m*M* and 0.03 m*M*, respectively. These values are in good agreement with those obtained by various radiometric assays (0.4—0.8 m*M* for Ch and 0.01—0.02 m*M* for acetyl-CoA) [11—13]. From the value of V_{max} obtained from Fig. 4, the specific activity of the enzyme in the soluble fraction of bovine caudate nucleus was calculated to be 0.0047 μmol ACh formed per min per mg protein, which is of the same order as the value of 0.0026 reported as the specific activity of the enzyme in the extract from bovine striatum [14].

CAT from bovine caudate nucleus has been reported to be inactive below pH 5.0 and showed a broad pH optimum from 7.5 to 10.0 [12]. A similar pH—activity curve was reported for the enzymes from human brain and placenta [15]. Our data (Fig. 5) agree well with these previous observations. On the other hand, Chao and Wolfgram [16] have demonstrated that the maximum activity was at about pH 7. The reason for this discrepancy is unclear.

From the data on the regional distribution of CAT activity in rat brain, the previous observation was confirmed that the enzyme activity is highest in the striatum and lowest in the cerebellum. It has been shown by immunohistochemical studies using cat brain [17] that cerebral cortex, olfactory bulb, and thalamus plus hypothalamus have no cholinergic cell bodies, although these regions are rich in cholinoceptive neurons. Therefore, the relatively high content of CAT in these regions is thought to be due to the nerve terminals of cholinergic neurons innervated from other parts of the brain.

Taking advantage of the high sensitivity of this method, it would be possible to study the changes in CAT activity in animal models of various diseases, or in human brain tissues from patients at autopsy.

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REFERENCES

1 M.N. Rossor, C. Svendsen, S.P. Hunt, C.Q. Mountjoy, M. Roth and L.L. Iversen, Neurosci. Lett., 28 (1981) 217.
2 L.T. Potter, Methods Enzymol., 17B (1971) 778.

3 B.K. Schrier and L. Shuster, J. Neurochem., 14 (1967) 977.
4 F. Fonnum, J. Neurochem., 24 (1975) 407.
5 L.T. Potter, in P.N. Campbell (Editor), The Interaction of Drugs and Subcellular Components on Animal Cells, Churchill, London, 1968, p. 293.
6 L.P. Chao and F. Wolfgram, Anal. Biochem., 46 (1972) 114.
7 L.B. Hersh, B. Coe and L. Casey, J. Neurochem., 30 (1978) 1077.
8 T. Kato, J. Neurochem., 42 (1984) 903.
9 P.E. Potter, J.L. Meek and N.H. Neff, J. Neurochem., 41 (1983) 188.
10 L.B. Hersh, B.H. Wainer and L.P. Andrews, J. Biol. Chem., 259 (1984) 1253.
11 H.L. White and C.J. Cavallito, Biochim. Biophys. Acta, 206 (1970) 343.
12 V.A.S. Glover and L.T. Potter, J. Neurochem., 18 (1971) 571.
13 H.L. White and J.C. Wu, J. Neurochem., 20 (1973) 297.
14 L.T. Potter and V.A.S. Glover, Methods Enzymol., 17B (1971) 798.
15 R. Roskoski, Jr., C.-T. Lim and L.M. Roskoski, Biochemistry, 14 (1975) 5105.
16 L.P. Chao and F. Wolfgram, J. Neurochem., 20 (1973) 1075.
17 H. Kimura, P.L. McGeer, J.H. Peng and E.G. McGeer, J. Comp. Neurol., 200 (1981) 151.

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MICROANALYSES OF β-CYCLODEXTRIN IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Procedures for the micro-determination of β-cyclodextrin (β-CyD) in plasma were investigated by four methods using high-performance liquid chromatography (HPLC). In methods A and B, underivatized β-CyD was detected with a refractive index detector and determined by the absolute calibration graph method. An NH_2-bonded silica/acetonitrile—water system was used in A and a C_{18}-bonded silica/methanol—water system in B. In method C, the percarbanilate of β-CyD was separated on a C_8-bonded silica column with acetonitrile—water and determined using γ-CyD as the internal standard with a UV detector at 231 nm. In method D, the per[1-^{14}C]acetate of β-CyD was fractionated on a silica column with *n*-hexane—ethanol containing 1% of water and the radioactivity of each fraction was measured with a liquid scintillation counter. γ-CyD was used as the internal standard. Interfering plasma proteins were removed by centrifugal ultrafiltration with an MPS-1 micropartition system. Method B was superior to the other methods with respect to ease of sample preparation, sensitivity and time required for analysis. The cumulative amount of β-CyD in the mesenteric vein absorbed from the rat intestinal lumen after administration of phenobarbital—β-CyD complex in a closed loop method was determined by the use of method B.

INTRODUCTION

β-Cyclodextrin (β-CyD) forms inclusion complexes with barbiturates (BA) [1—5]. The complexation improves solubility of BA and results in increase in gastrointestinal absorption and, consequently, in enhancement of bioavailability of BA [6, 7]. Nevertheless, the mechanism of gastrointestinal absorption of the complexes is still uncertain. Generally, it has been assumed that only free drug coexisting with the drug—β-CyD complex at equilibrium in

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gastrointestinal fluids is absorbed on oral administration of the complex:

Drug + β-CyD ⇌ Drug—β-CyD

In our previous in vitro experiment of absorption using rat everted intestinal sacs [8], however, β-CyD was also detected in serosal fluid, and hence it was suggested that β-CyD may be absorbed from the gastrointestine in the form of a complex or in the intact form.

To establish whether β-CyD can be absorbed from the gastrointestine in in situ or in vivo absorption studies, the development of methods for the micro-determination of β-CyD in plasma is necessary. This paper reports four high-performance liquid chromatographic (HPLC) methods for determining β-CyD in plasma.

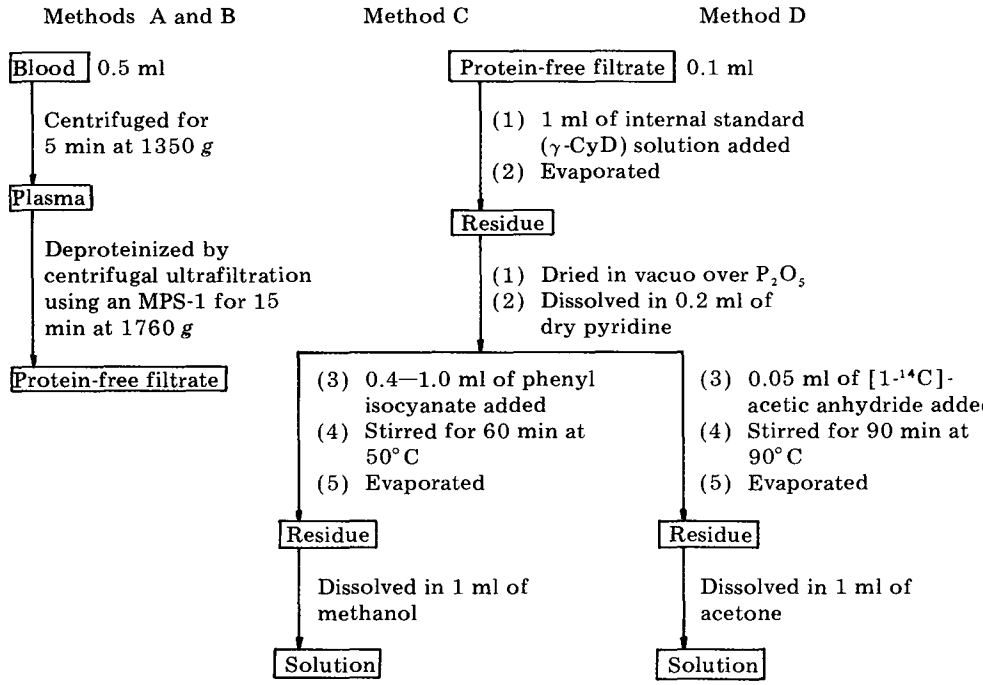
EXPERIMENTAL

Apparatus

A Tri Rotar SR-1 pump (JASCO, Tokyo, Japan) equipped with an SE-31 refractive index (RI) monitor (Showa Denko, Tokyo, Japan) and a VL-614 variable-loop injector (JASCO) was used for the determination of underivatized β-CyD. HPLC analyses of the β-CyD derivatives were conducted with a Model 6000A pump, a U6K injector (both from Waters Assoc., Milford, MA, U.S.A.) and a Uvidec-100III variable-wavelength detector (JASCO). The determination of [1-^{14}C]acetylated CyD was carried out using an LSC-700 liquid scintillation system (Aloka, Tokyo, Japan). The columns used were an ERC-NH-1171 (200 × 6 mm I.D.) (Erma Optical Works, Tokyo, Japan), a Hibar LiChrosorb RP-18 (250 × 4 mm I.D.) (Merck, Darmstadt, F.R.G.), a YMC-Pack A-212 (C_8) (150 × 6 mm I.D.) (Yamamura Chemical, Kyoto, Japan) and a Hibar LiChrosorb Si 60 (250 × 4 mm I.D.) (Merck). For the analysis of phenobarbital a semimicro liquid chromatograph equipped with a Familic-300S pump, an ML-425 injector, a Uvidec-100V detector and a μS-Finepak SIL C_{18} column (250 × 1.5 mm I.D.) (all from JASCO) was used. A Chromatopac C-RIA digital integrator (Shimadzu, Kyoto, Japan) was used to calculate peak areas. An MPS-1 micro-partition system (Amicon, Lexington, MA, U.S.A.) was used to remove plasma protein.

Materials

β-CyD was used after recrystallization from water, optical rotation, $[\alpha]_D^{18}$ +165.5°. γ-CyD, phenyl isocyanate (PHI) and acetic anhydride were of analytical-reagent grade. [1-^{14}C]Acetic anhydride (244 μCi/mg) and Scintisol EX-H as a scintillator were obtained from Amersham Japan (Tokyo, Japan) and Wako (Osaka, Japan), respectively. Phenobarbital (m.p. 176.3—176.8°C), of Japanese Pharmacopoeial standard, was purified by recrystallization. The organic solvents used for the preparation of mobile phase systems were of analytical-reagent grade and were freshly distilled before use. In all experiments distilled deionized water was used. The eluents were filtered through a 0.45-μm membrane filter and degassed.



Scheme 1. Preparation of sample solutions.

Preparation of sample solutions

Scheme 1 shows the preparation of each sample solution for HPLC analyses by the four methods. In methods A and B, 20 μl of protein-free filtrate was directly injected the chromatographic system. The amount of γ-CyD added as the internal standard (I.S.) was 2.5 or 10 μg in method C and 50 μg in method D. Pyridine used for derivatization of CyD, as a solvent having catalytic properties, was well dried by refluxing with anhydrous barium oxide and distilled before use. [1-^{14}C]Acetic anhydride (250 μCi) was used after dilution with 1 ml of acetic anhydride. At the end of the derivatization reactions excess of reagent and pyridine were removed by evaporation under reduced pressure. The methanol solution in method C and the acetone solution in method D were filtered through 0.45-μm membrane filters and subjected to HPLC.

Measurement of radioactivity in method D

Fractions of 1 ml were collected in vials and 6 ml of Scintisol EX-H were added to each vial for scintillation counting purposes. The radioactivities of [1-^{14}C]acetylated β-CyD and γ-CyD were measured as the sums of the radioactivities of corresponding fractions.

Proof of the structure of derivatized β-cyclodextrin

It was proved by elemental analyses of synthesized derivatives that all free hydroxy groups of β-CyD reacted with PHI or acetic anhydride.

Synthesis of β-CyD percarbanilate. β-CyD (1 g) was dissolved in 7.5 ml of dry pyridine at 50°C. PHI (4 ml, 42 mol.equiv.) was added and reaction was

carried out for 60 min at 50°C. Completion of the reaction was confirmed by thin-layer chromatography on a silica gel plate (Merck) with benzene—ethyl acetate (7:2) (R_F = 0.30). Most of the pyridine and PHI were evaporated under reduced pressure. The residue was dissolved in chloroform and washed with water. At that time most of the diphenylurea, the reaction product of water remaining PHI, was deposited between the water and chloroform layers. The chloroform layer was concentrated to dryness and purified by HPLC. The product was dissolved in hot isopropyl alcohol and reprecipitated on cooling, m.p. 216—217°C, $[\alpha]_D^{17}$ +71.0° (c = 1, pyridine) {lit. [9] m.p. 214—215°C, $[\alpha]_D^{25}$ +69.5° (c = 1, pyridine)}. Calculated for $C_{189}H_{175}O_{56}N_{21}$: C, 62.42; H, 4.85; N, 8.09%. Found: C, 62.46; H, 5.31; N, 8.00%.

Synthesis of β-CyD peracetate. β-CyD (1 g) was acetylated with acetic anhydride (5 ml) and dry pyridine (5 ml) for 90 min at 90°C, then the mixture was concentrated to dryness. The residual syrup was solidified by stirring with ice—water overnight. The amorphous powder obtained was crystallized from ethanol, m.p. 144—147°C. The crystals were convertible to other crystals having m.p. 211—212°C on recrystallization from a mixture of ethanol and methanol (1:1), and these two kinds of crystals showed the same optical rotation, $[\alpha]_D^{25.5}$ +123.5° (c = 2, $CHCl_3$) and had the same R_F value (0.72) on a silica gel plate with benzene—acetone (6:5), and were therefore polymorphic forms. Calculated for $C_{84}H_{112}O_{56} \cdot 2H_2O$: C, 49.12; H, 5.69%. Found: C, 49.01; H, 5.54%.

In situ intestinal absorption procedure

Male Wistar rats weighing between 250 and 260 g were fasted for 24 h prior to surgery and were anaesthetized with chloroform—diethyl ether (1:2). The small intestine was exposed by a midline abdominal incision, an intestinal segment (about 10 cm from ileal end) was cut and two glass cannulae were inserted at the both ends. The mesenteric arcades to adjacent portions were carefully tied off and the cannulae were ligated with silk suture. Heparin sodium solution was injected into the femoral vein. As a means of clearing the lumen, Krebs—Ringer solution warmed to 37°C was passed slowly from one end of the intestinal segment to the other. The remaining solution was carefully expelled from the lumen by means of an air pump and 0.5 ml of 0.1 *M* phenobarbital—β-CyD complex suspension in Krebs—Ringer solution, containing 1% CMC-Na and warmed to 37°C, was immediately introduced into the lumen. Both ends of the cannulae were connected to make a closed loop. The mesenteric vein was cannulated with an appropriate size of polyethylene tubing and venous blood was collected in centrifuge tubes in successive intervals, usually 2.5 min.

RESULTS AND DISCUSSION

Determination of underivatized β-cyclodextrin by HPLC with an RI detector

A few HPLC methods have been described for the separation of CyDs. A method using a cation-exchange resin column [Aminex 50W-X4 (Ca^{2+})] with water as the eluent [10] was applied at elevated temperature, but CyDs were very retarded, even at 90°C the retention time of β-CyD being over 30

min. Moreover, the relationship between peak area and β-CyD concentration, although demonstrating that the method can be used quantitatively, does not seem to be applicable to the micro-determination of β-CyD. HPLC analyses on an NH_2-bonded silica column (μBondapak-CH) with acetonitrile—water [11] and on a C_{18}-bonded silica column (Dextro-Pak) with methanol—water [12] are also thought to be unsuitable for the micro-determination of β-CyD, as the sharpness of the peaks in both chromatograms is insufficient. However, recent years have seen dramatic developments in the performance of columns for HPLC. In our previous study on the HPLC of cyclic (1→2)-β-D-glucans [13] excellent results could be obtained by using NH_2-bonded and C_{18}-bonded silica columns with 3—5 μm particles. Hence, HPLC analyses of β-CyD on these two types of columns were re-examined (methods A and B). In method A, an ERC-NH-1171 (3 μm) column was chosen as an NH_2-bonded silica column and acetonitrile—water (70:30) was used as the eluent. Method B was performed on a Hibar LiChrosorb RP-18 (5 μm) column with methanol—water (16:84). In order to minimize dilution of the sample, the removal of plasma protein was carried out by centrifugal ultrafiltration with an MPS-1 micro-partition system. Losses of β-CyD by this treatment were negligible. Typical HPLC results obtained by both methods for a standard β-CyD and a deproteinized plasma (blank) are shown in Figs. 1 and 2. In both chromatograms no peak in the plasma overlaps with that of β-CyD. Quantitative evaluation was carried out by

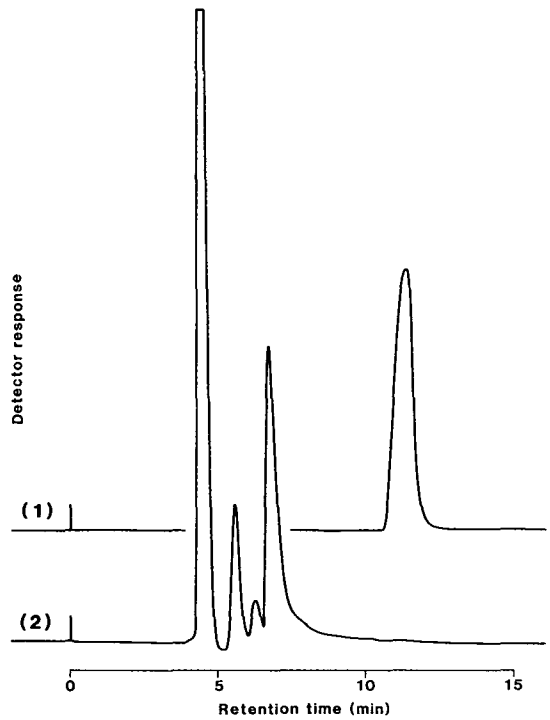


Fig. 1. Chromatograms of a β-cyclodextrin standard (0.2 μmol) (1) and a deproteinized plasma (20 μl) (2) on NH_2-bonded silica. Chromatographic conditions: column, ERC-NH-1171 (200 × 6 mm I.D.); eluent, acetonitrile—water (70:30); flow-rate, 1.0 ml/min; detector, Shodex RI SE-31 at $1.6 \cdot 10^{-4}$ RI units full scale; temperature, ambient.

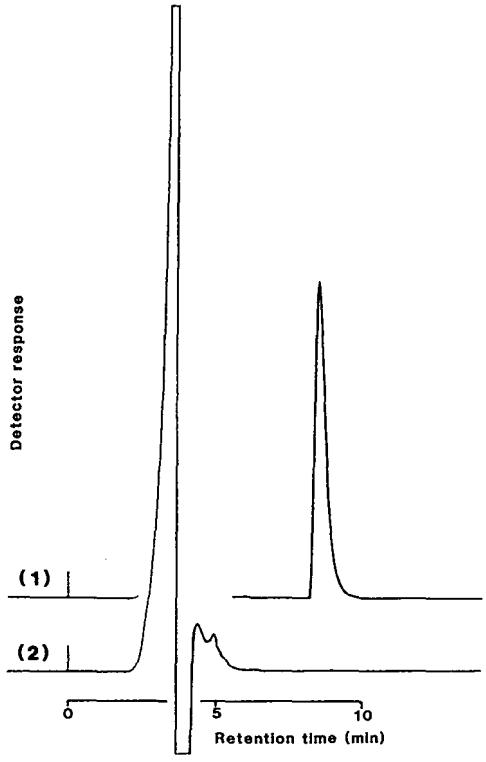


Fig. 2. Chromatograms of a β-cyclodextrin standard (0.06 μmol) (1) and a deproteinized plasma (20 μl) (2) on C_{18}-bonded silica. Chromatographic conditions: column, Hibar LiChrosorb RP-18 (250 × 4 mm I.D.); eluent, methanol—water (16:84); flow-rate, 0.6 ml/min; detector, Shodex RI SE-31 at $8 \cdot 10^{-5}$ RI units full scale; temperature, ambient.

TABLE I

CALIBRATION GRAPHS AND DETECTION LIMITS FOR THE FOUR METHODS

Method	Calibration graph			Detection limit for S/N* = 3 (nmol)
	Regression line	Correlation coefficient	Lower limit of determination (nmol)	
A	$y = 1.348x$	$r = 0.998$	0.4	0.09
B	$y = 1.913x$	$r = 0.999$	0.2	0.04
C	$y = 0.530x$	$r = 0.999$	0.2	0.04
D	$y = 0.153x + 0.247$	$r = 0.998$	0.9	0.20

*Signal-to-noise ratio.

the absolute calibration graph method using peak areas. The linearity of the detector response was investigated by injection of progressive dilutions of a β-CyD standard. Using method A the response was linear over the range 0.5—5 μg with the RI detector set at $0.25 \cdot 10^{-5}$ RI units full scale and over the range 4—40 μg at $2 \cdot 10^{-5}$, and using method B the linear range of the

detector response was 0.25—2.5 μg at $0.25 \cdot 10^{-5}$ and 2—20 μg at $2 \cdot 10^{-5}$. The regression lines and the correlation coefficients of the calibration graphs at higher sensitivity, the lower limits of determination and the detection limits at a signal-to-noise ratio of 3 are summarized in Table I. It is generally considered that RI monitoring is not suitable for micro-analyses. However, the use of a high-sensitivity RI detector together with a pump producing little pulsating flow allowed detection down to the 10^{-2} nmol level. Comparing methods A and B, the eluent in method B, methanol—water (16:84), offered a higher baseline stability of the RI detector at high sensitivity than that in method A, acetonitrile—water (70:30). Moreover, C_{18}-bonded phases are much more stable than NH_2-bonded phases.

Determination of phenyl isocyanate derivatized β-cyclodextrin by HPLC with a UV detector

Björkqvist [14] reported that the free hydroxy groups of saccharides and sugar alcohols react with PHI to yield very stable and strongly UV-absorbing derivatives, which possess good chromatographic properties in a reversed-phase system. He separated and determined some mono-, di- and trisaccharides, cello-oligomers up to octasaccharide and alditols on self-packed Spherisorb 5 ODS columns.

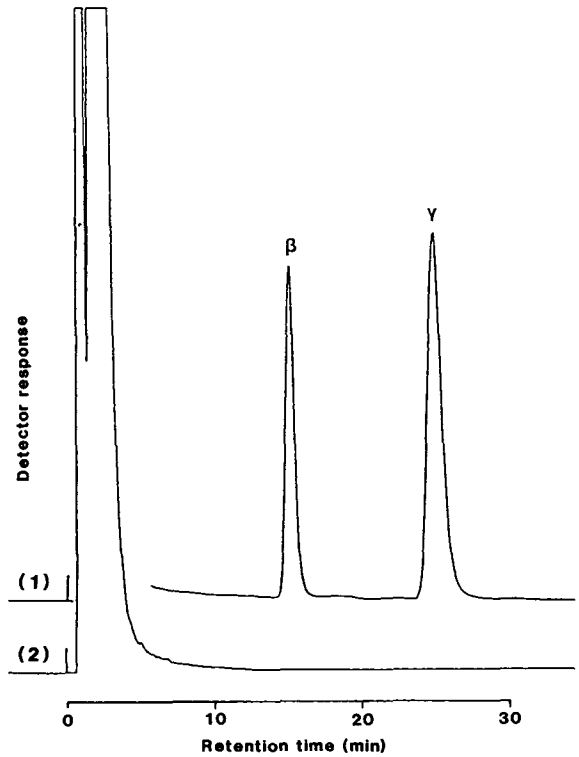


Fig. 3. Chromatograms of a phenyl isocyanate derivatized standard mixture of β-cyclodextrin (0.02 μmol) and γ-cyclodextrin (0.03 μmol) (1), and the blank (2) on C_8-bonded silica. Chromatographic conditions: column, YMC-Pack A-212 (150 × 6 mm I.D.); eluent, acetonitrile—water (89:11); flow-rate, 2.5 ml/min; detector, JASCO Uvidec-100III at 231 nm, 0.16 absorbance units full scale; temperature, ambient.

A modified method was applied to the determination of β-CyD. All free hydroxy groups of β-CyD reacted with PHI, as shown by elemental analyses of the synthesized derivative. Although Björkqvist [14] degraded the excess of PHI by addition of methanol, it turned out that most of the excess of PHI must be removed by evaporation under reduced pressure before addition of methanol, otherwise large amounts of the urethane derived from methanol and PHI interfere with the rapid analysis of β-CyD, because the urethane is eluted before the β-CyD derivative. To prevent the formation of diphenylurea, the reaction product of water and PHI, strictly anhydrous conditions should be maintained throughout the derivatization procedure. The internal standard method using γ-CyD was used for the determination. The β-CyD and γ-CyD percarbanilates were retained for a long period on C_{18}-bonded silica and a C_8-bonded silica column best fitted to determine β-CyD percarbanilate. Each of the CyD carbanilates yielded a single peak that did not overlap with peaks in the chromatogram of the blank (Fig. 3). The regression line and the correlation coefficient of the calibration graph obtained at 0.01 absorbance units full scale, the lower limit of determination, and the detection limit at a signal-to-noise ratio of 3 are shown in Table I.

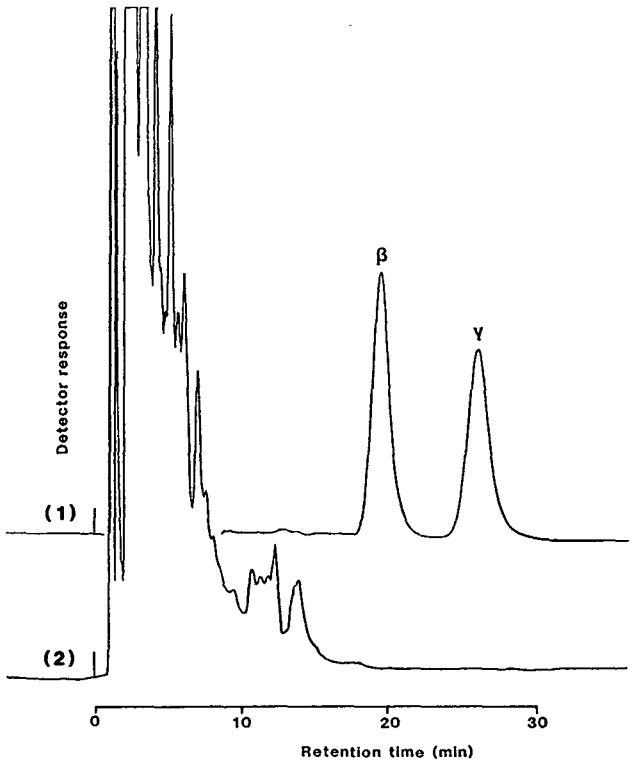


Fig. 4. Chromatograms of a peracetylated standard mixture of β-cyclodextrin (0.88 μmol) and γ-cyclodextrin (0.77 μmol) (1), and the blank (2) on silica. Chromatographic conditions: column, Hibar LiChrosorb Si 60 (250 × 4 mm I.D.); eluent, *n*-hexane—ethanol (73:27) containing 1% of water; flow-rate, 2.0 ml/min; detector, JASCO Uvidec-100III at 210 nm, 0.02 absorbance units full-scale; temperature, ambient.

Determination of [1-^{14}C]acetylated β-cyclodextrin with a scintillation counter after fractionation by HPLC

Derivatization of β-CyD with radioactive reagents should make this a very sensitive analytical technique. [1-^{14}C] Acetic anhydride was chosen as the radioactive reagent, as acetic anhydride readily reacts with all free hydroxy groups of CyD. First, the chromatographic conditions were investigated. By using a mobile phase consisting of *n*-hexane and ethanol, effective resolution of the acetates of β-CyD and γ-CyD, the I.S. and plasma components was achieved on a silica column. Tailing of peaks was prevented by addition of 1% of water to the eluent. Fig. 4 shows the elution profile of the mixture of peracetylated β-CyD and the I.S., γ-CyD, and a plasma sample treated with acetic anhydride in the same manner (blank). Quantitative analysis was carried out by fractionation of the eluate and scintillation counting of the fractions containing [^{14}C] acetylated β-CyD and γ-CyD. As shown in Table I, the calibration graph is sufficiently linear for quantitative analysis. The lower limit of determination and the detection limit are not very low, but could be lowered in theory by minimizing the dilution of [1-^{14}C] acetic anhydride with acetic anhydride. However, this is very costly and, moreover, the use of a reagent with high radioactivity should be avoided.

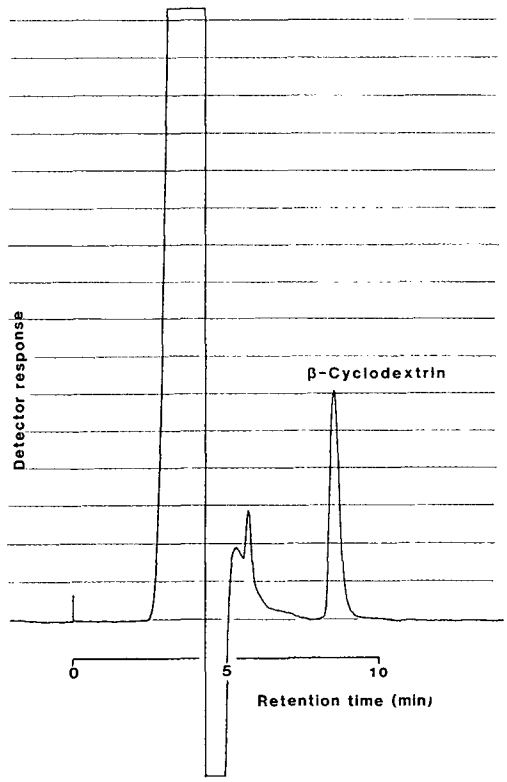


Fig. 5. Example of the determination of β-cyclodextrin in plasma collected during the second 2.5 min after administration of phenobarbital–β-cyclodextrin complex (50 μmol) in a closed-loop method. Chromatographic conditions: detector, Shodex RI SE-31 at $1 \cdot 10^{-5}$ RI units full scale; sample size, 20 μl; other conditions as in Fig. 2.

Determination of β-cyclodextrin in an in situ absorption study

Using method B, it is possible to detect 0.04 nmol of β-CyD. This result is comparable to the detection limit of percarbanilated β-CyD using UV detection (method C), and the lower limits of determination by both methods are the same. However, method B has the advantages of a shorter analysis time and no requirement for derivatization of β-CyD. Therefore, the amount of β-CyD in

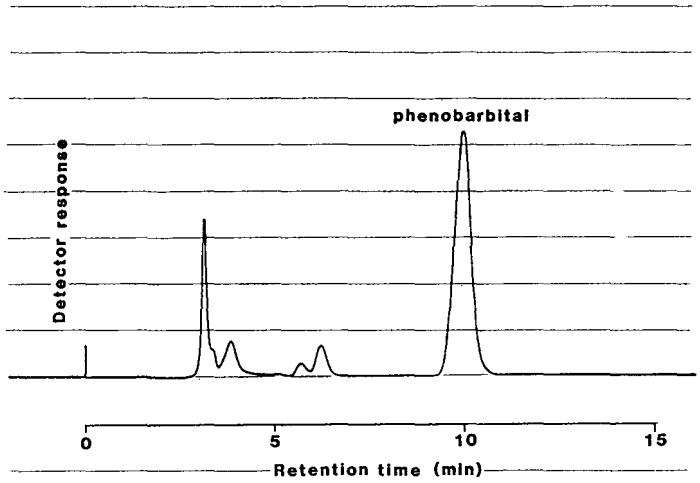


Fig. 6. Example of the determination of phenobarbital in plasma collected during the second 2.5 min after administration of phenobarbital—β-cyclodextrin complex (50 μmol) in a closed-loop method. Chromatographic conditions: column, μS-Finepak SIL C_{18} (250 × 1.5 mm I.D.); eluent, methanol—water (45:55); flow-rate, 0.1 ml/min; detector, JASCO Uvidec-100V at 220 nm, 0.64 absorbance units full scale; temperature, ambient; sample size, 3 μl.

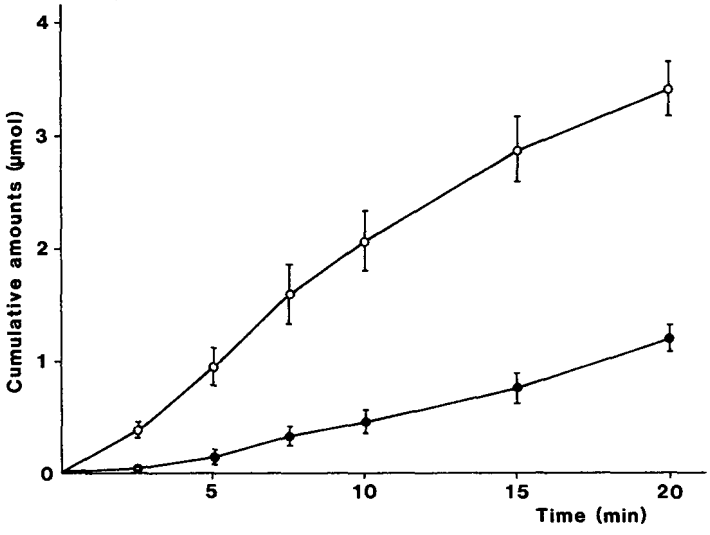


Fig. 7. Cumulative amounts of β-cyclodextrin (•) and phenobarbital (○) in mesenteric vein absorbed from rat intestinal lumen after administration of phenobarbital—β-cyclodextrin complex (50 μmol) in a closed loop method. Each point and vertical bar indicates the mean ± standard error for five rats.

the mesenteric vein absorbed from the rat intestinal lumen after administration of phenobarbital–β-CyD complex in a closed loop method was determined by the use of method B. For the purpose of comparison, phenobarbital in plasma was also determined by semimicro HPLC on a μS-Finepak SIL C_{18} (250 × 1.5 mm I.D.) column using a UV detector at 220 nm. Fig. 5 shows an example of the determination of β-CyD in plasma collected during the second 2.5 min after administration of the complex. Fig. 6 shows the determination of phenobarbital in the same plasma sample. As can seen in Fig. 7, although the rate of appearance of β-CyD in mesenteric blood, in situ, is slower than that of phenobarbital and the cumulative amount of β-CyD absorbed is smaller, β-CyD is undoubtedly able to be absorbed from the rat intestinal lumen.

In future work we shall carry out in situ absorption studies of β-CyD complexes and develop a method that is applicable to in vivo absorption studies.

REFERENCES

1 K. Koizumi and K. Fujimura, Yakugaku Zasshi, 92 (1972) 32.
2 A.L. Thakkar, P.B. Kuehn, J.H. Perrin and W.L. Wilham, J. Pharm. Sci., 61 (1972) 1841.
3 K. Koizumi, K. Mitsui and K. Higuchi, Yakugaku Zasshi, 94 (1974) 1515.
4 M. Otagili, T. Miyaji, K. Uekama and K. Ikeda, Chem. Pharm. Bull., 24 (1976) 1146.
5 T. Miyaji, Y. Kurono, K. Uekama and K. Ikeda, Chem. Pharm. Bull., 24 (1976) 1155.
6 K. Koizumi and Y. Kidera, Yakugaku Zasshi, 97 (1977) 705.
7 K. Koizumi, H. Miki and Y. Kubota, Chem. Pharm. Bull., 28 (1980) 319.
8 K. Koizumi and Y. Kubota, unpublished data.
9 I.A. Wolff and C.E. Rist, J. Amer. Chem. Soc., 70 (1948) 2779.
10 H. Hokse, J. Chromatogr., 189 (1980) 98.
11 B. Zsadon, K.H. Otta, F. Tüdős and J. Szejtli, J. Chromatogr., 172 (1979) 490.
12 K. Koizumi, Y. Okada, S. Horiyama, T. Utamura, M. Hisamatsu and A. Amemura, J. Chromatogr., 265 (1983) 89.
13 K. Koizumi, Y. Okada, T. Utamura, M. Hisamatsu and A. Amemura, J. Chromatogr., 299 (1984) 215.
14 B. Björkqvist, J. Chromatogr., 218 (1981) 65.

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RAPID AUTOMATED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF CYCLIC ADENOSINE 3′,5′-MONOPHOSPHATE SYNTHESIS IN BRAIN TISSUES

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SUMMARY

A rapid and sensitive automated system for measuring cyclic adenosine 3′,5′-monophosphate (cAMP) synthesized from either radiolabelled adenine or adenosine 5′-triphosphate (ATP) in intact and broken cell tissue preparations, respectively, is described. After incubation with radiolabelled precursor, tissue samples are deproteinized and then injected directly onto a reversed-phase high-performance liquid chromatographic column. The column effluent fraction which contains cAMP is collected into scintillation vials and assayed for tritium by liquid scintillation spectrometry. Since the high-performance liquid chromatographic and fraction collection procedures are automated, over fifty samples can be analyzed in duplicate in a single day. The utility of this assay is illustrated by investigations of the effects of β-adrenergic receptor stimulation on cAMP synthesis in tissue slices prepared from rat cerebral cortex and dopamine on cAMP synthesis in striatal membrane preparations.

INTRODUCTION

It is well established that cyclic adenosine 3′,5′-monophosphate (cAMP) is a second messenger which mediates many cellular responses to receptor stimulation [1, 2]. Changes in cAMP synthesis in response to various stimuli have been measured, using radioisotopic methods, in both intact and broken cell preparations. These methods are based upon the measurement of cAMP formation from either radiolabelled adenine or adenosine 5′-triphosphate (ATP) [3—10]. The use of high specific activity [^{32}P]ATP permits the sensitive analysis of cAMP synthesis, but its application is limited to studies with broken cell preparations, since ATP does not readily cross cell membranes. The use of radiolabelled adenine, which can be taken up by cells and converted to ATP,

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provides a useful alternative for measuring cAMP synthesis in intact cells or tissue slices [5—9]. Moreover, assays utilizing adenine can be applied to examine the effects of drugs on cAMP synthesis in ex vivo studies, whereas in methods which use ATP, drugs administered in vivo are washed out or greatly diluted when cell membrane fractions are prepared.

Typically, radioisotopic assays for cAMP synthesis have used one or more ion-exchange columns or other time-consuming chromatographic steps to separate cAMP from radiolabelled precursors and contaminants. Such tedious procedures are subject to errors if, for example, the elution profile of columns varies. Recently, Martinez-Valdez et al. [11] reported a high-performance liquid chromatographic (HPLC) method for separating purine and pyrimidine nucleotides. Using that chromatographic system as a starting point, we have modified it and developed a rapid system for separating cAMP from adenine, ATP, and other adenine-derived compounds, and we have automated and applied this system to the measurement of cAMP synthesis in brain tissue slices and broken cell preparations. While this manuscript was in preparation, Schulz and Mailman [10] presented an excellent method for measuring cAMP synthesis from [^{32}P]ATP in broken cell preparations. Their procedure also utilizes HPLC to purify cAMP. However, data were not presented on the resolution of cAMP from adenosine. Such resolution is necessary if cAMP synthesis is measured either in tissue slices using [^{3}H]adenine or in membrane preparations using [^{3}H]ATP. Using modifications of previously described incubation conditions for the measurement of adenylate cyclase [4, 5, 8, 9], both of these applications are described below.

EXPERIMENTAL

Measurement of cAMP synthesized from adenine in tissue slices

Male Sprague—Dawley rats (Hilltop Labs., Scottdale, PA, U.S.A.) weighing 170—230 g, are decapitated and tissues (e.g., occipital cortex) are immediately dissected out on an ice-cooled plate. Tissue slices (300 × 300 μm) are prepared using a McIlwain tissue chopper (Brinkman Instruments, Westbury, NY, U.S.A.). Then, the tissue slices are suspended in approximately 20 vols. (w/v) ice cold Krebs—Ringer bicarbonate buffer, pH 7.2 which is equilibrated with oxygen—carbon dioxide (95:5). After centrifugation (500 *g* for 1 min), the tissue pellet is resuspended in 20 vols. (w/v) of the Krebs—Ringer buffer.

[^{3}H]Adenine is mixed with the tissue suspension (5 μCi/ml; 287 n*M* final concentration in most experiments). Next, 1.2 ml of the suspension are incubated at 37°C in a shaking water bath under an oxygen—carbon dioxide (95:5) atmosphere. After a 45-min pre-incubation at 37°C to label ATP pools, samples are incubated with isoproterenol or other agonists in 10 μl of 0.1% (w/v) ascorbic acid (see Results). At the end of the incubation, 600 μl of 0.3 *M* sodium hydroxide and 10 μl water containing 50 μg cAMP are added to each sample and vortexed. After allowing samples to stand at room temperature (22°C) for 30 min, 100 μl are removed for protein analysis and 1.5 ml are transferred to 15-ml plastic centrifuge tubes which contain 0.5 ml of 0.15 *M* zinc sulfate. The samples are centrifuged at 10 000 *g* for 20 min at 4°C and aliquots of the supernatant (about 1.5 ml) are transferred to the sample vials

used by the HPLC automatic sample injector (see below). Care should be taken not to disturb the precipitate. Sample blanks are prepared using the above procedures, except that the tissue is boiled for 5 min and cooled to 4°C prior to the addition of [^{3}H] adenine.

Measurement of cAMP synthesized from ATP

Male Sprague—Dawley rats, weighing 170—230 g, are decapitated and tissues (e.g, striata) are rapidly dissected out on ice. Tissues are then homogenized with a ground glass pestle in 20 vols. (w/v) ice cold saline containing 0.5 m*M* ethyleneglycol-bis(β-aminoethyl ether)-N,N′-tetraacetic acid (EGTA) and buffered with 25 m*M* Tris · HCl, pH 7.5. After centrifugation at 300 *g* for 30 sec to remove debris, 100 μl supernatant are transferred to ice cold tubes which contain 70 μl reaction mixture (20 m*M* creatine phosphate, 5 U rabbit muscle creatine phosphate kinase, 1.5 m*M* 3-isobutyl-1-methylxanthine, and 2 m*M* cAMP in 100 m*M* Tris maleate buffer, pH 7.5) and 10 μl each of receptor agonists and antagonists or their diluent (generally 0.1% ascorbic acid). Sample blanks are prepared by boiling the sample homogenate for 5 min and then cooling the samples to 4°C prior to the above additions. The enzyme reaction is started by adding, at 10-sec intervals, 10 μl of a solution which contains 50 m*M* ATP (approximately 0.5 μCi [^{32}P] ATP or 1 μCi [^{3}H] ATP) and 200 m*M* magnesium chloride and immediately placing the samples in a shaking water bath at 37°C. After a 10-min incubation at 37°C, the reaction is stopped, at 10-sec intervals, with 0.2 ml of 0.3 *M* sodium hydroxide and the samples are vortexed. Then, 0.2 ml of 0.15 *M* zinc sulfate is added to each sample. After centrifugation at 5000 *g* for 50 min, 200 μl supernatant are transferred to HPLC autosampler vials. Proteins are determined using aliquots of the original homogenate.

Purification of cAMP by HPLC

cAMP is separated from other radiolabelled adenine derivatives on a 5-μm, 15 cm × 4.6 mm Ultrasphere ODS® reversed-phase column (Rainin Instruments, Woburn, MA, U.S.A.). A 5-μm, 4.0 cm × 4.6 mm precolumn is inserted between the analytical column and the sample injector to protect the analytical column from sample particulates. Samples (80 μl) are injected onto the columns using WISP 710B automatic sample injector (Waters Assoc., Milford, MA, U.S.A.). Mobile phase flow-rate is maintained at 1 ml/min using a Model 112 solvent delivery module (Beckman Instruments, Berkeley, CA, U.S.A.). The HPLC mobile phase consists of 0.2 *M* ammonium phosphate, pH 3.0—methanol (100:1, v/v).

The position of the HPLC column effluent fraction containing cAMP is monitored with a UV detector (254 nm; Model 153, Beckman Instruments). The output of the UV detector is connected to a peak separator (Model 2150, ISCO, Lincoln, NB, U.S.Al) which then triggers a programmable Foxy® fraction collector (ISCO). The fraction collector is equipped with a diverter valve which directs the cAMP fraction (1.5 ml) into 7-ml polypropylene scintillation vials and shunts the remaining effluent into a waste receptacle. Liquiscint® scintillation cocktail (5 ml) from National Diagnostics (Somerville, NJ, U.S.A.) is added to each vial and radioactivity is measured by liquid scintillation spectrometry. Counting efficiency is determined using ^3H_2O.

The output of the UV detector is also connected to an integrator, which is equipped with a printer/plotter (Model 3390A, Hewlett-Packard, Avondale, PA, U.S.A.). The integrator reports are used to verify the position of the cAMP peaks and to check sample recoveries (as measured by cAMP peak area owing to added cAMP carrier). Prior to each assay, 100 μl water containing 10 μg each of adenine, adenosine, ATP, and cAMP are injected into the HPLC system. This is carried out as a quality control to verify that the chromatographic system adequately resolves these compounds. Typically, the system, as described, can be used nearly continuously for many months without noticeable decreases in performance. If a decrease in HPLC column performance does occur, column performance can generally be returned to normal by washing the column according to the manufacturer's instructions.

Miscellaneous

2,3[^{3}H]Adenine (17.4 Ci/mmol), 2,8,5'[^{3}H]ATP (46.2 Ci/mmol), and [^{32}P]ATP (600 Ci/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.). Adenine, adenosine, and isobutyl methylxanthine were purchased from Sigma (St. Louis, MO, U.S.A.); creatine phosphate and rabbit muscle creatine phosphate kinase were purchased from Calbiochem (La Jolla, CA, U.S.A.). All other reagents are of the highest grade available. Proteins are assayed by the method of Lowry et al. [12]. cAMP synthesis rates were calculated by dividing the dpm present in the cAMP fraction by the specific activity of the added precursor (i.e. [^{3}H]adenine or [^{3}H]ATP) and are expressed as the molar concentration of cAMP formed per mg protein in the original sample aliquot per min of incubation at 37°C.

RESULTS

cAMP purification

Before undertaking measurements of cAMP synthesis, the conditions necessary for the separation of adenine and other adenine-derived compounds were determined. Modification of the chromatographic system of Martinez-Valdez et al. [11] provided an efficient and reliable separation of cAMP from adenine, adenosine, AMP, ADP, and ATP. Fig. 1 shows the elution profile obtained following the injection of a tissue sample incubated with [^{3}H]adenine. The cAMP containing fraction (peak 4) is well separated from the fractions containing ADP and ATP (peak 1), AMP and adenine (peak 2), and adenosine (peak 3). Similar elution profiles were observed when samples were incubated with [^{3}H]ATP, except that the adenosine fraction was reduced. When [^{32}P]ATP was used, no radioactivity was found in the adenosine fraction. Varying the composition of the HPLC mobile phase produced equivalent shifts in the retention times of the cAMP peak from tissue samples and authentic [^{3}H]cAMP, indicating that the radioactivity in the cAMP peak from tissue samples was attributable to cAMP. Pharmacological studies, described below, also confirm the identity of the cAMP peak from tissue samples.

Sodium hydroxide was used to stop cAMP synthesis as well as to solubilize tissues for protein analysis. Tissue solubilization with detergents, such as sodium dodecyl sulfate (SDS), should be avoided, since they can inactivate the HPLC

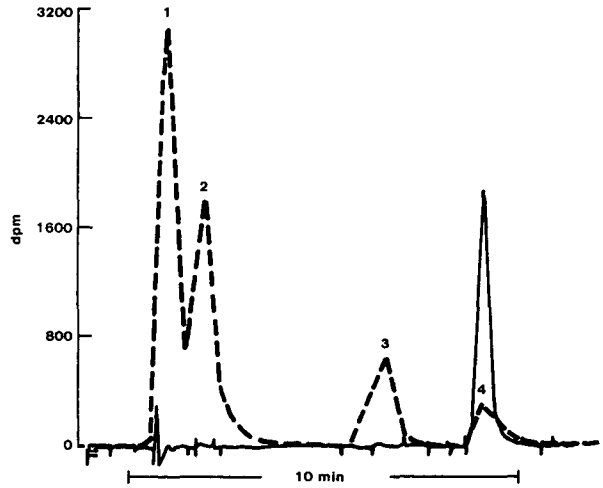

Fig. 1. Elution profile of radioactivity from HPLC column, after injection of deproteinized rat cortical tissue slices that had been incubated at 37°C for 60 min with [^{3}H]adenine. Peaks: 1 = ADP and ATP; 2 = AMP and adenine; 3 = adenosine; 4 = cAMP. Solid line is the UV tracing owing to the injection of unlabelled cAMP. See text for details.

column. After protein denaturation with sodium hydroxide, adenine and adenine-derived compounds other than cAMP were precipitated using zinc sulfate. However, adenosine was not precipitated under these conditions (data not shown). Samples deproteinized as described under Experimental were stable for at least 24 h at room temperature (22°C).

Isoproterenol-stimulated cAMP synthesis

The effects of isoproterenol, a β-adrenergic receptor agonist which stimulates cAMP synthesis [8], were examined in tissue slices prepared from rat occipital

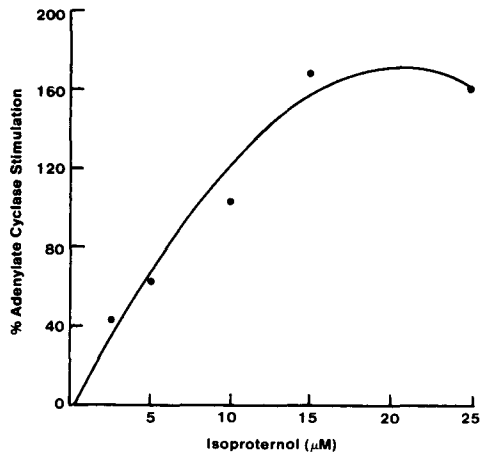

Fig. 2. Stimulation of cAMP synthesis in rat occipital cortical slices by isoproterenol. Slices were pre-incubated with [^{3}H]adenine for 45 min to label ATP stores, and then with isoproterenol for 15 min as described in Experimental. Basal cAMP rates were 27.9 pmol/mg protein per min.

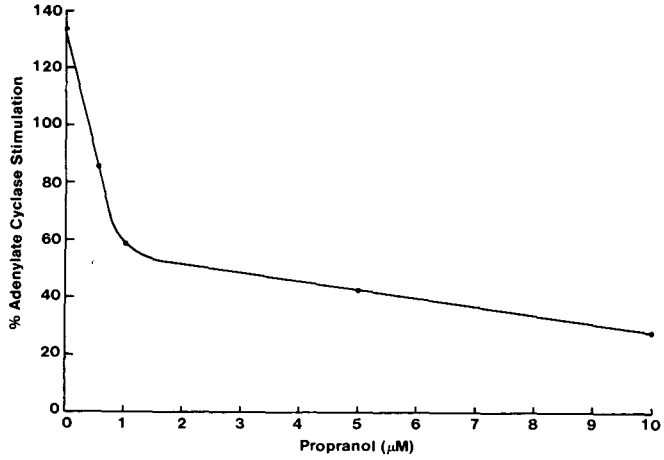

Fig. 3. Effect of propranolol on the response to isoproterenol. Occipital cortical slices were pre-incubated with [^{3}H]adenine for 45 min and then with various concentrations of propranolol for 10 min, followed by 25 μM isoproterenol for 15 min. Basal cAMP synthesis rates were 25.0 pmol/mg protein per min.

cortex. Slices were first pre-incubated for 45 min with [^{3}H]adenine and then with 0—25 μM isoproterenol. In agreement with others [8], using similar incubation conditions, isoproterenol increased tissue cAMP concentrations (Fig. 2) and this apparent stimulation of adenylate cyclase was attenuated in a dose-dependent manner by propranolol, a β-adrenergic receptor antagonist (Fig. 3).

Dopamine-stimulated cAMP synthesis

It is well known that dopamine (DA) receptor stimulation can promote cAMP synthesis [8, 10, 13, 14]. Fig. 4 illustrates the concentration-dependent stimulation of cAMP synthesis in rat striatal membrane preparations incubated with [^{32}P]ATP. Haloperidol, a DA receptor blocker, antagonized the

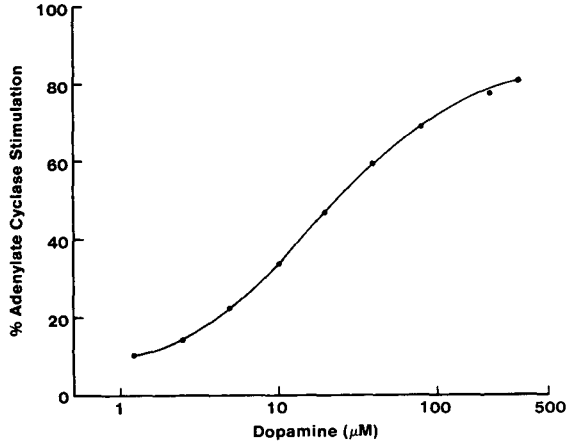

Fig. 4. Dopamine-stimulated cAMP synthesis in a rat striatal membrane preparation. Samples were incubated with [^{32}P]ATP and varying concentrations of dopamine, as described in Experimental. Basal cAMP synthesis rates were 283 fmol/mg protein per min.

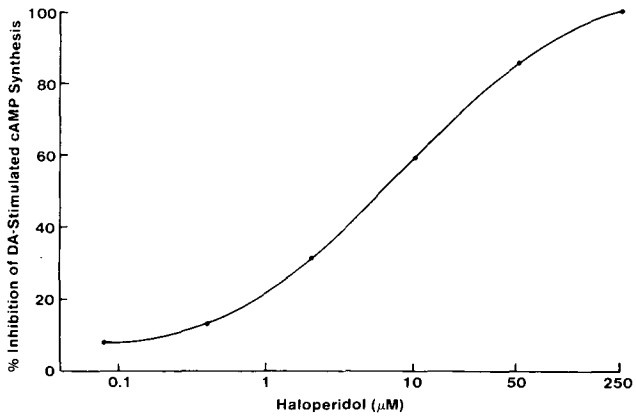

Fig. 5. Inhibition by haloperidol of dopamine-stimulated cAMP synthesis. Rat striatal membranes were incubated [^{3}H]ATP, 100 μM dopamine, and varying concentrations of haloperidol, as described in the text. Basal cAMP synthesis rates were 239.6 fmol/mg protein per min.

TABLE I

INTRA- AND INTER-ASSAY RELIABILITY

The ability of 25 μM isoproterenol to stimulate [^{3}H]cAMP formation from [^{3}H]adenine in occipital cortical tissue slices and the ability of 100 μM dopamine to stimulate [^{3}H]cAMP synthesis from [^{3}H]ATP in striatal membrane preparations were measured as described in Experimental.

Precursor	Single assay		Multiple assays	
	Percentage stimulation (mean ± S.E.M.)	Coefficient of variation (%)	Percentage stimulation (mean ± S.E.M.)	Coefficient of variation (%)
[^{3}H]Adenine	134 ± 2 (6)*	4.8	132 ± 2 (7)**	3.1
[^{3}H]ATP	31 ± 1 (8)*	3.2	44 ± 1 (28)**	8.1

*Number of determinations.
**Number of assays on separate days, triplicate determinations.

stimulation produced by 100 μM DA in a concentration-dependent manner when striatal membranes were incubated with [^{3}H]ATP (Fig. 5).

Assay reliability

The reliability of these assay procedures was investigated by determining the intra- and inter-assay coefficients of variation for tissue slices incubated in the presence and absence of isoproterenol and for membrane preparations incubated in the presence and absence of DA (Table I). The relatively low coefficients of variation indicate the high reliability of this assay.

DISCUSSION

The procedures described in this report offer several advantages over existing methods. These advantages are derived primarily from the HPLC system used

to separate radiolabelled cAMP from radioactive contaminants present in tissue extracts. Rather than using tedious and time-consuming extraction procedures, operator time is reduced through the use of an automated HPLC system. Likewise, the high efficiency and reproducibility of the HPLC purification procedures permits the reliable analysis of cAMP synthesis in small amounts of tissue (Table I). The extremely high efficiency of HPLC procedures relative to conventional column chromatography and other extraction procedures also ensures that the purification procedures are specific for cAMP (Fig. 1).

Barium hydroxide and zinc sulfate have been used to remove adenine and non-cyclic adenine nucleotides in several adenyl cyclase assays [3]. Sodium hydroxide is used in this assay, instead of barium hydroxide, to inactivate tissue enzymes. Barium hydroxide, under our assay conditions, produced some loss of cAMP (unpublished observations). In addition, the use of sodium hydroxide permits the solubilization of tissue slices for protein analysis. Although this assay is already highly sensitive, permitting the analysis of cAMP synthesis in only a few milligrams of tissue, lyophilization of deproteinized sample extracts after incubation with cAMP precursors could be used to increase assay sensitivity by concentrating samples prior to the HPLC purification of cAMP.

The investigations with isoproterenol and DA in the presence and absence of receptor antagonists illustrate the usefulness of this assay in studying receptor interaction with cAMP synthesis (Figs. 2—5). The use of tissue slices incubated with [^{3}H]adenine has the advantage that factors affecting cAMP metabolism can be examined using a relatively intact system. This method should also be directly applicable to use with tissue cultures. The use of ATP provides a highly sensitive assay for adenylate cyclase in broken cell preparations. Because adenosine is resolved from cAMP, [^{3}H]ATP instead of [^{32}P]ATP may be used for this type of assay. The use of high specific activity [^{3}H]ATP rather than [^{32}P]ATP has the advantage that tritium is a more stable isotope than ^{32}P, yet it still permits adequate sensitivity for most applications. When higher sensitivity is required, [^{32}P]ATP may be used and because adenosine, which is not radiolabelled when ^{32}P is used, would not interfere with the analysis, assay time may be considerably reduced by either increasing the HPLC mobile phase rates or increasing the concentration of methanol in the mobile phase (unpublished observations).

Modifications of the procedures described here also might be applied to the study of other aspects of cyclic nucleotide metabolism. For example, these procedures could be applied, as described, to purify cAMP from tissue samples prior to the analysis of endogenous cAMP levels by radioligand or enzymatic assays. In addition, preliminary investigations indicate that this methodology could be applied to studies of cyclic guanosine 3′,5′-monophosphate metabolism. Thus, these procedures provide a relatively rapid, sensitive, and versatile means for examining changes in cyclic nucleotide metabolism.

REFERENCES

1 J. Daly, Cyclic Neucleotides in the Nervous System, Plenum Press, New York, 1977.

2 G.A. Robinson, R.W. Butcher and E.W. Sutherland, Cyclic AMP, Academic Press, New York, 1971.
3 G. Brooker, in D. Glick (Editor), Methods of Biochemical Analysis, Wiley, New York, 1974, pp. 22, 95.
4 Y. Salomon, C. Londos and M. Rodhell, Anal. Biochem., 58 (1974) 541.
5 M. Chasin, I. Riukin, F. Mamrak, S.G. Saminiego and S.M. Hess, J. Biol. Chem., 240 (1971) 3037.
6 J. Forn and G. Krishna, Pharmacology, 5 (1971) 193.
7 G.C. Palmer, D.J. Jones, M.A. Medina, S.J. Palmer and W.B. Stavinoha, Neuropharmacology, 17 (1978) 491.
8 J. Schultz and J.W. Daly, J. Neurochem., 21 (1976) 1319.
9 H. Shimizu, J. W. Daly and C.R. Creveling, J. Neurochem., 16 (1969) 1609.
10 D.W. Schulz and R.B. Mailman, J. Neurochem., 42 (1984) 764.
11 H. Martinez-Valdez, R.M. Kothari, H.V. Hershey and M.V. Taylor, J. Chromatogr., 247 (1982) 307.
12 O.H. Lowry, N.J. Rosenbrough, A.L. Farr and R.J. Randall, J. Biol. Chem., 193 (1951) 581.
13 J. Bockaert, J.P. Tassin, A.M. Thierry, J. Glowinski and J. Premont, Brain Res., 122 (1977) 71.
14 J.W. Kebabian, G.L. Petzoldd and P. Greengard, Proc. Nat. Acad. Sci. U.S., 69 (1972) 2145.

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CHROMBIO. 2556

IMPROVEMENT OF ION CHROMATOGRAPHY WITH ULTRAVIOLET PHOTOMETRIC DETECTION AND COMPARISON WITH CONDUCTIVITY DETECTION FOR THE DETERMINATION OF SERUM CATIONS

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SUMMARY

Studies were made of the analytical conditions required for indirect photometric ion chromatography using ultraviolet photometric detection (UV method) for the determination of serum cations following a previously developed serum pre-treatment. The sensitivities of the conductivity detection (CD) and UV methods and the amounts of serum cations determined by both methods were compared. Attempts to improve the sensitivity of the conventional UV method are reported. It was found that the mobile phase previously reported by Small and Miller showed no quantitative response when more than 4 m*M* copper(II) sulphate pentahydrate was used. As a result, there was no significant difference in the amounts of serum cations shown by the CD and UV methods. However, by adding 0.5—5 m*M* cobalt(II) sulphate heptahydrate, nickel(II) sulphate hexahydrate, zinc(II) sulphate heptahydrate or cobalt(II) diammonium sulphate hexahydrate to 0.5—1.5 m*M* copper(II) sulphate pentahydrate, higher sensitivity and a quantitative response were attained.

INTRODUCTION

The author has previously reported an ion chromatographic method for the determintation of serum cations, used together with a conductivity detection (CD) method and a serum pre-treatment [1]. Recently, Small and Miller [2] reported an indirect photometric ion chromatographic method for the determination of ions using a UV detector (UV method) instead of a conductivity detector, in which the eluent salt with UV absorption in the mobile phase was replaced with sample ions without UV absorption, resulting in a reduction in

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UV absorption in the mobile phase. Serum cations were not determined by the UV method, nor were the CD and UV methods compared.

The author has now determined serum cations by the UV method after serum pre-treatment as previously described for the CD method [1]. Both sets of results were then compared. Various mobile phases other than those reported by Small and Miller [2] were studied in order to improve their UV method and, by using this improved UV method, a high sensitivity was attained.

EXPERIMENTAL

Reagents and materials

Reagents were as described previously [1], or were commercially available and chemically pure reagents. Serum was equine serum (Flow Labs., McLean, VA, U.S.A.) and the ultrafilter was an MPS-3 (Amicon, Danvers, MA, U.S.A.).

Equipment

The UV detector used was a Uvilog-8 (Oyo-Bunko, Tokyo, Japan). SGR-1A stepwise gradient elution and LC-3A high-performance liquid chromatographic (HPLC) equipment were obtained from Shimadzu (Tokyo, Japan). An ASC-4000 cation-exchange column (Oyo-Bunko) was used. All other instruments and equipment used were identical with those described previously [1].

Procedures

The CD method and the serum pre-treatment are shown in Table I. The

TABLE I

OPERATING CONDITIONS FOR ION CHROMATOGRAPHY WITH CONDUCTIVITY DETECTION

Parameter	Cation			
	Na	K	Mg	Ca
Injection volume (μl)	10	50	50	50
Column	Wescan cation-exchange column, 250 × 4.6 mm I.D. in all instances			
Temperature	Ambient	Ambient	Ambient	Ambient
Mobile phase	Aqueous HNO_3 (pH 2.1) for Na and K		0.07 ml of EDA*— 1.5 l of water (pH 6.1)	0.1 ml of EDA*— 1.5 l of water (pH 6.1)
Flow-rate (ml/min)	1.5	1.5	1.1	1.1
Detector	Wescan conductivity non-suppressor type detector, range × 10, course × 3 in all instances			
Serum treatment	Serum was diluted 100-fold with water for the determination of serum Na and 10-fold for the determination of serum K, Mg and Ca. Another treatment method was as follows: serum acidified to pH 3.0 with H_3PO_4 and non-acidified serum were ultrafiltered and the ultrafiltrate was neutralized and diluted for application to the HPLC system			

*Ethylenediamine hydrate.

**Acidified ultrafiltrate was neutralized with NaOH for divalent cation determination.

TABLE II

OPERATING CONDITIONS FOR ION CHROMATOGRAPHY WITH ULTRAVIOLET PHOTOMETRIC DETECTION

Parameter	Cation			
	Na	K	Mg	Ca
Injection volume (μl)	20	20	20	20
Column	ASC-4000 (250 × 4.6 mm I.D.), strong cation-exchange column in all instances			
Temperature	Ambient	Ambient	Ambient	Ambient
Mobile phase	1.28 m*M* $CuSO_4 \cdot 5H_2O$ for Na and K		3.2 m*M* $CuSO_4 \cdot 5H_2O$ for Mg and Ca	
Flow-rate (ml/min)	1.0	1.0	1.0	1.0
Detector	Uvilog-8 UV detector, detected at 218 nm, 0.02 a.u.f.s. in all instances			
Serum treatment	Non-acidified serum for the determination of free cations and serum acidified to pH 3.0 with the use of H_3PO_4 for the determination of free plus bound cations in serum were ultrafiltered. The ultrafiltrate was neutralized* and diluted 100-fold of serum with water for Na determination, 30-fold of serum with water for K determination, but was not diluted for Mg and Ca determinations. Sample treatment for the conducitivity detection method was the same as that used in the UV detection method in the comparison of the amounts of serum cations			
Retention time (min)	1.75	2.15	5.5	9.2

*Acidified ultrafiltrate was neutralized with NaOH (for divalent cation determination), with $Ca(OH)_2$ (for monovalent cation determination) and with $Sr(OH)_2$ (for simultaneous mono- and divalent cation determination).

analytical conditions for the conventional and the improved UV methods are shown in Tables II and Table VI, respectively.

RESULTS AND DISCUSSION

Small and Miller [2] have previously reported the simultaneous determination of mono- and divalent cations using a complicated system. This conventional UV method, using isocratic elution coupled with a single column, is unsatisfactory owing to incomplete separation of potassium from sodium and broadening of the calcium peak, none of which improved simultaneously. In this work, mono- and divalent cations have been determined separately by using different mobile phases to those used in the UV method.

Determination of monovalent cations

Analysis of standard aqueous solution. In the UV method, the logarithm of the retention time (t_R) of monovalent cations decreased linearly with increasing concentration of copper(II) sulphate pentahydrate (copper sulphate) in the mobile phase. A similar relationship was noted between the concentration of nitric acid in the mobile phase and log t_R of monovalent cations in the CD method.

The best separation of sodium from potassium and of sodium and potassium

from the vacant peak was obtained when the concentration of copper sulphate was around 1.28 m*M*. The vacant peak appeared around the void volume. The use of other copper sulphate concentrations was unsuccessful owing to poor separation. The highest sensitivity but with poor separation was attained when 0.5 m*M* copper sulphate was used, but concentrations lower than 0.5 m*M* showed decreased sensitivity with similarly poor separation.

Determination of free monovalent cations in serum. Non-acidified ultrafiltered serum was diluted 10—100-fold and injected into the HPLC system. The chromatogram shown in Fig. 1 was achieved when the injected ultrafiltrate was diluted 30-fold. It can be seen that the resolution of sodium from potassium was unsatisfactory owing to the concentration of serum sodium,

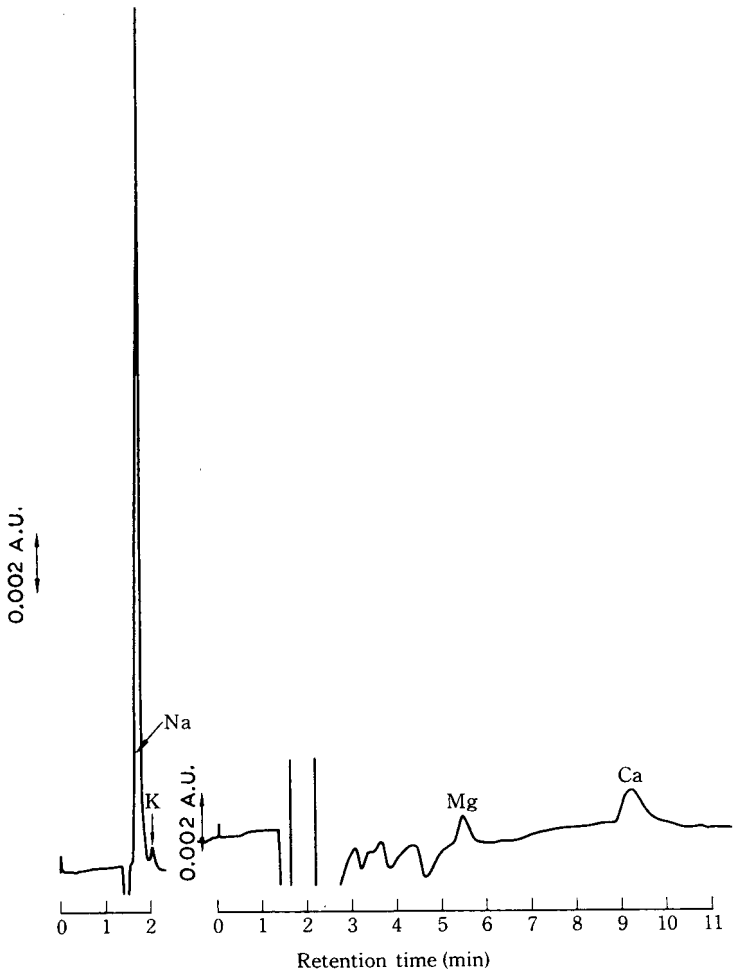

Fig. 1. Chromatograms of serum sodium, potassium, magnesium and calcium obtained by conventional ion chromatography with UV photometric detection. Volumes of 20 μl each of 30-fold diluted ultrafiltrate of serum for Na and K determination and of non-diluted ultrafiltrate of serum for Mg and Ca determination were applied. Concentrations of Na, K, Mg and Ca were 10.506, 0.506, 1.190 and 5.650 mg/dl, respectively. Other conditions as in Table II.

which was about 20 times that of serum potassium. In the present work using the conventional UV method, the ultrafiltrate of serum was diluted 100- and 30-fold for determining serum sodium and potassium, respectively.

The CD method (Fig. 2) is therefore superior to the conventional UV method for the determination of serum potassium, because potassium could not be clearly separated from sodium by the former method (Fig. 1) because the selectivity coefficient of copper(II) used in the mobile phase in the UV method is greater than that of the proton [3] used in the mobile phase of the CD method.

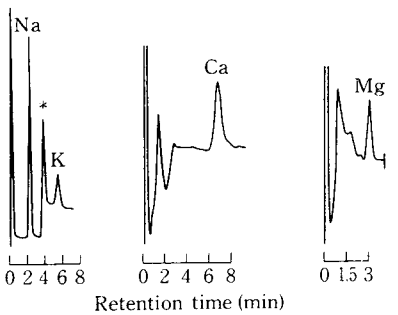

Fig. 2. Chromatograms of serum sodium, potassium, magnesium and calcium obtained by the conductivity detection method. A 100-μl volume of 100 fold diluted ultrafiltrate of serum for Na and K determination and 50 μl of 10-fold diluted ultrafiltrate of serum for Mg and Ca determination were applied. Concentrations of Na, K, Mg and Ca were 3.16, 0.15, 0.12 and 0.56 mg/dl, respectively. The asterisk indicates that the attenuation was changed from 3 for Na determination to 2 for K determination; the attenuation was 1 for Mg and Ca determinations. Retention times of Na, K, Mg and Ca were 2.50, 5.39, 2.95 and 6.40 min, respectively. Other conditions as in Table I.

Determination of the total amounts of free plus bound monovalent cations in serum. Serum was acidified to pH 3.0 with phosphoric acid and ultrafiltered. The ultrafiltrate was neutralized, diluted 10—100-fold with water and injected into the HPLC system. In the UV method, sodium and potassium could not be detected unless the ultrafiltrate was neutralized because the sample peaks were included in the vacant peak. In the CD method, the diluted ultrafiltrate was injected because the mobile phase was acidic (pH 2.1).

In comparing the total amounts with the free amounts of monovalent cations in serum, about 10% of serum sodium and potassium was found to be bound to serum protein.

Determination of divalent cations

Analysis of standard aqueous solution. Linearity was found between the concentration of ethylenediamine in the mobile phase in the CD method and log t_R of magnesium and calcium.

Magnesium and calcium could be quantified by the conventional UV method when the concentration of copper sulphate in the mobile phase was around 3.2 m*M*; the calibration graph for the determination of divalent cations was linear at 3.2 m*M*. The use of more than 4 m*M* of copper sulphate did not result in a quantitative response because the mobile phase at that concentration had an

excessive absorbance of more than 2, and therefore the absorbance difference between the eluent in the mobile phase and the sample ions was less than the detection limit. The use of concentrations other than ca. 3.2 m*M* was unsuccessful owing to the poor separation from the vacant peak and from serum admixtures and the difficulty in attaining a quantitative response.

The linearity range of mono- and divalent cations was from the detection limit (Table III) to around fifteen times the amount of each serum cation.

TABLE III

DETECTION LIMITS OF SODIUM, POTASSIUM, MAGNESIUM AND CALCIUM IN THE CONDUCTIVITY DETECTION AND UV PHOTOMETRIC DETECTION METHODS

Results in nanograms (mean of five determinations); coefficient of variation <0.5% in all instances. Signal-to-noise ratio = 2.

Cation	Conductivity detection method	Photometric detection method		
		Conventional	Improved	
			Stepwise	Isocratic
Na	3.8*	7.5§	7.5§	8.4§§
K	25.0*	11.7§	11.7§	12.8§§
Mg	15.0**	84.9§	10.6§§	9.8§§
Ca	28.0***	301.1§	34.0§§	34.0§§

*Attenuation 4; injection volume 10 µl.
**Attenuation 1; injection volume 50 µl.
***Attenuation 1; injection volume 20 µl.
§0.02 a.u.f.s.; injection volume 20 µl. Other conditions as in Table II.
§§0.02 a.u.f.s.; injection volume 20 µl. Other conditions as in Table VI.

Determination of free divalent cations in serum. The serum pre-treatment was the same as that used for free monovalent cations. Undiluted and ten-fold diluted ultrafiltrate were injected into the HPLC system for the determination by the conventional UV method and the CD method, respectively. The chromatograms of the free serum magnesium and calcium thus obtained by two methods are shown in Figs. 1 and 2, respectively, for comparison of UV and CD methods. The conventional UV method was less sensitive than the CD method, as shown in Table III. Taking into consideration this poor sensitivity to magnesium and calcium and broadening of calcium peak as shown in Fig. 1, the CD method was preferred for the determination of serum magnesium and calcium.

Determination of the total amounts of free plus bound divalent cations in serum. The serum pre-treatment was the same as that used for total monovalent cations. Undiluted and ten-fold diluted ultrafiltrate were injected into the HPLC system for the determination by the conventional UV method and the CD method, respectively. Comparing the total amounts with the free amounts of divalent cations in serum, about 30% of magnesium and about 50% of calcium in serum was bound to serum protein.

Comparison of the amounts of serum cations determined by the CD and the conventional UV methods

No substantial difference was found between the amounts determined using these two methods (Table IV). The within-run precisions of the amounts of free and total serum cations determined by the two methods were within 0.5% coefficient of variation (C.V., n = 10), the day-to-day precisions of both methods were within 1.1% C.V. (n = 10), and the month-to-month precisions were within 1.7% C.V. (n = 10) in all instances. The inter-group variation between these two methods was within 0.5% C.V. (n = 10).

TABLE IV

COMPARISON OF CONCENTRATIONS OF SERUM SODIUM, POTASSIUM, MAGNESIUM AND CALCIUM DETERMINED BY ION CHROMATOGRAPHY WITH UV AND CONDUCTIVITY DETECTION (CD)

Serum pre-treatment refers to Tables I and II. Amounts given are the means (mg/dl) (n = 3) ± coefficient of variation (%).

Cation	Free cations		Free plus bound cations	
	UV method	C.D. method	UV method	CD method
Na	316.0 ± 0.4	315.2 ± 0.5	350.0 ± 0.5	345.9 ± 0.4
K	15.4 ± 0.3	15.2 ± 0.3	18.2 ± 0.3	18.6 ± 0.4
Mg	1.3 ± 0.1	1.2 ± 0.0	1.8 ± 0.2	1.7 ± 0.1
Ca	5.5 ± 0.4	5.7 ± 0.2	11.0 ± 0.3	10.9 ± 0.4

Studies on the improvement of the use of copper sulphate only in the mobile phase in the conventional UV method

Small and Miller [2] used a complicated system for the determination of cations, in which the concentration of the eluent salt in the mobile phase, the column length, the exchange capacity and the detection wavelength were changed, and columns varying in length and exchange capacity were combined. Mono- and divalent cations were determined at 218 and 234 nm, respectively, with a mobile phase containing only copper sulphate in the first instance and copper(II) nitrate trihydrate (copper nitrate) in the second. When the concentration of copper sulphate exceeded 4 mM in order to shorten the t_R, a quantitative response was not attained. Although a quantitative response proportional to the concentration of cations was attained by reducing the concentration of the eluent in the mobile phase, for example to around 3.2 mM copper sulphate, the sensitivity was unsatisfactory (Table III). Accordingly, in order to establish a high sensitivity and rapid elution to avoid peak broadening at the same time as the simultaneous determination of mono- and divalent cations, eluent salts having little or no UV absorption (Table V) were added to the mobile phase, which contained either copper sulphate or copper nitrate at a low concentration of around 1 mM, and at that concentration of the eluent the UV absorbance was around 0.8. Several factors were considered for improving the mobile phase of the UV method reported by Small and Miller [2]. From the results shown in Tables III and V, the suitability of the molar

TABLE V

MOLAR ABSORPTIVITIES (ϵ) OF VARIOUS COMPOUNDS AT 220 nm

Compound	ϵ (l mol^{-1} cm^{-1})
$Zn(NO_3)_2 \cdot 6H_2O$	7000
$Ni(NO_3)_2 \cdot 6H_2O$	6925
$Co(NO_3)_2 \cdot 6H_2O$	6900
$Cu(NO_3)_2 \cdot 3H_2O$	7700
$ZnSO_4 \cdot 7H_2O$	6
$NiSO_4 \cdot 6H_2O$	3
$CoSO_4 \cdot 7H_2O$	10
$CuSO_4 \cdot 5H_2O$	798
$Co(NH_4)_2(SO_4)_2 \cdot 6H_2O$	0
K_2CrO_4	1202

absorptivity of the eluent for attaining an adequate UV absorbance of around 0.7 at low concentration was considered to be one factor. Copper sulphate solution afforded a more sensitive method for the determination of monovalent cations than divalent cations, as its concentration in the former instance was lower. Copper sulphate has a suitable molar absorptivity, as shown in Table V, and therefore has a suitable absorbance at a given eluent concentration; for instance, at a low concentration of around 0.75 m*M*, which is a suitable concentration for the mobile phase (Fig. 4), it has a suitable absorbance of around 0.7. As discussed above, the use of potassium chromate (Table V) in place of copper sulphate was successful except that potassium analysis became impossible. Additionally, salts having a selectivity coefficient [3] for the proton of the sulphonic acid of the cation-exchange resin in the range of those of sodium, potassium, magnesium and calcium, and having little or no UV absorption at around 220 nm, was considered to be another factor for eluent salts to be added to copper sulphate or copper nitrate in the mobile phase. As a result, in the improved UV method, mono- and divalent cations were determined using a mixed solution containing 0.5—1.5 m*M* copper sulphate, the concentration of which was kept low in order to attain adequate absorbance in the mobile phase, combined with 0.5—5 m*M* cobalt(II) sulphate heptahydrate (cobalt sulphate), nickel(II) sulphate hexahydrate (nickel sulphate), zinc(II) sulphate hexahydrate (zinc sulphate) or cobalt(II) diammonium sulphate hexahydrate (cobalt diammonium sulphate) as the eluent salts; all of these salts except copper sulphate have little or no UV absorbance at around 220 nm (Table V) and their selectivity coefficients are intermediate between those of sodium and calcium [3].

Compared with the conventional UV method reported by Small and Miller [2], the determination of mono- and divalent cations described above was far more sensitive (Table III) and simpler, with a quantitative response. The detection limits were improved over those of the conventional UV method, as shown in Table III, with no significant difference among the types and concentrations of the eluent salts when added to 0.75—1.0 m*M* copper sulphate solution in the mobile phase. Log t_R decreased almost linearly with increasing concentration of cobalt sulphate, nickel sulphate, zinc sulphate or cobalt

diammonium sulphate when added to copper sulphate solution. The greater the t_R of the cations, the greater is the decrease in t_R.

Depending on the molar absorptivity (Table V) and the UV absorbance in the mobile phase, a mobile phase containing copper nitrate in combination with cobalt sulphate, nickel sulphate, zinc sulphate or cobalt diammonium sulphate was used to determine mono- and divalent cations at 234 instead of 218 nm. In this instance, the sensitivity was around one fifth of that obtained by the use of copper sulphate with no significant difference among the types and concentrations of the eluent salts when added to 1 m*M* copper nitrate in the mobile phase. Otherwise, the results obtained by the use of copper nitrate were virtually identical with those obtained by the use of copper sulphate in the mobile phase.

Simultaneous determination of mono- and divalent cations by the improved UV method

Using the improved UV method, step-wise gradient elution and isocratic elution procedures were conducted in order to determine mono- and divalent cations simultaneously. The conditions for the stepwise gradient elution and isocratic elution procedures are given in Table VI and the chromatograms obtained are shown in Figs. 3 and 4, respectively. In the improved UV method stepwise gradient elution was carried out in order simultaneously to achieve an adequate separation of potassium from sodium and to improve the broadening of the calcium peak. The stepwise gradient procedure was found to be superior to the isocratic elution procedure, even though there was little effect on the baseline when the mobile phase was exchanged. However, there was no substantial difference in the detection limits of the two procedures (Table III).

TABLE VI

CONDITIONS FOR STEPWISE GRADIENT AND ISOCRATIC ELUTION PROCEDURES IN THE IMPROVED UV METHOD FOR DETERMINATION OF MONO- AND DIVALENT CATIONS

Parameter	Stepwise gradient elution procedure*	Isocratic elution procedure
Injection volume (μl)	20	20
Column	ASC-4000 (250 × 4.6 mm I.D.), strong cation-exchange resin in both instances	
Temperature	Ambient	Ambient
Flow-rate (ml/min)	1.0	1.0
Detector	Uvilog-8 UV detector, detected at 218 nm, 0.02 a.u.f.s. in both instances	
Mobile phase	1.28 m*M* $CuSO_4 \cdot 5H_2O$ for Na and K and a mixed aqueous solution of 1 m*M* $CuSO_4 \cdot 5H_2O$ combined with 2 m*M* $CoSO_4 \cdot 7H_2O$ for Mg and Ca determination	Mixed aqueous solution of 0.75 m*M* $CuSO_4 \cdot 5H_2O$ combined with 0.75 m*M* $CoSO_4 \cdot 7H_2O$ for Na, K, Mg, and Ca determination

*The mobile phase for Na and K determination was changed to that for Mg and Ca determination 3 min after injection and again after a further 10 min, then returned to that for the determination of monovalent cations.

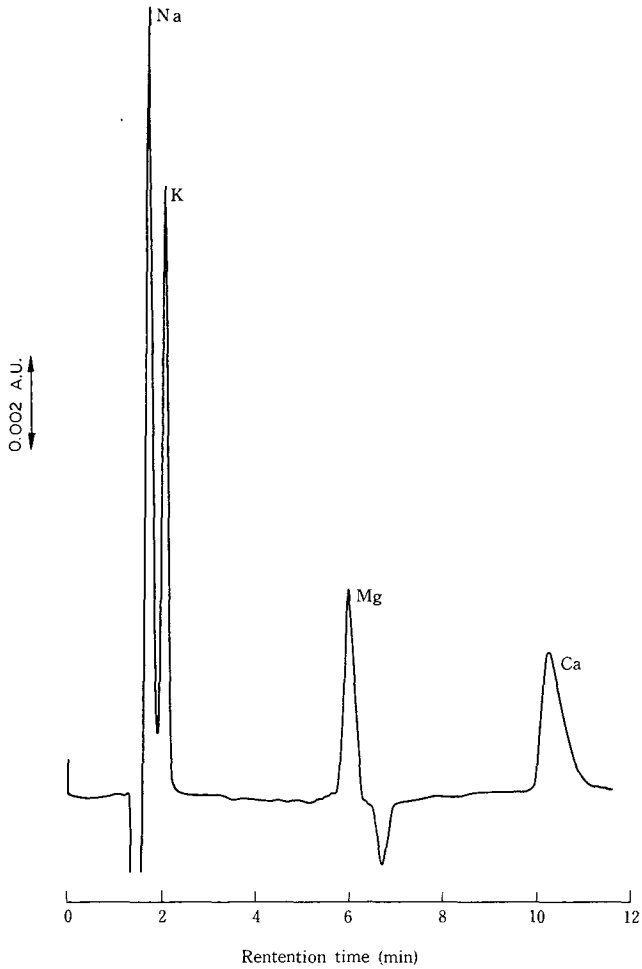

Fig. 3. Chromatogram of sodium, potassium, magnesium and calcium obtained by improved ion chromatography with UV photometric detection with the stepwise gradient elution procedure. Concentrations of Na, K, Mg and Ca were 1.814, 2.120, 0.976 and 2.108 mg/dl, respectively; 50 μl were applied. Other conditions as in Table VI.

Determination of free and total amounts of mono- and divalent cations in serum by the improved UV method

Free and total amounts of sodium, potassium, magnesium and calcium in serum were determined by using both the improved UV method with the isocratic elution and stepwise gradient elution procedures and the serum pretreatment described above. The results were almost the same as those determined by the conventional UV method, shown in Table IV. The C.V. of the intra- and inter-group analyses was less than 1.0% (n = 15) in both instances.

CONCLUSION

There was no substantial difference between the amounts of serum cations

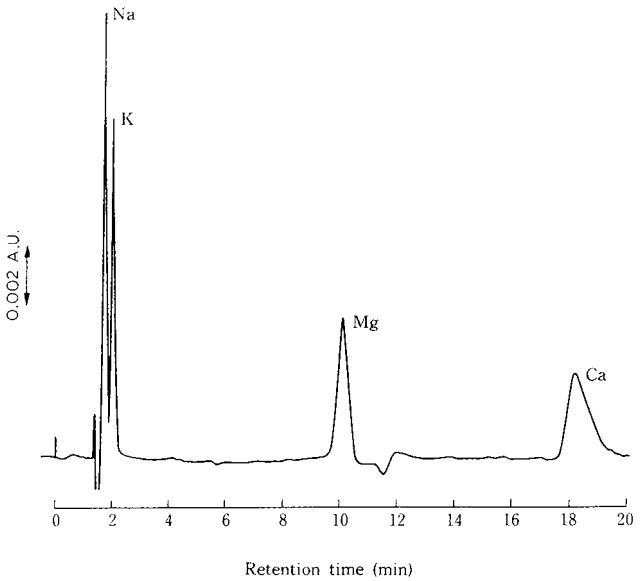

Fig. 4. Chromatogram of sodium, potassium, magnesium and calcium obtained by improved ion chromatography with UV photometric detection with the isocratic elution procedure. Concentrations of Na, K, Mg and Ca were 1.814, 2.120, 0.976 and 2.108 mg/dl, respectively; 50 μl were applied. Other analytical conditions as in Table VI.

determined by the CD and the indirect UV methods. The conventional UV method appears to be inferior to the CD method owing to the lack of a clear separation of potassium from sodium, low sensitivity and a limitation on the concentration of copper sulphate in the mobile phase. The sensitivity of the conventional UV method, however, could be improved by using an eluent containing copper sulphate at low concentration combined with either cobalt sulphate, zinc sulphate, nickel sulphate or cobalt diammonium sulphate.

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REFERENCES

1 H. Shintani, K. Tsuji and T. Oba, Bunseki Kagaku (Jap. Anal.), 33 (1984) 347.
2 H. Small and T.E. Miller, Jr., Anal. Chem., 54 (1982) 462.
3 O.D. Brunner and L.L. Smith, J. Phys. Chem., 61 (1967) 326.

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QUANTITATIVE DETERMINATION OF TRAMADOL IN HUMAN SERUM BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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SUMMARY

A gas chromatographic—mass spectrometric method for the quantitative determination of tramadol in human serum, plasma or whole blood samples is described. The method involves the use of [$^2H_2$,^{15}N]tramadol hydrochloride as an internal standard and chemical ionization with isobutane, employing single-ion monitoring for quantification. It is specific, sensitive and precise, and has high accuracy. The within-run coefficient of variation is about 1% between 25 and 200 ng/ml and 1.8—2.9% at the lowest concentrations tested (6.25 and 12.5 ng/ml). The between-run coefficient of variation increases from 1.6% to 5.2% with decreasing concentration from 200 to 12.5 ng/ml. The calibration graphs were linear in the tested concentration range, and the accuracy of the assay was not dependent on the sample volume used. The detection limit was about 4 ng/ml for serum samples of 1 ml. The method proved suitable for pharmacokinetic studies. Its high sensitivity allows measurements of serum concentrations for at least 30 h after the single administration of therapeutic doses of tramadol hydrochloride.

INTRODUCTION

Tramadol hydrochloride [*rac*-1(e)-(*m*-methoxyphenyl)-2(e)-(dimethylaminomethyl)cyclohexan-1(a)-ol hydrochloride, CG 315, Tramal; Grünenthal, Stolberg, F.R.G.] is a centrally acting analgesic [1—8] with an activity comparable to that of dextropropoxyphene, tilidine and codeine [2, 3, 9, 10], and is well tolerated [4—7, 9, 11, 12]. After single oral doses, tramadol is well and rapidly absorbed in man and animals, as was demonstrated by administration of [^{14}C]tramadol hydrochloride [13, 14]. The main metabolic pathways in man and all animal species tested are N- and O-demethylation followed by glucuronidation and sulphation of O-demethylated compounds [14, 15].

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Analytical methods for quantification of the unlabelled drug in body fluids have not previously been published.

This paper describes a gas chromatographic—mass spectrometric (GC—MS) method for the determination of tramadol in human serum, plasma and other biological fluids, which is suitable for pharmacokinetic studies following single administration of 50—100 mg tramadol hydrochloride. The method uses [$^2H_2$, ^{15}N]tramadol hydrochloride as an internal standard and the single-ion monitoring (SIM) technique after chemical ionization (CI) with isobutane.

EXPERIMENTAL

Standards

Drug standard. Tramadol hydrochloride of 99% purity (Grünenthal; Lot No. 1009/2) was used as a drug standard.

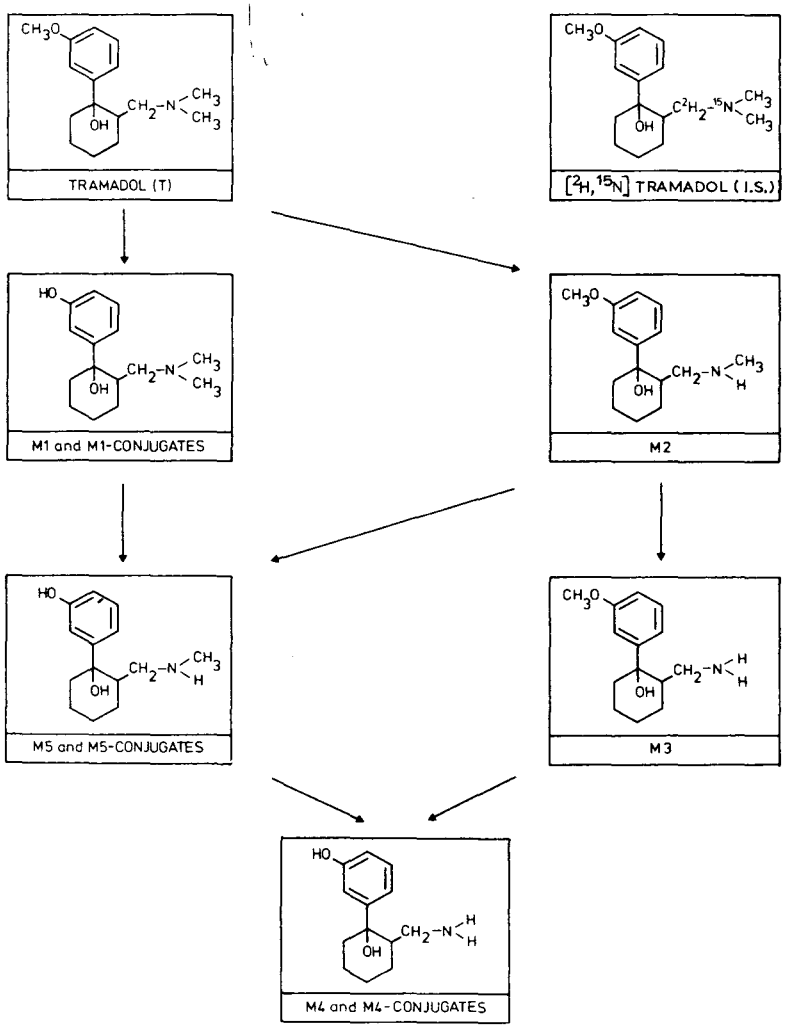

Fig. 1. Metabolic pathways of tramadol and structural formula of the internal standard.

Internal standard. [$^2H_2$,^{15}N]Tramadol hydrochloride (Fig. 1) as the internal standard was synthesized by the method originally described for unlabelled tramadol [1, 16]. The labelled intermediate compound was prepared via Mannich condensation from cyclohexanone, [$^2H_2$]paraformaldehyde (98 atom-% deuterium; Merck Sharp & Dohme, Montreal, Canada) and [^{15}N]dimethylamine hydrochloride (99 atom-% ^{15}N; Stohler Isotope Chemicals, Innerberg, Switzerland). Purification was performed by recrystallization with a

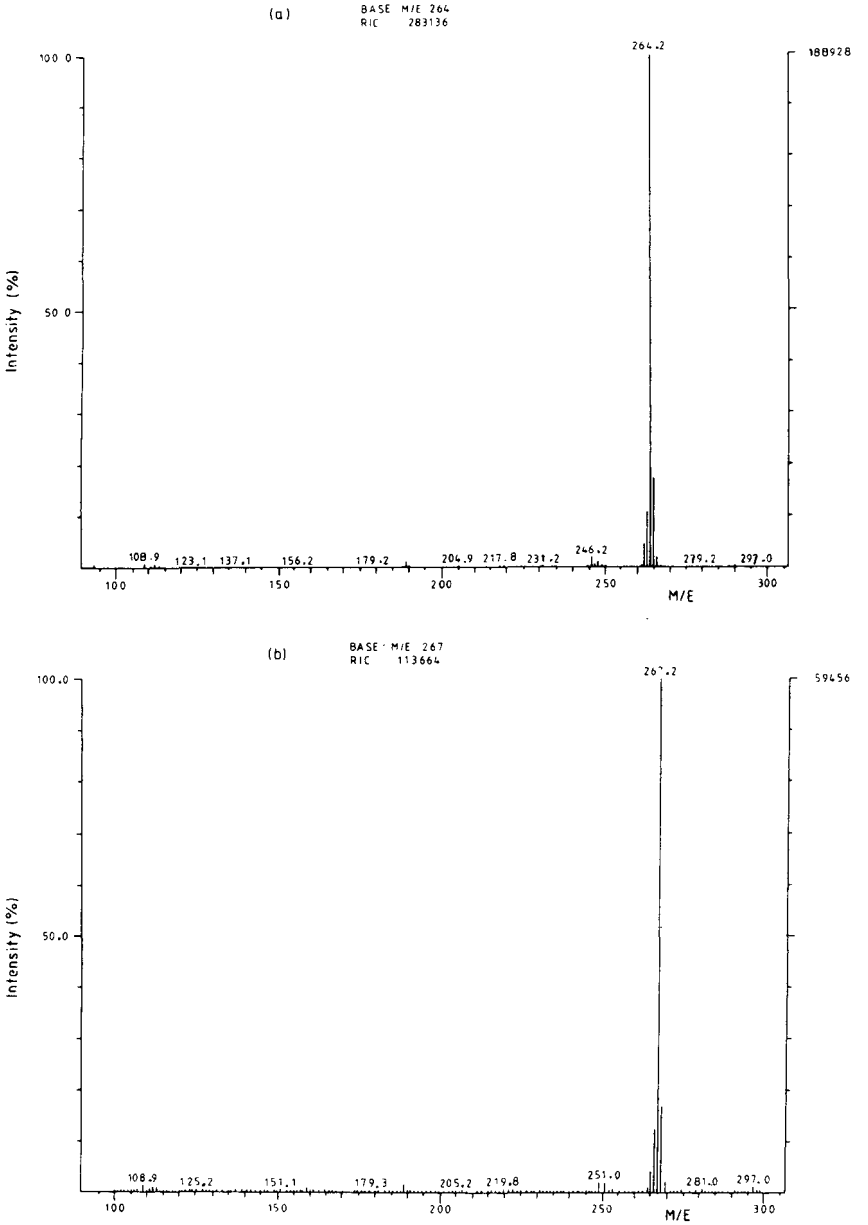

Fig. 2. Isobutane CI mass spectra of (a) tramadol, base peak *m/e* 264, and (b) [$^2H_2$,^{15}N]-tramadol, base peak *m/e* 267.

dioxane—water mixture (98:2). The chemical purity determined by thin-layer chromatography with chloroform—methanol—25% ammonia (90:8:2) as solvent system (hR_F = 74) exceeded 99%; the amount of the concomitant *cis*-isomer [1], as determined by high-performance liquid chromatography (HPLC), was 1—2%. The HPLC separation was achieved with a Nucleosil 10-C_{18} column (30 cm × 3 mm I.D.; 10 μm particle size; Macherey, Nagel & Co., Düren, F.R.G.) at an elution rate of 2.0 ml/min and a nominal pressure of 20.7 MPa. The elution system consisted of methanol—water (60:40) with 0.01% sodium bicarbonate, the temperature of the column was maintained at 40°C and the detector wavelength was 270 nm. The isotopic purity of [$^2H_2$,^{15}N]tramadol hydrochloride was about 99%, as shown by MS of pure standard samples (Fig. 2 and Table I).

TABLE I

RELATIVE ISOTOPE ABUNDANCE (RA) AND RECONSTRUCTED ION CURRENT (RIC) OF TRAMADOL AND [$^2H_2$,^{15}N]TRAMADOL (MASS RANGE: 264—269)

Mass	Tramadol (300 ng)		[$^2H_2$,^{15}N]Tramadol (300 ng)	
	RA (%)	RIC (%)	RA (%)	RIC (%)
264	100.00	66.73	0.97	0.50
265	17.19	11.47	4.41	2.31
266	2.00	1.34	12.59	6.59
267	0.20	0.14	100.00	52.31
268	0.04	0.03	17.17	8.98
269	0.06	0.04	2.32	1.21

Reference substances (drug metabolites). To determine the selectivity of the method, serum samples from several healthy volunteers, and all metabolites known so far, were used. The metabolites M1 to M5 were synthesized as described previously [14, 16]. The structures of these compounds are shown in Fig. 1.

Solvents

All solvents used in sample preparation were of analytical-reagent grade. Ammonia (25%), acetic acid (100%), *n*-hexane and chloroform were purchased from E. Merck (Darmstadt, F.R.G.). Doubly glass-distilled water was used to prepare the ammonia and acetic acid solutions.

Glassware

All glassware (tubes, stoppers and reaction vials) was rinsed with acetone and tap water, soaked overnight in 0.1 mol/l hydrochloric acid, thoroughly rinsed with tap water, then rinsed with doubly distilled water.

Sample preparation

Preparation of standard samples. Standard solutions of 1.00 mg/ml tramadol hydrochloride in water were prepared in 10-ml volumetric flasks and diluted to concentrations of 0.125, 0.25, 0.50, 1.00, 2.00 and 4.00 μg/ml. The diluted standard solutions of tramadol hydrochloride were then dissolved in pooled

drug-free serum to give final concentrations of 6.25, 12.5, 25, 50, 100 and 200 ng/ml tramadol hydrochloride. The final solutions were divided into 1.5-ml portions and stored at −20°C until used.

Preparation of quality control samples. Quality control samples with final concentrations of 12.5, 50 and 200 ng/ml tramadol hydrochloride were prepared and stored, as described for standard samples, by using independent standard solutions of tramadol hydrochloride in water and another serum pool.

Preparation of the internal standard solution. A 10.0-mg amount of [$^2H_2$,^{15}N]tramadol hydrochloride (internal standard, Fig. 1) was dissolved in 10.0 ml of water and diluted to a final concentration of 6.00 μg/ml. The stock solution was stored at −20°C, whereas the working solutions were kept at 4°C for two months at the most.

Extraction procedure

Samples of either 0.1 or 0.2 ml of serum were diluted to 1 ml with drug-free pool serum in 10-ml glass-tubes, whereas samples of 1.0 ml were used without dilution. About thirty unknown samples, six serum standards and three quality control samples were included in each extraction series. The internal standard solution (50 μl), containing [$^2H_2$,^{15}N]tramadol hydrochloride in water (6 μg/ml), was added to give a final concentration of 300 ng/ml. After mixing, the samples were alkalinized with 50 μl of ammonia (25%) and 3 ml of *n*-hexane were added. The samples were shaken for 20—30 min on a rotatory shaker and centrifuged for 5 min at 1400 *g*. As much as possible of the solvent phase was transferred into clean tubes containing 3 ml of 0.1 mol/l acetic acid.

The solvent phase was extracted with the acid by shaking for 20—30 min, followed by centrifugation as before. Thereafter the solvent phase was removed. The remaining acid phase was alkalinized with 200 μl of ammonia (25%) and extracted by gentle rotation (20—30 min) with 3 ml of *n*-hexane. After centrifugation the solvent phase was transferred into clean 10-ml glass tubes and evaporated to dryness under nitrogen at 37°C.

A 500-μl volume of chloroform was added and the samples were vortexed for 30 sec. The solutions were transferred into clean 0.3-ml V-shaped glass tubes (Micro Product V-vials, Wheaton Scientific, Melville, NJ, U.S.A.), evaporated under nitrogen at 37°C and the samples stored at 4°C. Prior to analysis, the samples were reconstituted in 20 μl of chloroform. Aliquots of 1—2 μl were injected into the gas chromatograph—mass spectrometer.

Gas chromatography—mass spectrometry

The measurements were carried out in a Finnigan 4000 gas chromatograph—mass spectrometer equipped with a multiple-ion monitoring device (Finnigan 6100 computer data system, Finnigan, Munich, F.R.G.). The injector of the gas chromatograph was a Grob-type split—splitless system for capillary columns, which operated in the splitless mode. The injector was equipped with valves that were programmed to vent the injector 48 sec after injection. The glass capillary column used was a 25-m ARNC-SE-30 glass capillary column (Macherey, Nagel & Co.) and a pressure of 345 kPa (50 p.s.i.) of helium was applied to the column, which was directly faced with the ion source.

For quantification of tramadol in body fluids, the following GC—MS

parameters and conditions were established: injection port temperature, 250°C; oven temperature, programmed from 80°C to 240°C at 25.5°C/min; transfer line temperature, 240°C; carrier gas (helium) flow-rate, approx. 2 ml/min; ionization gas, isobutane; ionization pressure, 47 Pa (0.35 Torr); ionizer temperature, 180°C; emission current, 0.25 mA; electron energy, 100 eV; electron multiplier voltage, 1800—2200 V; time interval between two injections, 7—8 min; scanning time, 0.419 sec per mass unit.

Quantification

Peak areas were integrated by using the "cross-hairs" method of the Finnigan 6100 computer data unit. The ratio of the peak area of *m/e* 264 (tramadol) to that of *m/e* 267 ([$^2H_2$,^{15}N]tramadol) at t_R = 5.2 min was calculated for each sample. Calibration graphs were constructed by linear regression analysis of the calculated ratios versus concentration of drug added. They were calculated using a $1/y^z$ weighting scheme for each set of standard samples, where y denotes the response (ratio of the peak areas) and z ($0<z<2$) represents the concentration-dependent within-run variations in the responses of the corresponding standard samples. Control and unknown samples were calculated by using the regression equations of each experimental series.

Selectivity, sensitivity, accuracy and precision of the method

Selectivity. In order to determine the selectivity criteria of the method, it was first applied to tramadol-free serum from ten healthy volunteers. Further, the GC separation of tramadol from its metabolites M1—M5 was tested.

Sensitivity. Sensitivity tests were performed by using blank samples and standard samples of known tramadol concentration, which were diluted to values near the detection limit with drug-free pooled serum.

Accuracy and precision (within-run, between-run). All studies to determine within-run and between-run variations in accuracy and precision were performed with identical serum samples prepared in the same manner as the standard samples. In addition, the influence of the metabolite M2 on the accuracy and precision of the tramadol determinations was investigated after the addition of this metabolite in quantities double those of tramadol.

Application of the method

To characterize the practical value of the method, the time course of the tramadol concentration in serum, after intravenous application of 32.8 and 100 mg of tramadol hydrochloride, was determined in two human volunteers. After the bolus injection of the dose within 1 min, blood samples were taken via an indwelling cannula over the first 4 h, and by venipuncture over the following 20 h. After clotting at room temperature the samples were centrifuged. The supernatant serum was then separated and stored frozen at −20°C until analysed.

RESULTS AND DISCUSSION

Gas chromatography

Amines and amino alcohols tend to adsorb on glass capillary columns. We

nevertheless considered it appropriate to develop a GC—MS method for tramadol itself because (a) derivatization involves more time and expense in the preparation of the samples, and (b) pilot tests showed that almost symmetrical GC peaks also result with non-derivatized tramadol after deactivation of acid centres on the surface of glass capillary columns.

Glass capillary columns with a liquid phase are just as suitable for quantitative determination of tramadol as glass capillary columns with chemically bound phases (e.g., SE-30 or other polydimethylsiloxane phases) if the active centres of the glass surface are sufficiently deactivated. All the columns tested were deactivated by pre-treatment of the glass surface with hydrochloric acid and then silylation of the OH groups. The results presented in this paper were obtained with an SE-30 column that was not optimally deactivated. Although better deactivation of the columns gives completely symmetrical tramadol peaks, the results as regards selectivity, sensitivity, precision and accuracy are not significantly better, as the internal standard used compensates for all the problems caused by chromatography, if its serum concentration is at least 50 ng/ml.

Internal standardization

[$^2H_2$,^{15}N]Tramadol (Fig. 1) is a highly satisfactory internal standard for the GC—MS determination of tramadol after CI with isobutane for the following reasons:

(1) The bonding of both deuterium atoms is sufficiently stable. Exchange reactions during sample preparation and GC were not observed.

(2) Owing to the relatively small difference in the molecular weight of tramadol and [$^2H_2$,^{15}N]tramadol, isotope effects that could considerably affect reaction rates, distribution coefficients and chromatographic results are certainly irrelevant in practice. [$^2H_2$,^{15}N]Tramadol additionally offers carrier

TABLE II

WITHIN-RUN COEFFICIENTS OF VARIATION (C.V., n = 10) AND ACCURACY FOR STANDARD SAMPLES OF TRAMADOL (ADDED AMOUNT: 25 ng) IN HUMAN POOL SERUM WITH AND WITHOUT THE TRAMADOL METABOLITE M2 (METHOD A), AND FOR THREE METHODS OF SAMPLE PREPARATION (METHODS B, C AND D)

Method B: without dilution; method C: after dilution to 1.0 ml with water; method D: after dilution to 1.0 ml with pooled human serum.

Method	Sample volume (ml)	Amount of M2 added (ng)	Amount of tramadol found (mean ± S.D.) (ng)	C.V. (%)	Accuracy (mean) (%)
A	0.2	50.0	25.0 ± 0.200	0.80	100.0
A*	0.2	—	25.1 ± 0.279	1.11	100.4
A	0.2	—	24.8 ± 0.327	1.32	99.2
B	0.1	—	25.4 ± 0.340	1.34	101.6
C	0.1	—	25.1 ± 0.434	1.73	100.4
D	0.1	—	25.1 ± 0.323	1.29	100.4

*Repeated injection of same sample.

functions that are advantageous to overcome adsorption losses in the lower detection range during sample preparation and GC.

(3) The *m/e* shift by the isotope labelling of tramadol is three mass units; for this reason, it is sufficiently large to overcome the natural isotope abundance of tramadol on the quasi-molecular ion $[M + H]^+ = 264$ (*m/e* 267 content of tramadol = 0.20%; Table I).

(4) The isotope purity of [$^2H_2$,^{15}N]tramadol was about 99%, i.e., the tramadol content of [$^2H_2$,^{15}N]tramadol is lower than 1%, as was shown by MS of pure standard samples (Table I).

(5) CI of tramadol with isobutane guarantees high sensitivity because the quasi-molecular ions *m/e* 264 and 267 are the base peaks [reconstructed ion current (RIC), 50—70%; Fig. 2 and Table I].

(6) The mass fragmentography of tramadol and [$^2H_2$,^{15}N]tramadol by isobutane CI can be reproduced with high precision. As shown by the results in Table II, the coefficients of variation for repeated injections of identical tramadol—[$^2H_2$,^{15}N]tramadol mixtures into the GC system are lower than 2%.

Calibration graphs

Calibration graphs obtained for serum samples spiked with increasing amounts of tramadol hydrochloride and a constant amount of internal standard (300 ng) were linear for concentrations ranging from 6.25 to 200 ng/ml. All calibration graphs were obtained by a weighted least-squares method of fitting (see *Quantification*). Results for the parameters a and b of ten weighted linear regression lines $y = a + bx$ are listed in Table III, where y is the peak-area ratio, x is the concentration of tramadol (ng/ml) and a and b are the y-intercept and the slope of the regression line, respectively. The mean values and standard deviations of a and b and also the correlation coefficients ($r > 0.999$) indicate reasonable linearity between the detector response and the amount added to serum.

TABLE III

COEFFICIENTS OF VARIATION (C.V.) OF TEN WEIGHTED LINEAR REGRESSION LINES, $y = a + bx$, FOR SIX STANDARD SAMPLES

y = Peak-area ratio; x = amount of tramadol added (ng/ml); tramadol concentrations of each calibration line, 6.25, 12.5, 25.0, 50.0, 100 and 200 ng/ml; weighting factor, $1/y^{1.0915}$.

Parameter	Mean ± S.D.	C.V. (%)
y-Intercept (a)	0.00935 ± 0.00440	47.1
Slope (b)	0.00365 ± 0.00011	2.92
Correlation coefficient (r)	0.99971 ± 0.00030	0.030

Selectivity

Theoretically a very high selectivity might be expected, because isotope-labelled tramadol was used as an internal standard and the quantification was performed by SIM. In fact, under these conditions tramadol-free human serum does not cause any signal in addition to the tramadol content of the internal standard, as was shown by testing blank serum samples from ten healthy volun-

teers against water samples (Fig. 3 and Table IV). For the same reason, interference by other drugs is extremely improbable, the method affording three selectivity stages with ascending separation efficacy: even the first stage, sample preparation by extraction, guarantees extensive separation of acidic and neutral compounds from the alkaline drug tramadol. GC using glass capillary columns in the second stage should be able to separate all alkaline drugs that differ in polarity from tramadol. Finally, the last stage should overcome the residual separation problems, especially as it is characterized by very high selectivity. Hence only substances with great structural similarity could interfere with tramadol.

To confirm these considerations, we checked the influence of all metabolites of tramadol so far known on the separation and quantification of tramadol. As expected in view of the partition coefficients [14], the metabolites M1, M4 and M5 (Fig. 1) were almost completely separated by the extraction procedure, owing to their amphoteric character. Furthermore, traces of M1, M4 or M5

TABLE IV

COEFFICIENTS OF VARIATION (C.V.) OF THE *m/e* 264 BACKGROUND (ng/ml TRAMADOL EQUIVALENTS) FOR AQUEOUS BLANK SAMPLES AND BLANK SERUM SAMPLES FROM TEN HEALTHY VOLUNTEERS

Samples	Mean ± S.D.	C.V. (%)
Blank water samples	1.75 ± 0.225	12.9
Blank serum samples	2.04 ± 0.740	36.2

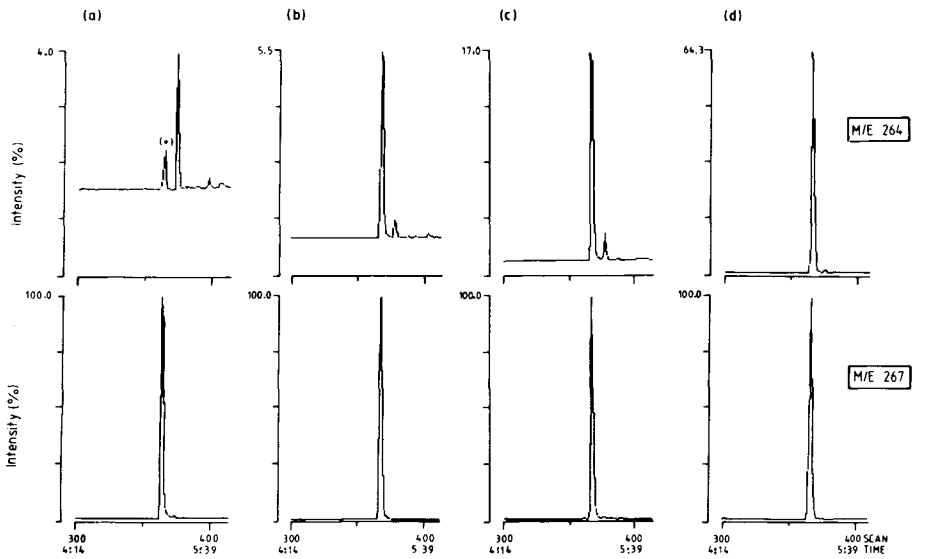

Fig. 3. Typical mass fragmentograms of human serum extracts spiked with different amounts of tramadol hydrochloride (*m/e* 264) (T), and 300 ng of internal standard (*m/e* 267): (a) blank serum; (b) 12.5 ng/ml T; (c) 50 ng/ml T; (d) 200 ng/ml T. The peak marked with an asterisk in mass fragmentogram (a) corresponds to the T content of the internal standard (cf., Table IV). Note the differences in the intensities at *m/e* 264 compared with those at *m/e* 267.

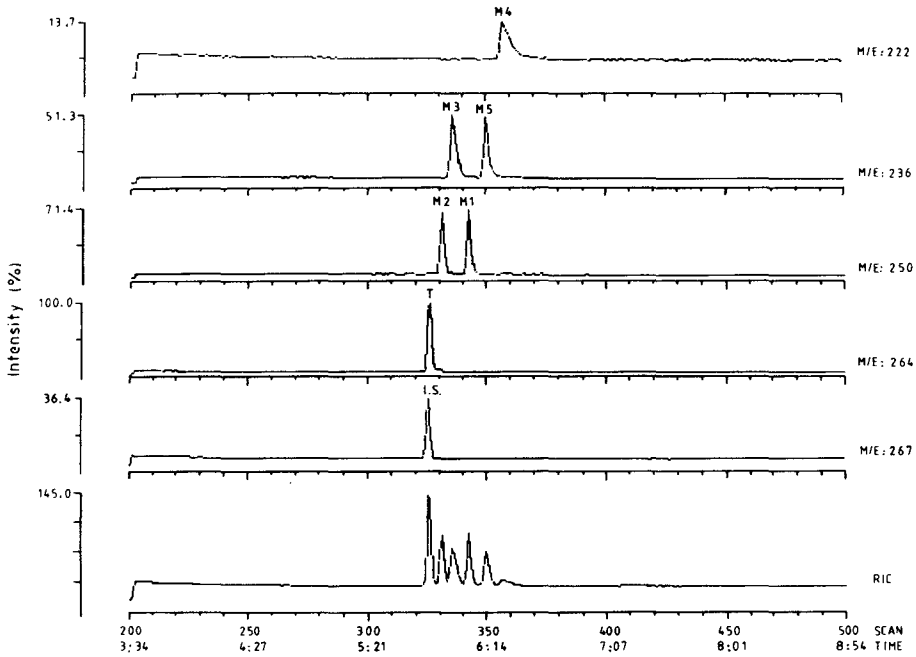

Fig. 4. Selected-ion monitoring (SIM) and reconstructed ion current (RIC) records after injection of 1 μl of a pure solution of tramadol, its metabolites M1—M5 and the internal standard [^{2}H,^{15}N]tramadol in chloroform. Concentrations, about 0.2 mg/ml; *m/e* values: 264 (T = tramadol); 267 (I.S. = [^{2}H,^{15}N]tramadol); 250 (M2 and M1); 236 (M3 and M5); 222 (M4).

extracted together with tramadol did not interfere with tramadol, the retention times of these metabolites under the GC conditions being greater than those of tramadol (Fig. 4). Appreciable amounts of the basic metabolites M2 and M3, however, are extracted together with tramadol; but there is no danger of interference in quantification, because the GC resolution between tramadol and these metabolites is satisfactory (Fig. 4). To confirm these considerations by experiment, we compared the results for tramadol-spiked serum samples with or without addition of a two-fold excess of M2. As shown in Table II, there was no significant difference between the samples containing M2 and those without M2. Hence the results confirm the expected high selectivity of the method, especially as M2 was added in amounts that are appreciably greater than those found in human serum following intravenous or oral administration of tramadol hydrochloride [14, 17].

Finally, glucuronides and sulphates of the metabolites M1, M4 and M5, which are known metabolites of tramadol in human serum [14], did not disturb the drug determination because they are not transferred to the solvent phase prior to enzymatic cleavage, and because they yield M1, M4 and M5, respectively, after cleavage.

Sensitivity

Maximum sensitivity for quantification was achieved by SIM using CI with isobutane and focusing on the base peaks of tramadol and [$^2H_2$,^{15}N]tramadol

at *m/e* 264 and 267, respectively (Fig. 2). The detection limit of the optimized MS detector with a freshly cleaned ion source is about 50 pg of tramadol, as was shown by injection of pure solutions of tramadol into the GC—MS system. This value corresponds to a theoretical detection limit of 0.5 ng/ml after extraction of 1 ml of serum and injection of one tenth of the final extract into the GC—MS system on condition that the overall recovery of tramadol is about 100%. As the recovery is reduced by the extraction procedure, and also by adsorption losses on the glassware and the GC column (see below), the actual detection limit would be about 1.0 ng/ml for 1-ml serum samples and injection of one tenth of the final extract, if the *m/e* 264 content of the internal standard were much lower than the actual detection limit. However, as [$^2H_2$,^{15}N]tramadol has an isotopic purity of about 99% and its tramadol content is about 1% (Table I), the amount of tramadol is about 3 ng when 300 ng of the internal standard are used, corresponding to 1.5 ng/ml at overall recoveries of about 50%.

Indeed, when pure water samples spiked with 300 ng of [$^2H_2$,^{15}N]tramadol hydrochloride were analysed, the background peak at *m/e* 264 corresponded to 1.75 ± 0.23 ng/ml of tramadol hydrochloride in water (Table IV). Similar results were obtained by using tramadol-free serum samples from ten volunteers instead of pure water samples (Table IV). This indicates that the background peak originates exclusively from the internal standard added as carrier and for exact quantification. Further, in the chromatograms of the blank samples no additional background peak was observed to interfere with tramadol. From the statistical results ($\bar{x}$ ± S.D.) for the background of the internal standard at *m/e* 264, and for the *y*-intercept (*a*) of the linear calibration graphs (see above), the 99% detection limit of the method ($\bar{x}$ ± 3 S.D.) is 4.3 and 3.6 ng/ml, respectively, if 300 ng of internal standard and 1 ml of serum are used, but can be lowered to about 1 ng/ml if only 50 ng of the internal standard are applied.

Accuracy and precision

Both the within-run and between-run accuracy and precision of the assay were investigated over a six-month period and the results are presented in Tables V and VI. Acceptable accuracy and precision were achieved even at the lowest concentration. The within-run coefficient of variation was 0.5—1.1% at concentrations between 25 and 200 ng/ml and 1.8—2.9% at the lowest concentrations

TABLE V

WITHIN-RUN COEFFICIENTS OF VARIATION (C.V., *n* = 10) AND ACCURACY OF TRAMADOL DETERMINATION IN HUMAN POOL SERUM

Concentration added (ng/ml)	Concentration found (mean ± S.D.) (ng/ml)	C.V. (%)	Accuracy (mean) (%)
6.25	6.28 ± 0.181	2.88	100.5
12.5	12.1 ± 0.213	1.76	98.6
25.0	25.4 ± 0.280	1.10	101.6
50.0	50.9 ± 0.440	0.864	101.8
100	100.4 ± 0.436	0.434	100.4
200	198.6 ± 1.45	0.728	99.3

TABLE VI

BETWEEN-RUN COEFFICIENTS OF VARIATION (C.V., n = 10) AND ACCURACY OF TRAMADOL DETERMINATION IN HUMAN POOL SERUM

Concentration added (ng/ml)	Concentration found (mean ± S.D.) (ng/ml)	C.V. (%)	Accuracy (mean) (%)
12.5	12.3 ± 0.645	5.27	98.4
25.0	24.6 ± 0.931	3.78	98.4
50.0	50.4 ± 1.21	2.41	100.8
100	100.7 ± 1.68	1.67	100.7
200	199.5 ± 3.21	1.61	99.8

tested (6.25 and 12.5 ng/ml) (Table V). For this reason, the within-run accuracy also depends slightly on the concentration and varies from 98.6% to 101.8% in the concentration range from 6.25 to 200 ng/ml. As expected, the results for the between-run coefficients of variation were slightly inferior to the within-run results, increasing from 1.6% to 5.3% with decreasing concentrations in the range from 200 to 12.5 ng/ml, whereas the accuracies were as good as within the same run (98.4—100.8%) (Table VI). Hence the within-run and between-run experiments confirm that the GC—MS method is characterized by good precision and high accuracy.

Further characterization of the method

Stability of tramadol in serum. Since tramadol is very stable in aqueous solutions at room temperature, good stability in serum, especially at −20°C, can be expected. In fact, no decrease in tramadol concentration was detectable after storage of samples at −20°C over a period of one year (see Table VII).

Extraction recovery. The extraction recovery was determined on samples of serum spiked with [^{14}C]tramadol hydrochloride (50 ng/ml). As expected in view of the partition coefficients [14], the extraction recovery was almost 100% if alkaline aqueous phases (pH > 9) were extracted with n-hexane. Nevertheless, the overall recovery of tramadol during sample preparation was about half that (50—55%). This relatively low overall recovery is due to incomplete phase separations during sample preparation, and also by inevitable adsorption losses to the glass surfaces during the extraction and concentration procedures. Hence the extraction procedure reduces the sensitivity of the assay, but this effect is compensated for, the three-step extraction providing clean extracts for analysis.

Protein binding. Binding of tramadol to human serum proteins is slight (about 14%) [18]. Further, it does not influence the extraction recovery, as was shown by comparison of water and serum samples. Using [^{14}C]tramadol hydrochloride we obtained identical and nearly complete recoveries of radioactivity in the solvent phases of both sample types after extractions without re-extraction. This result indicates that the protein binding of tramadol is broken by the extraction procedure. Consequently, the results of quantitative determination are independent of the sample size, as was shown by analysing samples containing equal amounts of tramadol hydrochloride in 0.1 and 1.0 ml of human serum (Table II).

TABLE VII

SERUM CONCENTRATIONS OF TRAMADOL HYDROCHLORIDE (ng/ml) AFTER INTRAVENOUS INJECTION OF 32.8 (A) AND 100 mg (B) TO TWO MALE VOLUNTEERS (A AND B), ASSAYED ON TWO DIFFERENT DAYS

Time delay between the first (c_1) and the second (c_2) tramadol determination; three days (A) and about one year (B); c_1/c_2 = ratio of first to second tramadol determination.

Time (h)	Serum concentration (ng/ml)					
	Volunteer A			Volunteer B		
	c_1	c_2	c_1/c_2	c_1	c_2	c_1/c_2
0.017	N.D.*	N.D.	N.D.	1149	1145	1.003
0.045	2075	2062	1.006	1011	962	1.051
0.080	484	475	1.019	941	981	0.959
0.112	340	328	1.037	N.D.	N.D.	N.D.
0.16	236	240	0.983	635	610	1.041
0.250	173	173	1.000	582	602	0.967
0.333	160	154	1.039	N.D.	N.D.	N.D.
0.500	138	140	0.986	579	578	1.002
0.750	110	113	0.973	N.D.	N.D.	N.D.
1.04	101	103	0.981	490	N.D.	N.D.
1.3	99.0	98.0	1.010	N.D.	N.D.	N.D.
1.5	89.9	91.4	0.984	447	410	1.090
2.0	84.2	80.5	1.046	394	407	0.968
2.5	78.7	80.5	0.978	N.D.	N.D.	N.D.
3.0	74.4	74.2	1.003	334	337	0.991
4.0	61.3	60.8	1.008	284	292	0.973
5.0	57.3	56.5	1.014	N.D.	N.D.	N.D.
6.0	52.1	54.4	0.958	232	237	0.979
7.0	49.2	48.1	1.023	N.D.	N.D.	N.D.
8.0	45.1	47.3	0.953	185	189	0.978
10.0	41.6	N.D.	N.D.	144	143	1.007
12.0	34.1	36.9	0.924	122	114	1.070
14.0	N.D.	N.D.	N.D.	104	106	0.981
24.0	N.D.	N.D.	N.D.	41	42	0.976
Mean			0.996			1.002
S.D.			0.031			0.040

*N.D. = Not determined.

Other biological fluids. Tests with blank samples of human urine, human bile, human milk and tissue samples from rats showed that the method described is also applicable to other biological fluids and tissue homogenates, without essential modifications to the sample preparation. In addition, identical blank chromatograms were obtained by using whole blood or plasma samples instead of serum samples. This means that it is possible to apply the method to blood or plasma in addition to serum.

Application of the assay

The utility of the method was demonstrated by applying it to the determination of tramadol in the serum of two healthy volunteers after intravenous

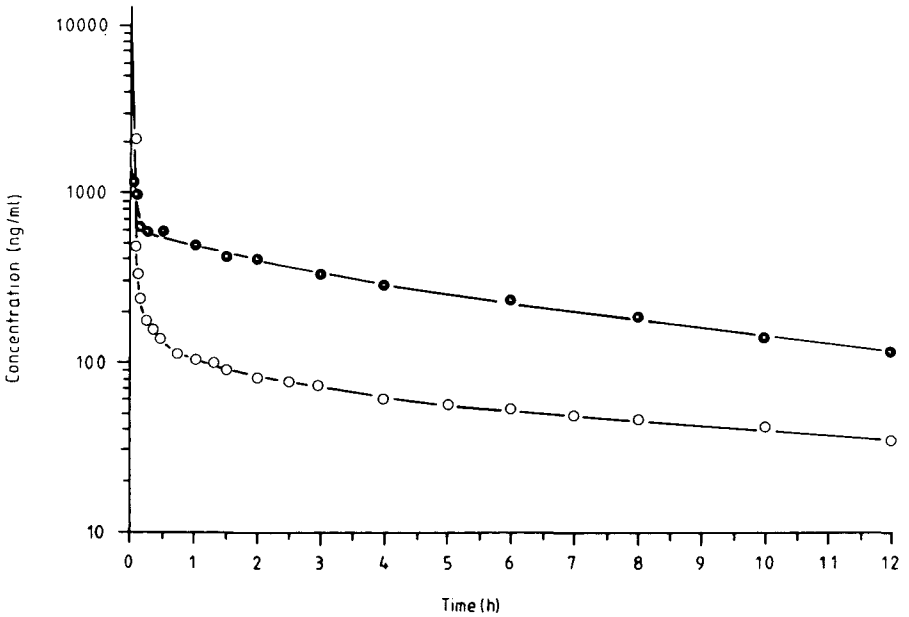

Fig. 5. Time course of serum concentrations of tramadol hydrochloride (mean values of two assays) following a single intravenous dose of 32.8 mg (volunteer A, ○) and 100 mg (volunteer B, ●) of tramadol hydrochloride administered to two healthy male volunteers. For exact values, see Table VII.

injection of tramadol hydrochloride. The results are given in Fig. 5 and Table VII. The serum concentration curves are fairly consistent, indicating a relatively short distribution phase with an apparent half-life of about 0.5 h and a terminal half-life of approximately 6 h. The time courses show that the technique described is sufficiently precise for the determination of serum concentrations of tramadol hydrochloride in humans, and that it is sensitive enough to allow the determination of tramadol over five to six half-lives, i.e., 30—36 h after the administration of therapeutic doses (50—100 mg).

CONCLUSION

With the GC—MS method described it is possible to determine tramadol quantitatively in serum, plasma, whole blood and other biological samples. The method offers the advantage of combining precision, accuracy, sensitivity and selectivity, and eliminates any interference from endogenic compounds in the biological fluids. Therefore, it appears satisfactory for all pharmacokinetic and drug monitoring studies after therapeutic dosage, allowing accurate determination of tramadol concentrations up to at least five biological half-lives.

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REFERENCES

1 E. Frankus, E. Friderichs, S.M. Kim and G. Osterloh, Arzneim.-Forsch., 28 (1978) 114.
2 E. Friderichs, F. Felgenhauer, P. Jongschaap and G. Osterloh, Arzneim.-Forsch., 28 (1978) 122.
3 A. Rost and E.G. Schenck, Arzneim.-Forsch., 28 (1978) 181.
4 E.G. Schenck and I. Arend, Arzneim.-Forsch., 28 (1978) 196.
5 I. Arend, B. von Arnim, J. Nijssen, J. Scheele and L. Flohé, Arzneim.-Forsch., 28 (1978) 199.
6 E.G. Schenck and I. Arend, Arzneim.-Forsch., 28 (1978) 209.
7 L. Flohé, I. Arend, A. Cogal, W. Richter and W. Simon, Arzneim.-Forsch., 28 (1978) 213.
8 H. Krueger, and W. Müller-Limmroth, Arzneim.-Forsch., 28 (1978) 176.
9 W. Richter, B. von Arnim and H. Giertz, Münch. Med. Wochenschr. , 123 (1981) 517.
10 D. Goroll, Med. Klin, 78 (1983) 173.
11 D. Paravicini, C. Schöngart and P. Lawin, Therapiewoche, 31 (1981) 2948.
12 W. Müller-Limmroth and H. Krueger, Arzneim.-Forsch., 28 (1978) 179.
13 W. Lintz, Naunyn-Schmiedeberg's Arch. Pharmacol., 313S (1980) R53.
14 W. Lintz, S. Erlacin, E. Frankus and H. Uragg, Arzneim.-Forsch., 31 (1981) 1932.
15 S. Erlacin, E. Frankus and W. Lintz, Naunyn-Schmiedeberg's Arch. Pharmacol., 313S (1980) R51.
16 K. Flick, E. Frankus and E. Friderichs., Arzneim.-Forsch., 28 (1978) 107.
17 W. Lintz, P.H. Hinderling and H. Uragg, Arzneim.-Forsch., in preparation.
18 W. Lintz, Arzneim.-Forsch., in preparation.

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USE OF DUAL-COLUMN FUSED-SILICA CAPILLARY GAS CHROMATOGRAPHY IN COMBINATION WITH DETECTOR RESPONSE FACTORS FOR ANALYTICAL TOXICOLOGY

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SUMMARY

Retention and detector response factor data have been given for 188 compounds on the DB1 capillary column using a dual nitrogen—phosphorus and flame ionization detection system. Factors affecting the detector response factor parameter in a dual-capillary column system have been discussed showing its advantage in drug screening.

INTRODUCTION

Recent developments in fused-silica capillary column manufacture have resulted in the increased use of capillary gas chromatography (GC) as a drug-screening technique in analytical toxicology [1—4]. The improved chromatography of the capillary column provides an excellent screening technique for drugs or poisons isolated from tissue extracts. The improvement in resolution in capillary GC can be tempered somewhat by the increased complexity of chromatogram generally experienced by the analyst in comparison to previous packed-column techniques. A recent report by Perrigo et al. [4] described the reproducibility of retention indices obtained from a capillary column system. The analyst still has the problem of differentiating between compounds with similar retention indices. These similarities in retention index occur between drug compounds themselves or between drugs and other products resulting from the extraction procedure.

It has been the practice in our laboratory with packed-column GC to use a variety of detectors and columns to handle various screening and quantitation problems [4—6]. Methods of dual-column screening have been reported using

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columns of differing polarity for drug identification [7, 8]. The relative ease with which two capillary columns can be inserted into a single injection port makes dual-column screening quite appealing.

The furter characterization of drugs in terms of retention time and a relative detector response ratio was reported by Baker [9]. Seventy-one drugs were characterized by their retention time on a packed 3% OV-17 column and by their relative response on a nitrogen-selective detector and a flame ionization detector. Caffeine was used as the internal standard for the detector response factors. This characterization of response ratio was successfully used to differentiate drugs having similar retention times.

This report discusses our recent investigations of using detector response factors (DRF values) in combination with retention indices (RI), achieved by temperature-programmed capillary GC, for improved toxicological analyses. Matched DB1 capillary columns are used to provide retention data with a concurrent determination of DRF values. Accordingly, the discriminating power [10, 11] for this combined approach was examined and tested.

EXPERIMENTAL

Equipment

A Hewlett-Packard Model 5880A gas chromatograph (Avondale, PA, U.S.A.), equipped with a flame ionization and a nitrogen—phosphorus detector was used to obtain the data in this report. The columns used were Durabond fused-silica DB1, 15 m × 0.32 mm I.D. with a film thickness of 0.25 μm (J & W Scientific, Rancho Cordova, CA, U.S.A.). DB1 is a bonded methyl polysiloxane equivalent phase that has been marketed to substitute for SE-30, OV-1 or SP-2100. Two closely matched columns were obtained by breaking a 30-m column in half.

The chromatograph was operated in the split mode, 10:1 using helium as the carrier and make-up gas to the nitrogen—phosphorus detector; make-up gas flow-rate was 20 ml/min.

The carrier gas linear velocity used was 29 cm/sec. This was slightly higher than the optimum velocity required for maximum column efficiency. The septum purge rate was 1 ml/min. Gas flow-rates to the nitrogen—phosphorus detector were hydrogen 4 ml/min, air 50 ml/min and to the flame ionization detector, hydrogen 20 ml/min, air 270 ml/min. The injection port temperature was 250°C and the injection port liner contained a 2-cm plug of 3% OV-101. The standard temperature programme used was 8°C/min from 120°C to 280°C with a 5-min hold at the upper temperature level. The detector temperature was 300°C.

The dual-column configuration was accomplished by inserting two 15-m columns into the same injection port. A good seal was obtained using a graphite ferrule with a slightly enlarged single hole. Retention times were matched on each column by injecting a test mixture and breaking off a small portion of the column having the later elution times until values were within ± 0.02 min. Once standard conditions had been set up the carrier gas flow-rate was adjusted slightly to keep eluting standards within ± 0.05 min of a reference value.

Method

The retention indices in Table I were determined by linear interpolation to retention time (t_R) values for hydrocarbons run under standard conditions as previously described [4]. The reference hydrocarbon t_R values used have been listed in Table II.

TABLE I

RETENTION INDICES (RI) AND DETECTOR RESPONSE FACTOR (DRF) DATA

Compound	RI (DB1)	DRF (ACB)	Compound	RI (DB1)	DRF (ACB)
Cyclopentamine	1085	4.02	Tryptamine	1681	3.80
Amphetamine	1118	1.62	Talbutal	1689	0.50
Methamphetamine	1173	2.08	Amobarbital	1697	0.45
Tropine	1183	3.24	Salol	1702	0.00
Ethosuximide	1193	0.44	Pentobarbital	1716	0.48
Arecoline	1195	3.72	Pethidine	1730	1.82
Tranylcypromine	1198	2.42	Norpethidine	1749	1.89
Fenfluramine	1220	1.68	Methohexital	1756	1.36
Mephentermine	1243	1.77	Meprobamate	1762	0.04
Phenylpropanolamine	1308	1.90	Caffeine	1768	11.7
Nicotine	1326	3.80	Secobarbital	1769	0.46
Chlorphentermine	1338	1.07	Pheniramine	1788	2.85
Ethinamate	1349	0.056	Alphaprodine	1788	1.52
Ephedrine	1350	2.38	Butrylaminophenol	1790	1.12
Pseudoephedrine	1360	2.14	Glutethimide	1806	0.20
Tyramine	1371	2.88	Prilocaine	1811	2.73
Hydroxyamphetamine	1404	1.94	Hexobarbital	1831	1.60
Salicylamide	1405	0.054	Ethoheptazine	1836	1.78
Metharbital	1417	1.96	Thiopentobarbital	1837	2.24
Phenmetrazine	1419	2.21	Carisoprodol	1847	1.99
Hordenine	1432	2.68	Diphenhydramine	1849	1.76
Methylenedioxyamphetamine	1443	1.98	Lidocaine	1854	2.66
Barbital	1465	0.69	Methylphenobarbital	1869	1.40
Tolazoline	1471	3.34	Aminopyrine	1879	5.54
Methyprylon	1497	1.41	Thiamyal	1886	2.11
Nikethamide	1497	4.39	Azapetine	1917	1.17
Benzocaine	1523	1.39	Theophylline	1917	14.1
3,4-Dimethoxyamphetamine	1537	1.62	Orphenadrine	1924	1.60
Chlorprenaline	1560	2.14	Phenyltoloxamine	1926	1.85
Allobarbital	1575	0.48	Phenobarbital	1928	0.52
Ibuprofen	1594	0.00	Butallylonal	1944	0.50
Aprobarbital	1594	0.56	Tripellenamine	1961	4.18
Methsuximide	1597	1.14	Methapyrilene	1965	4.74
Phenylephrine	1606	4.05	Pemoline	1968	1.45
Phensuximide	1607	1.44	Chlorpheniramine	1985	2.87
Bethanidine	1618	4.91	Aminochlorobenzophenone	1994	1.00
Acetaminophen	1631	1.43	Metoprolol	2023	2.20
Butabarbital	1634	0.55	Heptabarbital	2032	0.42
Butethal	1641	0.51	Mepivacaine	2041	2.73
Methoxymethylenedioxyamphetamine	1662	2.02	Oxytheophylline	2052	11.2
Mescaline	1663	2.19	Brompheniramine	2082	3.11
			Dicyclomine	2091	1.56
			Nomifenison	2108	2.07

(Continued on p. 84)

TABLE I (continued)

Compound	RI (DB1)	DRF (ACB)	Compound	RI (DB1)	DRF (ACB)
Methaqualone	2115	1.72	Hexahydrocannabinol	2407	0.00
Dextromethorphan	2116	1.49	Butacaine	2436	2.57
Aminodichlorobenzo-phenone	2119	1.00	Grey stopper artifact	2457	0.00
			Nordiazepam	2459	2.04
Methadone	2131	1.37	Tetrahydrocannabinol	2471	0.00
Propranolol	2136	1.94	Chlorpromazine	2474	2.82
Alverine	2137	1.50	Acetylcodeine	2480	1.47
Procyclidine	2154	1.62	Oxycodone	2483	1.80
Primidone	2159	2.87	Monoacetylmorphine (06)	2491	1.65
Hyoscyamine	2174	1.58	Oxethazine	2494	4.83
Cocaine	2175	1.79	Thebacon	2498	1.50
Propoxyphene	2178	1.33	Methotrimeprazine	2511	2.53
Amitriptyline	2179	1.50	Clobazam	2514	2.44
Atropine	2183	1.77	Norpropoxyphene amide	2527	1.29
Nortriptyline	2191	1.57	Trimethoprim	2534	5.33
Procainamide	2193	5.38	Cannabinol	2538	0.00
Trimipramine	2204	2.55	Nalorphine	2542	1.60
Imipramine	2205	2.40	Prenylamine	2546	1.26
Zimelidine	2206	3.17	Phenacaine	2546	2.15
Medazepam	2207	2.49	Temazepam	2554	2.27
Doxepin	2210	1.55	Midazolam	2559	3.36
Fluopromazine	2212	3.01	Bromazepam	2563	4.05
Tetracaine	2212	3.90	Flunitrazepam	2572	3.23
Desipramine	2217	2.52	Chloroquine	2600	4.33
Nordoxepin	2219	1.68	Amoxapine	2600	4.50
Norzimelidine	2223	3.28	Diamorphine	2602	1.51
Benzhexol	2226	1.55	Prazepam	2624	2.02
Protriptyline	2226	1.38	Hydroxyethylflurazepam	2630	2.33
Triprolidine	2236	2.88	Nimetazepam	2640	3.71
Benactyzine	2249	1.72	Naloxone	2644	1.92
Halazepam	2250	2.26	Trifluoperazine	2662	4.01
Promethazine	2254	2.61	Cinchocaine	2693	3.69
Carbamazepine	2259	1.01	Fentanyl	2701	2.06
Bupivicaine	2267	2.30	Nitrazepam	2714	2.61
Antazoline	2280	4.29	Flurazepam	2763	3.42
Trimeprazine	2283	2.87	Quinine	2773	2.73
Scopolamine	2286	1.93	Chlordiazepoxide	2778	3.55
Phenytoin	2289	0.85	Clonazepam	2795	2.89
Oxazepam	2293	2.25	Bisacodyl	2814	1.12
Benztropine	2302	1.56	Hydroxyzine	2874	3.85
Maprotiline	2315	1.41	Doxapram	2874	2.75
Levallorphan	2330	1.40	Alprazolam	2910	3.44
Cyproheptadine	2333	1.33	Haloperidol	2921	2.17
Phenylbutazone	2344	1.80	Diltiazem	2927	3.15
Codeine	2348	1.60	Triazolam	3008	3.70
Dihydrocodeine	2357	1.65	Meclozine	3030	3.08
Cannabidiol	2375	0.00	Etorphine	3033	1.23
Lorazepam	2375	2.31	Dimethothiazine	3050	4.54
Clomipramine	2397	2.47	Cholesterol	3081	0.00
Hydrocodone	2401	1.70	Strychnine	3109	2.36
Diazepam	2404	2.53	Thioridazine	3117	1.84
Desalkylflurazepam	2405	1.94	Noscapine	3168	1.49
Morphine	2406	1.71			

TABLE II

STANDARD RETENTION TIMES

Hydrocarbon RI value	t_R (min)	Hydrocarbon RI value	t_R (min)
1100	1.34	2200	12.21
1200	1.74	2300	13.30
1300	2.30	2400	14.34
1400	3.08	2500	15.35
1500	4.04	2600	16.32
1600	5.13	2700	17.26
1700	6.31	2800	18.17
1800	7.53	2900	19.04
1900	8.74	3000	19.88
2000	9.93	3100	20.76
2100	11.09		

The DRF values listed in Table I were calculated from the relative detector response of a compound X, nitrogen—phosphorus detection/flame ionization detection (NPD/FID), as compared to internal standard 2-amino-5-chlorobenzophenone (ACB). The formula for these calculations is

$$\text{DRF (ACB)} = \frac{\text{NPD area X/FID area X}}{\text{NPD area ACB/FID area ACB}}$$

Standards used for injection were made up in ethanol, methanol, or hexane to a concentration of 5—15 mg per 100 ml. Two to four runs of each drug compound were made with standard solutions of ACB, caffeine and prazepam to provide retention index and average DRF values. Some compounds that gave complex chromatograms and were not included in Table I owing to uncertainty as to the cause of these effects were tolbutamide, warfarin and carbromal, as well as the desmethyl metabolites of propoxyphene and chlordiazepoxide.

RESULTS AND DISCUSSION

Careful consideration was given to the choice of standard for DRF determinations. The data in Table III demonstrate the levels of precision calculated using four test compounds (nicotine, caffeine, ACB, and prazepam) for ten drugs with divergent retention indices. Although all four compounds gave good precision, ACB was chosen as the reporting standard for the following reasons: reluctance to co-inject caffeine with case material was expressed by some analysts queried in this regard owing to that compound's potential significance; ACB eluted in an appropriate mid-range position, chromatographically; ACB is readily available as a chemical compound and has proven stable in ethanol at room temperature for a period of at least one month; ACB has potential use as a DRF standard for the electron-capture detector.

As commented on by Baker [9], a distinction should be made between the level of reproducibility expected in short-term (i.e. daily) or long-term test

TABLE III

TEST STANDARDS FOR DRF CALCULATION (n = 15)

Test compound	RI	DRF using nicotine		DRF using caffeine		DRF using ACB		DRF using prazepam	
		Average	C.V. (%)	Average	C.V. (%)	Average	C.V. (%)	Average	C.V. (%)
Fenfluramine	1220	0.440	0.8	0.147	1.5	1.78	2.4	0.881	4.3
Nicotine	1326	—	—	0.333	2.0	4.04	2.9	1.99	4.3
Clorprenaline	1560	0.531	5.3	0.177	3.5	2.14	2.8	1.06	2.8
Pethidine	1730	0.469	1.7	0.156	0.6	1.90	1.4	0.939	3.5
Caffeine	1768	3.00	2.0	—	—	12.1	1.1	6.00	3.2
Diphenhydramine	1849	0.450	2.4	0.150	0.7	1.82	1.0	0.902	3.1
ACB	1994	0.247	3.1	0.082	1.1	—	—	0.495	2.4
Methaqualone	2115	0.424	2.7	0.141	3.4	1.71	2.7	0.847	3.3
Amitriptyline	2179	0.380	3.1	0.127	1.3	1.54	0.7	0.749	2.3
Carbamazepine	2259	0.256	4.6	0.085	2.4	1.03	1.5	0.513	1.4
Diazepam	2404	0.648	3.8	0.216	2.1	2.62	1.4	1.30	2.0
Prazepam	2624	0.500	5.3	0.167	3.4	2.02	2.5	—	—
Cinchocaine	2693	0.992	5.4	0.330	3.3	4.00	2.3	0.198	1.4
Triazolam	3008	0.971	7.2	0.324	5.4	3.92	4.5	1.94	3.6

TABLE IV

LONG-TERM VARIATION IN DRF VALUES

n = 75, over a ten-month period.

	Average	S.D.	C.V. (%)	Range
Area NPD/FID for caffeine	117	39	33	67—220
DRF for caffeine, using ACB reference	11.69	0.38	3.3	10.55—12.29
Area NPD/FID for ACB	10.1	3.4	34	5.5—19

situations. A comparison of DRF precision for data collected over a ten-month period for repeated injections of ACB and caffeine is shown in Table IV. The data presented in Tables III and IV are both representative of the level coefficient of variation (C.V., %) experienced when testing is carried out over a long-term period. In the single-test situation (i.e. runs on the same day) the choice of a standard may be optimized to easily produce a C.V. value of less than ± 3% for tested compounds. However, in the longer term or in comparisons of inter-laboratory data bases, a variation of 5—10% is likely to be more prudent when using the DRF parameter for drug screening. The absolute variation in individual NPD/FID response ratios for ACB and caffeine as presented in Table IV indicates the requirement of an internal standard for DRF calculations. Changing the bead does not affect DRF. The effect on concentration on DRF values was also briefly studied. A summary of this information in Table V shows there is little difference within injection of 5—1000 ng.

Discriminating power (DP) calculations have been used to demonstrate the benefits of various combinations of search data in the identification of drug compounds [10]. A DP calculation tests each member of a data set against all other members using designated error factors or search windows. For example, a peak with RI = 2100 would be considered unresolved from compounds eluting in the RI range 2080—2120 if the search window was set at ± 20 RI

TABLE V

EFFECT OF CONCENTRATION ON DRF VALUES

Compound	DRF (ACB) average values for ng injected		
	5—15	50—150	⩾ 1000
Fenfluramine	1.69	1.68	1.74
Nicotine	3.82	3.80	4.21
Clorprenaline	2.19	2.14	2.14
Phensuximide	1.36	1.44	1.45
Pethidine	1.80	1.82	1.97
Caffeine	11.3	11.7	11.5
Diphenhydramine	1.72	1.76	1.81
Methaqualone	1.62	1.72	1.64
Propoxyphene	1.30	1.33	1.45
Amitriptyline	1.50	1.50	1.51
Carbamazepine	1.05	1.01	1.01
Diazepam	2.79	2.53	2.42
Prazepam	1.95	2.02	1.91
Cinchocaine	3.99	3.69	3.75
Sample size (n)	3—5	8—12	3—5

units. The number of matches or unresolved members in the data base is then used to calculate DP as follows:

$$DP = 1 - 2M/n(n-1)$$

where M = number of matches; n = sample size. A DP value of 1.0 implies resolution of all compounds in the data base.

The value of DRF calculation using varied error factors for comparison is shown in Table VI, using a data sample of 188 compounds. The number of matches (M) is included to give a better perspective of the results.

The number of possible matches in a data base of this size is 17 578 if there was zero discriminating power. Because the variation in DRF value must be expressed as a percentage, rather than as an absolute number, a slightly

TABLE VI

DISCRIMINATING POWER (DP) CALCULATIONS

Sample size n = 188; M = number of matches.

RI values			Combination RI + DRF (ACB) values		
Error factor + RI units	M	DP	Error factor ± DRF (%)	M	DP
30	598	0.9659	10	142	0.9919
30	598	0.9659	5	73	0.9958
10	194	0.9890	10	50	0.9972
10	194	0.9890	5	21	0.9988
5	106	0.9940	10	28	0.9984
5	106	0.9940	5	9	0.9995

different approach was taken than for the determination of error factors for retention indices. The percentage error factors for DRF values listed in Table VI assume that the error is present in both values under test for discrimination. For example, two compounds which have DRF values of 1.00 and 1.50 are considered discriminated at the ± 10% level but not discriminated at the ± 20% level: i.e. 1.0 ± 10% = range 0.90—1.10; 1.5 ± 10% = range 1.35—1.65, indicating no match (or resolution is achieved); 1.0 ± 20% = range 0.80—1.20; 1.5 ± 20% = range 1.20—1.80, indicating a match where peaks are not resolved.

The combined DP values shown in Table VI compare favorably to combination values obtained from (packed) GC columns of differing polarity [11]. A range of error factors for both RI and DRF parameters has been calculated and is shown in Table VI for purposes of comparison and to show the benefits of increasing precision for RI and DRF calculations. The combined DP values of RI and DRF demonstrate the usefulness of the detector response qualifier in data base searching. A search window of ± 5 RI units in combination with a DRF variation of ± 10% has been routinely used in our laboratory. Future work in the authors' laboratory will involve the use of the electron-capture detector in a DRF context, as well as the choice of a secondary screening and quantitation column for further compound discrimination.

REFERENCES

1 W.H. Anderson and D.T. Stafford, J. High Resolut. Chromatogr. Chromatogr. Commun., 6 (1983) 247.
2 A. Eklund, J. Jonsson and J. Schuberth, J. Anal. Toxicol., 7 (1983) 24.
3 W. Jennings, Amer. Lab., 18 (1984) 15.
4 B.J. Perrigo, D.J. Ballantyne and H.W. Peel, Can. Soc. Forens. Sci. J., 17 (1984) 41.
5 B.J. Perrigo and H.W. Peel, J. Chromatogr. Sci., 19 (1981) 219.
6 H.W. Peel and B.J. Perrigo, J. Anal. Toxicol., 4 (1980) 105.
7 B. Newton and R.F. Foery, J. Anal. Toxicol., 8 (1984) 129.
8 G.W. Hime and L.R. Bednarczyk, J. Anal. Toxicol., 6 (1982) 247.
9 J.K. Baker, Anal. Chem., 49 (1977) 906.
10 A.C. Moffat, P. Owen and C. Brown, J. Chromatogr., 160 (1978) 179.
11 A.C. Moffat, A.H. Stead and K.W. Smalldon, J. Chromatogr., 90 (1974) 19.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY AND PRECLINICAL PHARMACOLOGICAL STUDIES OF PIBENZIMOL (BISBENZIMIDAZOLE)

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SUMMARY

A sensitive and selective high-performance liquid chromatographic assay with ultraviolet or fluorescence detection has been developed for the experimental antitumor agent pibenzimol. Drug is isolated from plasma or other aqueous solutions with reversed-phase C_{18} disposable extraction columns and chromatography afforded with a deactivated reversed-phase C_{18} column and phosphate buffer—methanol mobile phase. Plasma standard curves are linear for concentrations for pibenzimol from 0.01 to 5.0 μg/ml. Pibenzimol is stable in fresh human plasma and whole blood. Pibenzimol appears to bind to plasma proteins; however, drug adsorption to glass, plastic, membranes, and filters precludes accurate determination of pibenzimol plasma protein binding. Plasma concentrations of pibenzimol fall rapidly following rapid intravenous administration to rabbits, but parent drug is detectable in plasma 24 h after drug administration. The 24-h urinary recovery of pibenzimol is 10—20%.

INTRODUCTION

Pibenzimol (bisbenzimidazole, Hoechst 33258, NSC-322921, Fig. 1) binds to adenine—thymine base pairs of double-stranded DNA and is used as a water-soluble fluorescent stain in chromosomal analysis [1—3]. It has antitumor activity in vivo against intraperitoneally implanted murine L1210 and P388 leukemia [4]. Optimal activity is observed following intraperitoneal administration on a daily ×9 schedule [4]. Pibenzimol is currently undergoing preclinical pharmacologic and toxicologic evaluation by the National Cancer Institute prior to phase I clinical trials. As part of our preclinical pharmacologic studies a sensitive and selective high-performance liquid chromatographic (HPLC) assay has been developed using fluorescence detection for the drug. The assay has

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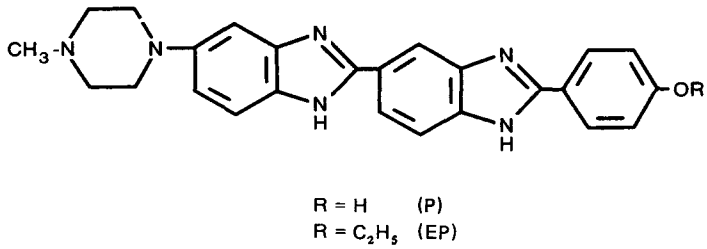

R = H (P)
R = C_2H_5 (EP)

Fig. 1. Structures of pibenzimol (P) and the O-ethyl (C_2H_5) analogue internal standard (EP).

been applied to drug stability studies, plasma protein binding studies, and rabbit pharmacokinetic studies with pibenzimol.

MATERIALS AND METHODS

Reagents

Pibenzimol was supplied as the trihydrochloride pentahydrate by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD, U.S.A.), and also purchased from Aldrich (Milwaukee, WI, U.S.A.). The O-ethyl (C_2H_5) analogue (Hoechst 33342, Fig. 1) was purchased as the trihydrochloride from Aldrich. Amicon Centriflo cone filters and Centrifuge micropartition filter assemblies were purchased from Amicon (Danvers, MA, U.S.A.). Equilibrium dialysis chambers and dialysis membrane were purchased from Technilab Instruments (Pequannock, NJ, U.S.A.). Disposable extraction columns (C_{18}, 1 ml size) were purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.). All solvents were glass-distilled and of chromatographic grade. All stock solutions of pibenzimol were prepared in water, methanol, dimethylsulfoxide, or mobile phase (see below) at concentrations of at least 100 μg/ml.

High-performance liquid chromatography

Analyses were performed on an IBM LC 9533 instrument equipped with an IBM Model LC 9505 autosampler. Detection was provided by a Waters 440 fixed-wavelength (340 nm) detector or a Varian Fluorichrom fluorescence detector with filters to provide excitation energy at 300—380 nm and to detect emission energy above 420 nm. The detector signal was analyzed by an IBM 9000 computer data system using the IBM CAPS software program. Chromatography was afforded by a Waters Nova-Pak C_{18} 5-μm column (15.0 × 0.39 cm) with 0.02 *M* potassium phosphate (pH 7.0)—methanol (38:62) as the mobile phase (flow-rate 1 ml/min).

Sample preparation

Aliquots of buffer, plasma, whole blood or urine (0.1—1.0 ml) were diluted to 2 ml with phosphate buffer (0.01 *M*, pH 11.0) and the internal standard added (0.5 μg in 10 μl water). Samples were applied to extraction columns which had been rinsed with 2 ml each of methanol, water, and buffer. Sample application was followed by a water wash (2 ml) and by elution with methanol

(4 ml). Methanol was evaporated under a gentle stream of nitrogen, the residue reconstituted in 100—200 μl mobile phase and 20 μl were injected for analysis. Quantitative analysis of pibenzimol was provided by comparisons of pibenzimol/internal standard peak area ratios of unknown samples to those of standard curves prepared by adding known amounts of pibenzimol (0.01—10.0 μg) and internal standard (0.5 μg) to the appropriate solution.

Pibenzimol stability studies

Pibenzimol (0.5, 1.5, 5.0 μg/ml) was incubated for various times at 4°C, 24°C, and 37°C in phosphate buffer (0.05 *M*, pH 7.4), fresh human plasma or fresh human whole blood. Aliquots were removed at appropriate times and analyzed as described above.

Pibenzimol plasma protein binding studies

Pibenzimol was added to fresh human plasma samples to provide final concentrations of 0.5, 1.5, and 5.0 μg/ml. For equilibrium dialysis chamber studies, the buffer employed was 0.02 *M* sodium phosphate (pH 7.4) in 0.9% saline. At the completion of filtration or equilibrium periods, aliquots were removed, internal was standard added, and samples were analyzed by the HPLC assay. Standard curves were prepared for all studies.

For equilibrium dialysis chamber studies, dialysis chambers (2.5 ml each side) were fitted with dialysis membranes (0.074 mm, molecular weight cut-off 6000 daltons) which had been soaked overnight in distilled water. Buffer was added to one side of the chambers, and drug—buffer or drug—plasma solutions added to the other side. Chambers were placed in a 37°C incubator for 24 h. For cone filter (molecular weight cut-off 25 000 daltons) studies, filters were soaked overnight in distilled water. Drug—buffer or drug—plasma samples (5 ml) were placed in the cones for centrifugation at 850 *g* at 37°C. Centrifugation periods were 2.5 and 4 min for drug—buffer and drug—plasma solutions, respectively, to allow approximately 30% of the sample to pass through filters into plastic collection tubes. For micropartition filter (molecular weight cut-off 30 000 daltons) studies, approximately 1.0 ml drug—buffer or drug—plasma solutions were placed in filter assemblies prior to centrifugation at 37°C. Centrifugation was accomplished at 120 *g* and 1935 *g* for the drug—buffer and drug—plasma solutions, respectively.

Rabbit studies

Male New Zealand white rabbits (2.0—3.0 kg) were administered pibenzimol (2 or 10 mg/kg in approximately 5 ml saline) by rapid intravenous administration (approximately 2 min) into a peripheral ear vein using a vein infusion set with winged adaptor (Miniset, Travenol, Deerfield, IL, U.S.A.). Blood was collected in heparinized tubes from a peripheral vein of the ear not used for drug infusion. Urine was collected using a pediatric Foley catheter (French, Size 10, Bard, Murray Hill, NJ, U.S.A.) inserted under light anesthesia into the bladder through the urethra. Collection for the following 18 h was by drip pan into glass containers.

RESULTS

Efforts to isolate and concentrate pibenzimol from aqueous solutions by extraction with organic solvents were not successful. Recoveries were variable and usually less than 50%. Satisfactory results were obtained with C_{18} disposable extraction columns when samples were applied at pH 11.0 and materials eluted with methanol. Recoveries of pibenzimol and internal standard from plasma were approximately 70% and 60%, respectively. During these studies, it was observed that pibenzimol was adsorbed on glass and other surfaces from most dilute aqueous solutions. When relatively concentrated (100 μg/ml) solutions of pibenzimol in water, buffer, methanol, or dimethylsulfoxide were sampled over time and analyzed by ultraviolet absorbance or HPLC peak area, little or no loss of parent drug was observed. However, when the same experiments were repeated at lower concentrations (0.3—10.0 μg/ml), significant loss of parent drug was observed in aqueous solutions, but not in methanol or dimethylsulfoxide solutions. Results for one set of experiments with pibenzimol at a concentration of 1.5 μg/ml are summarized in Table I. Similar results were obtained at other concentrations (data not shown). No loss of pibenzimol was observed when samples were concentrated, reconstituted in mobile phase and stored in HPLC injection vials for 24 h. Build-up and/or carry-over of pibenzimol on the HPLC system was not observed under routine assay conditions.

TABLE I

LOSS OF PIBENZIMOL IN SOLUTIONS OVER TIME

Solution*	HPLC area ($\cdot 10^{-3}$)			
	0 min	60 min	120 min	1200 min
Dimethylsulfoxide	1179	1282	1234	1137
Methanol	1537	1320	1594	1859
Mobile phase**	1304	1246	1375	1252
Phosphate buffer, 0.05 *M*, pH 7.4	470	146	117	0
Distilled water	411	192	43	16

*1.5 μg/ml final pibenzimol concentration.
**0.02 *M* phosphate buffer (pH 7.0)—methanol (38:62).

Preliminary chromatographic studies with reversed-phase columns (C_8 and C_{18}) and buffer—methanol mobile phases provided acceptable chromatograms, but with significant peak tailing. This problem was prevented by use of a deactivated C_{18} column designed for analysis of basic drugs. Chromatograms of two plasma samples, one containing the internal standard and the other containing pibenzimol and internal standard, are illustrated in Fig. 2. Sensitivity of the assay was increased five- to twenty-fold (depending on lamp and gain settings) by using fluorescence detection rather than ultraviolet absorption detection. The limit of detection under standard assay conditions was approximately 5 ng/ml. A series of ten plasma standard curves (0.01—5.0 μg/ml pibenzimol) was evaluated for linearity and inter-assay variation. The

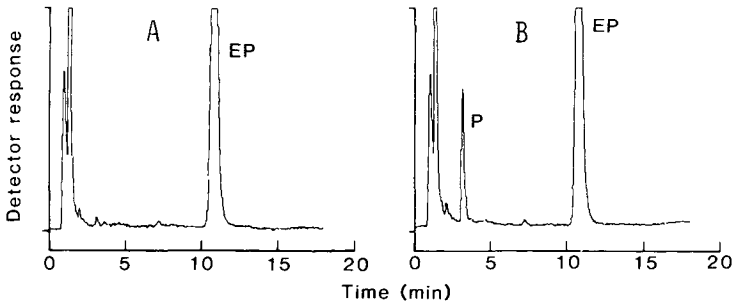

Fig. 2. Chromatograms from rabbit plasma samples (fluorescence detection) to which (A) 500 ng internal standard was added and (B) 500 ng internal standard was added and which contained 25 ng pibenzimol. Peaks: P = pibenzimol; EP = internal standard.

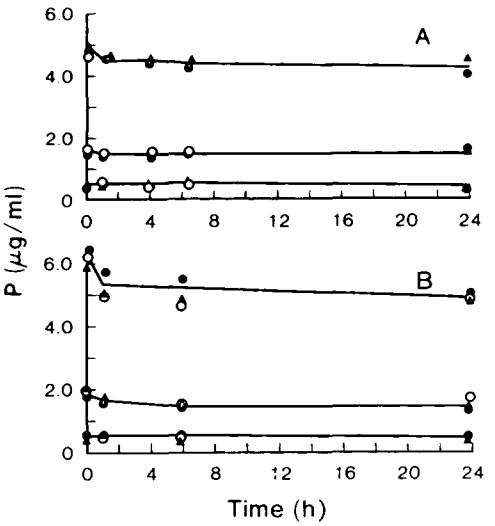

Fig. 3. Stability of pibenzimol (P) in fresh human plasma (A) and fresh human whole blood (B) at temperatures of 4° C (•), 25° C (○) and 37° C (▲).

correlation coefficient (r) was greater than 0.99 for all assays. The data were fitted to a linear model with mean slope ± S.E.M. and mean intercept ± S.E.M. values of 1.18 ± 0.03 and −0.07 ± 0.02, respectively. Coefficients of variation for concentrations of 1.0 μg/ml and 0.1 μg/ml were 4.2% and 8.7%, respectively.

Pibenzimol was stable in fresh human plasma and whole blood at concentrations from 0.5 to 5.0 μg/ml and temperatures from 4° C to 37° C (Fig. 3). There was no sequestration of pibenzimol in red blood cells. Binding of pibenzimol to human plasma proteins was studied using ultrafiltration and equilibrium dialysis chamber techniques. Control studies with buffer and drug only (in the absence of plasma) indicated adsorption of pibenzimol to filters, dialysis membranes, and dialysis chambers in that no drug was detected in ultrafiltrates and no drug crossed dialysis membranes into buffer-only chambers (Table II). There were also reduced concentrations of pibenzimol in unfiltered fractions (retentate) and in drug—buffer solutions added to dialysis chambers (Table II).

TABLE II

PIBENZIMOL PLASMA PROTEIN BINDING

Ultrafiltration and equilibrium dialysis chamber studies carried out as described in Materials and methods.

Drug concentration (μg/ml)	Percentage pibenzimol recovered			
	Equilibrium dialysis chamber		Amicon Centriflo cone filter	
	Drug chamber	Buffer chamber	Retentate*	Ultrafiltrate
Buffer studies				
0.5	0.0	0.0	47	0.0
1.5	1.1	0.0	46	0.0
5.0	3.0	0.3	27	0.0
Plasma studies				
0.5	16	0.0	68	1.3
1.5	28	0.0	69	0.0
5.0	34	0.0	57	0.0

*Recoveries corrected for reduced volume of retentate.

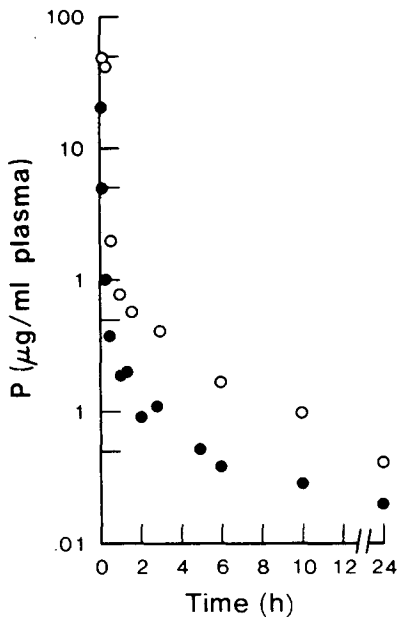

Fig. 4. Rabbit plasma time—concentration curves of pibenzimol (P) following rapid intravenous administration of 2 mg/kg (•) and 10 mg/kg (○).

Pibenzimol was detected when filters, dialysis chambers, and dialysis membranes were washed with methanol or dimethylsulfoxide and aliquots analyzed for the presence of drug. When drug—plasma solutions were substituted for buffer—drug solutions, little or no drug was detected in plasma ultrafiltrates or on the buffer side of equilibrium dialysis chambers (Table II).

However, concentrations of pibenzimol in unfiltered plasma fractions (retentate) or in the plasma—drug chambers were much higher than in the absence of plasma (Table II). Similar results for control and plasma studies were obtained when the studies were carried out with Amicon Centrifuge micropartition filters (data not shown).

Plasma concentrations and urinary recovery of pibenzimol were determined following rapid intravenous administration of 2 mg/kg and 10 mg/kg to rabbits. Plasma time—concentration curves are shown in Fig. 4. Plasma concentrations of pibenzimol fell rapidly in the first several hours after drug administration. Low concentrations of pibenzimol were detectable in plasma 10 and 24 h after drug administration. The 24-h recovery of parent drug in urine was 10—20%.

DISCUSSION

The HPLC method developed for the analysis of pibenzimol utilizes the fluorescent properties of the molecule. Fluorescence not only provides greater sensitivity than ultraviolet absorption detection, but eliminates detection of contaminating substances in plasma and urine which do not fluoresce under assay conditions. The sensitivity of the assay could be readily increased by injecting more than 20 μl of the 200 μl reconstituted sample.

The binding of pibenzimol to glass and other surfaces poses problems when working with relatively dilute aqueous solutions. When possible, methanol or mobile solvent were in contact with the drug, as in standard solutions and in the HPLC system. At concentrations used in our studies, this eliminated the adsorption problem. At even lower concentrations, others have found that dimethylsulfoxide prevents adsorption of pibenzimol to surfaces [5]. When aqueous solutions were used, concentrated stock solutions (1.0 or 0.1 mg/ml) were prepared, and small aliquots added to experimental samples. In addition, standard samples were prepared and handled in a manner identical to the experimental samples. While carryover has not been observed with HPLC injections, the least concentrated solutions were analyzed first, and blank samples were frequently analyzed as a check for this potential problem.

The loss of pibenzimol over time in aqueous solutions at concentrations of 0.3—10.0 μg/ml was not observed in stability studies with plasma and whole blood at similar concentrations of pibenzimol (0.5—5.0 μg/ml). These results suggest that pibenzimol binds to plasma proteins and other constituents of whole blood. Adsorption of drug to filters, dialysis membranes, and dialysis chambers in control studies unfortunately prevented a quantitative determination of plasma protein binding.

Following rapid intravenous infusion of pibenzimol to rabbits, pibenzimol appears to be rapidly distributed to tissues, and then more slowly eliminated as drug is still present in plasma 24 h after administration. Little parent drug is recovered in 24 h urine, owing either to metabolism and/or sequestration of pibenzimol in tissues. In preliminary studies, we have determined that pibenzimol is metabolized by rat hepatic microsomal preparations and that it is also found in the bile of cannulated rabbits [6]. Using the methodology described in this report, additional disposition and metabolism studies with pibenzimol are underway.

ACKNOWLEDGEMENTS

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REFERENCES

1 J. Hirschberg, U. Lavi, R. Goiten and M. Marcus, Exp. Cell Res., 130 (1980) 63—72.
2 M. Marcus and K. Sperling, Exp. Cell Res., 123.(1979) 406—411.
3 S.A. Latt, G. Stetten, L.A. Juergens, H.F. Willard and C.D. Sher, J. Histochem. Cytochem., 23 (1975) 493—505.
4 Pibenzimol (NSC 322921), Clinical Brochure, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD, 1984.
5 L. Malspeis, Ohio State University, personal communication.
6 K.J. Miller, D.M. Moertel, D.C. Anderholm, S.K. Gretsch and M.M. Ames, Proc. Amer. Assoc. Cancer Res., 26 (1985) in press.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF CICLETANIDE, A NEW DIURETIC, IN PLASMA, RED BLOOD CELLS, URINE AND SALIVA

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SUMMARY

A sensitive, selective and easy to use high-performance liquid chromatographic method for the determination of cicletanide, a new diuretic, in plasma, red blood cells, urine and saliva is described. After extraction of cicletanide together with an internal standard with diethyl ether, or diethyl ether—*n*-hexane (20:80) for urine, the sample extracts are chromatographed with water—methanol—acetic acid (50:50:0.3) as eluent on to a Nucleosil C_{18} column. Both compounds are detected by their ultraviolet absorption at 280 nm. The calibration graph was linear between 0.2 and 20 μg/ml for plasma and between 0.2 and 5 μg/ml for the other biological fluids. The sensitivity limit was 20 ng/ml for plasma, red blood cells and saliva and 30 ng/ml for urine. The coefficients of variation of the between-day assays did not exceed 4.6% in plasma, 8.3% in red blood cells, 7.8% in urine and 4.2% in saliva for the lowest concentrations studied. The application of the method to a pharmacokinetic study of cicletanide after a single oral therapeutic dose in humans is reported.

INTRODUCTION

Cicletanide [2-methyl-3-hydroxy-4*H*,5*H*-5-(4′-chlorophenyl)isofuropyridine hydrochloride] (PXD) is a new compound (IPSEN Labs.) that exhibits marked diuretic and anti-hypertensive properties [1]. Owing to these pharmacological properties, PXD is intended for use in the treatment of hypertension, a disease that is frequently associated with renal impairment. Therefore, the influence of renal insufficiency on the pharmacokinetics of PXD should be studied. Such a study can only be conducted with actual patients, so it requires a highly selective analytical technique with a sufficiently high sensitivity to allow the

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measurement of the drug concentration after the administration of only one therapeutic dose.

Until now, only one method has been described [2] for the determination of PXD in plasma and urine and it exhibits some drawbacks such as the lack of an internal standard for urine and a relatively poor reproducibility. As a consequence, it appeared of major importance to devise a technique that would allow the precise measurement of PXD in plasma and urine and also in red blood cells and saliva in order to obtain a larger amount of information concerning the kinetics and the disposition of the drug. In this paper, an easy to use high-performance liquid chromatographic technique is described and its application to the kinetic study of a single oral therapeutic dose of PXD in subjects with normal renal function is demonstrated.

EXPERIMENTAL

Standards and reagents

PXD and 2-methyl-3-hydroxy-4*H*-5-methyl-5-(4′-chlorophenyl)isofuropyridine hydrochloride, used as an internal standard (I.S.), were supplied by IPSEN Labs. (Paris, France).

Diethyl ether (Carlo Erba, Milan, Italy) and methanol (Prolabo, Paris, France) were distilled before use. *n*-Hexane (Merck, Darmstadt, F.R.G.) and acetic acid were of analytical-reagent grade. Diethyl ether was used as the extraction solvent for plasma, red blood cells and saliva and diethyl ether—*n*-hexane (20:80) was used for urine.

Stock solutions (100 μg/ml) of PXD and the internal standard were prepared in a mixture of methanol and twice-distilled water (1:9) and were found to be stable at 4°C for at least six months. Working solutions were prepared twice a month by diluting the stock solutions with the same solvent.

Biological fluids

Human blood obtained from the local blood bank was centrifuged at 4°C and 5-ml aliquots of separated plasma were stored at −20°C, while the remaining red blood cells were washed twice with sterile saline, the volume of which was equal to that of the discarded plasma. After the last centrifugation (600 *g*) and removal of sterile saline, 5-ml aliquots of haemolysate of red blood cells were obtained by freezing and then stored at −20°C. A human urine pool was collected from male and female volunteers and 10-ml aliquots were stored at −20°C. Human saliva was collected from a male volunteer shortly before analysis.

Apparatus and chromatographic conditions

Analyses were performed by using a chromatographic system that consisted of a Model 410 high-pressure pump (Kontron, Zurich, Switzerland), equipped with a Model 810 pulse damper and a Model 7125 100-μl loop injector (Rheodyne, Cotati, CA, U.S.A.). A Uvikon 720 LC variable-wavelength detector (Kontron) (2 nm slits) allowed the detection of both compounds at 280 nm. The stainless-steel column (25 cm × 4.6 mm I.D.) was packed with Nucleosil C_{18}, particle size 10 μm (Macherey, Nagel & Co., Düren, F.R.G.).

The mobile phase was a mixture of methanol and 0.1 *M* acetic acid in twice-distilled water (50:50), which was delivered at a flow-rate of 1 ml/min, producing a pressure of 75 bars at 22°C (air conditioning).

Procedure

Into a glass-stoppered centrifuge tube, 1 ml of internal standard solution (10 μg/ml), 1 ml of biological sample and 7 ml of extraction solvent were successively introduced. The mixture was shaken for 15 min on a rotating mixer (60 rpm) and then centrifuged (1800 *g*) for 15 min at 4°C. A 5-ml volume of the organic layer was transferred into a conical test-tube. This extraction step was applied again to the biological residue, then 5 ml of solvent were introduced and a further 5 ml of the organic layer was added to the previous one. The solvent was evaporated to dryness under a gentle stream of nitrogen in a water-bath at 37°C. Subsequently 0.25—0.5 ml of methanol—twice-distilled water (1:9) were added to the dry residue 30 min prior to injection and 100 μl of this solution were chromatographed.

RESULTS

Chromatographic data

Typical chromatograms obtained for blank plasma, red blood cells, urine and saliva before and after spiking with known amounts of PXD and for biological samples from a patient having received 300 mg of PXD 3 h before sampling, are shown in Fig. 1. The most important chromatographic characteristics obtained are given in Table I. The high value obtained for the resolution factor (2.6) between PXD and the internal standard with k' values of 2.9 and 4.4, respectively, and with an analysis time of less than 15 min are noteworthy. According to the low asymmetry factors (1.1 and 1.35 for PXD and the internal standard, respectively) both peak heights and peak areas could be used for the calculations.

In addition, it should be noted that no interference from endogenous substances were observed. However, for urine such a result could be obtained only when using diethyl ether—*n*-hexane (20:80) as the extraction solvent.

Linearity

For each biological fluid a calibration graph was generated by spiking samples of the corresponding fluid with various amounts of PXD (concentration range 0.2—20 μg/ml for plasma and 0.2—5 μg/ml for urine, red blood cells and saliva) and analysing them by the method already described. In each instance a linearity test was applied to the data obtained from six assays for each concentration studied and a linear relationship was found between the peak-height ratio of PXD to the internal standard and the PXD concentration. The results (Table II) indicate a high value of the regression coefficients associated with a low value of the intercept, which confirms the lack of endogenous interference, as already shown in Fig. 1. The slopes of the regression lines were similar for the various fluids tested, except for urine, owing to the different extraction solvent used.

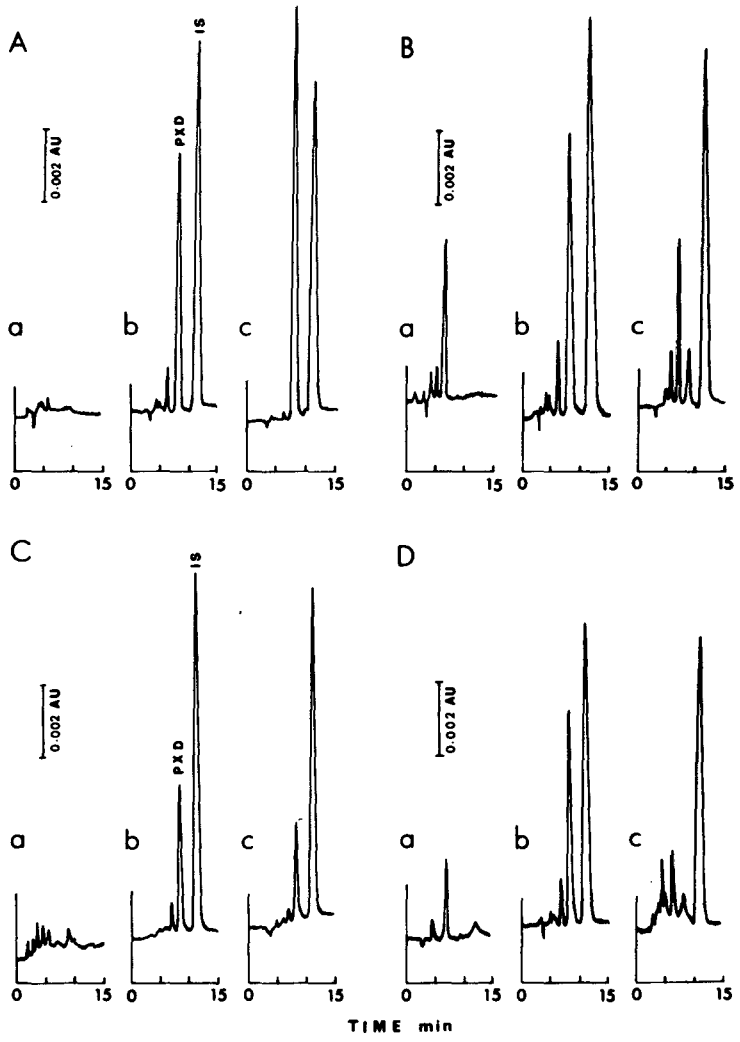

Fig. 1. Chromatograms obtained from extracts of (A) plasma, (B) red blood cells, (C) urine and (D) saliva. (a) Controls; (b) samples spiked with PXD (5 μg/ml) and I.S. (10 μg/ml); (c) samples from a subject having received a single oral dose of 300 mg of PXD 3 h before sampling.

TABLE I

CHROMATOGRAPHIC PARAMETERS FROM PXD AND THE INTERNAL STANDARD

Compound	Retention time (min)	k'	Resolution factor	Asymmetry factor
PXD	7.8	2.9	2.6	1.10
I.S.	10.8	4.4		1.35

TABLE II

PARAMETERS OF LINEAR CALIBRATION GRAPHS

n = 6 for all determinations.

Biological fluid	PXD concentration range (μg/ml)	Slope	S.D. of slope	Intercept	S.D. of intercept	Regression coefficient, r^2
Plasma	0.2—20	0.1314	0.0028	0.0063	0.0029	0.9996
Red blood cells	0.2— 5	0.1317	0.0056	—0.0005	0.0001	0.9982
Urine	0.2— 5	0.0875	0.0028	0.0069	0.0025	0.9990
Saliva	0.2— 5	0.1353	0.0014	—0.0071	0.0024	0.9988

Recovery

The overall recovery was determined by comparing the peak heights of PXD and the internal standard obtained after injection of standard solutions with those obtained after injection of solutions reconstituted from extracts of spiked biological samples. The results in Table III show that for plasma, red blood cells and saliva the recoveries of PXD are similar to those of the internal standard (around 78%), with low coefficients of variation. In contrast, for urine, owing to the change of extraction solvent, the recoveries of PXD and the internal standard were lower and higher, respectively, than those observed for the other fluids.

TABLE III

RECOVERY OF PXD AND OF THE INTERNAL STANDARD IN PLASMA, RED BLOOD CELLS, URINE AND SALIVA

n = 5 for all determinations.

Biological fluid	PXD concentration range (μg/ml)	Recovery (%)	C.V. (%)	I.S. concentration (μg/ml)	Recovery (%)	C.V. (%)
Plasma	0.2—20	81.5	2.1	10	73.8	1.9
Red blood cells	0.2— 5	79.1	2.4	10	75.2	2.3
Urine	0.2— 5	67.2	3.7	10	93.4	2.8
Saliva	0.2— 5	80.2	2.5	10	76.1	2.2

Sensitivity and precision

Under the experimental conditions described above, the sensitivity limit was 20 ng/ml for plasma, red blood cells and saliva and 30 ng/ml for urine. The assay sensitivity might be further increased by using 2 ml of biological fluid without modifying the procedure.

The precision of the assay was established by replicate analyses of samples over the concentration range defined for the study of linearity, which represents the entire range of PXD levels currently encountered after a single oral therapeutic dose in humans. For all the biological fluids studied, the within-day precision determined on six spiked PXD samples of each fluid was

TABLE IV

BETWEEN-DAY PRECISION OF PXD MEASUREMENT IN SPIKED PLASMA, RED BLOOD CELLS, URINE AND SALIVA

n = 6 for all determinations.

Biological fluid	Amount of PXD added (μg/ml)	Amoutn of PXD found (μg/ml) (mean ± S.D.)	C.V. (%)
Plasma	0.2	0.21 ± 0.01	4.6
	0.5	0.49 ± 0.01	2.3
	1	1.00 ± 0.01	1.3
	2	2.00 ± 0.03	1.6
	5	5.00 ± 0.10	2.0
	10	10.63 ± 0.28	2.6
	20	20.22 ± 0.17	0.8
Red blood cells	0.2	0.24 ± 0.02	8.3
	0.5	0.52 ± 0.02	3.8
	1	1.00 ± 0.04	4.0
	2	1.90 ± 0.07	3.7
	5	5.00 ± 0.02	4.0
Urine	0.2	0.18 ± 0.01	5.5
	0.5	0.51 ± 0.04	7.8
	1	1.00 ± 0.04	4.0
	2	2.00 ± 0.07	3.5
	5	5.00 ± 0.16	3.2
Saliva	0.2	0.24 ± 0.01	4.2
	0.5	0.53 ± 0.01	1.8
	1	1.00 ± 0.03	3.1
	2	1.90 ± 0.06	3.1
	5	5.00 ± 0.07	1.4

2.4 and 2.5% for concentrations of 5 and 0.5 μg/ml, respectively. The between-day precision is shown in Table IV. From these results, it appeared that the coefficients of variation did not exceed 4.6% in plasma, 8.3% in red blood cells, 7.8% in urine and 4.2% in saliva for the lowest concentrations studied.

DISCUSSION

As stated in the Introduction, previously only one method for determining PXD has been described [2] and it did not meet all the requirements for a precise pharmacokinetic study, especially for the determination of urinary excretion. The technique described here is simple and rapid, and can be applied to measure PXD accurately in four biological fluids.

Although PXD and the internal standard have very low solubilities in water, it must be emphasized that owing to their reversed-phase chromatographic behaviour both compounds must be injected as partly aqueous solutions (methanol—water) in order to obtain profiles with non-tailing peaks.

Although interferences from endogenous substances were easily avoided for

plasma, red blood cells and saliva, for urine this could only be achieved by using diethyl ether—*n*-hexane instead of pure diethyl ether as extraction solvent. This modification was necessary in order to reduce the front solvent peak so as to be able to determine amounts of PXD smaller than 0.5 μg/ml. For this purpose a proportion of 80% of *n*-hexane in the mixed solvent was required in order to remove the largest part of the interfering endogenous compounds within the limits of the extraction capacity of the solvent for PXD and the internal standard. In addition, the possible interference of several drugs that could be administered together with PXD, such as other diuretics (furosemide, hydrochlorothiazide), β-blockers (propranolol, atenolol), analgesics (aspirin, paracetamol) and tranquillizers (diazepam, lorazepam, oxazepam, nitrazepam) was tested by using samples drawn from patients treated with these drugs. Most of these compounds and their metabolites were not detectable under the chromatographic conditions used and the others were not extracted by the solvents used.

The reliability of the method was evaluated by carrying out fifty different analyses of two spiked samples used as controls with every batch of samples to be analysed over a six-months period. The stability of the chromatographic system was excellent as no change could be observed within this routine work period insofar, column clean-up being performed each forty to fifty assays. This was achieved by using methanol at a flow-rate of 1.5 ml/min for 1 h, after which the stability of the column was ensured by passing the mobile phase for 30 min.

The method was devised for pharmacokinetic studies and as a typical

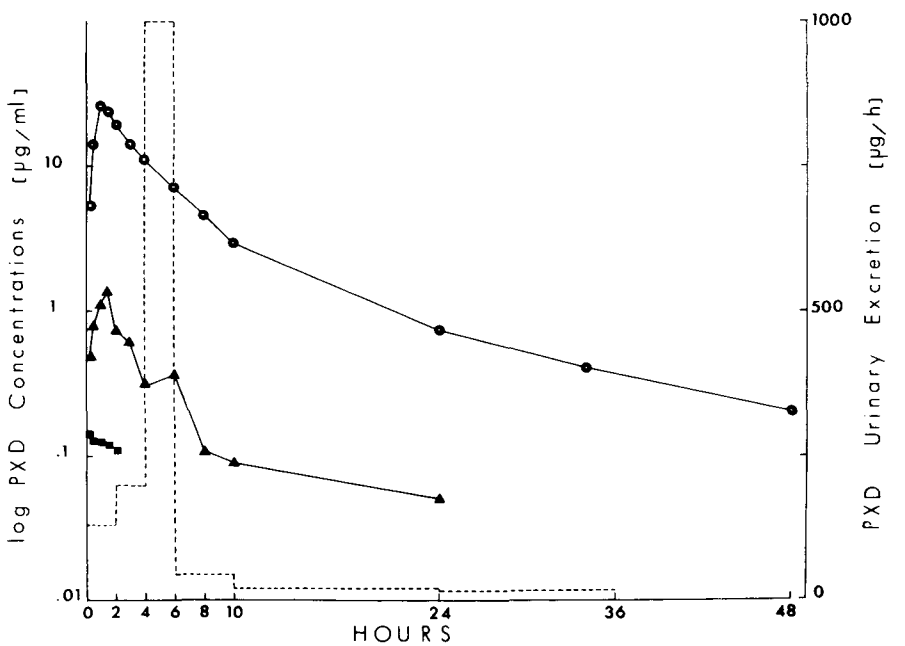

Fig. 2. Time course evolution of plasma (•), red blood cells (▲) and saliva (▪) concentrations and urinary excretion (- - - -) of PXD in a healthy volunteer given 300 mg of PXD orally at 8.00 a.m.

example Fig. 2 shows the concentrations of unchanged PXD observed in plasma, red blood cells, saliva and urine from one healthy volunteer given 300 mg of PXD orally at 8.00 a.m. The time course of the evolution of PXD concentrations in plasma and red blood cells showed a biphasic decline and could be fitted as the sum of three exponentials. The saliva levels remained near the sensitivity limit of the method.

In conclusion, the method described is easy to use, selective, highly sensitive and reliable. Therefore, it appears to be suitable for following the concentrations of PXD after a single oral therapeutic dose in humans, and should be of value in determining the pharmacokinetic parameters of PXD and their possible alterations in pathological states such as renal insufficiency.

ACKNOWLEDGEMENT

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REFERENCES

1 Confidential reports, IPSEN Labs., Paris, 1981—1982.
2 Human Pharmacokinetic and Metabolism Study of BN 1270, confidential report, Biomeasure, Hopkinton, MA, 1981.

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CHROMBIO. 2542

DETERMINATION OF PROQUAZONE AND ITS *m*-HYDROXY METABOLITE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

CLINICAL APPLICATION: PHARMACOKINETICS OF PROQUAZONE IN CHILDREN WITH JUVENILE RHEUMATOID ARTHRITIS

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SUMMARY

A method for the determination of proquazone and its *m*-hydroxy metabolite in serum and urine by reversed-phase high-performance liquid chromatography is described. The technique is based on a single extraction of the unchanged drug, its metabolite and an internal standard from serum or urine with chloroform. The column was packed with μBondapak C_{18} and the mobile phase was acetonitrile—water (50:50) (pH 3). The detection limits for proquazone and its metabolite were 0.02 μmol/l using 500 μl of sample. For the determination of the total *m*-hydroxy metabolite only 100 μl of sample are needed. The method described is suitable for routine clinical and pharmacokinetic studies. The clinical application of this method suggests that the pharmacokinetics of proquazone in adults and children are similar.

INTRODUCTION

Proquazone [1-isopropyl-7-methyl-4-phenyl-2(1*H*)-quinazolinone] is a non-acidic, synthetic non-steroid anti-inflammatory drug (NSAID), which has been shown to have significant anti-inflammatory and analgesic activity in patients with rheumatoid arthritis and other rheumatic disorders [1—16]. Proquazone

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has also been given to child patients for the treatment of juvenile rheumatoid arthritis [17]. The recommended daily doses for children range from 10 to 20 mg/kg, corresponding to a dose of about 400—800 mg/m^2 of body surface area; the total daily dose should not exceed 1000 mg [18].

Pharmacokinetic studies of proquazone have, however, been carried out only in adults [19] and no information is available about the pharmacokinetics of proquazone in children. The purpose of this study was to provide a preliminary pharmacokinetic profile of proquazone in children and to find a simple and relatively reliable means of controlling the levels of proquazone and/or its metabolites in serum and urine.

No suitable method for the measurement of proquazone was found in the literature. Therefore, a high-performance liquid chromatographic (HPLC) method for the determination of proquazone and its metabolites was developed. The technique is based on a single extraction of the unchanged drug, its metabolite and an internal standard from serum or urine with chloroform (Fig. 1).

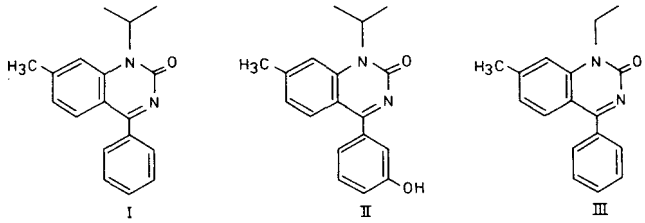


Fig. 1. Structures of proquazone (I), its *m*-hydroxy metabolite (II) and the internal standard (III).

EXPERIMENTAL

Reagents and standards

Pure samples of proquazone [1-isopropyl-7-methyl-4-phenyl-2(1*H*)-quinazolinone; Biarison], its *m*-hydroxymetabolite [1-isopropyl-7-methyl-4-(*m*-hydroxyphenyl)-2(1*H*)-quinazolinone] and the internal standard [1-ethyl-7-methyl-4-phenyl-2(1*H*)-quinazolinone] were supplied by Sandoz (Basle, Switzerland). The acetonitrile used was Baker Analyzed HPLC Reagent (J.T. Baker, Deventer, The Netherlands). Other reagents and solvents were of analytical-reagent grade obtained from E. Merck (Darmstadt, F.R.G.).

Stock solutions of 1000 μmol/l were prepared by dissolving 27.8 mg of proquazone or 29.4 mg of *m*-hydroxy metabolite in 100 ml of absolute ethanol. Working standard solutions were then prepared by diluting the appropriate volume of stock solution with drug-free serum to give final concentrations of proquazone and its metabolite of 0.10, 0.25, 0.50, 1.00 and 2.00 μmol/l. Standard solutions for the determination of the total *m*-hydroxy metabolite were made in the same manner to give final concentrations of 1.0, 5.0, 10.0, 20.0 and 30.0 μmol/l. A stock solution of the internal standard of 1000 μmol/l was prepared by dissolving 26.4 mg in absolute ethanol. A working standard solution of the internal standard of 20.0 μmol/l was prepared from the stock solution by dilution with distilled water. All standards were stored at —20° C.

Sample preparation

Serum and urine samples were stored at −20°C until assayed.

Chromatographic conditions

A Pye Unicam liquid chromatograph equipped with a variable-wavelength UV detector and a column (300 × 4 mm I.D.) packed with μBondapak C_{18}, 10 μm (Waters Assoc., Milford, MA, U.S.A.), was used with acetonitrile—water (50:50), adjusted to pH 3.0 with orthophosphoric acid, as the mobile phase. Isocratic elution was carried out at a flow-rate of 2.0 ml/min. The liquid chromatograph was connected to a Hewlett-Packard 3380A integrator.

Procedure

To 0.5 ml of serum or urine were added 0.5 ml of sodium acetate buffer (0.2 mol/l, pH 5.0) and 50 μl of internal standard solution (20 μmol/l). When the total amount of *m*-hydroxy metabolite in serum or urine was determined, any conjugated metabolite was first hydrolysed enzymatically. Sample (100 μl), acetate buffer (0.5 ml) and *Suc d'hélix pomatia* (50 μl) (β-glucuronidase activity, 100 000 U/ml; sulphatase activity, 1 000 000 U/ml; Pharmindustrie, l'Industrie Biologique Française, Clichy, France) were mixed in stoppered glass tubes and incubated at 37°C for 16 h. The tubes were cooled to room temperature and 100 μl of internal standard solution were added.

Proteins were precipitated by sulphuric acid—sodium tungstate precipitation and the mixture was extracted with 7 ml of chloroform. The sample was centrifuged and the aqueous layer and protein precipitate were aspirated. The chloroform extract was evaporated to dryness under a stream of air, the residue dissolved in 50 μl of methanol and 10 μl were injected into the liquid chromatograph.

A calibration graph was prepared by subjecting standards to the above procedure. The ratios of the peak area of proquazone or metabolite to that of the internal standard were plotted as a function of concentration. The peak-height ratio also gave sufficient accuracy with a linear relationship between peak-height ratio and concentration up to 2 μmol/l. A calibration graph for total *m*-hydroxy metabolite was constructed in the same manner and the linear range of the assay was 0—30 μmol/l (Fig. 2).

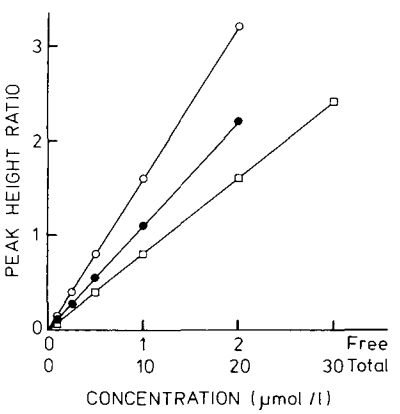


Fig. 2. Calibration graphs for proquazone (•), the free *m*-hydroxy metabolite (○) and the total *m*-hydroxy metabolite (□).

RESULTS

Selectivity

Representative chromatograms of serum specimens extracted according to our procedure are shown in Fig. 3. Chromatogram A is a typical pattern for serum from a normal individual not receiving drugs. Chromatogram B is obtained from the serum of an individual receiving proquazone. The concentrations of proquazone and its *m*-hydroxy metabolite in this sample were 0.59 and 0.14 μmol/l, respectively. Chromatogram C was obtained from the same serum sample as B after hydrolysis, with a total concentration of metabolite of 12.5 μmol/l. The retention times of proquazone, its *m*-hydroxy metabolite and internal standard were 4.9, 3.1 and 3.8 min, respectively. No interfering peaks in the same region were encountered from constituents of lipaemic or haemolytic sera. Paracetamol, salicylic acid, ketoprofen, indometacin, naproxen, flurbiprofen, ibuprofen and mefenamic acid do extract and chromatograph under these conditions, but they have retention times of 1.6, 2.2, 3.4, 3.5, 3.6, 5.2, 6.2 and 8.4 min.

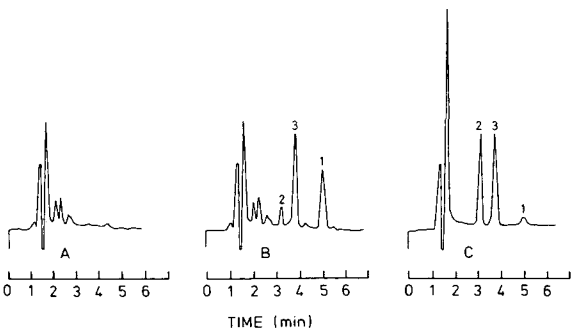


Fig. 3. Chromatograms of (A) serum blank (attenuation 0.005), (B) serum from a patient treated with proquazone (the serum was found to contain 0.59 μmol/l proquazone and 0.14 μmol/l *m*-hydroxy metabolite) (attenuation 0.005) and (C) the same serum as in B after hydrolysis (the serum was found to contain 12.5 μmol/l *m*-hydroxy metabolite) (attenuation 0.02). Peaks: 1 = proquazone; 2 = *m*-hydroxy metabolite; 3 = internal standard.

Sensitivity

In sensitivity studies, drug-free serum samples were spiked with proquazone and its *m*-hydroxy metabolite to a concentration of 0.02 μmol/l and processed as described above. The mean peak-area ratios were found to be 0.024 (n = 6, C.V. 11.2%) for proquazone and 0.021 (n = 6, C.V. = 11.5%) for the metabolite, indicating that 0.02 μmol/l of proquazone and its metabolite in serum can be determined with acceptable precision in 0.5-ml serum samples.

Recovery

Absolute recoveries of proquazone, its *m*-hydroxy metabolite and the internal standard were studied by adding known amounts of these compounds to a serum known to be drug-free. These samples were extracted as described above. A second set of standards were prepared in methanol at the same concentration. Volumes of 10 μl of each sample were chromatographed. Absolute

recoveries from serum were calculated by comparing the peak heights of the extracted serum samples with those of the non-extracted standards. The recoveries of proquazone and metabolite were 92.1 and 92.8%, respectively, at a concentration of 0.50 μmol/l. The recovery of internal standard was 92.6%. The relative recoveries of proquazone and metabolite were also determined. Known amounts of these compounds in ethanol were added to pooled serum to achieve the concentrations shown in Table I. These samples were processed as described above and at least ten samples were analysed. The relative recoveries are shown in Table I.

TABLE I

RELATIVE RECOVERIES OF PROQUAZONE AND ITS *m*-HYDROXY METABOLITE FROM SERUM (n = 10)

Compound	Initial concentration (μmol/l)	Calculated concentration (μmol/l)	Measured concentration (μmol/l)	Recovery (%)
Proquazone	0.42	0.82	0.80	97.6
Metabolite	0.22	0.62	0.61	98.4

TABLE II

PRECISION OF ASSAY FOR PROQUAZONE AND ITS *m*-HYDROXY METABOLITE (n = 20)

Parameter	Within-run		Day-to-day	
	Proquazone	Metabolite	Proquazone	Metabolite
Mean (μmol/l)	0.421	0.222	0.504	0.502
S.D. (μmol/l)	0.0133	0.0101	0.0206	0.0271
C.V. (%)	3.2	4.5	4.1	5.4

Precision

The within-run precision was evaluated by processing aliquots of pooled sera containing proquazone and the metabolite at the concentrations shown in Table II. The day-to-day precision was calculated from data obtained on samples analysed over a period of four months.

Clinical study

Subjects and procedure. Nineteen children with juvenile rheumatoid arthritis (JRA) were treated with daily doses of proquazone (Biarison) ranging from 9.4 to 21.4 mg/kg (274—600 mg/m^2). An initial pharmacokinetic short-term clinical study was performed by giving proquazone to eleven patients (eight girls and three boys) of age range 4—15 years (mean age 11.5 years). No other NSAIDs were allowed during the study.

Proquazone was administered to the patients as capsules containing 25, 50 or 200 mg of the drug. On the morning of day 1 (acute period) one single dose

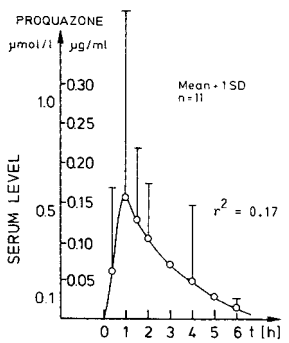
of about 5 mg/kg (range 4.7—7.5 mg/kg) was given to fasting subjects and the fasting was continued for a further 2 h. Blood samples were taken before (0 h) and 0.5, 1, 1.5, 2, 4 and 6 h after the administration of the drug. The duration of the constant repeated treatment period was fourteen days (days 2—15) and the total daily dose of about 10 mg/kg (range 9.4—10.8 mg/kg) was divided into three doses. All capsules were taken with food or a snack. During the elimination period (days 16—18) the patients took the last dose of proquazone in the morning of day 16 on an empty stomach and the fasting was continued for a further 2 h. On day 16 a blood sample was drawn at 8.00 a.m. before the administration of the last dose and thereafter at 0.5, 1, 1.5, 2, 4, 6, 24 and 48 h; 24-h urine samples were collected on days 11, 17 and 18.

Later, eight additional patients with JRA (three girls and five boys of age range 5—15 years (mean age 10.4 years) were included in the study. In order to achieve a better clinical effect, the daily dosage of proquazone was increased (range 13.0—21.4 mg/kg). In these patients the metabolism of proquazone was followed by collecting serum samples during a constant repeated dosing of the drug. In addition to the unchanged drug, its *m*-hydroxy metabolites (free and total) were also determined in the serum samples.

All patients fulfilled the diagnostic criteria of Brewer *et al.* [20]. The design and purpose of the study were explained orally to the patient (if old enough to understand) and to the parents in a simple and understandable way. The treatment was started only after written consent from the parent(s). The study was approved by the ethical committee of Turku University Central Hospital.

Results. Figs. 4 and 5 show the mean levels of unchanged proquazone in serum after a single dose and after the constant repeated dosing. Peak levels of unchanged proquazone in serum were attained 1—2 h after dosing. There was no unusual accumulation of unchanged proquazone in serum after two weeks of treatment.

The levels of the free *m*-hydroxy metabolite of proquazone in serum were generally very low. Peak serum levels of the total *m*-hydroxy metabolite



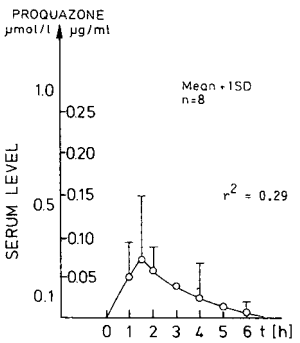


Fig. 4. Elimination profile after a single dose. Eleven patients (eight girls and three boys), age range 4—15 years (mean age 11.5 years). Mean dose of proquazone 5.3 mg/kg (range 4.7—7.5 mg/kg).

Fig. 5. Elimination profile after repeated dosing. Eight patients (six girls and two boys), age 10—15 years (mean age 12.6 years). Mean daily dose of proquazone 10.2 mg/kg (range 9.4—10.8 mg/kg/day).

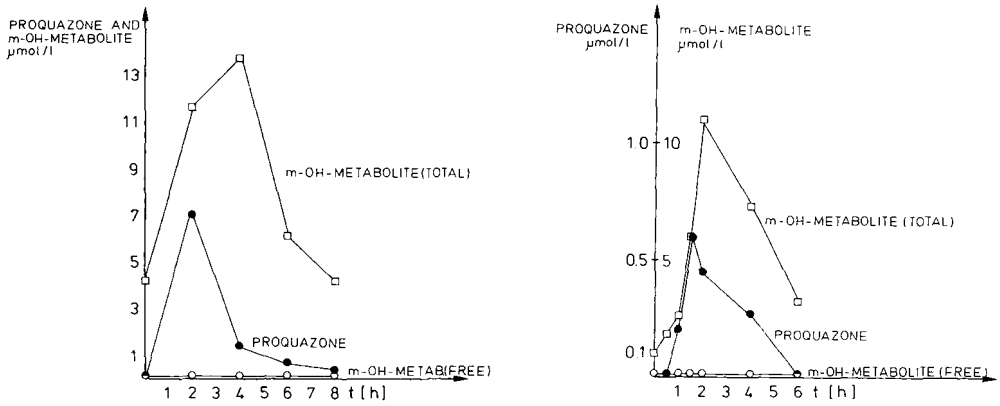


Fig. 6. Levels of proquazone and its *m*-hydroxy metabolite (free and total) during repeated dosing of proquazone. Dose: 13 mg/kg per day. Patient 15-year-old girl.

Fig. 7. Levels of proquazone and its *m*-hydroxy metabolite (free and total) during repeated dosing of proquazone. Dose: 17 mg/kg per day. Patient: 10-year-old boy.

(conjugated and unconjugated) were reached 2—3 h after dosing (Figs. 6 and 7). The peak values of 20—25 µmol/l (6—7 µg/ml of total *m*-hydroxy metabolite) were achieved with the repeated dosing of 15 mg/kg of body weight daily (divided into three equal doses).

The amounts of unchanged proquazone and of free *m*-hydroxy metabolite excreted in urine were low, being 0.01% and 0.06% of the total daily dose of the drug, respectively. During fourteen days' repeated dosing the amount of the total *m*-hydroxy metabolite in the urine ranged from 3.5% to 20.9% of the total daily dose. After the discontinuation of treatment on days 17 and 18 the amount of the total *m*-hydroxy metabolite excreted into urine decreased very quickly, being only 0.2—3% 24—48 h after the last dose.

DISCUSSION

Pharmacokinetic studies on adult healthy volunteers have shown that proquazone is rapidly absorbed from the gastrointestinal tract and before reaching serum nearly totally converted into its metabolites. About 90% of the orally administered dose is metabolized in the first liver passage. Owing to this high first-pass metabolism, the unchanged drug itself is very unstable in serum and gives large biological and inter-subject variations in the blood levels. In the liver of man proquazone is mainly hydroxylated to two active metabolites [19]. These two basic pathways are hydroxylation of the *m*-position in the phenyl ring and hydroxylation of the 7-methyl group, the former being by far the predominant one in man. Correspondingly, the *m*-hydroxy metabolite is the major metabolite in the blood and urine. It is present mainly in its conjugated form, with the proportion of unconjugated *m*-hydroxy metabolite accounting for only 5% of the total metabolite (Fig. 8).

According to the results of our study, the pharmacokinetics of proquazone in children correspond to the pharmacokinetics seen in adults. The levels of unchanged proquazone in serum and urine correlate very poorly with the daily

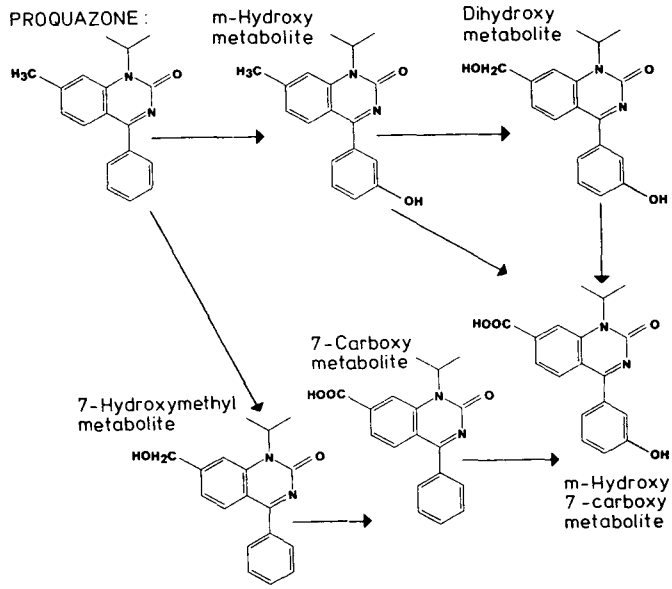


Fig. 8. The metabolism of proquazone.

dose of the drug. However, our study shows that the amount of total *m*-hydroxy metabolite correlates fairly well with the dosage of the drug and may offer a reliable basis for adjusting the dosage and following the compliance of the patients.

A dose of 15 mg/kg per day (500 mg/m^2 per day) gives *m*-hydroxy metabolite values of 20—25 μmol/l (6—7 μg/ml). In adults, when given proquazone in a dosage of 300 mg thrice daily, the corresponding value is about 5 μg/ml [21]. For those laboratories in which a high-performance liquid chromatograph with a UV detector is available, the present method is suitable for routine use. The advantages of the method are its simplicity and the small serum sample volume, combined with good accuracy and precision.

REFERENCES

1 R.Allan and M. Bleicher, J. Int. Med. Res., 5 (1977) 253.
2 G.L. Bach, Egypt. Rheumatol., 1 (Suppl.) (1980) 55.
3 G.L. Bach and P. Fotiades, Med. Klin. (Munich), 73 (1978) 1517.
4 F. Bachmann, Therapiewoche, 28 (1978) 6552.
5 R.V. Coombs, R.P. Danna, M. Denzer, G.E. Hardtmann, B. Huegi, G. Koletar, J. Koletar, H. Ott, E. Jukniewicz, J.W. Perrine, E.I. Takesue, J.H. Trapold, J. Med. Chem., 16 (1973) 1237.
6 M. Karakaya, Curr. Ther. Res., 22 (1977) 127.
7 G. Kupfrian and A. Zöller, Z. Allg. Med., 55 (1979) 1891.
8 M. Nissilä, and A. Kajander, Scand. J. Rheumatol., 21 (Suppl.) (1978) 36.
9 H. Ott and M. Denzer, J. Org. Chem., 33 (1968) 4263.
10 J. Puranen and H. Rönty, Scand. J. Rheumatol., 21 (1978) 21.
11 A. Ruotsi and B. Skrifvars, Scand. J. Rheumatol., 21 (Suppl.) (1978) 28.
12 A. Ruotsi and U. Vainio, Scand. J. Rheumatol., 21 (Suppl.) (1978) 15.
13 B. Skrifvars, Scand. J. Rheumatol., 21 (Suppl.) (1978) 40.

14 B. Skrifvars and M. Nissilä, Scand. J. Rheumatol., 9 (1980) 33.
15 J.T. Vainio and P.V. Lepistö, Scand. J. Rheumatol., 21 (Suppl.) (1978) 25.
16 H.-G. Velcovsky, R.G. Bretzel, H. Michels and K. Federlin, Therapiewoche, 29 (1979) 7103.
17 E.J. Brewer, E.H. Giannini, J. Baum, C.W. Fink, V. Hansen, J.C. Jacobs and J. Schaller, J. Rheumatol., 9 (1982) 135.
18 J.C. Jacobs, Pediatric Rheumatology for the Practitioner, Springer-Verlag, New York, 1982, p. 234.
19 H. Ott and J. Meier, Scand. J. Rheumatol., 21 (Suppl.) (1978) 12.
20 E.J. Brewer, J. Bass, J. Baum, J.T. Cassidy, Ch. Fink, J. Jacobs, V. Hanson, J.E. Levinson, J. Schaller and J.S. Stillman, Arthritis Rheum., 20 (1977) 195.
21 Sandoz, Basle, unpublished data.

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CHROMBIO. 2545

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF SULBACTAM USING PRE-COLUMN REACTION WITH 1,2,4-TRIAZOLE

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SUMMARY

A high-performance liquid chromatographic (HPLC) method for the determination of sulbactam in human and rat plasma and urine has been developed. Sulbactam was reacted with 1,2,4-triazole to yield a product having an ultraviolet absorption maximum at 326 nm. The product was separated using reversed-phase HPLC from the regular components of plasma and urine with an ion-pair buffer at 50°C and detected at the ultraviolet maximum. The limits of accurate determination were 0.2 and 1.0 μg/ml in plasma and urine, respectively. The coefficients of variation of inter- and intra-assays in human plasma spiked at 4.0 μg/ml (n = 5) were 1.02 and 3.05%, respectively. Coexisting cefoperazone, penicillins, or the alkaline degradation product(s) of sulbactam did not interfere in the sulbactam assay. The pharmacokinetic behaviour of sulbactam and cefoperazone coadministered to rats was estimated by moment analysis.

INTRODUCTION

Sulbactam, (2*S*,5*R*)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid 4,4-dioxide (I), is a potent semisynthetic β-lactamase inhibitor [1]. Microbiological assay [2] has mainly been used for the routine assay of sulbactam. Rogers et al. [3] reported a high-performance liquid chromatographic (HPLC) method for the determination of sulbactam in human plasma,

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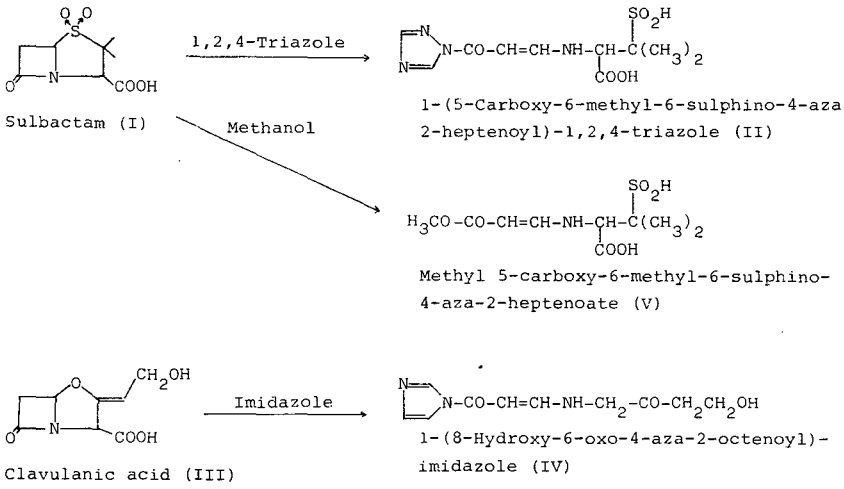

Fig. 1. Chemical structures of sulbactam, clavulanic acid and their degradation products.

saliva and urine. However, their method is not suitable for the routine monitoring of plasma or urine sulbactam levels because it involves a tedious extraction procedure and requires a long analysis time.

In a previous paper [4] we reported that sulbactam reacts with 1,2,4-triazole to yield 1-(5-carboxy-6-methyl-6-sulphino-4-aza-2-heptenoyl)-1,2,4-triazole (II) (Fig. 1), which shows an ultraviolet (UV) absorption maximum at 326 nm, and that the reaction can be used for the determination of sulbactam in pharmaceutical preparations.

This paper describes an HPLC method utilizing the above reaction for pre-column derivatization for the determination of sulbactam in plasma and urine. From the time course data obtained, the pharmacokinetic behaviour of sulbactam and cefoperazone intravenously coadministered to the rat is discussed.

EXPERIMENTAL

Reagents and materials

Sulbactam and cefoperazone were supplied by Pfizer Taito (Tokyo, Japan) and Toyama Chemical (Tokyo, Japan), respectively. 1,2,4-Triazole and tetrabutylammonium bromide (TBAB) were purchased from Nakarai Chemicals (Kyoto, Japan) and were used without further purification. Other chemicals of analytical-reagent grade were used.

A 2 *M* 1,2,4-triazole solution of pH 10.0 was prepared by dissolving 13.81 g of 1,2,4-triazole in 60 ml of distilled water, adjusting the pH to 10.0 ± 0.05 by addition of saturated sodium hydroxide solution and diluting to 100 ml with distilled water.

HPLC equipment and operating conditions

For sulbactam assay, a liquid chromatograph (Trirotar-V, Japan Spectroscopic, Tokyo, Japan) equipped with a variable-wavelength detector

(Uvidec-100-V, Japan Spectroscopic) with an 8-μl flow-through cell was used. A reversed-phase column (15 cm × 4.6 mm I.D.) packed with Develosil ODS-5 (5 μm) (Nomura Chemicals, Seto, Japan) was used with a pre-column (3 cm × 4.6 mm I.D.) packed with Develosil ODS-10 (10 μm). The pre-column was frequently repacked to guard the main column. The eluent was 5 m*M* TBAB + 1 m*M* disodium hydrogen phosphate + 1 m*M* sodium dihydrogen phosphate solution—acetonitrile (3:1) for both plasma and urine samples at a flow-rate of 1.0 ml/min. The detection wavelength was 326 nm. All chromatographic separations were carried out at 50° C.

For cefoperazone assay, a liquid chromatograph (LC-5A, Shimadzu, Kyoto, Japan) equipped with a variable-wavelength detector (SPD-2A, Shimadzu) was used with a stationary phase of Develosil ODS-10 packed in a 25 cm × 4.6 mm I.D. stainless-steel tube. The pre-column (3 cm × 4.6 mm I.D.) packed with the same material was used. The eluent was 5 m*M* disodium hydrogen phosphate + 5 m*M* sodium dihydrogen phosphate solution—methanol (2:1) at a flow-rate of 1.2 ml/min. The detection wavelength was 265 nm. All chromatographic operations were carried out at ambient temperature.

Derivatization procedure for sulbactam in plasma and urine

For plasma samples, a 50-μl aliquot of plasma was mixed with 150 μl of acetonitrile and shaken vigorously on a vortex-type mixer for 30 sec. The mixture was incubated at room temperature for 5 min. After centrifugation at 1500 *g* for 10 min, a 100-μl aliquot of the supernatant was reacted with an equal volume of the 2 *M* 1,2,4-triazole solution at 50°C for 15 min. After cooling to room temperature, the reaction solution was centrifuged at 1500 *g* for 5 min. A 20-μl aliquot of the supernatant was subjected to chromatography under the conditions described above.

For urine samples, a 100-μl portion of a neat urine was diluted ten-fold with distilled water and a 100-μl aliquot of the diluted urine was treated as described for plasma.

Rat experiments

Three male Wistar rats (285—295 g) were used for the experiments. Under pentobarbitone anaesthesia, sulbactam and cefoperazone (25 mg/kg each) dissolved in 0.9% sodium chloride solution were rapidly injected into the femoral vein. A blood sample (0.3 ml) was collected from the canulated jugular vein at 0, 5, 10, 20, 30, 40 and 60 min with a heparinized syringe. The plasma layer was obtained by centrifugation of the blood at 1500 *g* for 10 min, and stored at −20°C until assay.

For sulbactam assay, the plasma sample was treated according to the procedures described above. The standard solutions were prepared by dissolving known amounts of sulbactam in the control rat plasma to give five different concentrations (1—50 μg/ml) and were treated using the same procedures as for the plasma sample. A calibration graph of peak height versus concentration was constructed.

For cefoperazone assay, the plasma was treated as described for sulbactam, with the exceptions that methanol was used as protein precipitant and no derivatization was required. The five cefoperazone standards in plasma (1—50

μg/ml) were treated according to the procedures described above. A calibration graph of peak height versus concentration was constructed.

Pharmacokinetics

The area under the plasma concentration—time curve (AUC) and the mean residence time (MRT) in the systemic circulation are defined as

$$\mathrm{AUC} = \int_0^\infty C_p dt \quad (1)$$

$$\mathrm{MRT} = \int_0^\infty tC_p dt / \int_0^\infty Cp dt \quad (2)$$

where C_p is plasma concentration—time course [5]. AUC and MRT were calculated by linear trapezoidal integration with extrapolation to infinite time. The total body clearance (Cl_T) and steady-state volume of distribution (V_{ss}) [6] are given by

$$Cl_T = D/\mathrm{AUC} \quad (3)$$

$$V_{ss} = Cl_T\,\mathrm{MRT} \quad (4)$$

where D is a rapid intravenous dose.

RESULTS

HPLC conditions

It has been shown that the reaction of sulbactam with 1,2,4-triazole yields a product having strong UV absorption at 326 nm [4]. We tried to apply the reaction to the determination of sulbactam in human and rat plasma and urine. The reaction product (II) was separated from the regular components of plasma and urine on an ion-pair reversed-phase HPLC column. When the mobile phase did not contain an ion-pairing agent (TBAB), the retention time of II was markedly shortened, resulting in a poor separation from background peaks. The separation was also improved by elevating the column temperature to 50°C, at which II eluted as a single, sharp peak.

Reaction conditions

The effects of the concentration and pH of 1,2,4-triazole solution and the reaction temperature on the formation of II in human plasma and urine samples were examined in order to establish a routine assay procedure for sulbactam. The sample solution was reacted with an equal volume of the reagent solution, and a 20-μl portion was accurately removed at appropriate reaction times and subjected to the HPLC analysis. The reaction of sulbactam with 1,2,4-triazole in pooled human plasma (not deproteinized) followed by the precipitation of plasma proteins with methanol or acetonitrile gave no peak corresponding to II on the chromatogram. Also, the precipitation with methanol followed by the reaction with 1,2,4-triazole gave a lower response than the precipitation with acetonitrile. Therefore, sulbactam was recovered with three volumes of acetonitrile and reacted with 1,2,4-triazole. When a 2 *M* 1,2,4-triazole solution of pH 10.0 was used at a reaction temperature of 50°C, a maximum and constant peak height was obtained at 15 min. Hence the

conditions previously described [4] were selected for the routine assay of sulbactam in human plasma.

Corresponding results were obtained with urine samples, leading to the same reaction conditions. The reaction of sulbactam with the 2 *M* 1,2,4-triazole solution in neat urine gave a peak height only about 60% of that in distilled water. Therefore, the urine sample was diluted ten-fold.

The reaction conditions employed for the assay of sulbactam in rat plasma and urine were the same as those used for human plasma and urine.

HPLC analysis

Under the HPLC and reaction conditions established above, a 20-μl portion of a treated human plasma or urine sample was subjected to chromatography. Figs. 2 and 3 show that the derivatized sulbactam (II) was completely separated from the regular components of human plasma and urine within 8 min after injection. The chromatograms obtained from the treated rat plasma and urine samples were almost identical with those from human plasma and urine. The

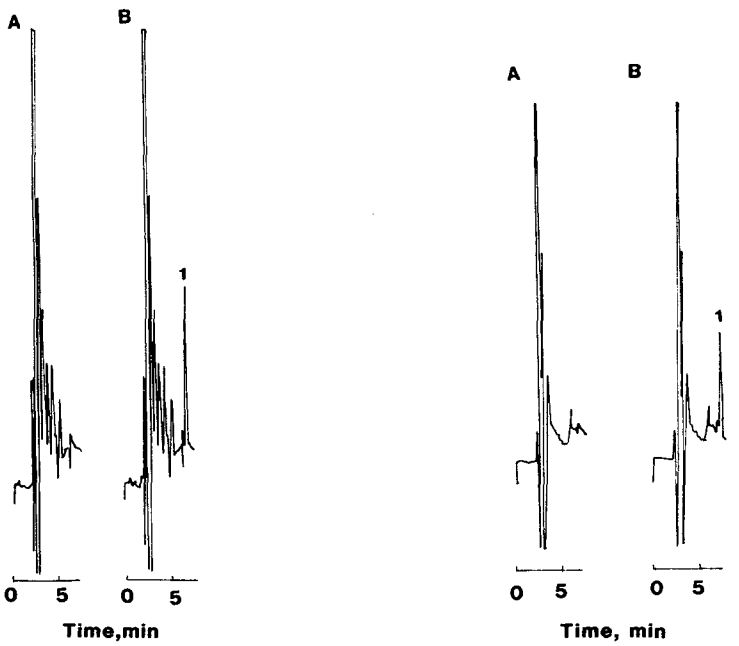

Fig. 2. Chromatogram of derivatized sulbactam in human plasma. The eluent was monitored at 326 nm and 0.04 a.u.f.s. Control plasma (A) and control plasma spiked with sulbactam (1.0 μg/ml) (B) were treated according to the derivatization procedures described under Experimental. A 20-μl portion of each sample solution was subjected to chromatography. Peak 1 is 1-(5-carboxy-6-methyl-6-sulphino-4-aza-2-heptenoyl)-1,2,4-triazole.

Fig. 3. Chromatogram of derivatized sulbactam in human urine. The eluent was monitored at 326 nm and 0.08 a.u.f.s. Control urine (A) and control urine spiked with sulbactam (5.0 μg/ml) (B) were treated according to the derivatization procedures. A 20-μl portion of each sample solution was subjected to chromatography. Peak 1 is 1-(5-carboxy-6-methyl-6-sulphino-4-aza-2-heptenoyl)-1,2,4-triazole.

lower limit of accurate determination was as low as 0.2 μg/ml for human plasma samples and 1.0 μg/ml for human urine samples. The calibration graphs for sulbactam with the concentrations ranging from 0.2 to 8 μg/ml for human plasma samples and from 5 to 80 μg/ml for human urine samples were linear and passed through the origin, with correlation coefficients of 0.999 and 0.998, respectively.

Recovery and interference

Table I shows the recoveries of sulbactam from spiked human plasma and urine, and the coefficients of variation.

The interferences of penicillins (ampicillin, amoxicillin, and penicillin G), cefoperazone and the alkaline degradation product(s) of sulbactam were examined. Sulbactam (5 μg/ml) and penicillins or cefoperazone (each at a concentration of 500 μg/ml) were reacted with 1,2,4-triazole under the assay con-

TABLE I

RECOVERY OF SULBACTAM FROM URINE AND PLASMA

Average and coefficient of variation (C.V.) for five analyses.

Sample	Added (μg/ml)	Inter-assay		Intra-assay	
		Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)
Urine	40.0	100.6	0.90	99.9	1.73
Plasma	4.0	100.5	1.02	105.8	3.50

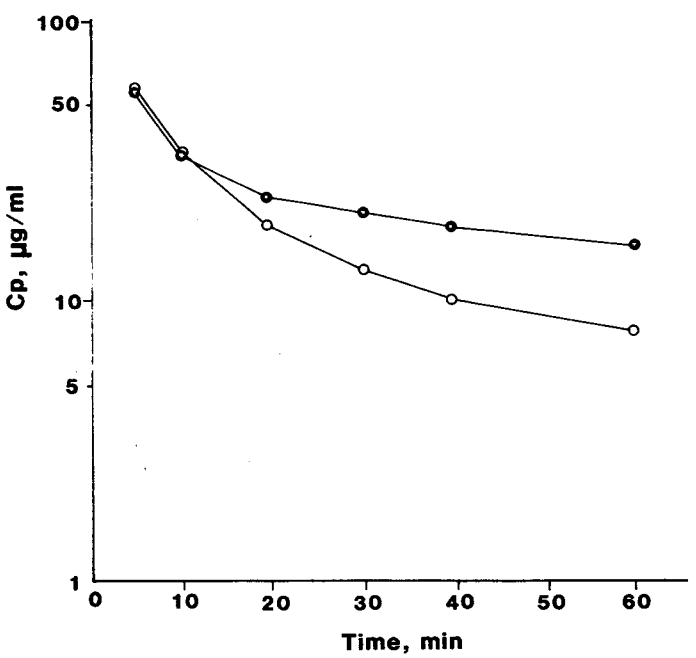

Fig. 4. Mean semi-logarithmic plots of plasma concentrations (C_p) of sulbactam (•) and cefoperazone (○) coadministered to three male rats.

ditions. There was no influence on the sulbactam quantitation in any instance. The alkaline degradation product(s) of sulbactam (1 mg/ml) (0.5 *M* sodium hydroxide solution for 10 min followed by neutralization) did not interfere in the sulbactam (5 μg/ml) assay.

Pharmacokinetic studies

Fig. 4 shows semi-logarithmic plots of the time courses following an intravenous combined dose of sulbactam and cefoperazone to rats. Table II shows the disposition properties of sulbactam and cefoperazone estimated by the non-compartmental method.

TABLE II

IN VIVO CHARACTERISTICS OF SULBACTAM AND CEFOPERAZONE IN RATS AFTER AN INTRAVENOUS COMBINED DOSE OF SULBACTAM AND CEFOPERAZONE

Data are given as mean values ± standard deviations (S.D.) for three rats.

Parameter	Sulbactam		Cefoperazone	
	Mean	S.D.	Mean	S.D.
AUC (mg min ml^{-1})	2.11	0.19	1.26	0.34
MRT (min)	56.9	8.4	29.5	3.4
V_{ss} (ml kg^{-1})	675	81	602	112
Cl_T (ml min^{-1} kg^{-1})	11.9	1.0	20.8	6.2

DISCUSSION

It has been reported that the reaction of clavulanic acid (III) (Fig. 1), which is a β-lactamase inhibitor isolated from *Streptomyces clavuligerus* ATCC 27064, with imidazole yields 1-(8-hydroxy-6-oxo-4-aza-2-octenoyl)imidazole (IV), having a UV absorption maximum at 312 nm [7]. Foulstone and Reading [8] applied the method to the HPLC determination of clavulanic acid in human plasma and urine. Bird et al. [7] reported that the *cis* and *trans* isomers of IV coeluted on a reversed-phase HPLC column. The same effect may be responsible for the broad peak with a shoulder that was observed when II was chromatographed in an ion-pairing mode at room temperature. For the purpose of HPLC quantitation, however, it is preferable that the isomers coelute as a single peak. This was achieved by elevating the column temperature to 50°C.

It has been found that sodium sulbactam was degraded to methyl 5-carboxy-6-methyl-6-sulphino-4-aza-2-heptenoate (V) in methanolic solution [9]. The recovery of sulbactam bound to plasma proteins with methanol followed by reaction with 1,2,4-triazole at 50°C might yield V. Therefore, the precipitation with methanol is unsuitable for this assay purpose.

It has been reported [10, 11] that the elimination of sulbactam from man and dog is slightly more rapid than that of cefoperazone, and that the coadministration of sulbactam and cefoperazone has no mutual influence on the pharmacokinetic features in man. However, as shown in Table II, the MRT of sulbactam in the rat is approximately double that of cefoperazone, that is,

sulbactam is eliminated more slowly than cefoperazone from the rat. As the V_{ss} value of sulbactam is almost the same as that of cefoperazone, the large difference in MRT values between sulbactam and cefoperazone is due to the differences in their Cl_T values. This coincides well with the previous result [12] that cefoperazone was eliminated rapidly from the rat but eliminated slowly from other species (man, rabbit, dog and monkey). Detailed consideration of the pharmacokinetic features of sulbactam and cefoperazone will be given elsewhere.

The proposed HPLC assay method for sulbactam in plasma and urine is specific to intact sulbactam without interferences from penicillins, cefoperazone and the alkaline degradation product(s) of sulbactam. It requires only simple treatment procedures and the elution time is as short as 8 min. Therefore, this method will be useful for the determination of sulbactam coadministered with penicillins or cefoperazone in clinical samples.

ACKNOWLEDGEMENT

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REFERENCES

1 A.R. English, J.A. Retsema, A.E. Girard, J.E. Lynch and W.E. Barth, Antimicrob. Agents Chemother., 14 (1978) 414.
2 United States Pharmacopoeia, Mack, Easton, PA, 20th ed., 1980.
3 H.J. Rogers, I.D. Brandbrook, P.J. Morison, R.D. Spector, D.A. Cox and L.J. Lees, J. Antimicrob. Chemother., 11 (1983) 435.
4 J. Haginaka, J. Wakai, H. Yasuda, T. Uno and T. Nakagawa, Analyst (London), 109 (1984) 1057.
5 K. Yamamoka, T. Nakagawa and T. Uno, J. Pharmacokinet. Biopharm., 6 (1978) 547.
6 K. Yamaoka, T. Nakagawa and T. Uno, J. Pharm. Pharmacol., 35 (1983) 19.
7 A.E. Bird, J.M. Bellis and B.C. Gasson, Analyst (London), 107 (1982) 1241.
8 M. Foulstone and C. Reading, Antimicrob. Agents Chemother., 22 (1982) 753.
9 J. Haginaka, J. Wakai, H. Yasuda, T. Uno and T. Nakagawa, Chem. Pharm. Bull., in press.
10 Sulbactam/Cefoperazone, Booklet of Clinical Data for a Combined Dose of Sulbactam and Cefoperazone, The Japan Society of Chemotherapy, Tokyo, 1982.
11 G. Foulds, J.P. Stankewich, D.C. Marshall, M.M. O'Brien, S.L. Hayes, D.J. Weider and F.G. McMahon, Antimicrob. Agents Chemother., 23 (1983) 692.
12 I. Saikawa, T. Yasuda, Y. Watanabe, H. Taki, N. Matsubara, T. Hayashi, K. Matsunaga and R. Tanaka, Chemotherapy (Tokyo), 28 (S-6) (1980) 163.

Journal of Chromatography, 341 (1985)123—130
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DETERMINATION OF DESFEROXAMINE AND A MAJOR METABOLITE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

APPLICATION TO THE TREATMENT OF ALUMINIUM-RELATED DISORDERS

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SUMMARY

A high-performance liquid chromatography method is described that permits separation and quantification of desferoxamine, a major metabolite, the iron(III) and the aluminum(III) chelates of desferoxamine. This method now facilitates pharmacokinetic studies on desferoxamine and derivatives designed to study side-effects and metabolite patterns in patients undergoing treatment.

INTRODUCTION

Aluminium has been implicated as a toxic factor in a number of human diseases. The dialysis encephalopathy syndrome, a progressive neurological disease, occurs in patients with impaired renal function undergoing chronic haemodialysis. This condition is associated with markedly elevated brain aluminium content [1—5]. An encephalopathy following chronic peritoneal dialysis has been reported [6] and increased serum aluminium levels occur during and after peritoneal dialysis [7]. Aluminium intoxication is implicated in dialysis osteomalacia in which aluminium accumulates in bone

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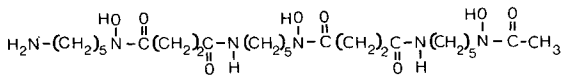
[3—5]. Increased levels of aluminium in bone, urine and plasma of patients on chronic total parenteral nutrition were reported [8]. Aluminium also appears to be a toxic factor in Alzheimer's disease, the most common cause of senile dementia. A significant increase of aluminium occurs in the cerebral cortex of brains affected by Alzheimer's disease [9—11]. Aluminium accumulation occurs in every neuron with Alzheimer's type neurofibrillary degeneration [12]. Yase [13] and Yoshimasu et al. [14] found elevated aluminium and calcium levels associated with amyotrophic lateral sclerosis in patients from the Kii penninsula of Japan and in Guam Parkinson dementia, amyotrophic lateral sclerosis syndromes. These latter neurodegenerative conditions are also associated with Alzheimer's type neurofibriliary degeneration.

One treatment strategy for patients suffering from diseases associated with elevated levels of aluminium is to remove aluminium with the chelating agent desferoxamine (DFO) (Desferal®, Ciba-Geigy). DFO is an effective trivalent metal-chelating agent capable of binding iron(III) (equilibrium constant, K 10^{31}) [15] and aluminium(III) (K ca. 10^{23}) [16] with high affinity and specificity. DFO has been widely employed in the treatment of conditions associated with iron overload [17, 18]. Ackrill et al. [19] reported successful removal of aluminium from patients with dialysis encephalopathy. More recently DFO was used to remove aluminium from patients with renal failure who were stabilized on standard haemodialysis [20]. Further DFO has been employed in an effort to remove aluminium from patients suffering from Alzheimer's disease [21]. Even though DFO has been established as a safe drug for short-term treatment [17—20], long-term exposure to the drug leads to undesirable side-effects in some elderly patients [21].

A



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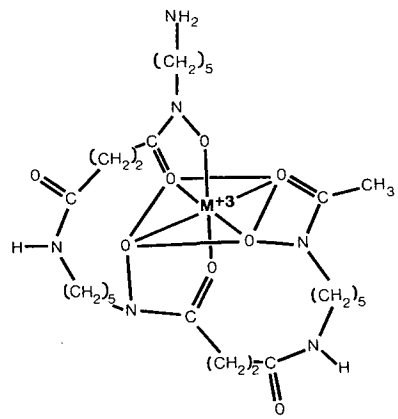

Fig. 1. (A) Molecular formula of desferoxamine, free base; (B) Suggested structure of metal^{3+} [iron(III) and aluminium(III)] chelate of desferoxamine.

Inter-individual differences in the metabolism of drugs are common occurrences [22]. In order to characterize the variability in patients' response to the drug treatment, it is essential to study the pharmacokinetics of DFO and its metabolites because long-term exposure to DFO may tend to cause deleterious side-effects.

Previously employed methods, based on the spectroscopic properties of the ferrioxamine (FO) chromophore (λ_{max} = 450 nm), are subject to interference by other iron(III)-chelating substances present in serum and urine in variable amounts. Further, the major metabolite of DFO having a chromophore similar to that of the parent compound cannot be distinguished spectroscopically, thus rendering these methods inappropriate for pharmacokinetic studies.

In this paper we describe a method that permits detailed studies on DFO and its derivatives. The molecular formula for DFO (free base) is given in Fig. 1A and a schematic representation of the metal (M^{+3}) complex is given in Fig. 1B.

EXPERIMENTAL

HPLC apparatus

An Altex variable-speed high-pressure pump fitted with a general-purpose manual valve, a 100-μl calibrated sample loop and micro-syringe injector was employed. Two chromatography columns were tested and used experimentally: (a) a commercial 20 cm × 4 mm HPLC stainless-steel column (Waters μPorasil) and (b) a 20 cm × 4 mm stainless-steel column packed with 10 μm particle size Dupont Zorbax silica gel. Sample detection was achieved at two wavelengths, 440 and 229 nm, using a Waters (Model 440) dual-fixed-wavelength detector equipped with separate flow-cells connected in tandem. The detector output was recorded on a Canlab dual-pen strip chart recorder (100 mV sensitivity).

Mobile phase

The mobile phase consisted of a mixture of 400 ml acetonitrile, 400 ml methanol, and 100 ml *n*-butanol, all HPLC grade, to which 100 ml deionized water were added and a buffering system was created by addition of 2.0 ml glacial acetic acid (BDH analytical grade) and 1.0 ml of 10 *M* sodium hydroxide (Fisher standard 10 *M* sodium hydroxide). This mixture was degassed under vacuum prior to use.

Desferoxamine, ferrioxamine and desferoxamine—aluminium complex

Standard solutons of DFO, ferric chloride (Fisher, analytical grade) and aluminium nitrate (BDH, analytical grade) were prepared by dissolving precisely weighed amounts in 10.0 ml deionized water to yield solutions of 0.0426 *M* desferoxamine mesylate, 0.0498 *M* ferric chloride, and 0.0501 *M* aluminium nitrate. Desferal was checked for purity by thin-layer chromatography (TLC) on 100-μm silica gel G-60 glass-backed plates developed in solvent of composition *n*-butanol—acetone—water—acetic acid (40:30:20:10). Detection of DFO was achieved by spraying developed TLC plates with freshly prepared 1% (w/v) solution of ferric chloride in water.

HPLC analysis of desferoxamine, ferrioxamine and desferoxamine–aluminium complex (aluminoxamine)

Solutions for HPLC analysis were prepared by dilution of appropriate amounts of stock solutions in the mobile phase. The amount of sample applied to the column was generally 100 μl.

Calibration of HPLC system for quantitative determination of ferrioxamine

Test solutions of FO were prepared by diluting DFO and ferric chloride stock solutions in mobile phase of pH 5.2. Known amounts of the calibration mixture were injected by micro-syringe into the sample loop and analysed. HPLC conditions were 135 bars at 2.0 ml/min flow-rate and chart speeds of either 10 or 20 cm/h were employed. The detection limit was arbitrarily taken to be three times the maximum noise level.

Patient sera and urine

Desferal was administered (intramuscularly 500 mg dissolved in distilled water) to a 63-year-old patient with normal renal function. Blood samples (5 ml) were withdrawn at intervals according to the schedule in Fig. 3. The blood was stored at 0°C and allowed to clot at room temperature immediately prior to analysis. Serum was separated from blood by centrifugation (1000 *g* for 10 min). Urine was collected and pooled for a 24-h period following injection of DFO.

Biological sample preparation for HPLC analysis

Method I. To 1.0 ml of serum in a 5.0-ml disposable glass test tube 1.0 ml of acetonitrile was added slowly with gentle rotation to achieve large flocculent precipitates. Then the tube was vortexed for 60 sec, twice, allowed to stand for at least 10 min and then centrifuged (3000 *g* for 5 min). The clear supernatant was suitable for direct injection into the HPLC system for determination of FO.

Method II. To 1.0 ml of serum in a 5-ml disposable glass test tube were added 300 mg sodium chloride (analytical grade) and the mixture was vortexed to achieve sodium chloride saturation. Then, 1.0 ml of benzyl alcohol was added and the mixture was vortexed for 60 sec, three times, followed by centrifugation (2000 *g* for 5 min) to achieve clear separation of layers. The benzyl alcohol layer contained the DFO and its metal ion complexes and was directly analysed by HPLC. The urine samples were treated similarly. Increased sensitivity could be obtained by increasing the sample to benzyl alcohol ratio without interference from increased extraction of coloured components absorbing in the visible region of the spectrum.

RESULTS

Determination of elution times of desferoxamine, ferrioxamine and aluminoxamine

Systematic variation of solvent composition in respect to dielectric constant, pH and ionic strength were employed to establish optimal elution volumes for separation of the three compounds, DFO, FO and aluminoxamine (AO). Then a mixture of these compounds was analysed by the HPLC system. The optimal conditions and results are given in Fig. 2. All compounds exhibited an

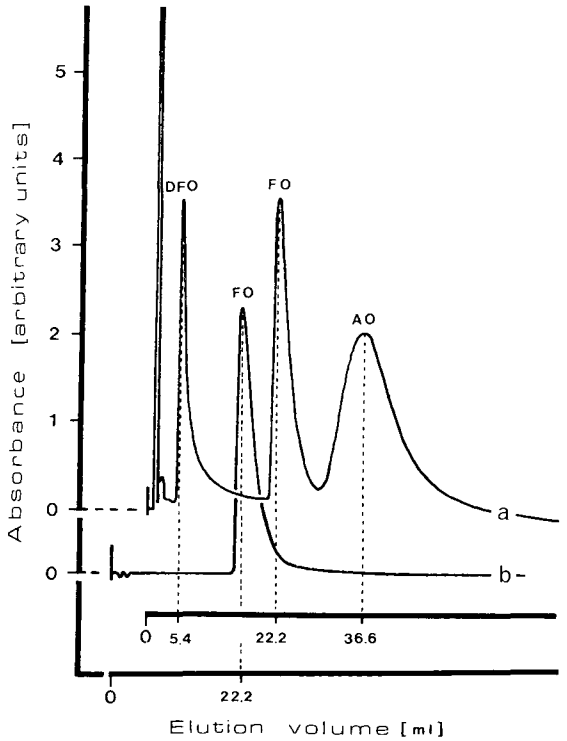

Fig. 2. Separation of a complex mixture of desferoxamine (DFO), ferrioxamine (FO) and aluminoxamine (AO) by HPLC. The input sample (20 μl) contained DFO, FO and AO in a molar ratio of 3:1:1 (150, 50 and 50 μM respectively). The absorbances of the column effluent are plotted as a function of eluting solvent volume (trace a, 229 nm, 0.2 a.u.f.s.; trace b, 440 nm, 0.05 a.u.f.s.). The elution maxima of DFO (5.4 ml), FO (22.2 ml) and AO (36.6 ml) are noted on the abscissa.

absorbance shoulder at 229 nm. FO showed an additional absorbance with a maximum at 435 nm. The UV tracing (Fig. 2) shows complete separation of DFO, FO and AO. Trace b, 440 nm, identified the FO complex. The elution volumes for DFO, FO and AO were 5.4 ml, 22.2 ml and 36.6 ml, respectively.

As concentrations of DFO in the analytical sample are decreased, increasing amounts of DFO are converted to FO presumably through a reaction with ferric ions liberated from the stainless-steel plumbing system of the HPLC apparatus by the acetic acid component of the solvent system. Generally at concentrations of less than 5 μg/ml all DFO was converted to FO.

Ferrioxamine calibration

The HPLC system was calibrated for standard normal serum by addition of varying amounts of FO. Plotting absorbance peak areas as a function of added amounts of FO resulted in a linear response (r = 0.99) and was reproducible for constant solvent composition (precision was S.D./x = 5.5% at the 10 μg/ml FO solvent level). No significant changes were observed on a day-to-day basis. The sensitivity of the test methods was defined in practical terms as the amount of FO giving rise to an absorbance peak of three times the average

maximum baseline noise. The detection limit taken as three times noise is 1.5 μg/ml for method I and 0.25 μg/ml for method II.

Pharmacokinetics of desferoxamine in a patient with Alzheimer's disease

Blood samples (5 ml) were analyzed according to methods I and II for disappearance of FO and DFO from blood following a single intramuscular injection of 500 mg DFO. Fig. 3 shows the amounts of FO determined in serum as a function of time in form of a semi-logarithmic plot. The half-life was 90 min. Extrapolation to zero time gave a value of 15 μg of FO per ml of serum, indicating a distribution volume of 33 l.

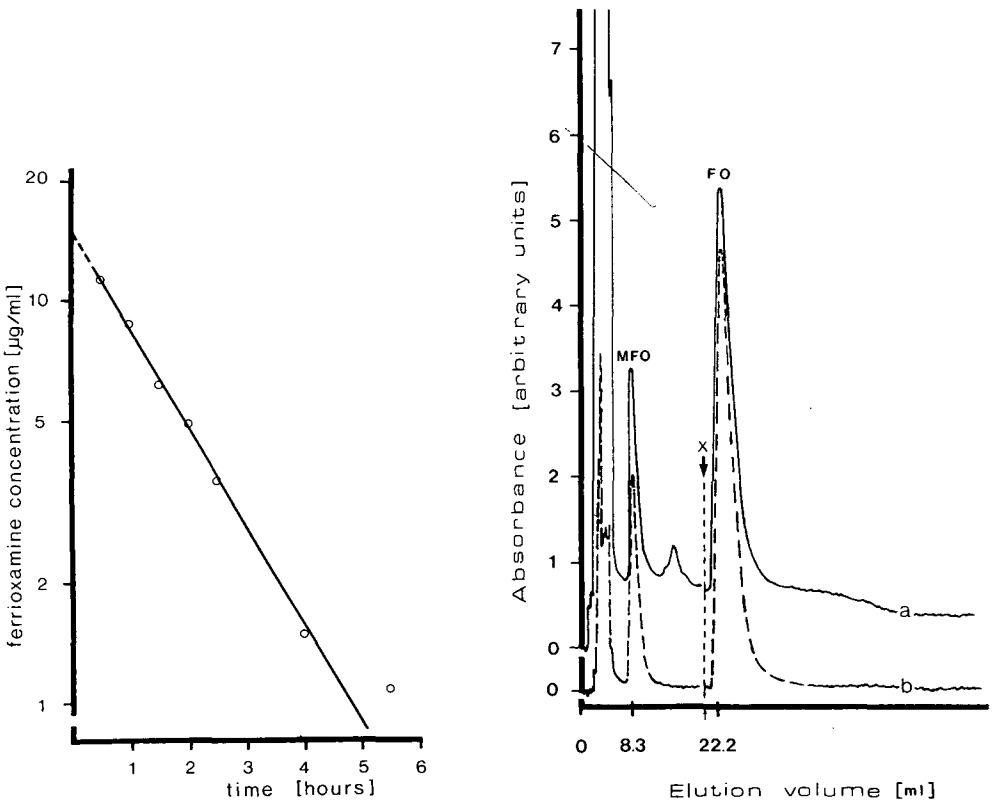

Fig. 3. Ferrioxamine elimination from the blood of an Alzheimer's disease patient (E.T.). Ferrioxamine concentrations in semilogarithmic form were plotted versus time lapsed after injection. The fitted line graph extrapolates to a desferal concentration of 15 μg/ml, indicating a volume of distribution of 33.3 l and it shows a drug half-life of 90 min.

Fig. 4. Determination of ferrioxamine and an iron(III)-binding metabolite in urine from patient with Alzheimer's disease. A urine sample was extracted with benzyl alcohol and the alcohol fraction was analyzed by HPLC. The position, marked X, on the abscissa designates where the sensitivity of the instrument was changed from 0.2 and 0.05 a.u.f.s. to 0.02 and 0.005 a.u.f.s. for 225- and 400-nm detectors, respectively. Traces a and b are the absorbance tracings at 229 and 440 nm, respectively. The peak appearing at 22.2 ml elution volume was identical to ferrioxamine (FO) and MFO (8.3 ml elution volume) was identified as an iron-binding metabolite.

Urinary excretion of desferoxamine

Initial experiments on urine employing method I showed that the UV absorbance of DFO eluting at 5.4 ml coincided with unidentified compounds absorbing also in the UV region. However, the FO peaks at 229 and 440 nm were well separated and free from interfering peaks. Method II yielded good resolution of the UV peaks. An early eluting iron(III)-binding compound, MFO, probably a DFO metabolite, was readily recognized (Fig. 4, trace a), since both compounds MFO and FO (Fig. 4) could be identified as iron(III) complexes through their absorbance at 229 and 440 nm.

DISCUSSION

Analytical methods employing spectroscopy failed to achieve DFO determination in serum of patients in the 0.5—50 μg/ml range necessary for monitoring patients undergoing long-term low-level DFO treatment. The HPLC method described here will separate both the iron(III) and the aluminium(III) complexes (Fig. 2) from other serum or urine components (Fig. 4). The method is limited in sensitivity primarily by the performance of the detection equipment. We achieved a sensitivity of 1.5 μg/ml FO using method I and 0.25 μg/ml using method II. The analytical response is linear and no zero bias is observed. Using this method to evaluate pharmacokinetic parameters of desferal in a patient with Alzheimer's disease (E.T.) we found in blood a half-life of FO of 90 min (Fig. 3), which is comparable to those reported by Keberle [15] and Wohler [17, 18]. DFO and FO are largely excreted by the kidney but lesser amounts are excreted in bile. Employing [^{14}C]DFO, Keberle [15] reported the production of three metabolites of DFO. The major component was shown to be an oxidized (COOH terminus) deamination product of DFO. Urine analysis by method II in this study revealed the presence of FO (Fig. 4) and another iron(III)-binding compound, MFO (Fig. 4) possibly the major metabolite reported by Keberle [15]. This tentative identification is suggested by the observation that both FO and MFO have 440-nm absorbance in addition to the 299-nm UV absorbance and the ratio of A_{229}/A_{440} is about equal (4—5) for both compounds.

The method described above provides a tool to study the pharmacokinetics of DFO and MFO. Further, the capability of this method to determine the aluminoxamine complex will provide a means of monitoring removal of aluminium from patients undergoing haemodialysis.

ACKNOWLEDGEMENTS

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REFERENCES

1 A.C. Alfrey, G.R. Le Gendre and W.D. Kaehny, N. Engl. J. Med., 294 (1976) 184.

2 J.A. Flendrig, H. Druig and H.A. Das, Proc. Eur. Dial. Transpl. Assoc., 13 (1976) 355.
3 I.S. Parkinson, M.K. Ward and D.N.S. Kerr, J. Clin. Pathol., 34 (1981) 1285.
4 S.W. King, J. Savory and M.R. Wills, Ann. Clin. Lab. Sci., 11 (1981) 337.
5 S. Sideman and D. Manor, Nephron, 31 (1982) 1.
6 D.G. Smith, J.A. Lewis, J.S. Burks and A.C. Alfrey, J. Amer. Med. Assoc., 244 (1980) 365.
7 A.D. Cumming, G. Simpson, D. Bell, J. Cowie and R.J. Winney, Lancet, i (1982) 103.
8 G.L. Klein, A.C. Alfrey, N.L. Miller, D.J. Sherrard, T.K. Hazlet, M.E. Ament and J.W. Coburn, Amer. J. Clin. Nutr., 35 (1982) 1425.
9 D.R. Crapper, S.S. Krishman and A.J. Dalton, Science, 180 (1973) 511.
10 D.R. Crapper, S.S. Krishnan and S. Quittkat, Brain, 99 (1976) 67.
11 D.R. Crapper, S. Quittkat, S.S. Krishnan, A.J. Dalton and U. De Boni, Acta Neuropathol. (Berlin), 50 (1980) 19.
12 D.P. Perl and A.R. Brody, Science, 208 (1980) 297.
13 Y. Yase, Excerpta Med. Int. Congr. Ser. 434, Neurology, 43 (1977) 413.
14 F. Yoshimasu, Y. Uebayaski, Y. Yase, S. Iwata and K. Sasajima, Folia Psychiatr. Neurol. Jpn., 30 (1976) 49.
15 H. Keberle, Ann. N. Y. Acad. Sci., 119 (1964) 758.
16 G. Anderegg, F. l'Eplattenier and G. Schwarzenbach, Helv. Chim. Acta, 46 (1963) 1409.
17 F. Wohler, Schweiz. Med. Wochenschr., 92 (1962) 1295.
18 F. Wohler, in F. Gross (Editor), Iron Metabolism, An International Symposium, Springer, Berlin, 1964, p. 551.
19 P. Ackrill, A.J. Ralston, J.P. Day and K.C. Hodge, Lancet, ii (1980) 629.
20 T.M.S. Chang and P. Barre, Lancet, ii (1983) 1051.
21 D.R. McLachlan, submitted for publication.
22 W. Kalow, Clin. Pharmacokin., 7 (1982) 373.

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CHROMBIO. 2560

RAPID CHROMATOGRAPHIC DETERMINATION OF CEFOTAXIME AND ITS METABOLITE IN BIOLOGICAL FLUIDS

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SUMMARY

A reversed-phase high-performance liquid chromatographic assay for the simultaneous determination of cefotaxime and its metabolite desacetylcefotaxime in plasma and urine was developed. Plasma was deproteinized with small amounts of acetonitrile. After separation of the proteins the supernatant was extracted with a mixture of chloroform and 1-butanol. A phase separation was obtained leaving the cephalosporin and its metabolite in the aqueous part and extracting most of the interfering endogenous material. The aqueous phase was injected directly into the chromatograph. As part of the plasma water was dissolved in the acetonitrile—1-butanol—chloroform layer, the concentration of the cephalosporin in the aqueous phase was significantly higher than in the original plasma sample. Therefore, the usual diluting effect of the deproteinization could be avoided. In a similar way the assay was applicable to measure cefotaxime and its metabolite in urine. Calibration curves were set up and were linear up to 25 μg/ml for desacetylcefotaxime and 250 μg/ml for cefotaxime. The assay was applied to study the pharmacokinetics of cefotaxime and its metabolite in a healthy volunteer. In a similar way this deproteinization and extraction method was also applied to assay for ceftazidime, cephalexin, cephazolin and cefoxitin.

INTRODUCTION

Pharmacokinetic studies of antimicrobial agents require sensitive assay methods that can be applied to detection of drug and metabolites in plasma and urine. Cefotaxime sodium is an intravenously administered third-generation cephalosporin antibiotic active against a wide variety of bacteria [1]. Studies of its pharmacokinetics have used microbiological and high-performance liquid chromatographic (HPLC) techniques for quantitation of the drug [2—6].

Microbiological assays frequently lack the sensitivity and specificity desired

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for pharmacokinetic studies. If the drug has an active metabolite, microbial assays may overestimate the concentration of the parent drug. Cefotaxime's metabolite, desacetylcefotaxime, has antimicrobial activity [1] and has been demonstrated to interfere with the determination of cefotaxime by microbiological assay techniques [5, 6]. Therefore, microbiological assays are not useful in the determination of the pharmacokinetic parameters for cefotaxime.

Difficulty in the HPLC analysis of cefotaxime and desacetylcefotaxime has been related to their highly polar nature and their instability. The primary difficulty in their HPLC analysis has been in obtaining suitable chromatographic separation of the metabolite from plasma components. Several methods have been described for precipitation of the plasma proteins that generally involve the use of an acid, e.g. trichloroacetic acid [5], perchloric acid [6] or phosphoric acid—methanol [7]. However, stability studies have shown that in strong acid a rapid hydrolysis of cefotaxime takes place and also a lactonization

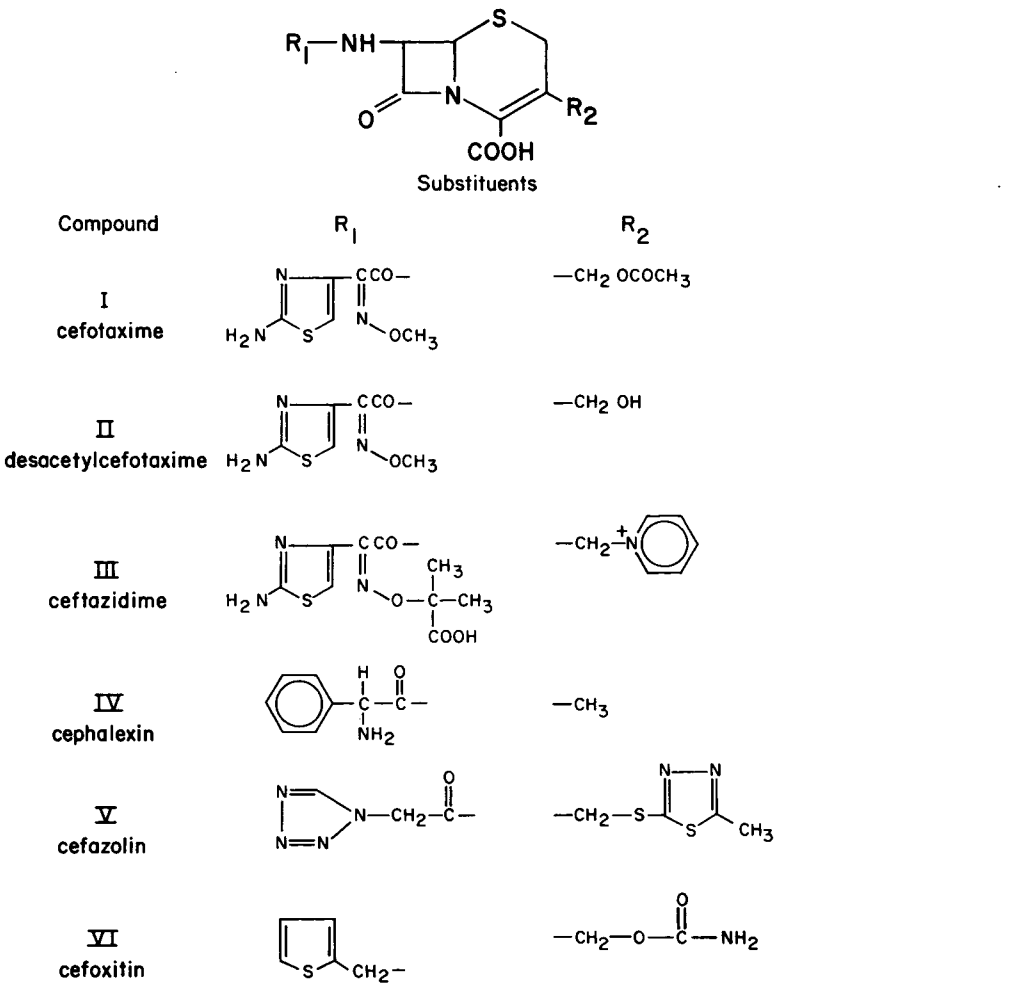

Fig. 1. Chemical structures of cefotaxime, desacetylcefotaxime, ceftazidime, cephalexin, cefazolin and cefotoxin.

of the desacetylcefotaxime occurs [8—10]. The optimum pH range of stability was reported to be 4.3—6.5 [8, 9]. Thus, acid deproteinization methods may convert significant amounts of cefotaxime to desacetylcefotaxime and to the lactone. One of the reported methods [6] even uses this conversion to the lactone to determine desacetylcefotaxime indirectly by measuring its degradation product. However, a direct determination of the unchanged compound would be a more desirable way to assay.

Another disadvantage of the reported deproteinization methods is the fact that the samples have to be diluted. Thus, sensitivity is decreased. For many drugs assay sensitivities can be increased by employing extraction techniques. However, neither cefotaxime nor its metabolite are extractable with organic solvents at neutral pH, and methods for extraction employing acid treatment and organic extraction [11] bear the danger of degradation and lactonization. A reported deproteinization method using chloroform—acetone to remove protein and freeze-drying to concentrate the sample [12] is feasible, but time-consuming and expensive.

This paper describes a simple assay method for cefotaxime and desacetylcefotaxime in plasma that does not degrade the parent drug or its metabolite, gives good separation, and is sensitive and linear over a wide range (0.5—250 μg/ml).

The assay procedure was also applied to ceftazidime, cephalexin, cefazolin and cefoxitin (Fig. 1).

EXPERIMENTAL

Materials

All chemicals were either USP, NF or ACS quality and were used without further purification. Cefotaxime sodium and desacetylcefotaxime were gifts from Hoechst-Roussel Pharmaceuticals (Somerville, NJ, U.S.A.). Ceftazidime was a gift from Glaxo (Greenford, U.K.). Cephalexin, cefazolin and cefoxitin were gifts from Eli Lilly (Indianapolis, IN, U.S.A.). These compounds were used as supplied.

Apparatus

For the HPLC assay the following instruments were used: high-pressure pump, Constametric III, LDC/Milton Roy (Riviera Beach, FL, U.S.A.), variable-wavelength UV detector, SpectroMonitor D, LDC/Milton Roy, autosampler, Model ISS-100, Perkin-Elmer (Norwalk, CT, U.S.A.), integrator, Model 3390A Hewlett-Packard (Palo Alto, CA, U.S.A.), and an octadecylsilane column, C_{18} μBondapak 30 cm × 4.5 mm I.D., (10 μm), Waters Assoc. (Milford, MA, U.S.A.) with a guard column, octadecyl-silane 37—50 μm, 4 cm, Waters Assoc. A laboratory centrifuge from International Centrifuge Equipment (Needham, MA, U.S.A.) was used in the separation of organic extracts from aqueous phases.

Chromatographic conditions

The mobile phase consisted of 0.007 *M* phosphoric acid in water—acetonitrile (85:15). The flow-rate was 1.3 ml/min, sensitivity 0.001 a.u.f.s., and

the wavelength 254 nm. The chart speed was 0.3 cm/min and all assays were performed at ambient conditions.

Sample preparation

Stock solutions of cefotaxime and desacetylcefotaxime were prepared by dissolving 25 mg of drug in 10 ml of methanol. Plasma standards were prepared for a range of 0.5—250 μg/ml for cefotaxime and 0.5—25 μg/ml for desacetylcefotaxime by spiking blank plasma with the appropriate amounts of the two solutions. Blank plasma was obtained from a single human donor. Urine standards were prepared by spiking urine from a drug-free, caffeine-free volunteer with appropriate amounts of the stock solutions. Urine standards were prepared for a range of 5—500 μg/ml for cefotaxime and 5—1000 μg/ml for desacetylcefotaxime. Stock solutions of the other cephalosporins were prepared in an analogous way.

Plasma (1.0 ml) and acetonitrile (3.0 ml) were mixed and vortexed for 5 sec. The mixture was centrifuged for 20 min at 1500 *g*. Of the resulting supernatant 1 ml was extracted with 1 ml of a mixture of chloroform—1-butanol (3:1). The sample was vortexed for 10 sec and then centrifuged for 5 min at 1500 *g*. A 20-μl aliquot of the resulting aqueous supernatant was injected into the HPLC system.

Urine (0.1 ml) was diluted with water (5.0 ml), vortexed for 5 sec and then centrifuged for 5 min at 1500 *g*. A 20-μl aliquot of the resulting supernatant was injected into the HPLC system.

Subject

The subject, a 42-year-old white female, 52.3 kg and 165.7 cm, was administered 1000 mg of cefotaxime sodium intravenously, Claforan R from Hoechst-Roussel Pharmaceuticals (Somerville, NJ, U.S.A.). The drug was dissolved in 12 ml of sterile water for injection and administered via Harvard infusion pump over 5 min. Blood samples were collected in sodium heparin tubes (Venoject Lot No. 13024, Terumo Medical Co., Elkton, NV, U.S.A.). The plasma was immediately separated and frozen at −20°C. Samples were collected immediately before and at 5, 10, 15, 30, 45, 60, 90, 120, 180, 240, 300, 360, and 480 min after the start of the infusion. Urine was collected prior to drug administration, and in intervals of 0—2, 2—4, 4—8, and 8—24 h. Total urine volumes were measured, the pH was recorded, and an aliquot was immediately frozen.

RESULTS AND DISCUSSION

Chromatographic separation

With a mobile phase of 0.007 *M* phosphoric acid in water—acetonitrile (85:15) cefotaxime and desacetylcefotaxime are well separated and can be assayed simultaneously (Fig. 2A). The retention times of all investigated compounds for a flow-rate of 1.3 ml/min are listed in Table I.

Sample preparation

Plasma samples were deproteinized with acetonitrile. This was advantageous

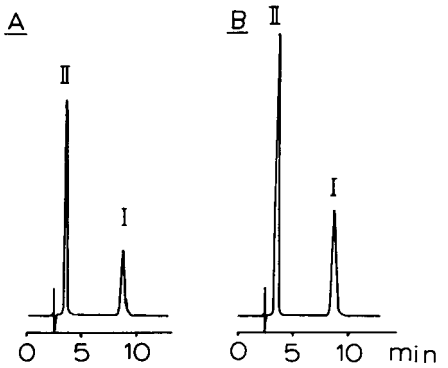

Fig. 2. Chromatograms of cefotaxime and desacetylcefotaxime in aqueous solution: (A) 5 μg/ml in water, injection volume 20 μl; (B) same sample as A after extraction of a mixture of 1 ml of the aqueous solution and 3 ml acetonitrile with the same volume of chloroform—1-butanol, injection volume 20 μl of the aqueous phase. Peaks: I = cefotaxime; II = desacetylcefotaxime.

TABLE I

RETENTION TIMES OF THE INVESTIGATED CEPHALOSPORINS

Chromatographic conditions: mobile phase 0.007 *M* phosphoric acid—acetonitrile (85:15), 10-μm octadecylsilane column, flow-rate 1.3 ml/min, UV detection at 254 nm.

Compound	Retention time (min)
Cefotaxime	8.7
Desacetylcefotaxime	3.5
Ceftazidime	4.2
Cephalexin	7.6
Cefazolin	12.0
Cefoxitin	16.3

as drastic pH changes as are obtained using trichloroacetic acid are avoided. However, two major problems remain using acetonitrile deproteinization. The sample is diluted to one fourth of its original concentration, thereby decreasing the sensitivity of the assay. Furthermore, it is very difficult to separate chromatographically the very polar desacetylcefotaxime from endogenous plasma components. Both of these problems can be overcome by a further sample preparation step. The supernatant—acetonitrile—plasma—water mixture is extracted with equal volumes of a mixture of chloroform—1-butanol (3:1). A phase separation is obtained with a small volume of an aqueous phase on top and a chloroform—1-butanol—acetonitrile—water mixture on the bottom. The cephalosporins concentrate in the aqueous phase whereas most of the interfering compounds are extracted into the organic. The final concentration of the cephalosporins in the supernatant is higher than the original plasma concentration (Fig. 2B), so the dilution step during the deproteinization is more than compensated. No evaporation of solvent is needed. The ratio 3:1 for the chloroform—1-butanol mixture was chosen as a further increase of 1-butanol prevents a good phase separation, whereas a decrease of the 1-butanol portion will lead to lower cephalosporin concentrations in the supernatant aqueous phase.

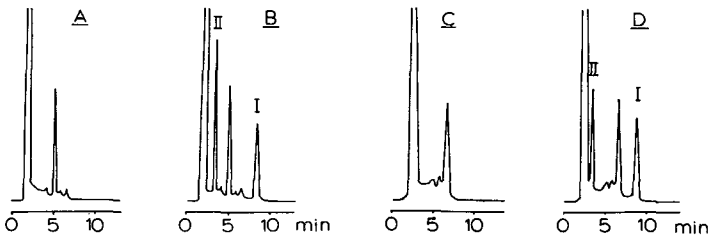

Fig. 3. Chromatograms of cefotaxime and desacetylcefotaxime in plasma and urine: (A) 1 ml of blank plasma, mixed with 3 ml acetonitrile, supernatant extracted with the same volume of chloroform—1-butanol, injection volume 20 μl of the aqueous phase; (B) 1 ml of plasma containing 5 μg/ml drug and metabolite, extracted and treated as in A; (C) 0.1 ml of blank urine, mixed with 5 ml water, injection volume 20 μl; (D) 0.1 ml of urine containing 20 μg of drug and metabolite, treated as in C. Peaks: I = cefotaxime; II = desacetylcefotaxime.

Typical chromatograms of cefotaxime and its metabolite in plasma after direct injection of the supernatant aqueous phase are shown in Fig. 3A and B. As the concentrations of the cephalosporins in urine are very high it is possible to assay urine by direct injection after dilution with water. Typical chromatograms of urine samples containing drug and metabolite are shown in Fig. 3C and D.

Simultaneous HPLC assay of cefotaxime and desacetylcefotaxime

With the described method calibration curves in plasma were set up over a range of 0.5—250 μg/ml for cefotaxime and 0.5—25 μg/ml for the metabolite. Under assay conditions no desacetylcefotaxime formed owing to degradation of parent drug. The calibration curves were linear over this wide range; their statistics are given in Table II. The limit of sensitivity was about 0.1 μg/ml for both cefotaxime and metabolite. The range for the urine calibration curves was 5—500 μg/ml for cefotaxime and 5—1000 μg/ml for its metabolite. The precision was determined by repetitive analysis of the same sample. The average relative standard deviation in the investigated concentration range was 1.8% for the parent drug and 1.1% for its metabolite.

Accuracy and linearity of the assay were challenged by measuring the concentrations of spiked plasma samples in the investigated range. Good agreements with an average error of 3.2% could be observed (Table III).

TABLE II

STATISTICS OF CALIBRATION CURVES

Concentrations (C) in μg/ml versus peak area (PA), $C \pm S_{x,y} = (m \pm s_m)PA + (b \pm s_b)$. Standard of error of estimate y on x, concentration μg/ml, on peak area.

Compound	Medium	Range	$m \pm s_m$	$b \pm s_b$	$S_{x,y}$
Cefotaxime	plasma	0.5—10	$1.5 \cdot 10^{-4} \pm 4.3 \cdot 10^{-6}$	0.20 ± 0.13	0.23
Cefotaxime	plasma	0.5—10	$1.7 \cdot 10^{-4} \pm 5.5 \cdot 10^{-6}$	0.37 ± 0.14	0.26
Cefotaxime	plasma	0.5—250	$1.2 \cdot 10^{-4} \pm 1.2 \cdot 10^{-6}$	1.17 ± 0.85	2.72
Cefotaxime	plasma	0.5—250	$1.7 \cdot 10^{-4} \pm 1.7 \cdot 10^{-6}$	2.11 ± 0.97	3.10
Desacetylcefotaxime	plasma	0.5—25	$9.0 \cdot 10^{-5} \pm 8.5 \cdot 10^{-7}$	—0.015 ± 0.10	0.21
Desacetylcefotaxime	plasma	0.5—25	$9.5 \cdot 10^{-5} \pm 1.0 \cdot 10^{-6}$	—0.077 ± 0.18	0.27
Cefotaxime	urine	5—500	2.55 ± 0.03	3.7 ± 2.1	4.8
Desacetylcefotaxime	urine	5—1000	1.93 ± 0.03	20.1 ± 15.6	28.9

TABLE III

ACCURACY OF THE ASSAY IN PLASMA

Compound	Concentration (μg/ml)	Assayed (μg/ml)	Error (%)
Cefotaxime	0.5	0.54	8.0
	2.0	1.94	3.0
	15.0	15.05	0.3
	100.0	96.67	3.3
	200.0	203.12	1.6
	250.0	253.04	1.2
Desacetylcefotaxime	0.5	0.53	6.0
	2.0	2.11	5.5
	15.0	14.81	1.3
	25.0	24.67	1.3

Application of the assay to pharmacokinetic studies

After intravenous administration a rapid decrease in the plasma level of cefotaxime can be observed (Fig. 4). Formation of the metabolite is fast, it reaches its maximum plasma concentration within a few minutes after administration of cefotaxime. The metabolite has a longer half-life than the parent compound. Analysis of the urine showed that about 35% of the given dose is

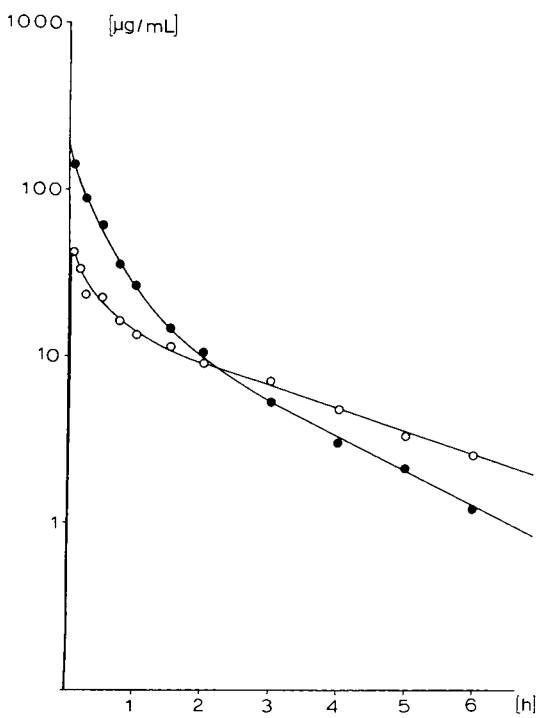

Fig. 4. Plasma levels for cefotaxime (•) and desacetylcefotaxime (○) after intravenous administration of 1 g of cefotaxime to a healthy volunteer.

TABLE IV

URINARY EXCRETION OF CEFOTAXIME AND DESACETYLCEFOTAXIME

Sample time (h)	Amount excreted (mg)	
	Cefotaxime	Desacetylcefotaxime
0—2	306.8	90.8
2—4	27.8	37.8
4—8	8.6	40.6
8—24	—	70.4
Total	343.2	239.6

excreted into the urine as unchanged cefotaxime and 25% as its metabolite (Table IV). As the metabolite also has microbiological activity [1], the results again underline the need for chromatographic assay methods to study the pharmacokinetics of cefotaxime, since microbiological assays will measure the antibacterial activities derived from the combination of drug and metabolite.

REFERENCES

1 R. Wise, P.J. Wills, J.M. Andrews and K.A. Bedford, Antimicrob. Agents Chemother., 17 (1980) 84.
2 R. Luthy, R. Munch, J. Blaser, H. Bhend and W. Siegenthaler, Antimicrob. Agents Chemother., 16 (1979) 127.
3 C. Harvengt, G. Wauters and H. Meunier, Chemotheraphy, 27 (1981) 173.
4 H.C. Neu, P. Aswapokee, K.P. Fu, I. Ho and C. Matthijssen, Clin. Pharmacol. Ther., 27 (1980) 677.
5 T. Bergan and R. Solberg, Chemotherapy, 27 (1981) 155.
6 F. Kees, E. Strehl, K. Seeger, G. Seidel, P. Dominiak and H. Grobecker, Arzneim.-Forsch. 31 (1981) 362.
7 J.B. Lecaillon, M.C. Rouan, C. Souppart, N. Febvre and F. Juge, J. Chromatogr., 228 (1982) 257.
8 S.M. Berge, N.L. Henderson and M.J. Frank, J. Pharm. Sci., 72 (1983) 59.
9 V. Das Gupta, J. Pharm. Sci., 73 (1984) 565.
10 H. Fabre, N. Hussam Eddine and G. Berge, J. Pharm. Sci., 73 (1984) 611.
11 A.M. Brisson and J.B. Fourtillan, J. Chromatogr., 223 (1981) 393.
12 D. Dell, J. Chamberlain and F. Coppin, J. Chromatogr., 226 (1981) 431.

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Note

Determination of fatty acids of the bacteria *Streptomyces* R61 and *Actinomadura* R39 by capillary gas chromatography—mass spectrometry

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(First received August 20th, 1984; revised manuscript received January 17th, 1985)

Actinomycetes have been used extensively by Ghuysen et al. [1] as sources of exocellular and membrane-bound DD-peptidases to study the mode of action of penicillin. The plasma membranes of various *Streptomyces* strains (R61, K15 and *rimosus*) have an atypical penicillin-binding protein (PBP) pattern characterized by the presence of a predominating low-molecular-weight (26 000-Mr) PBP which was characterized as a DD-transpeptidase [2]. The DD-transpeptidase of *Streptomyces* R61 exhibited unusual properties in that temperatures of −35°C (or below) were required to completely inhibit the enzyme when plasma membranes and the co-substrates Ac_2-L-Lys-D-Ala-D-Ala and Gly-Gly were incubated together in the frozen state. Under these conditions, the purified enzyme is devoid of activity [3]. Since the lipids could play an important role in providing the hydrophobic environment necessary for the membrane-bound enzyme to function, it was of interest to study the lipid composition of *Streptomyces* R61. The present paper reports the identification and quantitative analysis of the fatty acids of the phospholipids of *Streptomyces* R61 by gas chromatography—mass spectrometry (GC—MS) using high-resolution support-coated open tubular (SCOT) columns [4]. The same analysis was applied to another *Actinomycetes*, namely *Actinomadura* R39. The membrane-bound PBPs of this organism have not been investigated, but

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both *Actinomadura* R39 and *Streptomyces* R61 excrete during growth exocellular serine DD-peptidases that are able to catalyse concomitant carboxypeptidation and transpeptidation reactions [1].

EXPERIMENTAL

Growth of bacteria

Streptomyces R61, and *Actinomadura* R39, kindly provided by Professor J.M. Ghuysen, Université de Liège, were grown in batch culture in peptone—oxoid medium at 25°C and with vigorous shaking. At the onset of the stationary phase (65 h), cells were harvested by centrifugation, washed twice with water, and then lyophilised.

Extraction of lipids and separation of phospholipids

Lipids were extracted from the cells by the method of Bligh and Dyer [5] and fractionated on a silicic acid column (200—325 mesh), with elution by chloroform, acetone and methanol, to yield the total phospholipids in the methanol eluate. The total phospholipids were subjected to two-dimensional chromatography over Whatman SG-81 silica-impregnated paper, and two-dimensional thin-layer chromatography (TLC) over silica gel G. For both methods the solvent mixtures of Wurthier [6] were used, viz. chloroform—methanol—diisobutyl ketone—acetic acid—water (45:15:30:20:4) (first dimension) and chloroform—methanol—diisobutyl ketone—pyridine—0.5 *M* aqueous ammonium chloride (60:35:50:70:12) (second dimension). Rhodamine 6G solution (0.0012%), Zinzarde's reagent [7] and ninhydrin were used for detection. Individual phospholipid fractions were compared with phospholipid standards, using both solvent mixtures.

Conversion to fatty acid methyl esters and hydrogenation of unsaturated esters

The phospholipids of *Streptomyces* R61 and *Actinomadura* R39 were separately cleaved and converted to fatty acid methyl esters by refluxing over 14% boron trifluoride in anhydrous methanol [8] for 30 min in an all-glass apparatus, followed by pentane extraction after addition of water. In the present work, this treatment did not cause detectable decomposition of the fatty acids. Thus using GC the compositions of methyl esters were substantially the same when the following esterification methods were applied as tests to the phospholipids of *Actinomadura* R39: reaction with 14% boron trifluoride in methanol [8], with or without prior alkaline hydrolysis, and (for comparison) reaction with 10% boron trichloride in methanol [9], with or without prior alkaline hydrolysis. Furthermore, MS analysis showed that, when subjected to the identical treatment with boron trifluoride in methanol, methyl 3-hydroxydecanoate (a β-hydroxy ester) was not dehydrated.

Hydrogenation of unsaturated fatty acid methyl ester mixtures was carried out by bubbling hydrogen for 1 h into a solution in methanol in the presence of 10% palladium-on-charcoal. This treatment did not cause ring cleavage of cyclopropane rings (see Results and discussion).

Gas chromatography

GC was carried out on ethylene glycol succinate cross-linked to silicone (EGSS-X) 3% on Gas-Chrom Q (100—120 mesh) and flame ionization detection was used.

Gas chromatography—mass spectrometry

Fatty acid methyl esters were analysed by GC—MS using a 50-m SCOT SE-30 column and helium, methane and ammonia as chemical ionisation (CI) reactant gases. This system and its application to the analysis of fatty acid methyl esters have been described [4].

RESULTS AND DISCUSSION

Identification of fatty acid methyl esters derived from phospholipids

Streptomyces R61. GC—MS analysis under methane CI conditions of the fatty acid methyl esters from *Streptomyces* R61 before and after hydrogenation of unsaturated fatty acid methyl esters gave the chromatograms shown in Fig. 1. The following peaks were identified by comparison of their GC retention times and CI mass spectra (methane and helium gases) with those of authentic samples: peak 2 (14:0); peak 4 (anteiso-15:0); peak 5 (15:0); peak 7 (iso-16:0); peak 9 (16:0); peak 14 (17:0); peak 17 (iso-18:0); and peak 19 (18:0). Other peaks were identified by inspection of their helium CI mass spectra which yield information on the branching [4]. These are: peak 1 (iso-14:0); peak 3 (iso-15:0); peak 10 (10-Me-16:0); peak 12 (iso-17:0); peak 16 (10-Me-17:0); and peak 20 (10-Me-18:0). The remaining peaks in the chromatogram of the fatty acid methyl esters from *Streptomyces* R61 before hydrogenation (Fig. 1A) had mass spectra corresponding to mono-unsaturated or cyclopropane-containing esters. These peaks were not present on the chromatogram obtained after hydrogenation over palladium-on-charcoal (Fig. 1B) and must therefore correspond to unsaturated rather than cyclopropane esters. MS analysis showed that under the same hydrogenation conditions, the cyclopropane ring of methyl *cis*-9,10-methylenehexadecanoate was not opened. On the basis of the molecular ions observed in their mass spectra, peaks 6 and 8 corresponded to 16:1 isomers, peak 11 to 17:1, and peaks 15 and 18 to 18:1 isomers. Retention times indicated that peak 8 was probably 9-*cis*-16:1 and peak 18 was probably 9-*cis*-18:1.

The methane mass spectrum of peak 13 indicated that it corresponded to a co-chromatographing mixture of 17:0 and 17:1. This was confirmed by inspection of the ammonia CI mass spectrum of this peak. As described previously [4] the ammonia CI spectrum showed an enhanced peak area for the unsaturated fatty acid ester. After hydrogenation, which removed the 17:1 contribution, the helium CI mass spectrum of peak 13 showed enhanced cleavage ions at *m/z* 255 ($M - C_2H_5$) and 227 ($M - C_4H_9$) indicating the presence of anteiso-17:0.

Actinomadura R39. GC—MS analysis of the fatty acid methyl esters from *Actinomadura* R39 before and after hydrogenation gave chromatograms which were qualitatively identical to those described for *Streptomyces* R61.

A summary of the fatty acid methyl esters identified and the relative

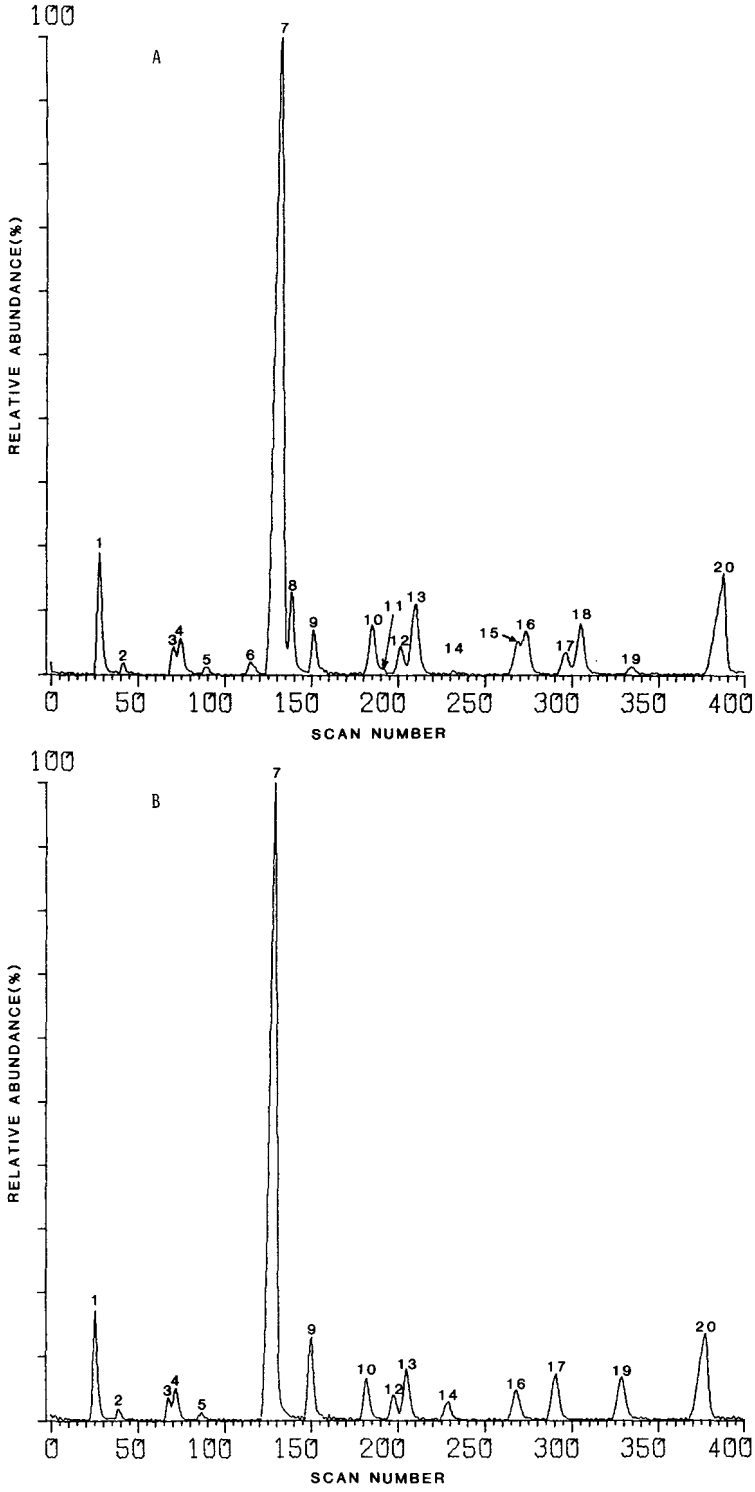

Fig. 1. Gas chromatograms obtained by methane CI GC—MS analysis of fatty acid methyl esters from *Streptomyces* R61 before hydrogenation over palladium-on-charcoal (A) and after hydrogenation (B). Peak numbers refer to methyl esters listed in Table I.

amounts of each present in extracts of *Actinomadura* R39 and *Streptomyces* R61 is given in Table I. The most striking feature of the results shown in Table I is that branched saturated esters accounted for approximately 72% and 84% of the total fatty acid methyl esters from *Actinomadura* R39 and *Streptomyces* R61 respectively, whereas straight-chain saturated esters contributed only 3% of the total in each case. In both strains, the most abundant fatty acid methyl ester was iso-16:0 which constituted almost half of the total.

Unsaturated esters accounted for approximately 25% and 13% of the total fatty acid methyl esters from *Actinomadura* R39 and *Streptomyces* R61, respectively. After hydrogenation, the proportion of branched-chain esters

TABLE I

FATTY ACID METHYL ESTERS DERIVED FROM PHOSPHOLIPIDS OF *ACTINOMADURA* R39 AND OF *STREPTOMYCES* R61

GC—MS peak no.* (Fig. 1)	Relative retention time**	Identity of fatty acid***	Composition§ (%)			
			Actinomadura R39		*Streptomyces* R61	
			Before hydrogenation	After hydrogenation	Before hydrogenation	After hydrogenation
1	0.407	iso-14:0	1.9	1.9	2.6	2.7
2	0.436	14:0	0.3	0.3	0.2	0.3
3	0.473	iso-15:0	0.6	0.7	1.0	0.9
4	0.479	anteiso-15:0	0.9	0.9	1.3	1.3
5	0.502	15:0	0.1	0.2	0.3	0.3
6	0.539	16:1	0.6	—	0.6	—
7	0.562	iso-16:0	41	40	48	49
8	0.574	16:1	4.0	—	3.5	—
9	0.596	16:0	1.5	5.7	2.0	5.2
10	0.636	10-Me-16:0	1.3	1.1	3.7	3.2
11	0.644	17:1	0.2	—	0.1	—
12	0.659	iso-17:0	1.8	1.8	2.2	2.1
13	0.672	anteiso-17:0 + 17:1	5.6	3.8 —	5.4	4.4 —
14	0.708	17:0	<0.1	2.3	< 0.1	1.7
15	0.757	iso-18:1	3.1	—	2.9	—
16	0.763	10-Me-17:0	1.7	1.8	3.9	3.4
17	0.798	iso-18:0	2.8	6.0	2.2	5.6
18	0.812	18:1	14	—	5.0	—
19	0.853	18:0	1.6	17	0.7	6.1
20	0.930	10-Me-18:0	16	16	15	15
		Branched-chain	72	74	84	87
		Straight-chain	3	26	3	13
		Unsaturated	25	—	13	—

*See Fig. 1 for chromatogram of *Streptomyces* R61 and Experimental for methane CI GC—MS conditions.

**Relative to docosane.

***First number, number of carbon atoms in the chain; second number, number of double bonds.

§Total does not come to exactly 100% due to rounding off.

increased only slightly whereas the proportion of straight-chain esters increased to 26% and 13%, respectively, which indicated that the unsaturated esters were predominantly straight-chain.

By comparison of the relative amounts of particular fatty acid methyl ester before and after hydrogenation it was possible to draw some inferences about the structure of some of the unsaturated esters. Peak 15 (3%, 18:1) decreased to zero after hydrogenation whereas peak 17 (iso-18:0) increased from 3% to 6% which implied that peak 15 represents iso-18:1. The same kind of argument may be used to show that peak 8 (16:1), peak 13 (17:1), and peak 18 (18:1) were straight-chain unsaturated fatty acid methyl esters.

The variation of fatty acid composition with phospholipid structure was assessed for the phospholipids of *Actinomadura* R39. Four individual phospholipids separated by preparative TLC were analysed by GC. The estimated fatty acid methyl ester compositions (expressed for groups of esters of similar retention time) as shown in Table II indicated that there were considerable variations. Phosphatidyl ethanolamine (a minor component) was different from the other phospholipids in not having iso-16:0 as the major fatty acid, but had instead an elevated 16:0 content. The level of 16:0 was also raised in cardiolipin. The results may reflect the dynamic nature of lipid turnover or the asymmetric nature of the bacterial membrane [10].

The fatty acids of most microorganisms including *Actinomycetes* species [11, 12] are known to contain branched and unsaturated fatty acids, but seldom do both types exist in appreciable quantities. The theory of homoviscous adaptation suggests that generally the degree of unsaturation and of

TABLE II

APPROXIMATE PERCENTAGE COMPOSITION OF GROUPS OF FATTY ACIDS FROM INDIVIDUAL PHOSPHOLIPIDS OF *ACTINOMADURA* R39

The approximate percentage composition is as analysed by GC under conditions given in Experimental.

Fatty acid	Phosphatidyl glycerol	Cardiolipin	Unidentified* phospholipid	Phosphatidyl ethanolamine
iso-14:0	1	7	1	1
14:0	0.2	2	1	3
iso-15:0, anteiso-15:0, 15:0	1	5	3	2
iso-16:0	42	38	45	17
16:0	3	13	6	18
17:0 isomers, 16:1**	11	2	6	3
18:0 isomers, 17:1**	5	3	5	15
10-Me-18:0, 18:1 isomers	37	29	33	41

*Representing 25% of the total phospholipids; stained negative to ninhydrin, ammoniacal silver nitrate and Dragendorff's reagents.

**Probably one of two isomers found in *Actinomadura* R39.

branching may be adjusted to maintain membrane fluidity [13, 14]. The membrane lipids of *Escherichia coli* K12 grown at 13°C was shown to be considerably more unsaturated than those of cells grown at 37°C [15]. Incorporation of branched fatty acids into *Acholeplasma laidlawii* membrane lipids resulted in non-appearance of particle aggregation on cooling of the modified cells [16]. It had been suggested that the branched chains could be more loosely packed than straight chains, and consequently had a lower crystalline state transition temperature [13, 16]. A number of studies support the idea [17] that a liquid—crystalline state for cell membrane lipids is necessary to support growth [18, 19], membrane transport [19, 20], and the activity of membrane-associated enzymes [21]. The fatty acid composition of both *Streptomyces* R61 and *Actinomadura* R39 is unusual in that the branched and unsaturated members together contribute as much as 97% of the total fatty acids. The 26000-Mr membrane-bound DD-transpeptidase of *Streptomyces* R61 remains active at temperatures below 0°C [2, 3]. It might be speculated that this is a consequence of the maintenance of membrane lipid fluidity by the reinforcing effects of the branched and unsaturated fatty acids.

REFERENCES

1 J.M. Ghuysen, J.M. Frère, M. Leyh-Boville, O. Dideberg, J. Lamotte-Brasseur, H.R. Perkins and J.L. De Coen, in A.S.V. Burgen and G.C.K. Roberts (Editors), Topics in Molecular Pharmacology, Elsevier/North-Holland, New York, 1981, p. 63.
2 M. Nguyen-Distèche, M. Leyh-Boville and J.M. Ghuysen, Biochem. J., 207 (1982) 109.
3 J. Dusart, A. Marquet, J.M. Ghuysen and H.R. Perkins, Eur. J. Biochem., 56 (1975) 57.
4 J. Vine, J. Chromatogr., 196 (1980) 415.
5 E.G. Bligh and J.W. Dyer, Can. J. Biochem. Physiol., 37 (1959) 911.
6 R.E. Wurthier, J. Lipid Res., 7 (1966) 544.
7 U. Beiss, J. Chromatogr., 13 (1964) 104.
8 W.R. Morrison and L.M. Smith, J. Lipid Res., 5 (1964) 600.
9 W.E. Klopfenstein, J. Lipid Res., 12 (1971) 773.
10 M.R. Salton and P. Owen, Ann. Rev. Microbiol., 30 (1976) 451.
11 S.G. Batrakov and L.D. Bergelson, Chem. Phys. Lipids, 21 (1978) 1 and references cited therein.
12 M.L. Zyuzina, T.P. Efimova and I.M. Tereshin, Mikrobiologiya, 48 (1979) 620.
13 R.N. McElhaney, in M.R. Henrich (Editor), Extreme Environments: Mechanisms of Microbial Adaptation, Academic Press, New York, 1976, p. 225.
14 H. Hauser, G.P. Hazlewood and R.M.C. Dawson, Nature, 279 (1979) 536.
15 C.W.M. Haest, J. De Gier and L.L.M. Van Deenan, Chem. Phys. Lipids, 3 (1969) 413.
16 C.W.M. Haest, A.J. Verkleij, J. De Gier, R. Scheck, P.H.J. Ververgaert and L.L.M. Van Deenan, Biochim. Biophys. Acta, 356 (1974) 17.
17 V. Luzzati and F. Husson, J. Cell Biol., 12 (1962) 207.
18 R.N. McElhaney, J. Mol. Biol., 84 (1974) 145.
19 P. Overath, H.U. Schairer and W. Stoffel, Proc. Natl. Acad. Sci., U.S., 67 (1970) 606.
20 N.A. Machtiger and C.F. Fox, Ann. Rev. Biochem., 42 (1973) 575.
21 H.K. Kimelberg and D. Papahadjopoulos, Biochim. Biophys. Acta, 282 (1972) 277.

Journal of Chromatography, 341 (1985) 146—153
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Note

Unusual fatty acids from amniotic fluid phospholipids

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While looking at the possibility of using high-performance liquid chromatography (HPLC) to obtain accurate data on phospholipids contained in amniotic fluid, particularly phosphatidyl glycerol, the HPLC phospholipid fractions were collected, hydrolysed and their fatty acids were identified and quantified by gas—liquid chromatography (GLC) or gas chromatography—mass spectrometry (GC—MS) to see if there was any significant variation in fatty acid content with gestational age.

The expected $C_{14:0}$, $C_{16:0}$ and $C_{18:0}$ fatty acids were identified as their methyl esters as well as methyl oleate; however, in addition there appeared to be present two unsaturated fatty acids whose retention times suggested that their sites of unsaturation were not at the C-9—C-10 position usually reported for phospholipids.

EXPERIMENTAL

Materials and methods

Amniotic fluid was collected by trans abdominal amniocentesis as part of the routine management of complicated pregnancies in King George V Maternity Hospital and other New South Wales hospitals. Amniotic fluid (2 ml) was extracted following the method of Brown et al. [1]. About 0.5 ml of the phospholipid extract was used for thin-layer chromatographic (TLC) determination of lecithin/sphingomyelin (L/S) ratios and the remainder (about 1 ml) of the chloroform extract was evaporated to dryness at room temperature under a stream of nitrogen. The sides of the tube were washed with methanol and the washings also blown down to dryness with nitrogen, then 25 μl of methanol were added to the dried residue, vortex-mixed for 30 sec and 20 μl injected as single injection onto the HPLC column.

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Separation

The method used was based on that of Briand et al. [2] using a gradient system composed of acetonitrile and acetonitrile—water (80:20) set to deliver a water gradient from 2.4% to 15% at a flow-rate of 2 ml/min. After 9 min the elution was complete, the column was re-equilibrated and the next injection was made after 16 min. The UV detector was set at 206 nm. The purity of the separated phospholipids was checked by collecting the individual peaks and running them against the appropriate standards using two-dimensional silica gel TLC; solvent 1: chloroform—methanol—ammonium hydroxide—water (65:30:2.5:2); solvent 2: chloroform—methanol—glacial acetic acid—water (80:25:6:2); the spots were visualized by dipping the plates into 10% ethanolic sulphuric acid solution and heating at 150°C for 5—10 min. Phospholipids and other organic material charred dark brown.

Identification of phospholipid fatty acids

Separated peaks from the HPLC procedure were collected individually and evaporated to dryness in vacuo at room temperature. The residues were dissolved in 14% boron trifluoride—methanol complex and heated at 80°C for 20 min [3]. The resulting fatty acid methyl esters were extracted using a light petroleum (40—60°C)—water mixture (2:1). The organic layer was washed, dried, and evaporated to dryness under a stream of nitrogen at room temperature. The fatty acid methyl esters were dissolved in hexane (1—2 μl) and separated by GLC using 3% OV-101 on Gas-Chrom Q 120—140 mesh packed into a glass column (1.8 m × 6 mm I.D.).

A temperature programme of 160°C—230°C (6°C/min) was run and the column was held at 230°C until all the fatty acid esters were eluted.

Fatty acids were identified by comparison with retention times of reference standards obtained under the same conditions.

Gas chromatography—mass spectrometry

Analysis of fatty acid methyl ester mixtures was also carried out on a Finnigan 3200 quadrupole mass spectrometer fitted with a chemical ionisation (CI) source and interfaced to a Finnigan 9500 gas chromatograph. Data acquisition and processing were carried out on line using an Incos 2300 data system. The GLC column (2 m × 2 mm) used was packed with 3% OV-101 Gas-Chrom Q (120—140 mesh). Helium was used as the carrier gas at a flow-rate of 2.5 ml/min and held at 230°C until elution of peaks ceased. Injection port and interface temperatures were kept at 250°C. CI mass spectra were generated by adding reactant gas (methane) through a make-up T-piece at the end of the column. The reactant gas flow-rate was adjusted to give a source pressure of 67 Pa for helium and 133 Pa for methane.

Mass spectra were generated using an electron beam energy of 135 eV and source temperature was kept at 100°C.

2- and 3-dodecenoic acid

A mixture of 2- and 3-dodecenoic acids was produced via bromination of dodecanoic acid [4]. However, HPLC was found to be a more appropriate method to obtain small quantities of pure isomers than the fractional

distillation used in the reference [4]. Replicate injection of a mixture of isomers onto an ODS 10-μm HPLC column (250 × 4.5 mm), mobile phase acetonitrile—water (90:10), UV detector set at 206 nm, flow-rate 1.8 ml/min, gave a good separation of the components of the reaction mixture. Combining appropriate fractions gave sufficient material for GLC, GC—MS and UV spectral examination which were used to identify the 2- and 3-dodecenoic acids as their methyl esters.

2-Hexadecenoic acid

Tetradecanal (1.6 g) was refluxed with granulated zinc metal (0.5 g) and ethylbromoacetate (1.0 g) in benzene—diethyl ether (3:1) (15 ml) until the zinc had dissolved. The product, ethyl-3-hydroxyhexadecanoate, was obtained following the usual Reformatsky reaction product work up [5].

Ethyl-3-hydroxyhexadecanoate (1.0 g) was refluxed with 10% sulphuric acid—methanol (1:1) (40 ml) for 30 min. The reaction mixture was cooled, extracted with diethyl ether, the ether extract was then extracted with 10% sodium carbonate solution. The alkaline extract was acidified with hydrochloric acid and extracted with diethyl ether. The ether extract was washed with water, dried, and evaporated to dryness. The residue was refluxed with thionyl chloride (5 ml) for 60 min and the cooled mixture poured into ice water, then extracted with hexane. After removal of the solvent a portion of the reaction product (0.05 g) was reacted with boron trifluoride—methanol complex and separation of the esters by GLC and GC—MS showed the presence of 2- and 3-hexadecenoic acid methyl esters together with a small amount of starting material and an unidentified reaction product.

Purification of 2-hexadecenoic acid

The reaction mixture containing the hexadecenoic acid isomers was purified by HPLC following the procedure outlined above for dodecenoic acids. The mobile phase used was acetonitrile—water (95:5). The major HPLC peak was collected and methylated (boron trifluoride—methanol) and shown by the UV spectrum (λ_{max} 210 nm, log ϵ 3.9) and GC—MS to be methyl-2-hexadecenoate, molecular weight 268 a.m.u.

RESULTS AND DISCUSSION

Phospholipid fatty acids

The individual phospholipid classes extracted from amniotic fluid obtained from 36 patients were separated by HPLC, the collected fractions were hydrolysed, and the fatty acids chromatographed by GLC using the C_{12}—C_{22} even-numbered saturated fatty acids as reference standards, Fig. 1.

The expected tetradecanoic, hexadecanoic, octadecanoic and 9-octadecenoic acids were identified as their methyl esters. In some samples one or two unidentified fatty acids were noticed in addition to the normally occurring compounds.

Further investigation by GC—MS showed that the unknown compounds had molecular weights of 268 and 296 a.m.u., therefore they appeared to be hexadecenoic and octadecenoic acid methyl esters. However, their chromato-

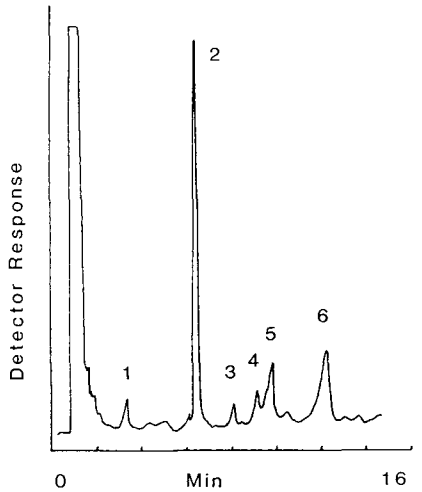

Fig. 1. Gas chromatogram of fatty acid methyl esters obtained from hydrolysis of phosphatidyl choline fraction. Column 3% OV-101 on Gas-Chrom Q (120—140 mesh), temperature programme 160°C—230°C at 6°C/min. Peaks: 1 = tetradecanoic acid methyl ester; 2 = hexadecanoic acid methyl ester; 3 = $C_{16:1}$ acid methyl ester; 4 = 9-octadecenoic acid methyl ester; 5 = octadecanoic acid methyl ester; 6 = $C_{18:1}$ acid methyl ester.

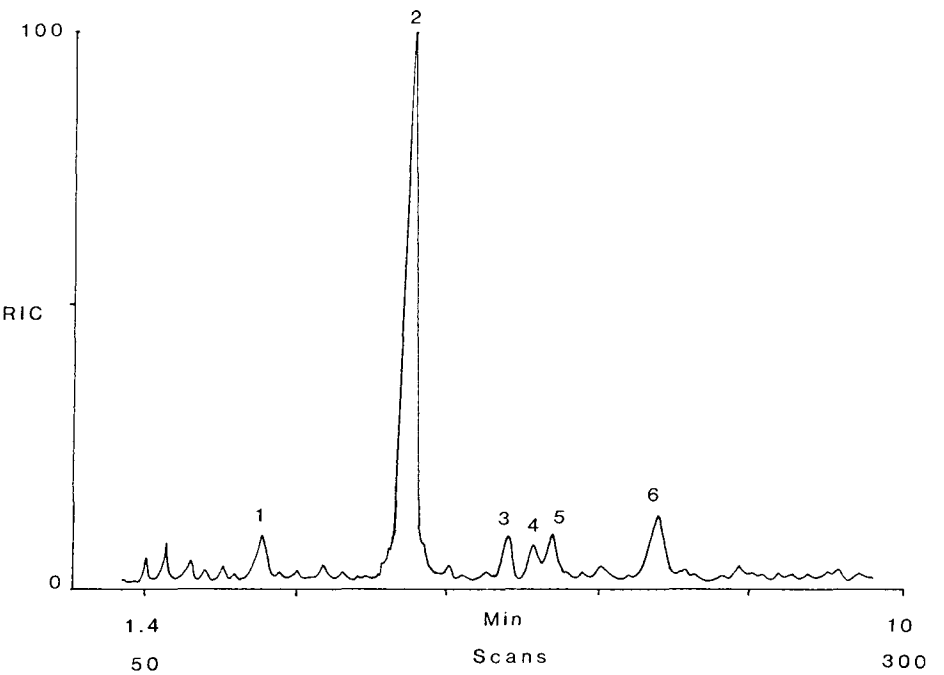

Fig. 2. GC—MS scan of fatty acid methyl esters obtained from hydrolysis of phosphatidyl choline fraction $(M + H)^+$. Peaks: 1 = *m/z* 243; 2 = *m/z* 271; 3 = *m/z* 269; 4 = *m/z* 297; 5 = *m/z* 299; 6 = *m/z* 297.

graphic characteristics did not point to their sites of unsaturation being at the usual C-9—C-10 position as in oleic acid (9-octadecenoic acid) since these isomers generally have shorter retention times than the saturated analogues (Fig. 2).

Comparison of the mass spectra by GC—MS of the two unknown compounds and their corresponding saturated analogues shows that the prominent peak *m/z* 74 ascribed to a McLafferty rearrangement in saturated fatty acid methyl esters is absent in the mass spectra of the unknown compounds indicating that the double bond is adjacent to the ester carbonyl group.

The readily accessible 2- and 3-dodecenoic acids [4] were synthesised for use as model compounds and it was found that the conjugated methyl-2-dodecenoate had a longer retention time than either the 3-dodecenoic or dodecanoic acid methyl esters. Using the Reformatsky procedure [5], ethyl-3-hydroxyhexadecanoate was synthesised from tetradecanal, then dehydration and hydrolysis produced a mixture of 2- and 3- hexadecenoic acids.

Separation of the mixture was easily followed since the conjugated chromophore in 2-hexadecenoic acid (λ_{max} 206 nm, log ϵ 3.9) allows this isomer to be readily distinguished from the unconjugated 3-hexadecenoic acid. Fig. 3 shows gas chromatograms of a mixture of hexadecanoic and 2-hexadecenoic acid methyl esters and Fig. 4 shows a GC—MS trace of a mixture containing hexadecanoic, octadecanoic, 2- and 3-hexadecenoic acid methyl esters together with specific ion scans at *m/z* 269 and 271 which show that the conjugated 2-hexadecenoate is clearly separated from the other components. The retention times for the unknown and synthetic samples relative to hexadecanoic acid methyl ester were the same and the CI mass spectra are shown in Fig. 5.

The mass spectrum of the second unknown component showed the

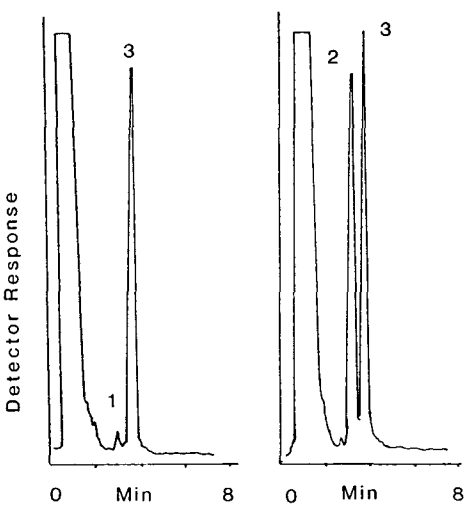

Fig. 3. Gas chromatograms of methyl esters of 2-hexadecenoic acid and hexadecanoic acid. Column 3% OV-101 on Gas-Chrom Q (120—140 mesh), 210°C. Peaks: 1 = 3-hexadecenoic acid methyl ester; 2 = hexadecanoic acid methyl ester; 3 = 2-hexadecenoic acid methyl ester.

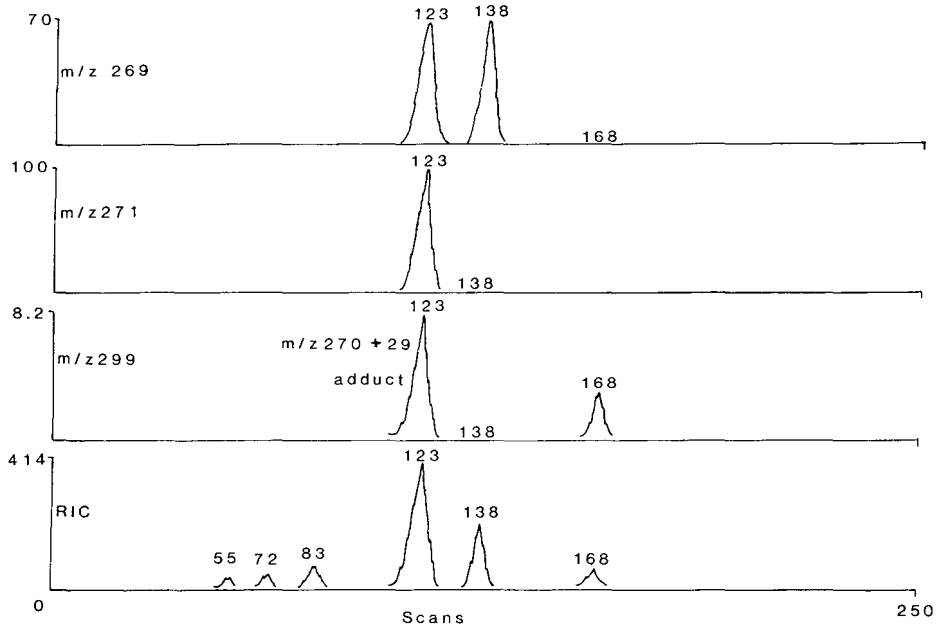

Fig. 4. Limited ion mass scan chromatogram of methyl esters of hexadecanoic acid, octadecanoic acid, 2- and 3-hexadecenoic acid.

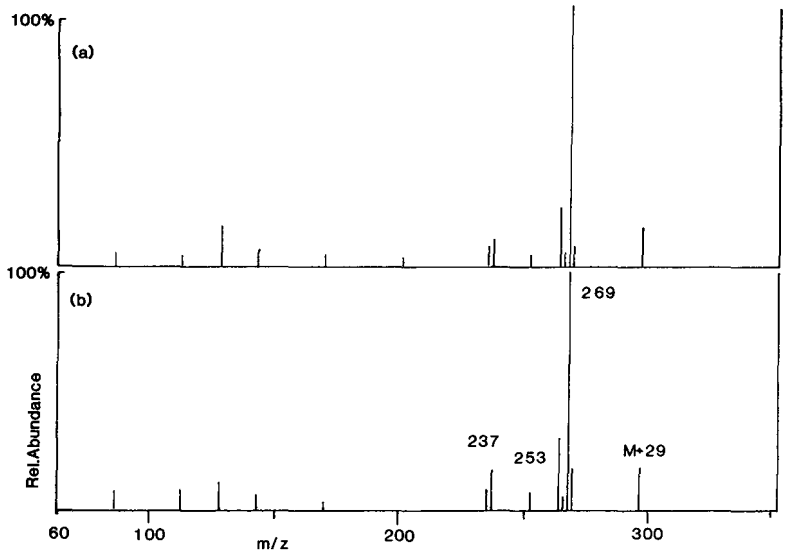

Fig. 5. Chemical ionisation mass spectra. (a) Unknown from amniotic fluid peak 3, Fig. 2; (b) synthetic 2-hexadecenoic acid methyl ester.

MH^+—CH_3OH peak characteristic of methyl esters but no *m/z* 74 peak, differed from the saturated C_{18} acid by 2 a.m.u., had a longer retention time analogous to 2-hexadecenoic acid methyl ester, and was separated from methyl-9-octadecenoate. Therefore it was concluded that the second unknown component was 2-octadecenoic acid.

TABLE I

PERCENTAGE COMPOSITION OF THE INDIVIDUAL PHOSPHOLIPID FATTY ACIDS COLLECTED FROM THE HPLC OF AMNIOTIC FLUIDS AT DIFFERENT STAGES OF GESTATION

Data are from 36 samples. Abbreviations: PG = phosphatidyl glycerol; PI = phosphatidyl inositol; PE = phosphatidyl ethanolamine; PC = phosphatidyl choline; SPH = sphingomyelin.

Fatty acid composition	< 35 Weeks gestation					35—37 Weeks gestation					> 37 Weeks gestation				
	PG	PI	PE	PC	SPH	PG	PI	PE	PC	SPH	PG	PI	PE	PC	SPH
$C_{14:0}$	3.5	8.5	20.0	2.5	4.5	11.0	12.1	13.6	6.0	9.2	6.0	11.6	9.4	6.9	11.4
$C_{16:0}$	45.7	43.7	40.0	72.6	64.0	43.4	41.1	41.1	74.8	64.0	47.0	37.1	44.1	74.6	74.1
$C_{16:1}$*				0.3		2.2	1.3	2.5	1.9	1.5	1.9	1.5	1.7	2.7	1.1
$C_{18:1}$	15.9	19.4	20.0	9.2	8.1	10.4	5.3	8.0	3.5	5.0	12.0	10.9	8.0	3.7	3.7
$C_{18:0}$	34.9	28.4	16.0	9.2	22.6	19.7	20.8	18.1	6.0	12.0	16.1	18.3	18.1	4.8	6.8
$C_{18:1}$*			4.0	6.2	0.8	13.3	19.4	16.7	7.8	8.3	17.0	20.6	18.7	7.3	2.9
Total saturated	80.6	80.6	76.0	84.3	91.1	74.1	74.0	72.8	86.8	85.2	69.1	67.0	71.6	86.3	92.3
Total unsaturated	19.4	19.4	24.0	15.7	8.9	25.9	26.0	27.2	13.2	14.8	30.9	33.0	28.4	13.7	7.7

*α—β Unsaturated fatty acids.

Table I shows the saturated and unsaturated acids identified in phospholipids from amniotic fluid. It is apparent that the conjugated unsaturated fatty acids are found in a range of phospholipids and in some phospholipids reach a significant proportion of the fatty acids present.

It is not clear how these fatty acids arise, however 2- and 3-hydroxy fatty acids are known to be intermediates in plant and animal metabolism of lipids and 3-hydroxy fatty acids are biosynthetic intermediates.

It is unlikely that the conjugated fatty acids identified arose by chemical degradation in the sample work-up since the synthetic work has shown that 3-hydroxy fatty acids are not as easily dehydrated as might be expected and when dehydration does occur chemically a mixture of mono unsaturated acids are formed. There was no sign of C_3—C_4 unsaturated isomers in the phospholipid extracts that would be expected to accompany the C_2—C_3 unsaturated acids if they were the result of chemical reactions.

It is not known what clinical or physiological significance, if any, can be attributed to these conjugated unsaturated fatty acids in amniotic fluid phospholipids; however, it is generally accepted that saturated phospholipids, especially dihexadecanoylphosphatidyl choline, are physiologically the most important phospholipids. This is reflected in the amounts of saturated phospholipids found in the samples analysed (Table I).

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REFERENCES

1 L.M. Brown, C.G. Duck-Chong and W.J. Hensley, Clin. Chem., 28 (1982) 344.
2 R.L. Briand, S. Harold and K.G. Blass, J. Chromatogr., 223 (1981) 277.
3 W.R. Morrison and L.M. Smith, J. Lipid Res., 5 (1964) 600.
4 N. Rabjohn (Editor), Organic Syntheses Collectivé, Vol. 4, Wiley, New York, 1963, p. 398.
5 A.I. Vogel, Practical Organic Chemistry, Longman, London, 3rd ed., 1956, p. 874.

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Note

Rapid separation of plasma lipoproteins by gel permeation chromatography on agarose gel Superose 6B

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Agarose gel has been used successfully in the separation of plasma lipoproteins [1—5]. However, the conventional method of agarose gel chromatography in separating plasma lipoproteins requires a long elution time (> 16 h) and a large elution volume [1—3, 5]. The eluted fractions are usually pooled, concentrated or extracted to enable analysis of the chemical components of lipoproteins [1, 2, 5].

In this paper, a rapid separation of plasma lipoproteins using an extensively cross-linked agarose gel matrix is described. The cholesterol and triacylglycerol concentrations distributed in the different lipoproteins are also measured. A comparison is made between the separation of lipoproteins in the plasma of man, rabbit and rat.

EXPERIMENTAL

Equipment

A fast protein (peptide, polynucleotide) liquid chromatography (FPLC) system (Pharmacia Fine Chemicals, Uppsala, Sweden) was used for carrying out the separation of plasma lipoproteins on a column of Superose 6B. The system consisted of two P-500 high-precision, high-pressure pumps (only one was necessary in these experiments), a GP-250 gradient programmer, V-7 injection valve and a 5-ml sample loop. For these experiments, the system was connected to a single-path UV monitor (UV-1), a fraction collector (FRAC-100) and a chart recorder (Pharmacia Fine Chemicals).

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Superose 6B (Pharmacia Fine Chemicals) was packed onto a K 16/70 column (56 cm × 1.6 cm) equipped with a protective outer jacket.

Separation of standard and total lipoprotein fractions from human, rabbit and rat plasma was carried out on a Beckman L5-50 ultracentrifuge in a Ti 50.3 rotor.

Samples

Plasma samples were obtained from normal 12—16 h fasted men, female NZW rabbits and male Porton rats. Standard lipoprotein fractions were prepared by sequential ultracentrifugation using solid potassium bromide to obtain the required densities [6, 7]. Very-low-density lipoproteins (VLDL) were isolated as the fraction of density < 1.006 g/ml, low-density lipoproteins (LDL) in the density interval 1.006—1.063 g/ml, high-density lipoproteins (HDL) in the density interval 1.063—1.21 g/ml and the total lipoprotein as the fraction of density < 1.21 g/ml.

Gel permeation chromatography on Superose 6B

A new breed of agarose gel, Superose 6B, was used for separating plasma lipoproteins. The gel was packed onto a K 16/70 column and water was pumped into the outer protective jacket to prevent sudden temperature changes during the elution.

Total plasma lipoproteins were isolated from 2.5—3.5 ml plasma by ultracentrifugation at density 1.21 g/ml, and made up to a volume of 1.5—2.0 ml with a pre-filtered and degassed solution containing 0.15 *M* sodium chloride, 0.01% (w/v) Na_2EDTA and 0.02% (w/v) sodium azide, pH 7.2. The sample was injected onto the column through a V-7 valve attached to a 5-ml sample loop. The column was pre-equilibrated and the sample eluted with the same saline solution.

The P-500 pumps, fraction collector and recorder were controlled by the gradient programmer. A constant-elution flow-rate of 0.75 ml/min was achieved and fractions of 2.0 ml were collected after discarding the first 32 ml. The absorbance of the eluent was monitored continuously at 280 nm using the UV-1 monitor. The tracing was printed on the recorder, set at a sensitivity of 100 mV and a chart speed of 1 mm/min. Absorbance of the selected fractions was calibrated on a Gilford spectrophotometer 250 (Instrument Labs., Oberlin, OH, U.S.A.).

Chemical analyses

The cholesterol concentration in each of the fractions collected was analysed by an enzymatic method (CHOD-PAP method, Boehringer, Mannheim, F.R.G.) adapted for multiple determination in a CentrifiChem System 400 (Union Carbide, U.S.A.). Triacylglycerol concentration was analysed in the same way but with a different enzymatic method (Triglycerides GPO-PAP, Boehringer).

RESULTS

The elution profiles of lipoproteins in the plasma fraction of density < 1.21 g/ml show three major peaks which eluted with decreasing particle size

corresponding to VLDL, LDL and HDL, respectively (Fig. 1). The human HDL peak eluted later than that of rabbit and rat indicating a smaller particle size of human HDL. The peaks labelled P may represent albumin, prealbumin or potassium bromide salt used for the ultracentrifugation.

There is a species difference in the proportion of each lipoprotein class; in all cases the strongest absorbance occurred in HDL. The proportion of LDL is highest in man, lower in rabbit and negligible in rat (Fig. 1).

Cholesterol concentration was measured in each of the fractions collected. Again three distinct peaks corresponding to VLDL, LDL and HDL were observed (Fig. 2). The greatest proportion of human plasma cholesterol is found in the LDL fraction. In rabbit, LDL and HDL are the major cholesterol carriers, whereas in rat, HDL is the major carrier of plasma cholesterol.

Triacylglycerol concentration profiles also show the presence of three

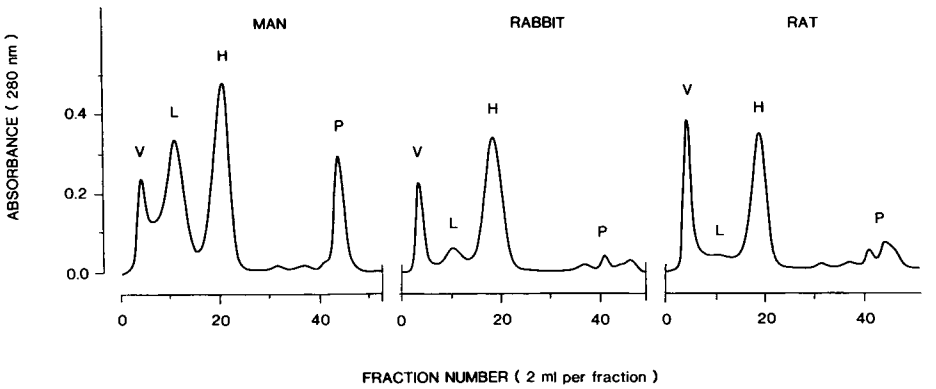

Fig. 1. Elution profile of lipoproteins on Superose 6B. Total lipoproteins were isolated by ultracentrifugation at density 1.21 g/ml from 3.5 ml plasma. A final volume of 1.5—2.5 ml was injected onto a column of Superose 6B (56 cm × 1.6 cm) and eluted at 0.75 ml/min with 0.15 *M* sodium chloride, 0.01% Na_2EDTA and 0.02% sodium azide, pH 7.2. Fractions (2 ml) were collected after discarding the first 32 ml. The monitor sensitivity was set at 0.1, the recorder sensitivity at 100 mV and the chart speed 1 mm/min. The operation was carried out at room temperature (ca. 23°C). Peaks: V = VLDL, very-low-density lipoproteins; L = LDL, low-density lipoproteins; H = HDL, high-density lipoproteins; P = unidentified peaks.

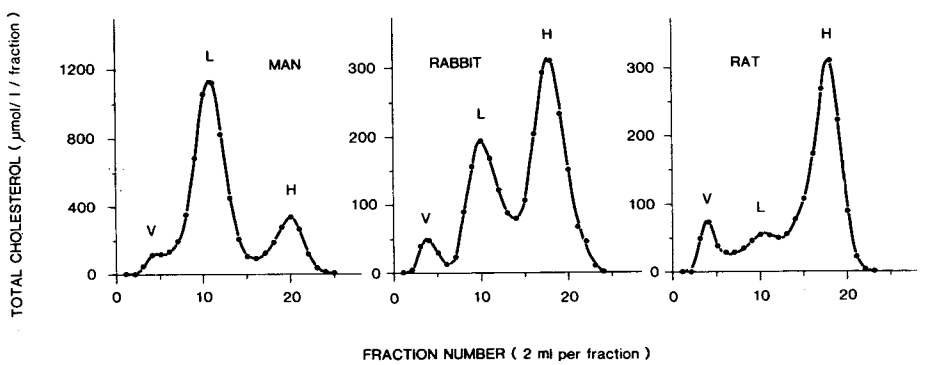

Fig. 2. Cholesterol profiles of lipoproteins. Cholesterol concentration in each of the lipoprotein fractions shown in Fig. 1 was measured enzymatically as described in Experimental. The concentration scale for rabbit and rat is different from that of man.

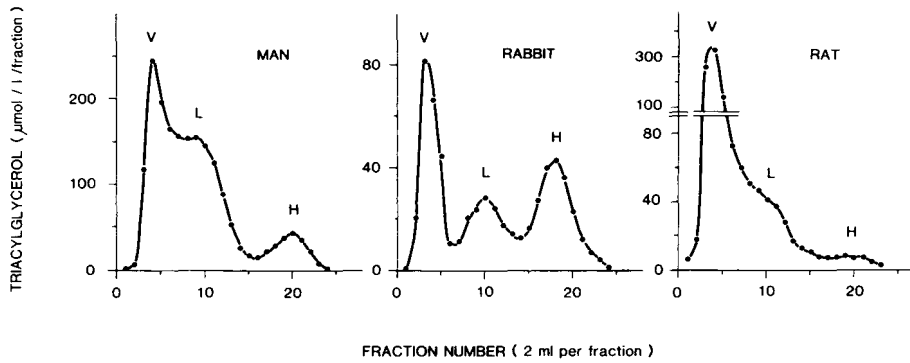

Fig. 3. Triacylglycerol profiles of lipoproteins. Triacylglycerol concentration in each of the lipoprotein fractions shown in Fig. 1 was measured enzymatically as described in Experimental. The concentration scale is different for each of the three animal species.

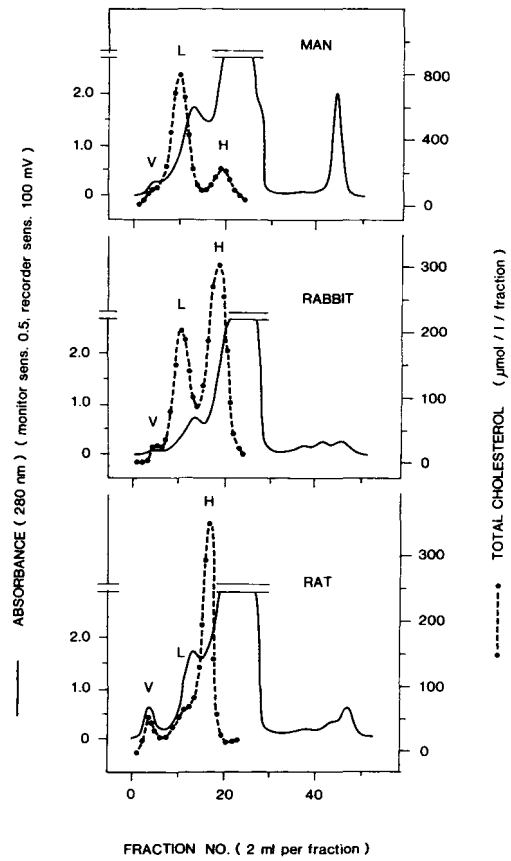

Fig. 4. Elution profiles of whole plasma. A 3-ml volume each of fasted rat and rabbit plasma and 2.5 ml of human plasma were chromatographed on a column of Superose 6B as described in Fig. 1. The absorbance was monitored continuously. The monitor sensitivity was set at 0.5 and the recorder sensitivity at 100 mV. The fractions collected were assayed for cholesterol concentration using enzymatic methods as described in Experimental.

lipoprotein regions, but the VLDL and LDL peaks overlapped in the case of man and rat owing to the high concentration of triacylglycerol in VLDL (Fig. 3). While most of the plasma triacylglycerol is found in VLDL and LDL in all three animal species, there is a marked difference in the proportion of triacylglycerol distributed in HDL. The proportion of triacylglycerol in HDL is highest in rabbit, less in man and negligible in rat (Fig. 3).

The absorbance profiles of whole plasma of the three animal species are shown in Fig. 4. While the VLDL peak is visible in all cases, there are no obvious peaks corresponding to LDL and HDL, as these are masked by the bulk of plasma proteins. However, the individual lipoprotein peaks can be identified by measuring the total cholesterol concentration in each fraction collected (Fig. 4).

DISCUSSION

The classification of plasma lipoproteins by their hydrated densities after ultracentrifugation has its drawbacks, especially when inter-species comparison is concerned. Often, the density range defined for human plasma lipoprotein classes may not correspond exactly to that of rabbit or rat, e.g. the overlapping of LDL and HDL_1 density range in rat [8]. The difference in the particle size of human HDL and that of rabbit or rat (Fig. 1) indicates that the density range of HDL may vary betweeen these animal species. The other disadvantage of prolonged ultracentrifugation is the dissociation of some apoproteins from HDL [9—11]. For these reasons, a more gentle separation of plasma lipoproteins according to their particle size by gel permeation chromatography offers a viable alternative.

The separation of lipoprotein classes by conventional agarose gel chromatography offers a considerable saving in time compared to the sequential ultracentrifugation; however, a typical chromatographic run still requires 16 h or longer [1—5]. Furthermore, the fractions collected are often too dilute for direct measurement of the chemical components. The present method uses a new agarose gel matrix, superose 6B, which greatly reduces the separation time and improves the analysis of lipoproteins. The highly cross-linked nature of the individual agarose bead ensures its overall rigidity. As a consequence of the small bead size (20—40 nm), an increase in eluent flow-rate can be achieved. A flow-rate of 0.75 ml/min is possible without encountering excessive back-pressure and a good lipoprotein separation can be achieved within 2—3 h (Fig. 1). The fractions collected from the column are concentrated enough to be assayed directly for cholesterol (Fig. 2) and triacylglycerol (Fig. 3) without any requirement for prior concentration of the sample. Unless the absorbance profile of lipoproteins is required, lipoproteins can be separated from whole plasma directly without prior separation by ultracentrifugation (Fig. 4). Because lipoproteins can be separated rapidly by this method, the procedure can be carried out at room temperature with reduced chances of protein denaturation. The control of the operation by the FPLC system enables automation and reproducibility.

This method is particularly well suited for metabolic studies of lipoproteins. The distribution of lipid or protein components of lipoprotein classes can be

compared directly [12]. Examples of such usage is shown in this study: the smaller human HDL particle size compared to those of rabbit and rat is obvious from the difference in elution volumes (Fig. 1). The cholesterol distribution among the lipoproteins is quite different in the three animal species (Fig. 2). The predominence of triacylglycerol in rabbit and human HDL compared to that of rat (Fig. 3) is consistent with the reported lipid transfer protein activity in these animal species [13, 14].

Despite the usefulness of this technique, one should be aware of its limitations. An obvious disadvantage of the method is that only one lipoprotein sample can be handled at a time. The recovery of samples from the process is about 90% owing to a small extent of non-specific absorption by the agarose gel. However, these are minor problems when compared to other available techniques. This method should provide a useful additional technique for studies of plasma lipoprotein metabolism.

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REFERENCES

1 F.V. Hooft and R.J. Havel, J. Biol. Chem., 256 (1981) 3963.
2 M. Fainaru, R.J. Havel and K. Imaizumi, Biochem. Med., 17 (1977) 347.
3 S. Margolis, J. Lipid Res., 8 (1967) 411.
4 J.A. Bowden and M. Fried, Comp. Biochem. Physiol., 32 (1970) 391.
5 L.L. Rudel, J.A. Lee, M.D. Morris and J.M. Felts, Biochem. J., 139 (1974) 89.
6 R.J. Havel, H.A. Eder and J.H. Bragdon, J. Clin. Invest., 34 (1955) 1345.
7 F. Hatch and R.S. Lees, Adv. Lipid Res., 6 (1968) 1.
8 G.S. Getz and R.V. Hay, in A.M. Scanu, R.W. Wissler and G.S. Getz (Editors), The Biochemistry of Atherosclerosis, Marcel Dekker, New York, 1979, p. 151.
9 M. Fainaru, R.J. Havel and K. Imaizumi, Biochim. Biophys. Acta, 490 (1977) 144.
10 J.C. Gibson, A. Rubinstein, P.R. Bukberg and W.V. Brown, J. Lipid Res., 24 (1983) 886.
11 C.B. Blum, L. Aaron and R. Sciacca, J. Clin. Invest., 66 (1980) 1240.
12 Y.C. Ha, L.B.F. Chang and P.J. Barter, Comp. Biochem. Physiol., 78B (1984) 675.
13 Y.C. Ha and P.J. Barter, Comp. Biochem. Physiol., 71B (1982) 265.
14 G.J. Hopkins and P.J. Barter, Metabolism, 29 (1980) 546.

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Note

Routine determination of unconjugated 3-methoxy-4-hydroxyphenylglycol in plasma using high-performance liquid chromatography with electrochemical detection

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3-Methoxy-4-hydroxyphenylglycol (MHPG) is the major metabolite of noradrenaline [1] and it has been reported that unconjugated (free) MHPG in plasma may closely reflect central noradrenaline turnover [2]. Free plasma MHPG levels have been reported to be altered in both depression [3—5] and anxiety [6], thus suggesting that MHPG may be used as a clinical marker.

Plasma MHPG is generally determined by gas chromatography with either electron-capture detection [7, 8] or mass spectrometry [9, 10]. These techniques require derivatization of MHPG prior to analysis and although they are very selective, they are either too expensive or too slow to be used routinely. High-performance liquid chromatography (HPLC) with electrochemical detection (ED) has been used to measure MHPG in urine [11, 12] and cerebrospinal fluid [13, 14].

The rather complex nature of plasma has made the determination of free MHPG potentially more difficult. A search of the relevant literature revealed three methods using HPLC with amperometric detection. Prior to extraction, the first method [15] involves deproteination of the plasma and subsequent pH adjustment with two buffers whilst the other two [16, 17] require lengthy and complex column separation and purification steps.

The recent improvement in the coulometric detector as opposed to the traditional amperometric detector has given the chromatographer increased versatility. The coulometric detector oxidises 100% of the analyte passing through the cell while an amperometric detector cell oxidises only 1—5% [18]. By incorporating a guard cell between the delivery system and the injector the

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expected increase in background noise owing to the solvent can be eliminated. In addition the detector cell itself contains two electrodes and some potentially interfering compounds may be eliminated by their complete oxidation or reduction at the first electrode. In this instance the necessity for a tedious purification and extraction procedure has been avoided. In this paper we describe a fast and simple method for the routine determination of free MHPG in plasma.

EXPERIMENTAL

Reagents and standards

All the standards apart from the internal standard 4-methoxy-3-hydroxyphenylglycol (iso-MHPG) were supplied by Sigma (Poole, U.K.). Iso-MHPG was kindly donated by Dr. H.J. Gaertner, although it can be easily prepared [11]. Methanol and sodium acetate (HPLC grade) were purchased from Fisons (Loughborough, U.K.). The remaining reagents (AnalaR grade) were obtained from BDH (Poole, U.K.).

All water was deionised and glass-distilled prior to use.

HPLC instrumentation

The HPLC system comprised of a Model 302 pump fitted with an 802 Manometric module (Gilson SA, Villiers Le Bel, France), a Kontron Model MSS1 660 autosampler with a 20-μl loop (Kontron Instruments, St Albans, U.K.,), an ODS reversed stationary phase column (Rainin, 3 μm particle size, 100 × 4.6 mm, Anachem) protected by a 5-μm ODS HPLC guard column (Brownlee, Anachem). The detection system consisted of a Model 5100A Coulochem^{TM} detector and a Model 5020 guard cell (ESA, Bedford, MA, U.S.A.) protected by a 5-μm ODS HPLC guard column (Brownlee). The detector was linked to an LDC CI-10 integrator with an NEC printer—plotter (LDC, Stone, U.K.).

HPLC conditions

The mobile phase consisted of a mixture of 0.1 *M* sodium acetate—methanol (90:10). The pH was adjusted to 5.0 with glacial acetic acid and the solvent degassed under vacuum prior to use. The flow-rate was set at 1.0 ml/min. The potentials for detectors 1 and 2 were selected after injection of fixed amounts of MHPG and the internal standard over the range 0.1—0.6 V for each detector (Fig. 1). The potentials for the guard cell and detectors 1 and 2 were +0.5, +0.1 and +0.4 V, respectively. The response time was 2 sec.

Collection and storage of blood for analysis

There have been several reports of sample deterioration under different storage conditions [15, 19]. To date samples have been stored in the manner described below for up to three months without any evidence of decay.

Whole blood (10 ml) was added to a 10-ml lithium heparin tube containing 100 μl of 10 μM iso-MHPG and 0.2 *M* sodium metabisulphite. This was gently mixed and then centrifuged at 1500 *g* for 10 min at 4°C. The plasma was removed and stored at −20°C until analysis. Prepared samples were found to be

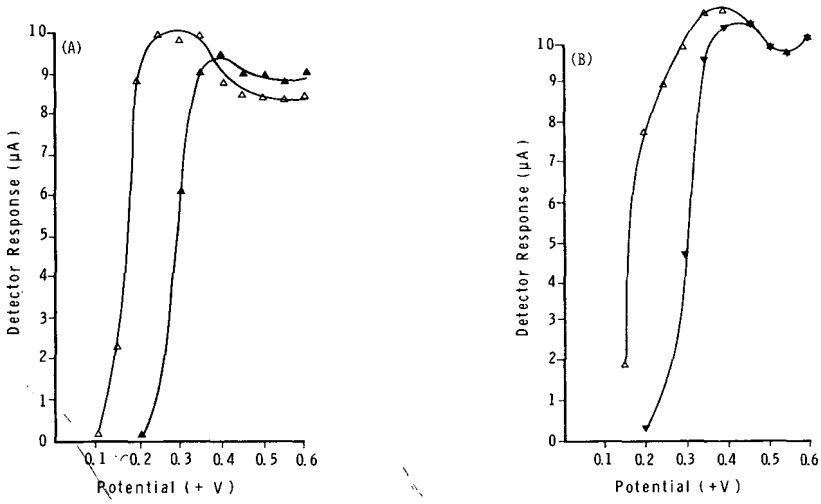

Fig. 1. Voltagrams of MHPG and iso-MHPG from both detectors at different electrode potentials. Each voltagram was determined whilst the other electrode was set at zero potential. (A) Voltagram of MHPG from detector 1 (△) and detector 2 (▲). (B) Voltagram of iso-MHPG from detector 1 (△) and detector 2(▼).

stable for more than 24 h at room temperature. This also allowed the system to be automated.

Extraction procedure

To 1 ml of plasma spiked with internal standard contained in a 15-ml glass stoppered centrifuge tube, were added 5 ml of ethyl acetate. The tube was vortex-mixed for 1 min and then centrifuged for 5 min at 1500 *g* at 4°C. The organic phase was aspirated into a 15-ml glass centrifuge tube containing 1 ml of 0.1 *M* potassium bicarbonate. This was vortex-mixed, centrifuged and separated as above. The organic phase was transferred to a third glass centrifuge tube, and vortex-evaporated to dryness at 30°C under vacuum in a Buchler Vortex Evaporator (Fortlee, NJ, U.S.A.). The residue was re-dissolved in 100 μl of mobile phase and 20 μl were injected onto the column. Standards and blanks were extracted in the same manner.

RESULTS

Resolution and sensitivity were determined by an injection of an extracted plasma standard (Fig. 2b). The retention times of MHPG and the internal standard, iso-MHPG, were 4.1 and 7.6 min, respectively. The linearity of both the extraction procedure and detector response (determined from the peak height) was verified over the anticipated range of the assay (1—200 nmol/l). The linearity was determined by assaying pooled plasma that had been dialysed for 24 h against 200 vols. of water and then spiked with known amounts of MHPG. A calibration curve was calculated for MHPG and a linear relationship was observed between MHPG concentration and the peak height ratio over the concentration range studied. The equation for the calibration curve was $y = 0.01833x + 0.00037$; $r = 0.9999$. Each point on the calibration curve was

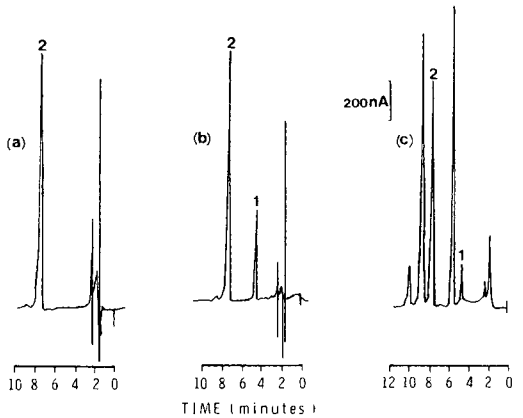

Fig. 2. Chromatography of MHPG and iso-MHPG, internal standard. (a) Chromatogram of a dialysed plasma extract spiked with 100 nmol/l iso-MHPG. (b) Dialysed plasma extract spiked with 100 nmol/l iso-MHPG and 20 nmol/l MHPG. (c) Plasma extract from a normal male subject 30 min after insertion of a catheter (12.8 nmol/l MHPG). Peaks: 1 = MHPG and 2 = iso-MHPG.

TABLE I

INTRA- AND INTER-DAY COEFFICIENTS OF VARIATION (C.V.) (n = 5)

MHPG Concentration (nmol/l)	Intra-day C.V. (%)	Inter-day C.V. (%)
1	9.0	22.5
5	6.5	22.5
10	2.8	11.0
20	2.0	13.3
50	2.0	9.6
100	1.8	11.1
150	2.4	11.5
200	1.2	12.3

calculated from the means of the inter-day assay variation data (Table I).

The actual recovery of MHPG over the anticipated concentration range was 35% (this value is not corrected for volume losses), which is lower than that reported elsewhere [7, 8, 15, 17]. Further study has shown that this can be improved to 55% with a second ethyl acetate extraction, but this was thought to be unnecessary as the minimum quantifiable concentration calculated from a peak height of twice the baseline noise was 0.5 nmol/l (2 pg of MHPG injected).

Several catecholamines, indoleamines, their metabolites and precursors have been examined for possible interference with the assay; their retention times are given in Table II. MHPG values form normal volunteers are given in Table III and are within the range reported by others [15—17, 19].

The methodology has been used in a recent research study. In this study normal healthy male volunteers received diazepam for a period of three weeks, the dose being increased over the first three days to a maximum of 25 mg per day. Subjects were sampled the morning prior to the investigation and during the third week.

TABLE II

CHROMATOGRAPHIC CHARACTERISTICS OF CATECHOLAMINES, INDOLEAMINES, THEIR PRECURSORS AND METABOLITES

Each value is for a single extraction from water of a 1 μmol/l solution prepared on the day of analysis.

Compound	Capacity factor, k
3-Methoxy-4-hydroxyphenylglycol	0.46
4-Methoxy-3-hydroxyphenylglycol	1.00
5-Hydroxytryptamine	N.D.*
4-Hydroxy-3-methoxyphenylacetic acid	N.D.
4-Hydroxy-3-methoxyphenethanol	1.84
Tryptophan	N.D.
4-Hydroxy-3-methoxymandelic acid	N.D.
3,4-Dihydroxyphenylglycol	N.D.
Noradrenaline	N.D.
Adrenaline	N.D.
Dopamine	N.D.
Normetanephrine	N.D.
Metanephrine	N.D.
5-Hydroxyindoleacetic acid	N.D.
3,4-Dihydroxymandelic acid	N.D.
Tryptamine	N.D.
Tryptophol	N.D.
5-Hydroxytryptophol	N.D.

*N.D. = Not detected.

TABLE III

FREE MHPG CONCENTRATIONS IN PLASMA OF NORMAL MALE VOLUNTEERS

All the subjects were sampled in a supine position, 30 min after insertion of a cannula.

Subject	Free MHPG (nmol/l)
1	17.4
2	15.4
3	12.8
4	20.6
5	8.4
6	17.5
7	26.5
8	19.8
Mean	17.3 ± 5.41

Subjects, in a supine position, were sampled three times at 15-min intervals via a butterfly cannula. Table IV shows that there was a significant increase ($p > 0.001$) in baseline MHPG levels during the third week of diazepam treatment as compared to normals.

TABLE IV

BASELINE MHPG LEVELS BEFORE AND DURING DIAZEPAM TREATMENT (25 mg PER DAY)

Subject	MHPG concentration (mean ± S.D., nmol/l)	
	Before diazepam treatment	After diazepam treatment
1	21.4 ± 2.0	28.5 ± 0.4
2	7.9 ± 0.5	25.5 ± 2.4
3	14.9 ± 0.9	16.7 ± 0.5
4	11.5 ± 1.2	16.5 ± 1.1

DISCUSSION

The use of the coulometric detector as opposed to the amperometric detector has several advantages, which enable it to be performed in the manner described. A full and comprehensive treatise of the potential use of coulometric detectors is given by Matson et al. [20]. The detector cell which oxidises 100% of the eluent, compared with 1—5% oxidised by the amperometric cell [18, 20] produces a substantial increase in sensitivity. Fortunately, the sensitivity is not matched by a corresponding increase in noise for two reasons. Firstly, the guard cell oxidation potential is set higher than that of the detecting cell, which removes most of the background noise associated with the solvent system. Secondly, by setting the potential of the first detector cell at 0.1 V, it reduces the response of the second detector cell (the analytical cell) to the solvent front and thus enables it to attain baseline stability earlier.

The 3-μm 10-cm column was found to decrease the assay time whilst giving good resolution of the extracted compounds. One possible disadvantage of this system is that the pore sizes of the cells are very small and could block. However, this has not yet been a problem.

It is recognized that the recovery of MHPG is relatively low, but it is felt that this is more than adequately compensated for by the greater sensitivity of the coulometric detector. The possibility of sample deterioration has been reduced by adding the internal standard to the sample prior to storage at —20°C. This approach is justified because samples have been stored for three months without any evidence of breakdown. Free plasma MHPG concentrations found in our normal volunteers compare well with those reported elsewhere [15—17, 19].

CONCLUSIONS

A novel technique using HPLC with ED has been described. It is a reliable, quick and inexpensive alternative to gas chromatography—mass spectrometry and other HPLC methods available, as a method for the determination of free MHPG in plasma. These factors render the method suitable for routine clinical analysis and neurochemical research.

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REFERENCES

1 J.W. Maas and D.H. Landis, J. Pharmacol. Exp. Ther., 163 (1968) 147.
2 J.W. Maas, MHPG: Basic Mechanisms and Psychopathology, Academic Press, New York, 1983, p. 38.
3 J.J. Schildkraut, P.J. Orslak, A.F. LaBrie, A.F. Schatzberg, J.E. Gudman, J.O. Cole and W.A. Rohde, Arch. Gen. Psychiatry, 35 (1978) 1436.
4 D.C. Jimerson, T.R. Insel, V.I. Reus and I.J. Kopin, Arch. Gen. Psychiatry, 40 (1983) 173.
5 E.M. De Met, A.E. Halaris, H.E. Gwirtzman, R.M. Reno and P.I. Becker, Psychopharmacol. Bull., 18 (1982) 221.
6 G.N. Ko, J.D. Elsworth, R.H. Roth, B.G. Rifkin, M. Leigh and D.E. Redmond, Arch. Gen. Psychiatry, 40 (1983) 425.
7 H. Dekirmenjian and J.W. Maas, Clin. Chim. Acta, 52 (1974) 203.
8 G.E. Halaris, E.M. DeMet and M.E. Halaris, Clin. Chim. Acta, 78 (1977) 285.
9 J.W. Maas, S.E. Hattox, D.H. Landis and R.H. Roth, Brain Res., 118 (1976) 167.
10 D.R. Sweeney, J.F. Leckman, J.W. Maas and G.R. Heniger, Arch. Gen. Psychiatry, 37 (1980) 1100.
11 J.R. Shipe, J. Savory and M.R. Wills, Clin. Chem., 30 (1984) 140.
12 A. Krstulovic, C.T. Matzura, L. Bertani-Dziedzic, S. Cerqueira and S.E. Gitlow, Clin. Chim. Acta, 103 (1980) 109.
13 P.J. Langlais, Clin. Chem., 26 (1980) 786.
14 G.M. Anderson, J.G. Young, D.J. Cohen, B.A. Shaywitz and D.K. Batter, J. Chromatogr., 222 (1981) 112.
15 M. Scheinen, W.-H. Chang, D.C. Jimerson and M. Linnoila, Anal. Biochem., 132 (1983) 165.
16 H. Ong, F. Capet-Antonini, N. Yamaguchi and D. Lamontagne, J. Chromatogr., 233 (1982) 97.
17 P.A. Shea and J.B. Howell, J. Chromatogr., 306 (1984) 358.
18 Environmental Sciences Associates, Model 5100A Coulochem TM Instruction Manual, 1983, p. 7.
19 J.W. Maas, MHPG: Basic Mechanisms and Psychopathology, Academic Press, New York, 1983, p. 88.
20 W.R. Matson, P. Langlais and L. Volicer, Clin. Chem., 30 (1984) 1477.

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CHROMBIO. 2526

Note

Determination of urinary 5-hydroxyindole-3-acetic acid using solid-phase extraction and reversed-phase high-performance liquid chromatography with electrochemical detection

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(First received August 1st, 1984; revised manuscript received January 3rd, 1985)

5-Hydroxyindole-3-acetic acid (5-HIAA), a metabolite of serotonin (5-hydroxytryptamine) measured in urine, has been documented as a biochemical marker of carcinoid tumor [1, 2]. 5-HIAA analysis has also been suggested as an aid in the diagnosis of certain neurological [3] and psychiatric [4] disorders. Traditionally, this compound is assayed by diazotization with nitrosonaphthol to form a purple color [2, 5, 6]. However, it is well known that many medications and their metabolites interfere in this reaction yielding incorrect 5-HIAA results [7—10].

Recently, liquid chromatographic procedures for 5-HIAA with either ultraviolet (UV) [11—13] or fluorometric [14—18] detection have been described. Most of these procedures involve solvent extraction. In addition, some procedures have been referred to as lacking selectivity [18]. Various electrochemical detection procedures have been published [19—23]. These procedures generally involve extensive specimen preparation. Most of the reported electrochemical detection procedures lack an internal standard to monitor extraction efficiency and injection variation. The only exception is the work reported by Petruccelli et al. [23].

The present system is designed to simplify the extraction procedure and incorporate an internal standard to improve the analytical precision.

EXPERIMENTAL

Instrumentation

A high-performance liquid chromatographic (HPLC) pump (Model 8800;

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DuPont, Wilmington, DE, U.S.A.) was used to deliver the solvent (flow-rate 1 ml/min) through a 250 × 4.6 mm column of Biophase ODS, 5-μm spherical C_{18} particles (MF6017; Bioanalytical Systems, West Lafayette, IN, U.S.A.) at room temperature. Samples were introduced through an automatic sample injector (Model 710B; Waters Assoc., Milford, MA, U.S.A.) and the effluent was monitored by an electrochemical detector with a glassy carbon electrode cell (Model LC4B; Bioanalytical Systems). The oxidation potential was maintained at 0.55 V (versus Ag/AgCl reference electrode), while the detector and recorder ranges were adjusted to 20 nA and 1 V full scale deflection, respectively. A Baker-10 extraction system (No. 70180; J.T. Baker, Phillipsburg, NJ, U.S.A.) equipped with a vacuum gauge was used for the extraction.

Reagents

The 5-HIAA (H8876, Sigma, St. Louis, MO, U.S.A.) stock standard solution concentration was 0.5 g/l. 5-HIAA working standard solution was prepared daily by diluting the stock standard solution 100-fold with deionized water.

The 5-hydroxyindole-3-propionic acid (5-HIPA, provided by Dr. J. Stephen Kennedy, Neurosciences Research Branch, National Institute of Mental Health, Rockville, MD, U.S.A.) stock standard solution concentration was 0.5 g/l. Working standard solution was prepared by diluting the stock standard solution 100-fold with deionized water.

The HPLC mobile phase was prepared by adding 50.7 ml of concentrated ammonium hydroxide, 64.7 ml of glacial acetic acid and 0.2 g of disodium EDTA to 1760 ml of deionized water and adjusting the pH to 5.1 with either 6 *M* acetic acid or 6 *M* ammonium hydroxide. To this mixture 325 ml of methanol were added and the solution was filtered and degassed.

Procedure

The Baker C_{18} extraction columns should be conditioned prior to the addition of specimens. The columns were washed with two column volumes of HPLC-grade methanol under a vacuum. As the level of methanol approached the top of the packing, the vacuum was discontinued. Following the methanol wash, two column volumes of deionized water were added. The vacuum was discontinued before the solution had been totally aspirated through the columns. Reconditioning was performed if drying occurred before sampe introduction.

Centrifuged urine (100 μl) together with 400 μl of the working internal standard solution were added to 4 ml of acetate buffer (0.1 *M*, pH 5.0). Of this solution 1 ml was allowed to pass through the C_{18} disposable extraction column (7020-1; J.T. Baker). The column was washed three times with 1 ml of mobile phase. The void volume and eluates were collected in a 10 × 75 mm test tube. Of this mixed solution 50 μl were introduced into the HPLC system. Standards were prepared by adding 200, 400, and 600 μl of working 5-HIAA standard solution to 3.9, 3.7, and 3.5 ml of acetate buffer (0.1 *M*, pH 5.0). After the addition of 400 μl of working internal standard solution, all standards were treated in the same manner as the urine specimens.

RESULTS AND DISCUSSION

The percentage cumulative recovery for the extraction of 5-HIAA and 5-HIPA is depicted in Fig. 1. These results demonstrate that in addition to the void volume, 3 ml of the eluate must be collected in order to maximize the recovery of both components.

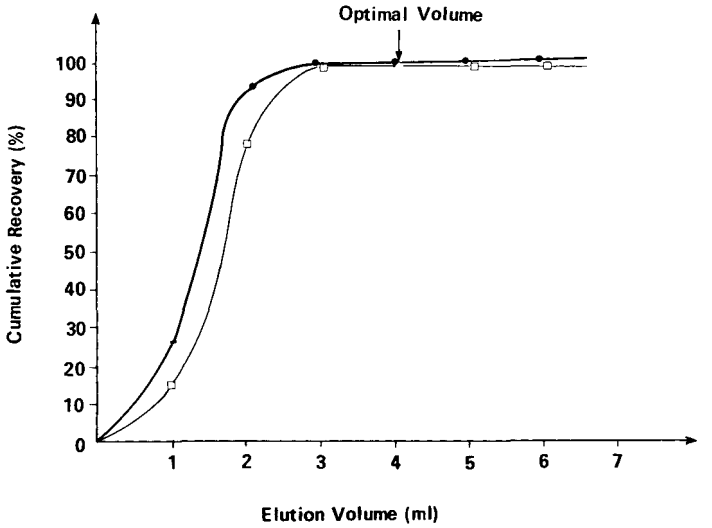

Fig. 1. Percentage cumulative recovery of 5-HIAA (•) and 5-HIPA (□) obtained from a solid-phase extraction column. "1" ml represents the fraction containing the void volume.

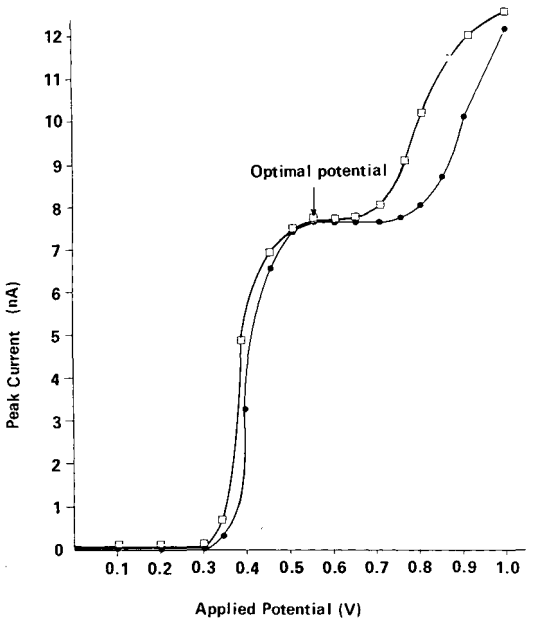

Fig. 2. Hydrodynamic voltammograms of 5-HIAA (•) and 5-HIPA (□). The potential readings are oxidizing voltage versus a Ag/AgCl reference electrode.

Fig. 2 illustrates the voltammograms for both 5-HIAA and 5-HIPA. In order to maximize the signal strength and minimize the variation from amperometric measurements, a potential of 0.55 V versus a Ag/AgCl reference electrode was selected.

The intra-assay and inter-assay coefficients of variation (C.V.) at a level of 6.7 mg/l were 4.8% (n = 15) and 7.1% (n = 9), respectively, while the minimum detection limit was 1 mg/l (signal-to-noise ratio is 2.5). Specimens spiked with various amounts of 5-HIAA when analyzed by this method resulted in an analytical recovery ranging from 97% to 102% (Table I) with absolute recovery ranging from 90% to 98%. A reference range of 1.9—10.4 mg per 24 h

TABLE I

RESULTS OF RECOVERY STUDY

Number of measurements, n = 4.

Standard added (μg)	Amount measured (μg)	Standard recovered (μg)	Percentage recovery
0	0.12	0	—
1	1.14	1.02	102
2	2.11	1.99	99.5
3	3.04	2.92	97.3

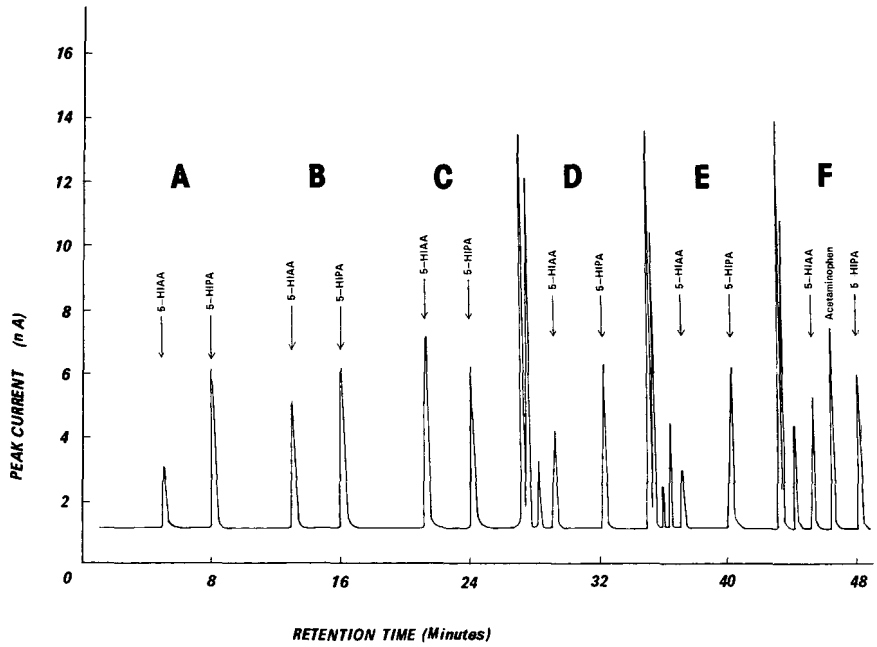

Fig. 3. Chromatograms of standard solutions and three urine extracts. A, B and C represent 1, 2 and 3 μg of 5-HIAA in 200, 400 and 600 μl of working 5-HIAA standard solution, respectively (quality sufficient to 4.1 ml with acetate buffer followed by the addition of 400 μl of working 5-HIPA standard solution and solid phase extraction). D and E represent two normal urine specimens while F demonstrates one urine specimen containing acetaminophen.

was established by analyzing specimens collected from 23 apparently healthy control subjects.

Thirty-one specimens demonstrating no apparent interferences in a colorimetric procedure [2] (x) when analyzed by the present method (y) yielded a slope of 0.992, an intercept of —0.35 mg per 24 h and 0.990 for the correlation coefficient.

The linearity of the present procedure has been established as 0—10 μg (absolute quantity). Chromatograms obtained from a typical analysis are demonstrated in Fig. 3.

A common interference for urinary 5-HIAA determination by colorimetric procedures is the presence of acetaminophen [6]. A chromatogram of a specimen containing acetaminophen is presented in Fig. 3. The separation between 5-HIAA and acetaminophen will not cause identification difficulty with the present method.

In the current procedure, the HPLC separation provides high selectivity while the electrochemical detector improves the minimum detection limit. However, there are numerous electrochemical active compounds present in urine specimens which will prolong the chromatographic separation time. Therefore, the specimen preparation step is very important. With the use of solid-phase extraction columns, the average retention time can be reduced from 20 to 8 min.

In summary, the proposed method is sensitive, selective and reduces the time required for analysis when compared to many existing procedures. Ten specimens can be processed within 1 h. This procedure is adaptable for routine analysis.

REFERENCES

1 A. Hanson and F. Serin, Lancet, ii (1955) 1359.
2 S. Udenfriend, E. Titus and H. Weissback, J. Biol. Chem., 216 (1955) 499.
3 D.L. Garves and J.M. Davis, Life Sci., 24 (1979) 383.
4 M. Pines, Science 83, 10 (1983) 55.
5 H. Goldenberg, Clin. Chem., 19 (1973) 38.
6 Z.K. Shihabi and E.L. Wilson, Clin. Biochem., 15 (1982) 106.
7 D.S. Young, D.W. Thomas, R.B. Friedman and G. Pestaner, Clin. Chem., 18 (1972) 1268.
8 A.C. Deacon and W.A. Bartlett, Clin. Chem., 28 (1982) 251.
9 C.I. Bhaget and M. Dick, Clin. Chem., 28 (1982) 1240.
10 V.A. John, J.P. Monk and G. Dorhofer, Clin. Chem., 29 (1983) 743.
11 P.S. Draganac, S.J. Steindel and W.G. Trawick, Clin. Chem., 26 (1980) 910.
12 R.P. Tracy, L.E. Wold, J.D. Jones and M.F. Burritt, Clin. Chem., 27 (1981) 160.
13 V. Skrinska and S. Hahn, Clin. Chem., 29 (1983) 1205.
14 A.P. Graffeo and B.L. Karger, Clin. Chem., 22 (1976) 184.
15 O. Beck, G. Palmskog and E. Hultman, Clin. Chim. Acta, 79 (1977) 149.
16 J.P. Garnier, B. Bousquet and C. Dreux, J. Chromatogr., 204 (1981) 225.
17 T.G. Rosano, J.M. Meola and T.A. Swift, Clin. Chem., 28 (1982) 207.
18 E.J. Kiser and A.R. Long, Clin. Chem., 29 (1983) 1202.
19 D.D. Koch and P.T. Kissinger, J. Chromatogr., 164 (1979) 441.
20 Z.K. Shihabi and J. Scaro, Clin. Chem., 26 (1980) 907.
21 M.H. Joseph, B.V. Kadam and D. Risby, J. Chromatogr., 226 (1981) 361.
22 B.H.C. Westerink, F.J. Bosker and J.F. O'Hanlon, Clin. Chem., 28 (1982) 1745.
23 B. Petruccelli, G. Bakris, T. Miller, E.R. Korpi and M. Linnoila, Acta Pharmac. Toxicol., 51 (1982) 421.

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CHROMBIO. 2551

Note

Assay of urinary phenylacetic acid by high-performance liquid chromatography

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Phenylacetic acid (PAA) is a normal constituent of human urine, where more than 90% of it occurs as the glutamine conjugate [1—4], and ca. 150 mg is excreted by a healthy adult every 24 h. Urinary PAA is derived from L-phenylalanine either by decarboxylation to 2-phenylethylamine (PEA) and further deamination by monoamine oxidase to PAA, or by transamination to phenylpyruvic acid followed by decarboxylation to PAA.

PEA is a trace amine occurring in mammalian brain [5], which according to the PEA hypothesis of affective behaviour [6] acts as an endogenous amphetamine and may be involved in alertness, excitement and mood. Consequently a change in PEA concentration or in PEA turnover in the brain may influence affective state, or is even considered to be an aetiological factor in the pathogenesis of certain forms of schizophrenia [7]. In a recent report, Sabelli et al. [8] suggest that low urinary excretion of PAA, the major metabolite of PEA, may be a reliable marker for diagnosis of some forms of unipolar major depressive disorders. Other workers [4] observed a decreased PAA excretion in a group of chronic schizophrenic patients, particularly of the non-paranoid subtype. In the cerebrospinal fluid of unmedicated schizophrenics free PAA is significantly reduced [7].

In order to evaluate the importance of urinary PAA as a diagnostic aid in psychiatric disorders, we developed a high-performance liquid chromatographic (HPLC) assay of total PAA in urine. We believe that there is a place for this assay alongside gas chromatographic methods [2, 3, 9].

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EXPERIMENTAL

Materials

PAA (98.5% pure) was obtained from Janssen Chimica (Beerse, Belgium) and 3-phenyl-1-propanol (98% pure) was from Fluka (Buchs, Switzerland). Disposable extraction columns (1-ml bed volume) packed with octadecylsilane (C_{18}) bonded silica gel were from Baker Chemicals (Deventer, The Netherlands). Sephadex G-10 was purchased from Pharmacia (Uppsala, Sweden). All other chemicals were of analytical grade.

High-performance liquid chromatography

The liquid chromatograph consisted of an M6000A pump, a Model 440 absorbance detector (254 nm) and a Model U6K injector (Waters Assoc., Milford, MA, U.S.A.). Peak areas were measured with a CDP1 digital integrator from Pye Unicam (Cambridge, U.K.). The solvent system consisted of 0.01 mol/l acetic acid in methanol—water (2:3), pH 3.6. The flow-rate was 1.5 ml/min. Analyses were carried out at room temperature on a LiChrosorb 10 RP-18 column (25 cm × 4.6 mm I.D.; 10 μm particle size) obtained from Chrompack (Middelburg, The Netherlands).

Sample collection

Random urine samples from unselected hospitalized psychiatric patients were frozen at −20°C immediately after collection. None of the patients had serious somatic illness.

Method

PAA in urine was quantitated by HPLC after consecutive purification on a C_{18} extraction column and a Sephadex G-10 column. Urine was centrifuged after thawing and 2.0 ml of the supernatant fluid was added to 2.0 ml of 6 mol/l hydrochloric acid in a screw-cap test tube. Conjugated PAA was hydrolysed by placing the tube in a water bath at 90°C for 4 h. After cooling and centrifugation for 5 min, 3.5 ml of the supernatant were applied to a C_{18} extraction column, which had been conditioned by aspirating 1 ml of methanol and 1 ml of water through the column prior to use. The column was subsequently washed with 1.0 ml of water and 0.1 ml of methanol. The organic fraction containing PAA was eluted with 0.25 ml of methanol. This fraction was diluted with 0.75 ml of water, and 0.9 ml of the resulting mixture was applied to a Sephadex G-10 column (8 × 0.4 cm I.D.) equilibrated with 0.01 mol/l formic acid prior to use. The column was washed with 1.0 ml of 0.01 mol/l formic acid, and PAA was eluted with 1.0 ml of the same eluent. As we were unsuccessful in finding a standard that behaved identically with PAA on the Sephadex G-10 column, the standard 3-phenyl-1-propanol was added to the eluate after Sephadex G-10 chromatography. PAA was measured with the HPLC system as described above, typically by a 100-μl injection. PAA excretion was calculated from calibration curves of known amounts of PAA added to the external standard. The amount of PAA in urine was expressed per millimole of creatinine. Urine samples containing more than 10.0 mmol/l creatinine were diluted (1:1) with water before processing. Creatinine in urine was determined by the Jaffé reaction with alkaline picrate reagent [10].

RESULTS AND DISCUSSION

Under standard HPLC conditions, PAA and 3-phenyl-1-propanol have retention times of 8.3 and 17.0 min, respectively. 3-Phenyl-1-propanol was selected from a number of compounds (including 2-phenylpropionic acid, 3-phenylpropionic acid and benzoic acid) as the most suitable standard. As shown in Fig. 1, PAA and the standard are well resolved from interfering peaks. The limit of detection of PAA, as defined by a signal-to-noise ratio of 3 at 0.005 a.u.f.s., is 16 ng. This corresponds with a limit of detection of 28 ng/ml PAA in urine. The linearity of the assay was determined from the HPLC response of various known amounts of PAA to the external standard. The assay is linear up to at least 3 mmol/l PAA in the original solution. This value is approximately equivalent to four times the mean expected value of the PAA concentration in a 24-h urine of a healthy adult [8]. The major part of PAA in urine is conjugated with glutamine. The optimal time of hydrolysis in acidified urine, studied by varying the time of hydrolysis at 90°C from 0.5 to 5 h, is 4 h. This is in full agreement with the results of Gusovski et al. [9].

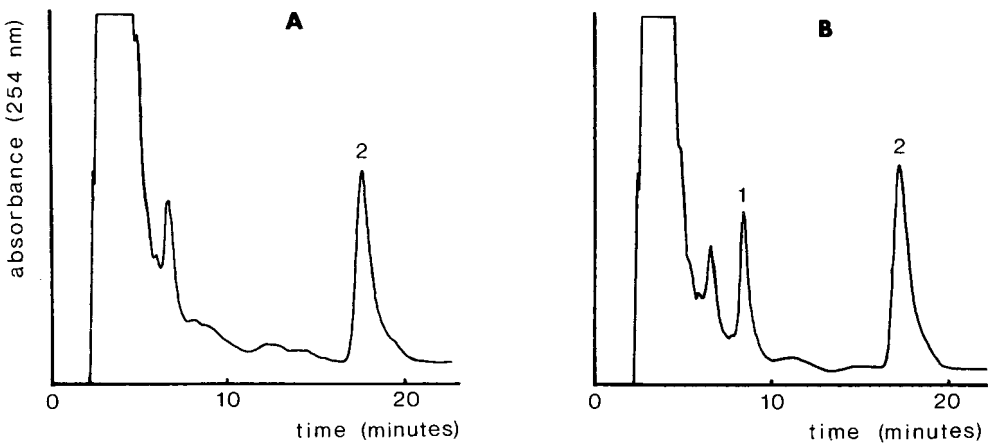

Fig. 1. HPLC profile of phenylacetic acid in urine before (A) and after (B) acid hydrolysis of conjugates. 3-Phenyl-1-propanol was used as the external standard. Conditions for HPLC as described in Experimental. Peaks: 1 = phenylacetic acid: 2 = 3-phenyl-1-propanol.

Recoveries

To C_{18} extraction columns and Sephadex G-10 columns, 2 ml of known solutions of PAA in water were applied. Before HPLC, 3-phenyl-1-propanol was added to the eluates as the standard and PAA was quantitated by the HPLC system as described above. Recovery (mean ± S.D.; n = 5) of PAA from the C_{18} extraction column is 93% ± 2% and from the Sephadex G-10 column it is 63% ± 3%, which is acceptable as this recovery is reproducible and because further elution of the Sephadex G-10 column gives rise to interfering peaks in the final chromatogram.

Reproducibility

For the determination of the intra-assay coefficient of variation (C.V.) five

samples were processed on the same day. The inter-assay C.V. was determined from five samples assayed on different days within one week. The concentration of PAA in the samples was 0.44 mmol/l of urine. Intra-assay and inter-assay C.V. values are 1% and 3%, respectively.

Interferences

Systematic interference studies were not carried out. From the investigation of urine samples from patients we conclude that the following drugs do not interfere with the assay: lithium carbonate, carbamazepine, flurazepam, flunitrazepam, lorazepam, temazepam, pimozide, mianserin, sulpiride, clomipramine, thioridazine, promethazine, *cis*-clopenthixol, glibenclamide, furosemide, triamterene, phenprocoumon and digoxin.

PAA excretion in urine

Differences depending on sex were not observed. Therefore, values of PAA excretion (mean ± S.E.M.) are given for the whole group. The urinary PAA excretion was 96.6 ± 10.8 μmol/mmol of creatinine (n = 35). Comparison with other published methods is complicated by the fact that other workers present their results as milligrams of PAA excreted per 24 h. Calculation of our results on the basis of a mean creatinine excretion of 12.8 mmol per 24 h [11] gives an approximate 24-h excretion of PAA, as measured by the method presented here, of 168.0 ± 18.8 mg per 24 h. This is in excellent agreement with the reported values (mg PAA per 24 h): 137.4 ± 15.8 [3], 162 ± 19 [4] and 141.1 ± 10.2 [8, 9] obtained by other methods.

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REFERENCES

1 M.O. James, R.L. Smith, R.T. Williams and M. Reidenberg, Proc. Roy. Soc. Ser. B, 182 (1972) 25.
2 B.L. Goodwin, C.R.J. Ruthven and M. Sandler, Clin. Chim. Acta, 62 (1975) 443.
3 M.E. Martin, K. Karoum and R.J. Wyatt, Anal. Biochem., 99 (1979) 283.
4 F. Karoum, S. Potkin, L.W. Chuang, D.L. Murphy, M.R. Liebowitz and R.J. Wyatt, Biol. Psychiatry, 19 (1984) 165.
5 A.A. Boulton and A.V. Juorio, in A. Lajtha (Editor), Handbook of Neurochemistry, Vol. 1, Plenum Press, New York, 2nd ed., 1982, pp. 189—222.
6 H.C. Sabelli and A.D. Mosnaim, Amer. J. Psychiatry, 131 (1974) 695.
7 H. Beckmann, G.P. Reynolds, M. Sandler, P. Waldmeier, J. Lauber, P. Riederer and W.F. Gattaz, Arch. Psychiat. Nervenkr., 232 (1982) 463.
8 H.C. Sabelli, J. Fawcett, F. Gusovsky, J. Javaid, J. Edwards and H. Jeffries, Science, 220 (1983) 1187.
9 F. Gusovsky, H. Sabelli, J. Fawcett, J. Edwards and J.I. Javaid, Anal. Biochem., 136 (1984) 202.
10 R.J. Henry, Clinical Chemistry, Harper and Row, New York, 1964.
11 R.D. Eastham, Biochemical Values in Clinical Medicine, John Wright and Sons, Bristol, 6th ed., 1979.

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CHROMBIO. 2536

Note

Simple and sensitive assay of dopamine β-hydroxylase in human cerebrospinal fluid by high-performance liquid chromatography with electrochemical detection

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Dopamine β-hydroxylase (DBH, E.C. 1.14.17.1) catalyses the hydroxylation of dopamine (DA) to norepinephrine (NE). The enzyme is released from nerve endings together with NE by exocytosis and appears in the blood [1, 2]. Therefore, many physiological and clinical studies on blood DBH levels have been reported. DBH activities have also been measured in cerebrospinal fluid (CSF) [3—7]. It has been suggested that DBH in CSF is of central origin [6], and its measurement is thought to be valuable for studying the central noradrenergic activity.

Several procedures are available for the assay of DBH [8]: radioassays [9—13], spectrophotometry [14—18], fluorometry [19, 20], gas chromatography—mass spectrometry [21], high-performance liquid chromatography (HPLC) [22, 23]. The limitations and advantages of these various procedures have been reviewed [8].

Recently, a number of methods using HPLC have been introduced in the assay of DBH. HPLC with fluorescence or ultraviolet absorbance detection using tyramine as substrate [22—26] and HPLC with electrochemical detection

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(ED) using DA as substrate [27—29] have been used for the assay of DBH activity.

In the present study, we developed a sensitive and simple HPLC method with electrochemical detection using tyramine as substrate. Under the optimal conditions, tyramine is enzymatically converted to octopamine which is then oxidized with periodate to *p*-hydroxybenzaldehyde. The latter compound is extracted into diethyl ether and determined by HPLC—ED. As our method has the advantages of sensitivity and simplicity, it can be applied to the measurement of very low DBH activity in CSF from children.

EXPERIMENTAL

Materials

Tyramine hydrochloride, octopamine hydrochloride, pargyline hydrochloride and N-ethylmaleimide were obtained from Sigma (St. Louis, MO, U.S.A.); isovanillin was from Tokyo Kasei (Tokyo, Japan), catalase from Boehringer (Mannheim, F.R.G.), and Dowex-50W-X4 from Dow Chem. (Midland, MI, U.S.A.). All other chemicals used were analytical grade.

Procedures

The incubation mixture contained (total volume 1.0 ml, each final concentration in parentheses): 500 μl of enzyme solution (CSF), 100 μl of 2 *M* sodium acetate buffer, pH 5.0 (0.2 *M*), 150 μl of 0.2 *M* N-ethylmaleimide (30 m*M*), 50 μl of 0.2 m*M* copper sulphate (10 μ*M*), 25 μl of aqueous solution (20 mg/ml) of catalase (500 μg, 25 000 I.U.), 25 μl of 40 m*M* pargyline hydrochloride (1 m*M*), 50 μl of 0.2 *M* ascorbic acid (10 m*M*), 50 μl of 0.2 *M* sodium fumarate (10 m*M*); 50 μl of 2 m*M* fusaric acid (100 μ*M*) were included for the blank (control). The reaction mixture was preincubated at 37° C for 5 min, and then the reaction was started by addition of 50 μl of 0.4 *M* tyramine hydrochloride (20 m*M*). After 45 min, the reaction was stopped by adding 1 ml of ethanol containing 1 m*M* fusaric acid in an ice-bath. The mixture was left at 0° C for at least 30 min and then centrifuged at 25 000 *g* for 10 min. The supernatant was immediately transferred to a small glass column (0.5 × 1.0 cm) of Dowex-50-X4 (H^+, 200—400 mesh, packed volume 0.2 ml). Octopamine was eluted with 1 ml of 3 *M* ammonia and converted to *p*-hydroxybenzaldehyde by addition of 20 μl of 2% sodium metaperiodate. The excess sodium metaperiodate was decomposed by addition of 20 μl of 10% sodium metabisulphite. The mixture was neutralized with 0.5 ml of 6 *M* hydrochloric acid and 1.0 nmol of isovanillin was added as an internal standard. Isovanillin and *p*-hydroxybenzaldehyde formed from octopamine were extracted with diethyl ether. After evaporating the diethyl ether, the residue was dissolved with 1.0 ml of the mobile phase and 50 μl were injected into the HPLC system (Yanaco PN-101) with a Yanaco VMD-101 electrochemical detector and with a column of Nucleosil 7 C_{18} (particle size 7.5 μm, 25 cm × 0.4 cm I.D.). The mobile phase was 0.05 *M* potassium phosphate buffer (pH 7.2) containing 20% methanol with a flow-rate of 1.0 ml/min. The detector potential was set at 0.9 V versus the Ag/AgCl electrode.

RESULTS

p-Hydroxybenzaldehyde and isovanillin (internal standard) can be measured with very high sensitivity by the present HPLC—ED system. A linear response of the peak height of the electrochemical detector for the injected amounts of *p*-hydroxybenzaldehyde was observed from 500 fmol to 10 nmol. The recovery of octopamine by cation-exchange resin, Dowex-50-X4, was 92 ± 2% (S.E.M.).

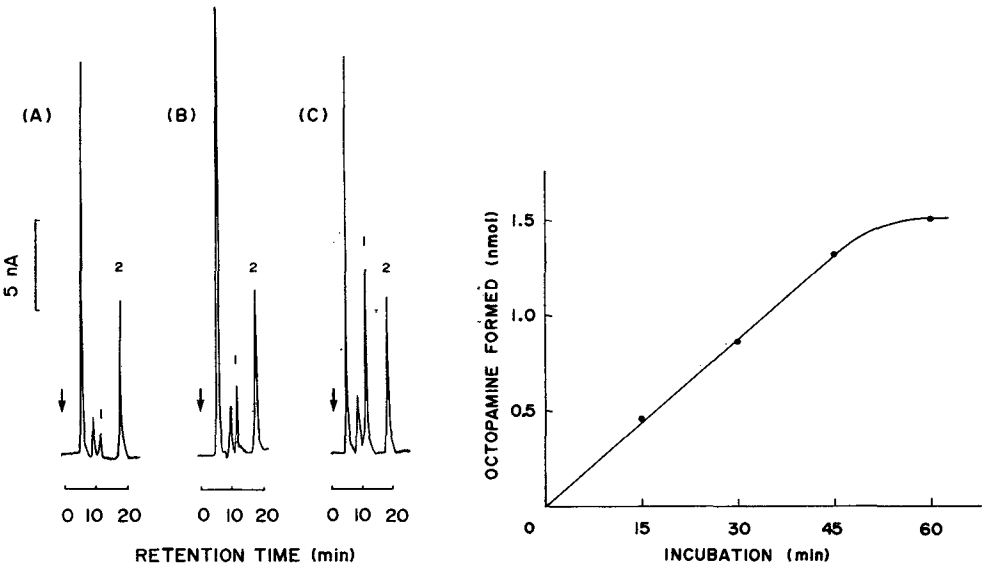

Fig. 1. Chromatographic patterns using DBH incubation mixture of human child CSF as enzyme. (A) Fusaric acid blank incubation; (B) experimental incubation; (C) 50 pmol of octopamine were added to the fusaric acid blank. Isovanillin (50 pmol) was added to each sample as internal standard. DBH activity was calculated to be 12.0 pmol per min per ml CSF. Peaks: 1 = *p*-hydroxybenzaldehyde; 2 = isovanillin.

Fig. 2. The rate of octopamine formation using human adult CSF (500 μl) as enzyme.

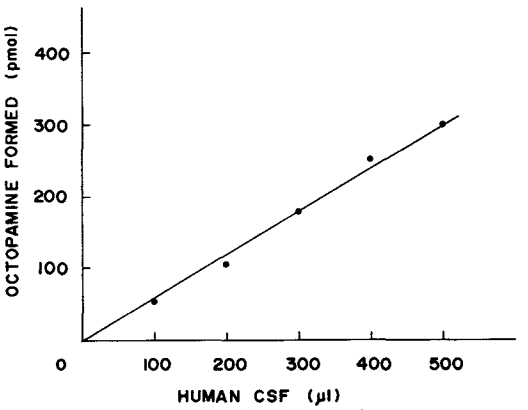

Fig. 3. Relation between the amount of octopamine formed in 45 min and the amount of human adult CSF as enzyme.

A typical chromatogram of the DBH reaction with human CSF as enzyme is shown in Fig. 1. The peak of *p*-hydroxybenzaldehyde (peak 1) derived from enzymatically formed octopamine in the experimental incubation (B) was 3.3 times higher than that in the blank incubation (A). The peak of the chromatogram in the blank incubation has almost the same retention time as that of *p*-hydroxybenzaldehyde and increases in height when the amount of tyramine is increased without enzyme sample. This peak may be due to *p*-hydroxybenzaldehyde, indicating that octopamine is present in commercial tyramine hydrochloride, as already mentioned by Nohta et al. [25]. The contamination of the commercial tyramine with octopamine was shown to be approximately 0.001% by the HPLC—ED assay.

The rate of octopamine formation using human CSF as enzyme proceeded linearly for 45 min, as shown in Fig. 2.

DBH activity in human CSF as a function of enzyme concentration is shown in Fig. 3. Complete linearity was observed between human CSF in the range 100—500 μl and *p*-hydroxybenzaldehyde formed from tyramine.

The precision of this enzyme assay was tested with CSF as samples in ten simultaneous assays (within-assay) or in five consecutive assays (between-assay), the coefficients of variation being 9.0% and 9.5%, respectively.

Finally, we applied this method to measure DBH activity in CSF from children including epileptic patients. The results are summarized in Table I.

TABLE I

DBH ACTIVITIES IN CSF OF CHILDREN

Diagnosis	Sex	Age (years)	DBH activity (pmol per min per ml CSF)
Epilepsy	F	8 months	5.5
	F	8 months	5.3
	M	2	6.4
	F	2	43.5
	F	2	12.4
	F	5	15.2
	F	5	6.9
	F	5	37.9
	M	13	9.5
	F	14	8.5
Guillain-Barre syndrome	F	5	21.5
	F	5	23.3
Malignant lymphoma	F	9	28.8

DISCUSSION

The assay of DBH activity by HPLC—ED using tyramine as substrate has many advantages.

First, it is very sensitive. The limit of sensitivity for *p*-hydroxybenzaldehyde derived from octopamine and internal standard isovanillin is 500 fmol. Therefore, it is more sensitive than previously published methods and can be applied to measure very low DBH activities in CSF from children. Under such a high

sensitivity, the sensitivity of the assay is determined solely by the blank value. The blank peak appears at the same retention time as *p*-hydroxybenzaldehyde. It increased in height as the amount of the substrate tyramine was increased without an enzyme sample. Nohta et al. [25] also reported such a blank peak and speculated that it may be octopamine contaminating the commercial tyramine. We proved by HPLC—ED assay that tyramine was contaminated by approximately 0.001% of octopamine. Dowex-50 cation-exchange resin will retain other amines and metabolites endogenous to the CSF and present in the enzyme incubation mixture. These amines will be present with octopamine in a similar fashion as octopamine. The resulting products of this reaction are not shown in the chromatograms of Fig. 1. Perhaps the peak found in enzyme blanks, which co-chromatographs with *p*-hydroxybenzaldehyde, originates from a source other than octopamine. The blank peak, however, does not disturb the measurement of DBH activity in CSF. The limit of sensitivity of octopamine formed enzymatically was about 10 pmol.

Secondly, it is reproducible. The coefficient of variation in both simultaneous and consecutive assays was less than 10%.

Thirdly, it is simple and rapid, because this assay includes fewer preliminary steps before the HPLC—ED. Octopamine, which is also electroactive and capable of being detected by the HPLC—ED method, was not directly measured after enzymatic conversion from tyramine, because a large amount of tyramine, the substrate, is also extracted from the incubation mixture by the cation-exchange resin and interferes with the assay of octopamine in HPLC—ED. In the present method, tyramine can be almost completely removed prior to HPLC by converting octopamine to *p*-hydroxybenzaldehyde and by extracting it with organic solvent.

We applied this method to the measurement of low DBH activity in human CSF, especially from children. It has been suggested that DBH in CSF is of central origin [6], and its measurement is thought to be valuable for studying the central noradrenergic activity in physiological and pathological conditions. Recently, several authors reported the DBH activities in CSF of humans, but not of children. As an application of the present DBH assay, we measured DBH activities in CSF of children including epileptic patients. The DBH activities in CSF of children were lower than those of adults, as reported before [30]. Although no tendency towards changes in DBH activities in CSF could be found in epileptic children, this remains to be investigated further.

REFERENCES

1 J. Axelrod, Pharmacol. Rev., 24 (1972) 233.
2 T. Nagatsu, Trends Biochem. Sci., 2 (1977) 217.
3 D.J. Goldstein and L.X. Cubbedu, J. Neurochem., 26 (1976) 193.
4 T. Okada, T. Ohta, T. Shinoda, T. Kato and T. Nagatsu, Neuropsychobiology, 2 (1976) 139.
5 W.P. DePotter, J. Physiol., 258 (1976) 26P.
6 W.P. DePotter, C.P.-H. Chanh, F. DeSmet and A.F. DeSchepdryver, Neuroscience, 1 (1976) 523.
7 P. Lerner, F.K. Goodwin, D.P. van Kammen, R.M. Post, L.F. Major, J.C. Ballenger and W. Lovenberg, Biol. Psychiatry, 13 (1978) 685.

8 T. Nagatsu, in S. Parvez, T. Nagatsu, I. Nagatsu and H. Parvez (Editors), Methods in Biogenic Amine Research, Elsevier, Amsterdam, 1983, p. 329.
9 E.Y. Levin, B. Levenberg and S. Kaufman, J. Biol. Chem., 235 (1960) 2080.
10 P.B. Molinoff, R. Weinshilboum and J. Axelrod, J. Pharmacol. Exp. Ther., 178 (1971) 425.
11 M. Goldstein, L.S. Freedman and M. Bonnay, Experientia, 27 (1971) 632.
12 T. Nagatsu, P. Thomas, R. Rush and S. Udenfriend, Anal. Biochem., 55 (1973) 615.
13 T.H. Joh, R.A. Ross and D.J. Reis, Anal. Biochem., 62 (1974) 248.
14 J.J. Pisano, C.R. Creveling and S. Udenfriend, Biochim. Biophys. Acta, 43 (1960) 566.
15 T. Nagatsu, H. Kuzuya and H. Hidaka, Biochim. Biophys. Acta, 139 (1967) 319.
16 T. Nagatsu and S. Udenfriend, Clin. Chem., 18 (1972) 980.
17 T. Kato, H. Kuzuya and T. Nagatsu, Biochem. Med., 10 (1974) 320.
18 T. Kato, Y. Wakui, T. Nagatsu and T. Ohnishi, Biochem. Pharmacol., 27 (1978) 829.
19 Y. Ohkura, K. Ohtsubo, K. Kohashi and K. Zaitsu, Chem. Pharm. Bull., 25 (1977) 748.
20 M. Yamaguchi, Y. Yutani, S. Miyamoto and Y. Ohkura, Anal. Biochem., 104 (1980) 402.
21 Y. Hashimoto and H. Miyazaki, J. Chromatogr., 168 (1979) 59.
22 K. Fujita, T. Nagatsu, K. Maruta, R. Teradaira, H. Beppu, Y. Tsuji and T. Kato, Anal. Biochem., 82 (1977) 130.
23 T. Flatmark, T. Skotland, T. Ljones and O.C. Ingebretsen, J. Chromatogr., 146 (1978) 433.
24 H. Nohta, M. Yamaguchi, K. Zaitsu and Y. Ohkura, J. Chromatogr., 233 (1982) 324.
25 H. Nohta, K. Ohtsubo, K. Zaitsu and Y. Ohkura, J. Chromatogr., 227 (1982) 415.
26 N.B. Feilchenfeld, H.W. Richter and W.H. Waddell, Anal. Biochem., 122 (1982) 124.
27 P.T. Kissinger, C.S. Bruntlett, G.C. Davis, L.J. Felice, R.M. Reggin and R.E. Shoup, Clin. Chem., 23 (1977) 1449.
28 G. Sperk, I. Galhaup, E. Schlögl, H. Hörtnagl and O. Hornykiewicz, J. Neurochem., 35 (1980) 972.
29 H. Matsui, T. Kato, C. Yamamoto, K. Fujita and T. Nagatsu, J. Neurochem., 37 (1981) 289.
30 K. Fujita, K. Maruta, R. Teradaira, H. Beppu, M. Ikegami, K. Kawai and T. Nagatsu, Clin. Chem., 28 (1982) 1408.

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Note

Quantitative high-performance liquid chromatography of bases and nucleosides in cerebral DNA of rat foetus

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Recently, the analysis of DNA constituents has progressed rapidly with the use of high-performance liquid chromatography (HPLC). The latest advances in the HPLC analysis of nucleotides, nucleosides and bases in biological fluids and tissues were reviewed by Zakaria and Brown [1]. HPLC has been applied to the determination of the relationship between modified nucleoside levels in biological fluids and the disease status in patients [2] in clinical studies and to the detection of the methylated bases in the liver DNA of animals treated with carcinogens [3]. For the brain, Heizmann et al. [4] determined the deoxyribonucleoside composition of DNA from cortex neurons of foetal and postnatal rats by HPLC after digestion with a combination of nucleic acid-degrading enzymes. There are no other reports on the analysis of DNA constituents in the developing brain. It is well known that faulty DNA metabolism in some heritable disorders or imperfect repair of environmentally induced damage to DNA can lead to an increased risk of progressive degeneration of the developing brain. This paper describes the application of reversed-phase HPLC to the quantitative analysis of base and nucleoside compositions in cerebral DNA isolated from rat foetuses.

EXPERIMENTAL

Apparatus

All studies were conducted with an Atto (Tokyo, Japan) Model SF 0709 high-speed liquid chromatograph with an Atto UV monitor II. The injection

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valve was a Rheodyne (Cotati, CA, U.S.A.) Model 7125 with a 100-μl fixed-volume sample loop. A Waters Assoc. (Milford, MA, U.S.A.) μBondapak C_{18} reversed-phase column (300 × 3.9 mm I.D., particle size 10 μm) was used, preceded by a Whatman (Clifton, NJ, U.S.A.) Co:Pell ODS guard column. The peaks were identified by the addition of the corresponding standard to the hydrolysates, but the standard was not added in the quantitative analysis of samples. The areas of indistinct peaks were divided vertically and all peaks were integrated by a System Instruments (Tokyo, Japan) Model 5000E integrator.

Chemicals

Adenine (Ade), cytosine (Cyt), guanine (Gua), thymine (Thy), deoxyadenosine (dAdo), deoxycytidine (dCyd), deoxyguanosine (dGuo), deoxythymidine (dThd), deoxyinosine (dIno), deoxyribonuclease (DNase I, E.C. 3.1.4.5), snake venom phosphodiesterase (PDase I, E.C. 3.1.4.1) and calf thymus DNA (Type I) were purchased from Sigma (St. Louis, MO, U.S.A.). Alkaline phosphatase (APase, grade I, E.C. 3.1.3.1) was obtained from Boehringer (Mannheim, F.R.G.). Theobromine (Thb), xanthine (Xan), acetonitrile (chromatographic grade), acetone (UV grade), hydrochloric acid (super special grade) and all other chemicals of analytical-reagent grade were obtained from Wako (Osaka, Japan).

Calibration standards

All base and nucleoside stock solutions except for those of dGuo and Gua were prepared at a concentration of 10 m*M*; dGuo stock solution was prepared at 5 m*M* by adding 0.01 *M* phosphate buffer, pH 5.65 (buffer A), and Gua was resolved in a small amount of 1 *M* sodium hydroxide and finally adjusted to 10 m*M* with buffer A. These stock solutions were diluted to each concentration accurately and used as calibration standards. The range of calibration standards used in this study was decided depending on the level in the hydrolysates. Thb as an internal standard was prepared at a concentration of 2 m*M* by adding buffer A, and 4 μl of this solution were injected together with 96 μl of a standard or sample.

Sample preparation

Cerebral DNA was isolated from rat foetuses on gestational day 21 using the methods of Lǿvtrup-Rein and McEwen [5] and Marmur [6] with minor modifications. The acidic and enzymatic hydrolysis of cerebral DNA were performed by the methods of Sharma and Yamamoto [7] and Breter et al. [8] with minor modifications. A 0.5-mg amount of freeze-dried DNA was hydrolysed in 0.5 ml of 6 *M* hydrochloric acid for 3 h at 100°C for base analysis and in 1.0 ml of an enzyme mixture (DNase I, 100 U/ml; PDase I, 0.0019 U/ml; APase, 0.05 U/ml) for 4 h at 37°C for nucleoside analysis. Enzymatic hydrolysates were precipitated with 5 vols. of acetone and centrifuged at 7700 *g* for 10 min, and the supernatants were removed. Both hydrolysates were evaporated to dryness under a stream of nitrogen at 60°C, and the residues were dissolved in 0.5 ml of buffer A for the acidic hydrolysate and 1.0 ml of buffer A for the enzymatic hydrolysate and then filtered through 0.45-μm membrane filters (Millipore, Bedford, MA, U.S.A.).

Chromatographic conditions

A 100-μl volume of a sample or standard mixture was injected and eluted with a linear gradient of acetonitrile to 10% in buffer A after 30 min. The flow-rate was set at 1.0 ml/min and the column effluent was monitored at 254 nm.

TABLE I

PRECISION OF THE ASSAY FOR THE ACIDIC HYDROLYSATE OF FOETAL CEREBRAL DNA

Compound	Concentration (nmol/ml)	Mean ± S.D.* (nmol/ml)	C.V.** (%)	Recovery (%)
Cytosine	31.25	36 ± 3	8.1	113.6
	62.5	64 ± 4	6.1	102.0
	125	132 ± 5	4.0	105.4
	250	257 ± 5	2.1	103.0
Thymine	37.5	38 ± 3	7.4	101.3
	75	72 ± 6	8.2	96.0
	150	145 ± 5	3.5	96.3
	300	292 ± 12	4.0	97.2
Guanine	31.25	45 ± 3	6.4	145.3
	62.5	71 ± 5	6.4	113.2
	125	131 ± 14	10.6	104.8
	250	267 ± 6	2.1	106.9
Adenine	37.5	38 ± 2	5.3	101.3
	75	71 ± 5	6.7	94.0
	150	153 ± 5	3.3	101.7
	300	305 ± 8	2.7	101.7

*Each value represents the mean ± S.D. for four samples assayed at each compound concentration.

**C.V. denotes coefficient of variation.

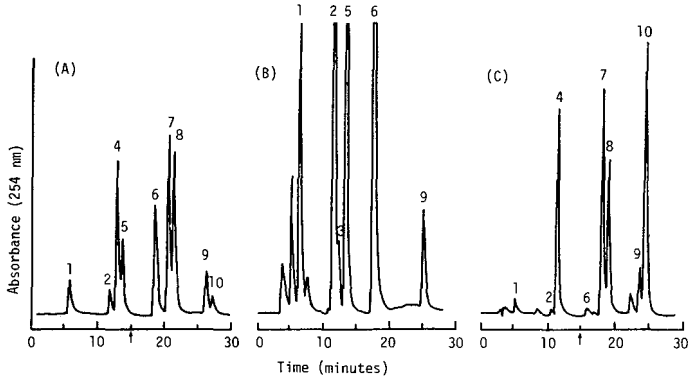

Fig. 1. Chromatograms of bases and nucleosides (A) in the standard mixture, (B) in the acidic hydrolysate and (C) the enzymatic hydrolysate of cerebral DNA. The chromatographic conditions are given in the text. Peaks: 1 = Cyt; 2 = Gua; 3 = Xan; 4 = dCyd; 5 = Thy; 6 = Ade; 7 = dGuo; 8 = dThd; 9 = Thb; 10 = dAdo. The arrows show the time of switching the sensitivity range from 0.05 to 0.10 a.u.f.s.

The sensitivity range used was usually 0.05 a.u.f.s. and switched to 0.10 a.u.f.s. after 15 min in the separation of the enzymatic hydrolysate. Chromatography was carried out at ambient temperature.

RESULTS AND DISCUSSION

The separation of the eight standard bases and nucleosides is shown in Fig. 1A. Fig. 1B and C show chromatograms of acidic and enzymatic hydrolysates

TABLE II

PRECISION OF THE ASSAY FOR THE ENZYMATIC HYDROLYSATE OF FOETAL CEREBRAL DNA

Compound	Concentration (nmol/ml)	Mean ± S.D.* (nmol/ml)	C.V.** (%)	Recovery (%)
Cytosine	3.75	—	—	—
	7.5	7.6 ± 1.6	2.1	101.7
	15	15.3 ± 0.5	3.3	101.7
	30	30.3 ± 0.5	1.7	100.8
	60	60.0 ± 2.3	3.8	100.0
Guanine	1.25	1.1 ± 0.3	25.5	90.0
	2.5	2.3 ± 0.3	14.9	89.0
	5	5.9 ± 0.3	5.1	117.5
	10	9.4 ± 1.3	14.0	93.5
Deoxycytidine	37.5	38 ± 3	6.8	101.3
	75	71 ± 4	6.1	94.7
	150	147 ± 3	2.1	97.7
	300	298 ± 14	4.8	99.3
Deoxythymidine	50	53 ± 11	20.7	106.0
	100	95 ± 7	7.9	95.0
	200	187 ± 6	3.2	93.5
	400	381 ± 17	4.4	95.2
Adenine	3.75	3.7 ± 0.3	6.9	97.4
	7.5	6.8 ± 0.2	2.4	89.7
	15	13.9 ± 0.6	4.0	92.4
	30	28.8 ± 1.2	4.3	95.8
	60	—	—	—
Deoxyguanosine	37.5	44 ± 7	16.0	117.4
	75	78 ± 5	6.1	104.0
	150	161 ± 6	3.7	107.0
	300	329 ± 22	6.6	109.5
Deoxyadenosine	50	50 ± 3	6.9	100.0
	100	95 ± 6	6.3	94.5
	200	195 ± 4	2.1	97.5
	400	391 ± 32	8.1	97.7

*Each value represents the mean ± S.D. for four samples assayed at each compound concentration.

**C.V. denotes coefficient of variation.

of cerebral DNA, respectively. Heizmann et al. [4] assayed dAdo as dIno with addition of excess of adenosine deaminase because the commercial preparation of APase contained small amounts of adenosine deaminase, which converted dAdo to dIno. In our experiments, the peak of dIno would be virtually negligible because the APase level was very low (0.05 U/ml).

In order to determine the linearity and limits of detection of the present quantitative HPLC method, we made three non-consecutive injections of each of the base and nucleoside mixtures. The responses for bases and nucleosides were found to be linear, with correlation coefficients of 0.997—1.000. The limits of detection in the present analysis were 2.5 nmol/ml for Gua, 7.5 nmol/ml for Ade, 15 nmol/ml for Cyt, 75 nmol/ml for Thy, dCyd and dGuo and 100 nmol/ml for dThd and dAdo. These values for bases and nucleosides were slightly higher than the reported limits [9] because of the detection of some small modified peaks.

The coefficients of variation (C.V.) and recoveries of standard mixtures added to acidic and enzymatic hydrolysates of prepared foetal cerebral DNA are shown in Tables I and II. Base and nucleoside levels showed good precision, C.V. of 2.1—10.6% and recoveries of 89.7—113.6%, except for Gua in the enzymatic hydrolysate and at the lowest concentrations of Gua, dGuo and dThd. These unauthentic data were partly due to the low level of Gua, the interference of Xan with the Gua peak and the indistinct separation of dGuo and dThd. Consequently, it is possible to determine the base and nucleoside levels of a sample within the authentic range. Hence the present method has the advantage that all DNA bases and nucleosides can be identified and determined under the same HPLC conditions, although the limits of detection are not so low. It is thought that this method is useful for clarifying the quantitative and qualitative changes of DNA base and nucleoside compositions in the developing brain of animal models for brain dysfunction.

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REFERENCES

1 M. Zakaria and P.R. Brown, J. Chromatogr., 226 (1981) 267.
2 R.W. Zumwalt, K.C.T. Kuo, P.F. Agris, M. Ehrlich and C.W. Gehrke, J. Liquid Chromatogr., 5 (1982) 2041.
3 D.C. Herron and R.C. Shank, Anal. Biochem., 100 (1979) 58.
4 C.W. Heizmann, R. Hobi, G.C. Winkler and C.C. Kuenzle, Exp. Cell Res., 135 (1981) 331.
5 H. Lφvtrup-Rein and B.S. McEwen, J. Cell Biol., 30 (1966) 405.
6 J. Marmur, J. Mol. Biol., 3 (1961) 208.
7 R.C. Sharma and O. Yamamoto, Biochem. Biophys. Res. Commun., 96 (1980) 662.
8 H.-J. Breter, G. Seibert and R.K. Zahn, J. Chromatogr., 140 (1977) 251.
9 R.A. Hartwick and P.R. Brown, J. Chromatogr., 126 (1976) 679.

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Note

Simple high-performance liquid chromatographic method for the separation of retinoids including N-(4-hydroxyphenyl)-all-*trans*-retinamide

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Many retinoids, both natural and synthetic, have a demonstrated prophylactic and in some cases therapeutic effect in a variety of chemically induced epithelial cancers in experimental animals [1, 2]. Earlier studies from our laboratory have provided evidence that the inhibition of carcinogenesis by retinoids is target organ specific, for example, 13-*cis*-retinoic acid is effective in suppressing urinary bladder carcinogenesis but ineffective against mammary cancers. Similarly, retinyl acetate can effectively inhibit mammary carcinogenesis but has little effect against two-stage skin tumorigenesis [2]. Among the retinoids tested against mammary and urinary bladder carcinogenesis, N-(4-hydroxyphenyl)retinamide (4-HPR) appears to be the most promising in terms of its effectiveness relative to toxicity [3]. The usefulness of a retinoid for protection against carcinogenesis depends not only on the lack of toxicity but also upon the tissue distribution, blood level and metabolism of the compound. Essential information on these pharmacokinetic parameters with respect to the breast and urinary bladder is lacking. Although Swanson et al. [4] have examined the pharmacokinetics of 4-HPR in male rats, such studies in the female rat have not been performed. Since 4-HPR is an effective inhibitor of breast cancer, it is of critical importance to study the distribution and metabolism of 4-HPR in female rats and mice.

Several methods exist for the high-performance liquid chromatographic (HPLC) separation of retinoids, however the majority of these procedures involve complex mobile phases and flow-rate changes [5—7]. Swanson and co-workers [4, 8] reported two separate HPLC systems to analyze the processing of 4-HPR in tissues. However, the first method failed to separate retinol from the 4-HPR metabolites [4] whereas in the second procedure, separation of polar metabolites of 4-HPR could not be achieved [8]. In this

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study, we describe a simple, sensitive HPLC method for the separation and quantitation of 4-HPR and its potential metabolites, as well as the separation of these compounds from retinol, retinyl acetate, and retinyl palmitate.

MATERIALS AND METHODS

All manipulations were carried out under yellow light or with the use of amber glassware or aluminum foil. All the solvents used for extraction and chromatography were chromatoquality grade and were purchased from E.K. Industries (Addison, IL, U.S.A.).

HPLC instrument and conditions

A Spectra Physics Model 8700 high-pressure liquid chromatograph equipped with a Model 8440 variable-wavelength detector set at 350 nm was used. This wavelength was intermediate between the maximum absorption wavelengths of several of the retinoids of interest. Peak areas were calculated with a Model 4100 computing integrator.

Separations were performed on a 250 mm × 4.6 mm I.D., 10-μm, bonded octodecylsilane, reversed-phase column (Partisil 10 ODS-2; Whatman, Clifton, NJ, U.S.A.). A 70 mm × 2.1 mm I.D. guard column containing Co:Pell ODS was positioned between the injector and the analytical HPLC column.

The column was eluted with a 30-min linear gradient of methanol—water (70:30) (pH ≈ 6) to 100% methanol (pH ≈ 7) at a flow-rate of 1.2 ml/min. Chromatography was continued at the final conditions for 40 min.

Analytical standards

All-*trans*-4-HPR, 13-*cis*-4-HPR, and all-*trans*-4-ethoxyphenylretinamide (4-EPR) were obtained from Southern Research Institute (Birmingham, AL, U.S.A.) through the National Cancer Institute; all-*trans*-retinoic acid, all-*trans*-retinol, and all-*trans*-retinyl palmitate from Sigma (St. Louis, MO, U.S.A.); all-*trans*-retinyl acetate and 13-*cis*-retinoic acid from BASF (Ludwigshafen, F.R.G.); and all-*trans*-4-methoxyphenylretinamide (4-MPR) from McNeil Pharmaceuticals (Springhouse, PA, U.S.A.). All-*trans*-5,6-epoxyretinoic acid was generously provided by Drs. Andre LaCroix and Pangala Bhat (Clinical Research Institute of Montreal, Montreal, Canada). All standards were stored under argon in amber vials at − 20 °C.

A solution of all standards was made by dissolving 2 mg of each dry compound and 10 μl retinyl palmitate in 200 ml of methanol. Of the standard solution 1 ml was concentrated to 0.5 ml by evaporation under nitrogen and 10 μl (200 ng of each standard) were injected onto the HPLC column.

Treatment of animals

Female Sprague—Dawley rats, 55—60 days of age, were injected intraperitoneally with 5 mg/kg 4-HPR per day for five days. Control rats received dimethyl sulfoxide (DMSO) vehicle alone. Rats were sacrificed 6—8 h after the last 4-HPR injection at the end of day 5. Liver, mammary gland, urinary bladder, and serum were collected, frozen under liquid nitrogen and stored at − 80 °C.

Extraction of retinoids

Tissues were lyophilized overnight, ground to a fine powder or cut into small pieces, and added to 100 x 16 mm glass-stoppered tubes. Retinyl acetate (80 μg) was added as an internal standard to control for end volume differences and potential spillage. The tissues were extracted twice with several volumes (6—10) of chloroform—methanol (2:1) by rotating on a Labquake shaker (Labindustries, Berkeley, CA, U.S.A.) for 1 h each. The samples were centrifuged at 800 *g* for 10 min, the liquid was transferred into amber vials and evaported immediately under a gentle stream of nitrogen. The residue was redissolved in 0.5 ml methanol (concentrated to 0.1 ml for mammary gland and urinary bladder) and a 10-μl aliquot was analyzed.

In order to calculate the concentration of 4-HPR in the samples, known amounts of 4-HPR were injected into isolated tissues from untreated rats prior to lyophilization. Retinyl acetate was added to dry tissue as the internal standard. Peak area ratios of 4-HPR relative to retinyl acetate were calculated, and a linear peak area versus 4-HPR concentration curve was constructed. This curve was linear from approximately 200 ng to 20 μg 4-HPR. However, below this level, peak height ratios were used in the construction of the standard curve. This curve was linear down to 10 ng 4-HPR. Amounts of 4-HPR in biological samples were quantitated by reference to the standard curves.

RESULTS AND DISCUSSION

Although several systems exist for the separation of retinoids, they involve complex assay conditions. In addition, suitable systems for the separation of

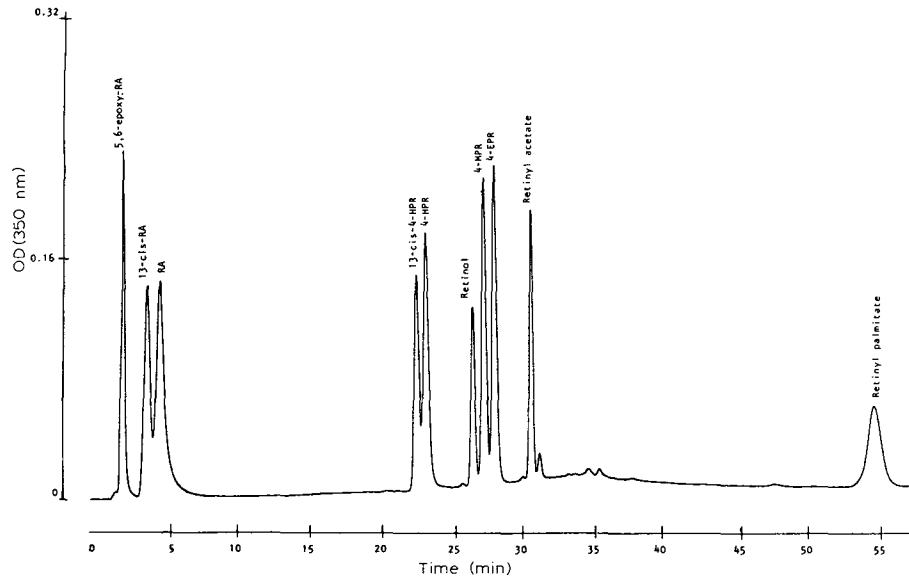

Fig. 1. HPLC separation of a mixture of retinoid standards. Retinoids were eluted from a Partisil 10 ODS-2 reversed-phase column with a 30-min linear gradient of methanol—water (70:30) to 100% methanol at a flow-rate of 1.2 ml/min. Chromatography was continued at the final conditions for 40 min. Each peak represents 200 ng of retinoid standard. RA = retinoic acid.

4-HPR and its metabolites are unavailable. This study was undertaken to develop a simple, sensitive method for the separation of 4-HPR, its possible metabolites, and the endogenous retinoids.

The separation of a mixture of ten retinoid standards is shown in Fig. 1. The retinoids were all clearly separable and eluted with retention times shown in Table I. The detection limit for retinoids using this procedure was approximately 5—10 ng. The signal-to-noise ratio at this level for 4-HPR was approximately 2—2.5, and the intra- and inter-assay variability was less than 5%.

TABLE I

HPLC ELUTION OF RETINOID STANDARDS

Retinoid	Retention time (min)
5,6-Epoxyretinoic acid	2.3
13-*cis*-Retinoic acid	4.0
All-*trans*-retinoic acid	4.9
13-*cis*-4-HPR	22.7
All-*trans*-4-HPR	23.4
Retinol	26.6
4-MPR	27.4
4-EPR	28.1
Retinyl acetate	30.7
Retinyl palmitate	54.6

Previous investigators have concentrated their efforts on separating only 4-HPR and/or on separating 4-HPR esters which are more hydrophobic than 4-HPR. The conditions used in those studies did not distinguish potential polar metabolites of 4-HPR such as retinoic acid [3, 4, 8]. Using the conditions described here retinoic acid and 4-HPR separated by about 18 min. This allows one to evaluate the polar metabolites of 4-HPR, if any, in the tissue. Moreover, all the phenyl retinamides tested separated very well under the conditions described here (Fig. 1).

Retention times for most of the retinoids were constant. However, all-*trans*-retinoic acid and 13-*cis*-retinoic acid showed variability (up to 1.5 min) in their elution profiles. Nevertheless, they were always separable from each other and clearly distinguishable from neighboring peaks. If necessary, a better separation of the two compounds with longer, more constant retention times could be accomplished either by increasing the amount of water or by including ammonium acetate, or another appropriate buffer, in the mobile phase [6].

Three small peaks were visible in the profile of standards (Fig. 1). One peak eluted immediately after retinyl acetate and appeared to be a minor contaminant in the retinyl acetate standard. In addition, two small peaks eluted approximately 4 min after retinyl acetate and corresponded with the end of the gradient and the beginning of 100% methanol mobile phase. These two peaks appeared to be due to a change in the percentage of organic modifier and were visible in every HPLC assay carried out using this gradient procedure.

The separation of retinoids extracted from the liver of 4-HPR-treated female rats is shown in Fig. 2. Liver was used as the example to demonstrate the separation in the presence of large amounts of endogenous retinoids found in

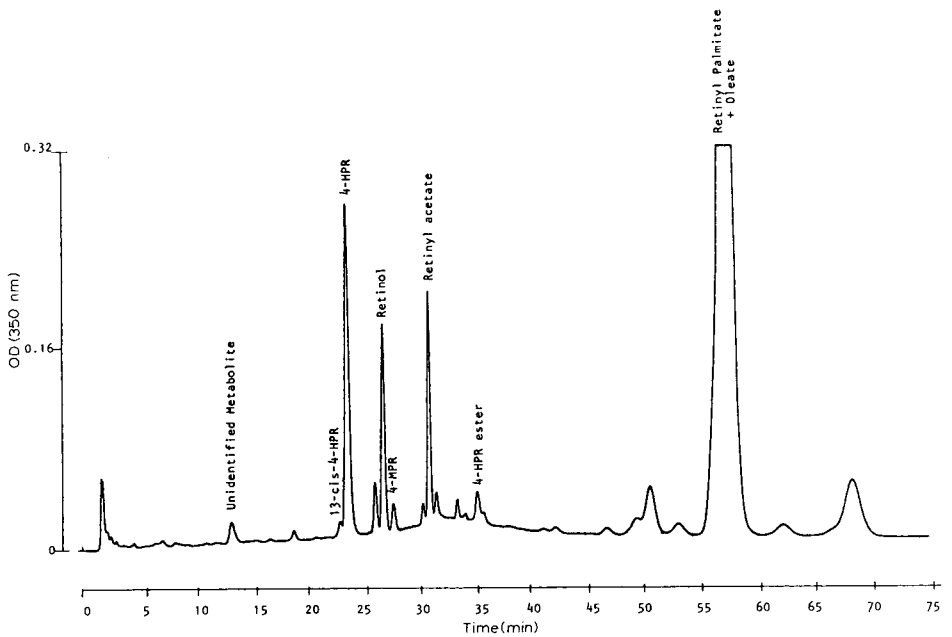

Fig. 2. Liver extract from 4-HPR-treated female rats. Rats were injected intraperitoneally with 5 mg/kg 4-HPR per day for five days and sacrificed on day 5, 6—8 h after the last injection. Liver was lyophilized, extracted with chloroform—methanol (2:1), and analyzed by HPLC as described in Fig. 1.

this tissue. Again, all retinoids were separable and exhibited a similar elution profile and retention times as shown in Fig. 1 and Table I. Compounds which coeluted with retinol and retinyl palmitate were extracted from the liver. In addition, several other peaks which appeared to be retinyl esters eluted with 100% methanol. Unfortunately, 100% methanol does not separate retinyl palmitate from retinyl oleate, but Bhat and LaCroix [9] have shown that separation on a 5-μm ODS column could be achieved with methanol—water (98:2). A 4-HPR peak was readily detectable as were peaks corresponding to 4-MPR, 13-*cis*-4-HPR, and a 4-HPR ester, all probable metabolites of 4-HPR. A more polar compound which peaked at 13.6 min, and which was not present in vehicle-treated animals was considered to be an unidentified metabolite of 4-HPR. All other peaks, including retinyl acetate which was added as the internal standard, were present in vehicle-treated animals.

In summary, we have developed a simple, sensitive method which separated 4-HPR from its metabolites, from retinol and retinyl palmitate, and from the internal standard, retinyl acetate. Currently, this procedure is being utilized successfully in our laboratory to study pharmacokinetics of 4-HPR in several tissues including normal and cancerous breast and urinary bladder samples [10].

REFERENCES

1 M.B. Sporn, N.M. Dunlop, D.L. Newton and J.M. Smith, Fed. Proc., Fed. Amer. Soc. Exp. Biol., 35 (1976) 1332.
2 R.C. Moon, D.L. McCormick and R.G. Mehta, Cancer Res., 43 (1983) 2469s.

3 R.C. Moon, H.J. Thompson, P.J. Becci, C.J. Grubbs, R.J. Gander, D.L. Newton, J.M. Smith, S.L. Phillips, W.R. Henderson, L.T. Mullen, C.C. Brown and M.B. Sporn, Cancer Res., 39 (1979) 1339.
4 B.N. Swanson, D.W. Zaharevitz and M.B. Sporn, Drug Metab. Dispos., 8 (1980) 168.
5 P.V. Bhat and A. LaCroix, Biochim. Biophys. Acta, 752 (1983) 451.
6 C.A. Frolik, T.E. Tavela and M.B. Sporn, J. Lipid Res., 19 (1978) 32.
7 T. Annesley, D. Giacherio and K. Wilkerson, J. Chromatogr., 305 (1984) 199.
8 B.N. Swanson, D.L. Newton, P.P. Roller and M.B. Sporn, J. Pharmacol. Exp. Ther., 219 (1981) 632.
9 P.V. Bhat and A. LaCroix, J. Chromatogr., 272 (1983) 269.
10 T.A. Hultin, R.G. Mehta and R.C. Moon, unpublished results.

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Note

Ion-pair high-performance liquid chromatographic determination of isoniazid and acetylisoniazid in plasma and urine

Application for acetylator phenotyping

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Isoniazid can be used for the determination of acetylator phenotype either by measuring the plasma half-life of the drug or by determining the ratio of the metabolite, acetylisoniazid, to the parent drug [1]. Plasma concentrations of isoniazid have been measured spectrophotometrically [2] and the metabolic ratio has been determined in urine colorimetrically [3], in serum and urine with fluorometry [1], and in serum, plasma and urine with high-performance liquid chromatography (HPLC) [4—6]. In our laboratory we have previously used a spectrophotometric method [2] to determine the plasma half-life. This method is rather time-consuming, the sensitivity is in some instances inadequate, and it requires several blood samples from the patient. We have now developed a new method, based on ion-pair HPLC, with high sensitivity and simple purification and derivatization steps. It enables determination of acetylator phenotype with a single blood or urine sample, by measurement of the ratio of acetylisoniazid to isoniazid. The method is currently used both for phenotyping and for determination of isoniazid plasma concentrations.

EXPERIMENTAL

Materials

Isoniazid (INH) and isonicotinic acid were obtained from Ferrosan (Malmö,

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Sweden). Acetylisoniazid (AINH) solution was made from INH according to the method of Eidus et al. [3]. Isonicotinuric acid was synthesized from INH according to the method used by Rohrlich [7] for preparation of nicotinuric acid, and identified by mass spectrometry. Centrifree ultrafilters were obtained from Amicon (Danvers, MA, U.S.A.). Sep-Pak C_{18} cartridges were obtained from Waters Assoc. (Milford, MA, U.S.A.). 1-Dodecyl sulphate (sodium salt) was chromatography-grade (Beckman, Berkeley, CA, U.S.A.). Acetonitrile was HPLC-grade. All other chemicals were analytical reagents. The water used was deionized.

Apparatus

The chromatographic equipment consisted of a Constametric I pump (Laboratory Data Control, Riviera Beach, FL, U.S.A.), a Model 7120 injector (Rheodyne, Berkeley, CA, U.S.A.) with a 10-μl loop, an Ultrasphere ion-pair 150 × 4.6 mm I.D. reversed-phase column (5-μm particles; Beckman) and a Spectromonitor III variable-wavelength UV detector (Laboratory Data Control).

Chromatographic conditions

The eluent was a 10 m*M* sodium dihydrogen phosphate buffer (pH 3.0, adjusted with phosphoric acid) containing 1 m*M* dodecyl sulphate and 25% acetonitrile. The flow-rate was 1.5 ml/min. The temperature was ambient, and the detector wavelength was set at 270 nm.

Sample preparation

Plasma. The centrifree ultrafilters were filled with ca. 1 ml of plasma, and centrifuged for 30 min at 2600 *g* in a 35° rotor angle centrifuge. Then 100 μl of the ultrafiltrate were mixed with 10 μl of 25% phosphoric acid and 1 μl of propionic anhydride. After 10 min reaction time, 10 μl of the mixture were injected on to the column.

Urine. A 1.0-ml volume of urine was mixed with 3.0 ml of 0.1 *M* hydrochloric acid and 50 μl of propionic anhydride. After 10 min reaction time the mixture was passed through a Sep-Pak C_{18} cartridge (pretreated with 5 ml of methanol and 5 ml of water) at a flow-rate of 10—15 ml/min. The last millilitre of the eluate was collected, and 10 μl were injected on to the column.

Standard curves

Standard curves were obtained by analysis of plasma and urine spiked with INH and AINH. Peak areas (peak height multiplied by peak width at half height) were measured. The standard concentration ranges for INH were 3—150 μmol/l in plasma, and 12 μmol/l to 12 mmol/l in urine. The standard concentration ranges for AINH were: plasma, 2.85—142.5 μmol/l, and urine, 11.4 μmol/l to 11.4 mmol/l. We found linear relationships between peak areas and concentrations.

RESULTS AND DISCUSSION

Owing to severe tailing it was found necessary to derivatize INH before chro-

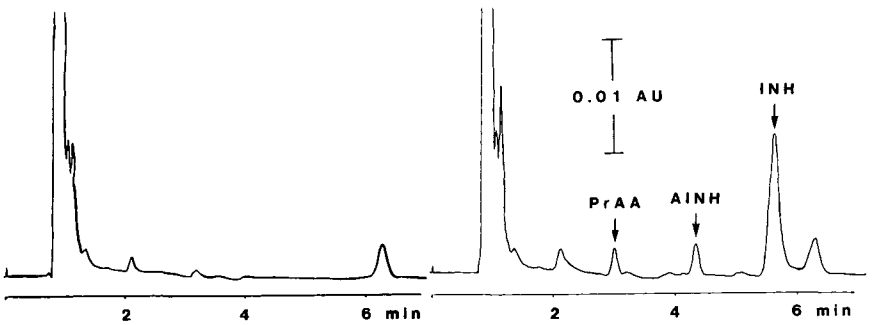

Fig. 1. Chromatograms from plasma before and 4 h after dosing. The peaks correspond to propionic anhydride (PrAA), 11.9 μmol/l acetylisoniazid (AINH), and 101 μmol/l (propionyl) isoniazid (INH).

matography. Preparation of the propionyl derivative, PrINH, considerably improved the peak shape and the sensitivity of the method.

Ultrafiltration of the plasma samples proved to give sufficient purification. INH has no protein binding [8]. Recovery in the ultrafiltrate is 100% for both INH and AINH. Ultrafiltration gives lower protein content (more than 99.9% are removed) than other deproteinization techniques, and there is no protein denaturation causing liberation of protein-bound substances. Phosphoric acid is added to the ultrafiltrate to give a suitable pH for chromatography.

The propionylation is complete after 10 min and the concentrations of AINH and PrINH are stable for at least 6 h. Unchanged propionic anhydride gives a peak in the chromatogram (Fig. 1). However, within 30 min the anhydride is completely hydrolysed and the peak disappears.

In urine, ca. two thirds of the INH is present in the form of acid-labile hydrazones [1, 9]. Eidus et al. [3] hydrolysed these hydrazones by adding 0.5 ml of 0.5 *M* hydrochloric acid to 1.0 ml of urine, and keeping it at room temperature for 15 min. We found that hydrolysis and derivatization could be done simultaneously, giving the same result as with pre-hydrolysis. The propionylation is complete after 10 min and the concentrations of AINH and PrINH are stable for at least 6 h. After propionylation of INH, the solution was cleaned by running it through a Sep-Pak C_{18} cartridge. As both AINH and PrINH are charged at this pH (ca. 1.0), they are very water-soluble and virtually unretained on the Sep-Pak. In the eluate from the Sep-Pak the concentrations of the two compounds have reached the concentrations in the incoming solution after 2.5 ml.

The detector wavelength was set at 270 nm, where both AINH and PrINH show absorption maxima.

The coefficients of variation were: for INH in plasma, 1.5% at 60 μmol/l (n = 11); for AINH in plasma, 0.7% at 57 μmol/l (n = 13); for INH in urine, 1.0% at 385 μmol/l (n = 5); and for AINH in urine, 1.1% at 2.19 mmol/l (n = 5). The minimum detectable concentrations were: for INH and AINH in plasma, ca. 1 μmol/l; in urine, ca. 4 μmol/l.

Thirty patients without severe renal disease have been tested for acetylation phenotype, both with the new HPLC method and with the spectrophotometric method [2]. Blood samples were drawn before the dose of INH (ca. 10 mg per

kg body weight, range 5—18.1 mg/kg), and after 2, 4, 6 and 8 h. A blank urine sample was taken before the dose, when the patient emptied the bladder, and then urine was collected 0—4 h after the dose.

Chromatograms from plasma before, and 4 h after a dose of INH are shown in Fig. 1. Chromatograms from urine collected before dosing and 0—4 h after dosing, are shown in Fig. 2. The INH metabolites isonicotinic acid and isonicotinuric acid gave peaks in the chromatograms from urine, whereas their concentrations in plasma were normally below the detection limit. No interfering peaks from concomitant drug therapy or endogenous compounds have been observed in plasma or urine.

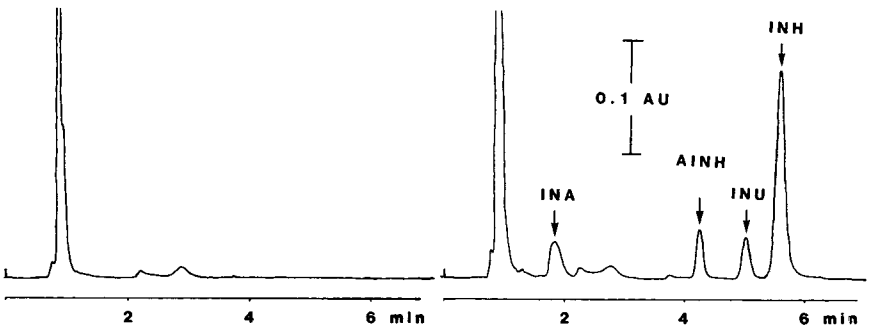

Fig. 2. Chromatograms from urine sampled before dosing and collected 0—4 h after dosing. The peaks correspond to isonicotinic acid (INA), 640 μmol/l acetylisoniazid (AINH), isonicotinuric acid (INU) and 4.33 mmol (propionyl) isoniazid (INH).

The INH concentrations in plasma varied from less than 1 to 160 μmol/l. The spectrophotometric and HPLC methods correlated well ($y = 1.01x - 0.63$). When the HPLC method was used, twenty-one patients were classified as slow acetylators ($t_{½} > 2.1$ h) and nine patients were classified as rapid acetylators ($t_{½} < 2.1$ h). The spectrophotometric method gave the same result, except that for two rapid acetylators the half-life could not be accurately determined because of low INH concentrations at 6 and 8 h (Table I). The AINH concentrations in plasma varied between 4.6 and 73 μmol/l.

Calculation of the ratio of AINH to INH in plasma at 2, 4, 6 or 8 h gave the same discrimination between the phenotypes as determination of the INH plasma half-life (Table I).

TABLE I

COMPARISON OF DIFFERENT METHODS FOR PHENOTYPING

Twenty-one patients were classified as slow acetylators and nine as rapid acetylators.

Phenotype	$t_{½}$ (h)		AINH/INH ratio				
	Spectr.	HPLC	Plasma				Urine
			2 h	4 h	6 h	8 h	0—4 h
Slow acetylators	2.5—5.4	2.4—5.8	0.058—0.22	0.10— 0.39	0.16— 0.58	0.22— 0.79	0.093—0.47
Rapid acetylators	1.2—2.0*	0.9—1.7	0.56 —2.0	1.7 —10.0	3.5 —19.5	5.7 —25.4**	0.94 —3.4

*Seven patients ($t_{½}$ was not measurable in two patients).
**Three patients (INH concentrations were below the detection limit in six patients).

In urine the INH concentrations varied between 185 μmol/l and 12 mmol/l and the AINH concentrations varied between 114 μmol/l and 9.3 mmol/l. Phenotyping by calculation of the ratio of AINH to INH in urine gave the same result as that determined from INH plasma half-life (Table I).

CONCLUSION

A method for simultaneous determination of isoniazid and acetylisoniazid in plasma and urine is described. It involves simple purification steps, with ultrafiltration of plasma, and removal of interfering matter in urine with a Sep-Pak C_{18} cartridge. Isoniazid is derivatized with propionic anhydride to propionylisoniazid. The compounds are separated on a reversed-phase HPLC column with ion-pair formation, using 1-dodecylsulphate as counter ion, and quantitated with a UV detector. The method has been used for determination of acetylator phenotype.

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REFERENCES

1 G.A. Ellard and P.T. Gammon, J. Pharmacokinet. Biopharmaceut., 4 (1976) 83.
2 L. Eidus and A.M.T. Harnanansingh, Clin. Chem., 17 (1971) 492.
3 L. Eidus, P. Varughese, M.M. Hodgkin, A.H.E. Hsu and K.B. McRae, Bull. W. H. O., 49 (1973) 507.
4 S.J. Saxena, J.T. Stewart, I.L. Honigberg, J.G. Washington and G.R. Keene, J. Pharm. Sci., 66 (1977) 813.
5 M.A. Moulin, F. Albessard, J. Lacotte and R. Camsonne, J. Chromatogr., 226 (1981) 250.
6 M. Guillaumont, M. Leclercq, U. Frobert, B. Guise and R. Harf, J. Chromatogr., 232 (1982) 369.
7 M. Rohrlich, Arch. Pharm., 284 (1951) 6.
8 G.S. Avery (Editor), Drug Treatment, ADIS Press, Sydney, 1976, Appendix A.
9 M.G. Boxenbaum and S. Riegelman, J. Pharm. Sci., 63 (1974) 1191.

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Note

Simultaneous determination of serum vitamin A and E by liquid chromatography with fluorescence detection

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Several papers have been published [1—9] on the separate determination of serum/plasma vitamins A and E; relatively few have described the simultaneous determination. Manual, fluorimetric methods based upon the difference in fluorescence characteristics of the two vitamins have been reported [10, 11]. Two procedures for the simultaneous determination have been described using reversed-phase liquid chromatography and UV absorption detection with the wavelength set at 292 nm [12] or at 250 nm [13]. As the absorption spectra (and fluorescence emission) of the vitamins differ considerably [14], monitoring at one wavelength is a compromise. In this paper is described the use of a fluorescence detector which automatically changes the excitation and emission wavelengths with respect to retention time so optimising the selectivity and sensitivity of the analysis.

Normal-phase chromatography is used both for providing compatibility with the solvent extraction procedure and for increasing the sensitivity compared with the high-polarity solvents used in reversed-phase chromatography.

EXPERIMENTAL

Reagents

All-*trans*-retinol and *d*-α-tocopherol were obtained from Sigma (St. Louis, MO, U.S.A.). *n*-Hexane special for liquid chromatography was obtained from Fisons (Loughborough, U.K.), and ethanol, 99.7—100% (AnalaR) was obtained from BDH (Poole, U.K.). Stock standards were made up in ethanol and diluted in ethanol in the range 6.0—60 μmol l^{-1} for *d*-α-tocopherol and 0.4—4.0 μmol l^{-1} for *d*-1-retinol.

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Apparatus

Chromatography was carried out using a Perkin-Elmer Series 4 pump and a Rheodyne 7125 injector fitted with a 20-μl loop. The fluorescence detector was a Perkin-Elmer Model LS-4. The silica column was an HS-3 100 × 4.6 mm column with 3-μm silica. Elution was performed with *n*-hexane—ethanol (99:1) at 2 ml min^{-1} at ambient temperature. The Model LS-4 was programmed to monitor first at 295 nm excitation, 390 nm emission and after 2.5 min to change to 325 nm excitation, 480 nm emission.

Sample preparation

In a PTFE-capped 100 × 10 mm centrifuge tube 200 μl ethanol were added to 200 μl of serum sample. After vortex-mixing to ensure effective precipitation of the proteins, 200 μl of water were added followed by 200 μl of the mobile phase. The samples were further vortex-mixed for 1 min and then centrifuged at 4500 *g* for 3 min. A 20-μl aliquot of the clear top organic phase was injected directly onto the silica column. Sample recovery was tested by using spiked serum samples, substituting the 200 μl of ethanol with 200 μl of the standards.

Standard calibration

A similar procedure to that of the sample preparation was used substituting 200 μl of water for the serum sample and 200 μl of the ethanolic standards for the 200 μl of ethanol. Calibration curves were made by plotting peak height against vitamin A and E concentrations.

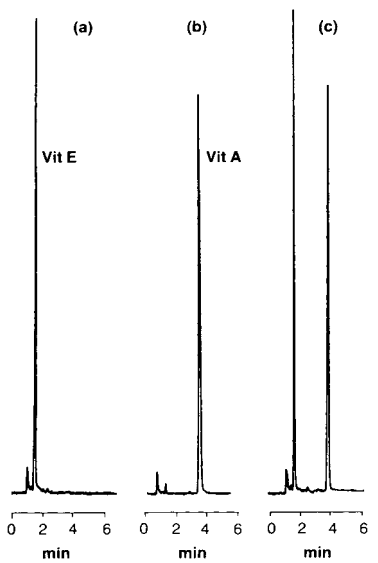

Fig. 1. Liquid chromatograms of serum extracts using procedures described in the text. (a) With the detector set at excitation 295 nm, emission 390 nm; (b) with the detector set at excitation 325 nm, emission 480 nm; (c) with the detector set initially at excitation 295 nm, emission 390 nm and programmed to change to excitation 325 nm, emission 480 nm 2.5 min after injection of the sample. The serum vitamin concentrations were 31.1 and 1.52 μmol l^{-1} for *d*-α-tocopherol and *d*-1-retinol, respectively.

RESULTS

Fig. 1 shows a typical chromatogram obtained from a serum extract with capacity factors (k') of 0.6 and 3.0 for *d*-α-tocopherol and *d*-1-retinol, respectively. A linear relationship between peak height and concentration was observed over the working range. The coefficients of the equations (a = intercept, b = slope and r = regression coefficient) were as follows: *d*-α-tocopherol, $a = 0.0433$, $b = 0.295$, $r = 0.991$; *d*-1-retinol, $a = 0.0478$, $b = 0.3996$, $r = 0.998$. The mean extraction recoveries from four spiked samples were 96.9% for *d*-1-retinol and 98.5% for *d*-α-tocopherol with coefficients of variation of 2.0% and 4.5%, respectively. Concentrations of *d*-α-tocopherol and *d*-1-retinol in various serum samples agreed well with those found by previous studies (Table I). Values ranged from 24.31 to 63.99 μmol l^{-1} for *d*-α-tocopherol and 1.88 to 3.26 μmol l^{-1} for *d*-1-retinol. The coefficient of variation analysing ten samples of a serum pool was 4.90% for *d*-α-tocopherol and 2.02% for *d*-1-retinol at mean values of 31.1 and 1.52 μmol l^{-1}, respectively. The sensitivity of detection using 200-μl samples and 20-μl injection volume was 0.02 μmol l^{-1} for *d*-1-retinol and 0.37 μmol l^{-1} for *d*-α-tocopherol using the basis of a signal twice the noise level.

TABLE I

d-α-TOCOPHEROL AND *d*-1-RETINOL LEVELS IN DIFFERENT SERUM (n = 10)

d-α-Tocopherol (μmol l^{-1})	*d*-1-Retinol (μmol l^{-1})
31.1 (24.31—63.99)	2.08 (1.88—3.26)

DISCUSSION

The choice of normal-phase chromatography was based on two main criteria. These were (a) to obtain the most sensitive assay and (b) to maintain compatability between the extraction procedure and the chromatography. Many organic compounds exhibit quite large changes in fluorescence intensity when measured in solvents of varying polarity [15]. Both *d*-α-tocopherol and *d*-1-retinol show this effect with a five- to six-fold decrease in intensity on going from *n*-hexane to acetonitrile—water (50:50). Repeated injections of *n*-hexane extracts onto reversed-phase columns result in a deterioration of the chromatography. This is probably due to the build-up of lipids and other fat-soluble products on the column. The combination of fluorescence detection (for better sensitivity) and normal-phase chromatography removes the necessity of evaporating to dryness the organic extract and its uptake in a different mobile phase. Ethanol was used as a precipitant in preference to methanol as it gave a better recovery for *d*-α-tocopherol.

The mobile phase composition chosen gave a good separation of the two vitamins in the shortest convenient time. Although peaks could be distinguished between the unretained peak and that of the *d*-α-tocopherol, a lower solvent polarity would ensure absolute separation. However, lowering the polarity significantly increases the retention of the *d*-1-retinol. As the

procedure gave a reasonable degree of precision no internal standard has been used. Previously published methods have used retinyl esters as internal standards. However, these are not suitable for normal-phase chromatography as they would elute in the void volume. *d*-1-Tocol would appear to be useful as an internal standard [12] as it elutes between the two vitamins. This or any other suitable internal standard would improve the precision of the assay as would standardization using serum samples.

The examination of the fluorescence characteristics of the two vitamins shows that they are widely different. *d*-α-Tocopherol has an excitation and emission maximum of 295 and 330 nm, respectively, whereas, those of *d*-1-retinol are 325 and 480 nm, respectively. However, for serum samples containing mean values of both vitamins, an emission wavelength of 390 nm for the *d*-α-tocopherol gives peaks of comparable intensities. If the correct emission wavelength is used the *d*-α-tocopherol peak would be 25-fold more intense than that of *d*-1-retinol.

The effect of automatically changing the excitation and emission wavelengths during chromatography can be seen in Fig. 1. The first two chromatograms are of a serum sample monitored at the appropriate wavelengths of each vitamin. The third chromatogram is of the same sample but with the detection initially at 295 nm excitation, 390 nm emission and then automatically changed after 2.5 min to 325 nm excitation, 480 nm emission.

The sensitivity of the method is such that the serum sample could be reduced from 200 μl to 25—50 μl, but was kept at 200 μl for ease of handling. The overall procedure of extraction and chromatography is relatively simple and the optimization of detection through wavelength programming reduces the assay time to less than 10 min per sample.

REFERENCES

1 D.E. Duggan, Arch. Biochem. Biophys., 84 (1959) 116.
2 J. Kahan, Int. J. Vit. Nutr. Res., 43 (1973) 127.
3 M.G.M. De Ruyter and A.P. De Leenheer, Clin. Chem., 22 (1976) 1593.
4 A.P. De Leenheer, V.O.R.C. De Bever, A.A. Cruyl and A.E. Claeys, Clin. Chem., 24 (1978) 585.
5 L.J. Hatam and H.J. Kayden, J. Lipid Res., 20 (1979) 639.
6 C.H. McMurray and W.J. Blanchflower, J. Chromatogr., 178 (1979) 525.
7 L. Jansson, B. Nilsson and R. Lindgren, J. Chromatogr., 181 (1980) 242.
8 J. Lehmann and H.L. Martin, Clin. Chem., 28 (1982) 1784.
9 H.G.C.F. Nelis, J. De Roose, H. Vandenbaviere and A.P. De Leenheer, Clin. Chem., 29 (1983) 1431.
10 J.N. Thompson, P. Erodody and W.B. Maxwell, Biochem. Med., 8 (1973) 403.
11 L.G. Hansen and W.J. Warwick, Amer. J. Clin. Pathol., 51 (1969) 538.
12 A.P. De Leenheer, V.O.R.C. De Bevere, M.G.M. De Ruyter and A.E. Claeys, J. Chromatogr., 162 (1979) 408.
13 J.G. Bieri, T.J. Tolliver and G.L. Catignani, Amer. J. Clin. Nutr., 32 (1979) 2143.
14 J.J. Aaron and J.D. Winefordner, Talanta, 19 (1972) 21.
15 R.F. Chen, Arch. Biochem. Biophys., 120 (1969) 609.

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CHROMBIO. 2535

Note

Determination of propisomide, a new antiarrhythmic agent, in biological samples by gas chromatography with a thermionic detector

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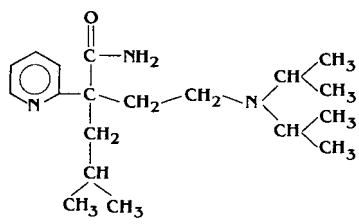
Propisomide, *dl*-2[2-(diisopropylamino)ethyl]-4-methyl-2-(2-pyridyl)-pentamide (CM-7857, Fig. 1), displayed a potent antiarrhythmic activity as demonstrated on a model of myocardial infarction in the conscious dog [1]. Cellular electrophysiology of this compound has shown an efficiency on experimental models of arrhythmia and in patients with rhythm disturbance [2]. Safety and tolerance of propisomide were assessed in man after intravenous and oral administration. The drug appeared to be particularly safe and well tolerated [3]. Tolerance studies were completed by pharmacokinetic studies. Therefore a quantitation technique of the parent drug in plasma and urine was developed.

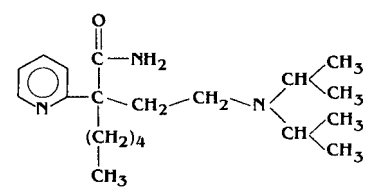
The chemical structure of propisomide and the use of the thermionic detector for gas chromatographic determination of disopyramide in the literature [4, 5] suggested this method for the determination of propisomide in biological samples.

Propisomide



MW = 319

Internal standard



MW = 333

Fig. 1. Structures of propisomide and internal standard.

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EXPERIMENTAL

Reagents

Solutions of propisomide and internal standard (CM-7973, Fig. 1) were prepared in acetonitrile (Uvasol; Merck, Darmstadt, F.R.G.) in the concentration range 1 mg/ml to 1 μg/ml. R.P. Normapur sodium carbonate, R.P. Normapur potassium dihydrogen phosphate and sodium hydroxide (10 *M*) were obtained from Prolabo (Paris, France). Analytical grade acetonitrile, methanol and methylene chloride were obtained from Merck, Carlo Erba (Milan, Italy) and Solvant Documentation Synthese Compagny (Peypin, France), respectively.

Extraction procedure in plasma

Plasma (500 μl) and 25 μl of internal standard solution (0.1 μg/ml) were put into a centrifuge tube (Du Pont de Nemours, Newtown, CT, U.S.A.); 200 μl of a buffer solution pH 12 (0.5 *M* sodium hydroxide, 1 *M* sodium carbonate) and 6 ml of methylene chloride were added and the mixture was shaken for 15 min. After centrifugation (5 min, 12 000 *g*) the organic layer was recovered and 4 ml of a buffer solution pH 2 (0.01 *M* potassium dihydrogen phosphate) were added. After agitation (15 min) and centrifugation (5 min, 12 000 *g*), the aqueous layer was separated and adjusted to alkaline pH by addition of 200 μl of a basic solution (5 *M* sodium hydroxide, 1 *M* sodium carbonate). The mixture was extracted with 6 ml of methylene chloride (15 min) and, after centrifugation (5 min, 12 000 *g*), the organic layer was removed and evaporated to dryness under a dried nitrogen stream. The residue was reconstituted in 50 μl of methanol and 2 μl of this solution were injected.

Extraction procedure in urine

Extraction was realized without clean-up; 200 μl of internal standard solution (10 μg/ml) were added to 1 ml of urine. After addition of 1 ml of buffer solution pH 12 (0.5 *M* sodium hydroxide, 1 *M* sodium carbonate) and 6 ml of methylene chloride, the mixture was shaken for 15 min and then centrifuged (5 min, 12 000 *g*). The organic layer was separated and evaporated to dryness under nitrogen; 500 μl of methanol were added to the residue and 2-μl aliquots were submitted to analysis.

Chromatographic conditions

All analyses were run on a Varian 4700 gas chromatograph (Palo Alto, CA, U.S.A.) with a thermionic detector. The chromatographic separations were performed on a packed column (OV-17, 3% on Chromosorb W AW, 100—120 mesh, 2 m × 18 mm I.D.). Helium was used as the carrier gas at a flow-rate of 30 ml/min. The injector and detector temperatures were 300°C, the oven temperature was 250°C. The combustants for the thermionic detector were hydrogen (1.4 Pa) and air.

Peak areas were obtained using a Varian CDS 401 Vista data station.

Preparation and storage of plasma and urine samples

Blood samples were collected into Vacutainer tubes (Becton Dickinson,

France) containing sodium heparin as anticoagulant. Plasma was separated immediately by centrifugation and stored at −20°C. Urine samples were immediately frozen and stored at −20°C until analysed. The use of heparin as anticoagulant did not change the repartition of propisomide in blood samples — the drug was equally distributed in plasma and red blood cells. Any possible plasticizers in the rubber stopper of Vacutainer tubes did not affect the chromatographic traces.

Calculations

Plasma concentrations were calculated from standard curves established from chromatograms of spiked plasma samples containing known amounts of propisomide (0.2, 0.5, 1, 2.5 and 5 μg/ml).

Urine concentrations were calculated from standard curves established after analysis of spiked urine samples in the concentration range of propisomide (25, 75, 150, 200 and 500 μg/ml).

Standard curves were generated daily by plotting peak area ratios (propisomide/internal standard) versus propisomide concentrations.

Mass spectrometry

Mass spectra of propisomide and internal standard were recorded using a Ribermag R10-10B mass spectrometer coupled on-line to the SIDAR data system (Rueil-Malmaison, France)

Gas chromatographic conditions were the same as described above. Helium was used with a flow-rate of 20 ml/min. The oven temperature, injector temperature and interface temperature were, respectively, 250°C, 280°C and 280°C. Under these conditions the retention time of propisomide and internal standard were, respectively, 4.5 min and 6.7 min. Mass spectra were obtained in the chemical ionization mode with ammonia as reagent gas, ionization energy of 70 eV, ionization current of 200 μA and ion source pressure of 0.1 Torr.

RESULTS

Typical gas chromatograms obtained from spiked human plasma are shown in Fig. 2. No interfering peaks were observed under the chromatographic conditions adopted.

The plasma standard curve was linear over the range 0.2—5 μg/ml, the

TABLE I

PRECISION OF THE GAS CHROMATOGRAPHIC ASSAY AND EXTRACTION EFFICIENCY OF PROPISOMIDE IN PLASMA

Concentration (μg/ml)	Peak area ratios (± S.D., n = 5)	C.V. (%)	Extraction efficiency (%)
0.2	0.131 (± 0.009)	6.8	63.6
0.5	0.326 (± 0.024)	7.4	65.5
1	0.680 (± 0.027)	3.9	59.3
2.5	1.564 (± 0.044)	2.8	64.2
5	3.351 (± 0.174)	5.2	67.5

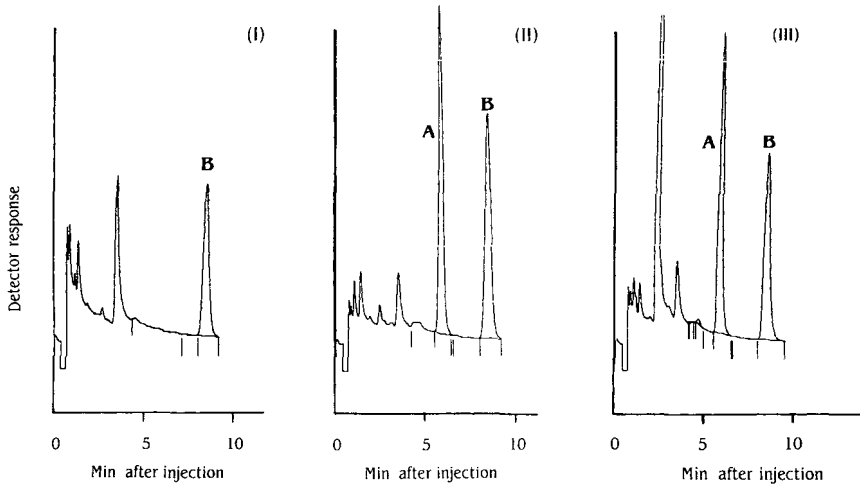

Fig. 2. Chromatograms of extracted human plasma: (I) blank human plasma sample; (II) human plasma sample spiked with 2 μg/ml propisomide; (III) human plasma sample after administration of propisomide to the patient (concentration found: 2.89 μg/ml). Peaks: A = propisomide, B = internal standard.

correlation coefficient was better than 0.998 and the regression curve passed through the origin. The reproducibility of the method was tested by repeated analysis of spiked human plasma samples (range 0.2—5 μg/ml propisomide) daily for five days. The results of the reproducibility test and the extraction recoveries in the same concentration range are presented in Table I. For each concentration, the coefficient of variation is less than 7.5% and the extraction recovery ranges from 59.3% to 67.5%. The losses occurred during clean-up with buffer solution pH 2, the remaining drug was in the aqueous phase (about 10%). The limit of quantitation in plasma was about 25 ng/ml.

The urine standard curve was linear over the range 25—500 μg/ml, the correlation coefficient was better than 0.997 and the regression curve passed through the origin. Reproducibility, tested using the same procedure as for plasma, and the extraction recoveries are reported in Table II. The coefficient of variation is better than 4% and the extraction recovery ranges from 84.3% to 93.5%. The limit of quantitation in urine was about 50 ng/ml.

Mass spectra of the compounds were recorded from a plasma extract obtained from a patient receiving propisomide. The sample was assayed by gas

TABLE II

PRECISION OF THE GAS CHROMATOGRAPHIC ASSAY AND EXTRACTION EFFICIENCY OF PROPISOMIDE IN URINE

Concentration (μg/ml)	Peak area ratios (± S.D., n = 5)	C.V. (%)	Extraction efficiency (%)
25	0.139 ± 0.002	1.3	88.4
50	0.284 ± 0.003	1.1	89.3
100	0.585 ± 0.007	1.2	84.3
200	1.213 ± 0.040	3.3	93.5

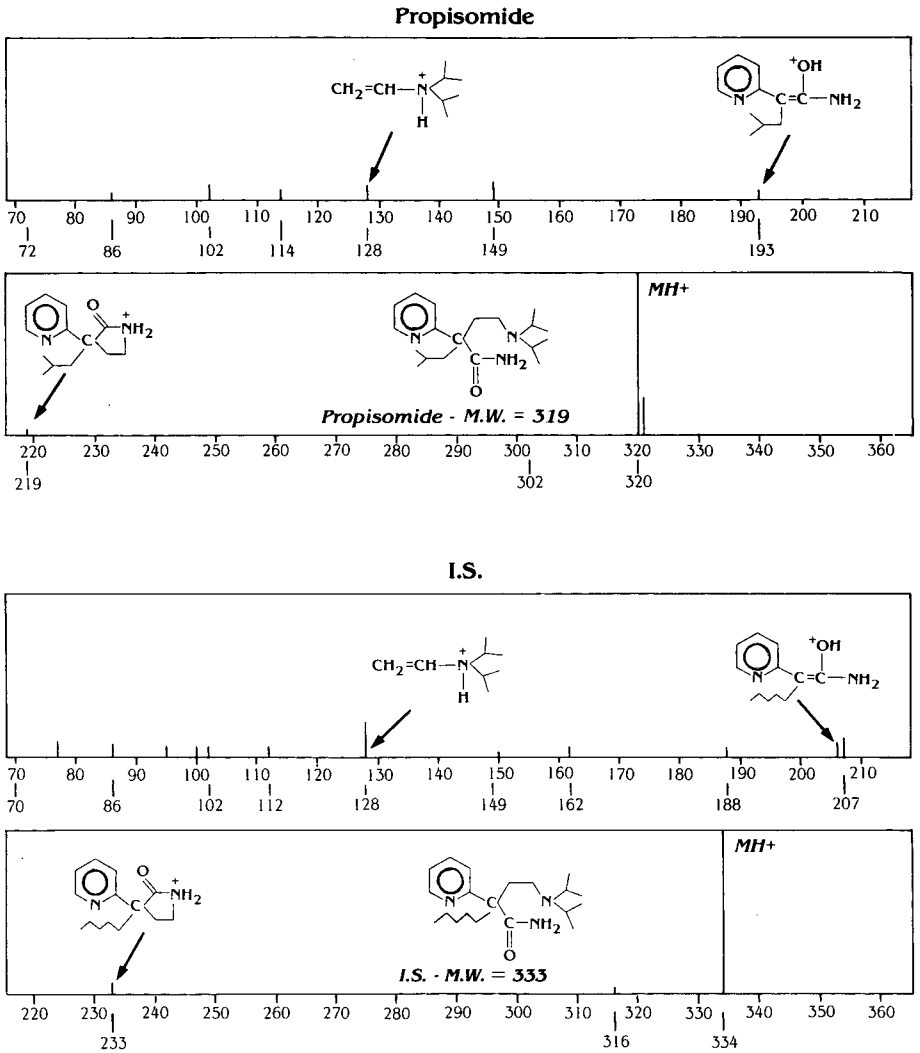

Fig. 3. Mass spectra of propisomide and internal standard (conditions as described in the text).

chromatography with thermionic detection before analysis by gas chromatography—mass spectrometry. Mass spectra of the compounds are shown in Fig. 3. They were completely consistent with mass spectra obtained from the reference compounds. This procedure confirms the specificity of the gas chromatographic method developed.

Application to biological samples

The method described was applied for the determination of plasma levels of propisomide following intravenous administration of 280 mg of the drug during 30 min to cardiac patients and after oral administration of 600 mg of propisomide.

Fig. 4 illustrates the pharmacokinetic profiles of the drug as obtained after

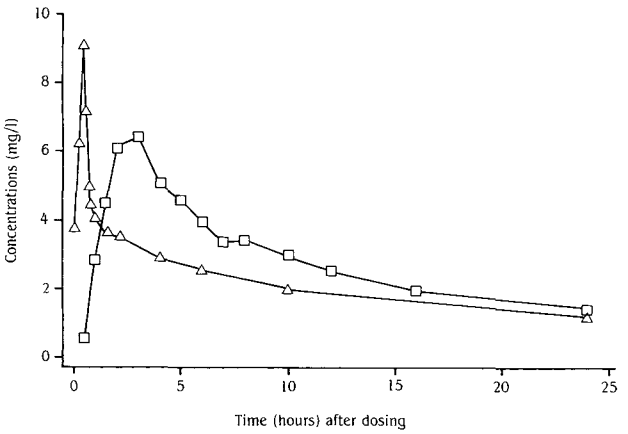

Fig. 4. Concentrations of propisomide in human plasma after oral (□) (600 mg) or intravenous (△) (280 mg for 30 min) administration.

analysis of the plasma samples and confirms the applicability of the method in human pharmacokinetics.

CONCLUSION

A selective, reproducible, and accurate assay was developed for propisomide in plasma and urine. The sensitivity of the method allows its application for the elucidation of human pharmacokinetics after intravenous and oral administration. The method is currently being applied for monitoring the drug in clinical trials. In routine use, the characteristics of the method remain at the level described in this paper.

REFERENCES

1 C.A. Bernhart, C. Condamine, H. Demarne, R. Roncucci, J.P. Gagnol, P.J. Gautier and M.A. Serre, J. Med. Chem., 26 (1983) 451.
2 P. Gautier, F. Pezziardi, P. Guiraudou, C. Bernhart and J.P. Gagnol, Naunyn-Schmiedeberg's Arch. Pharmacol., 324 (Suppl.) (1983) 125.
3 J.D. Arnold, M.E. Smith, A. Berger, L. March, D. Moore, E. McCafferty and J.P. Gagnol, Clin. Res., 31 (1983) 627A.
4 J. Vasiliades, C. Owens and D. Pirkle, Clin. Chem., 25 (1979) 311.
5 T. Kwong, J. Keller, A. Kalburgi and F. Mirabella, Clin. Chem., 29 (1983) 1249.

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Note

Simultaneous determination of ketanserin and ketanserinol in biological fluids using ion-pair liquid chromatography and fluorometric detection

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Among novel 5-hydroxytryptamine antagonists (5-HT_2) ketanserin, a quinazoline derivative, is presently under clinical investigation as an antihypertensive [1—4]. Following oral doses of 40 mg of intravenous injection of 10 mg of ketanserin a decrease in the supine systemic arterial pressure can be achieved [4]. Furthermore, ketanserin increases cardiac output even in patients already under digitalis treatment [5]. Pharmacokinetic studies have been performed recently with young healthy volunteers [6] as well as with elderly subjects [7]. Ketanserin is converted to its major metabolite ketanserinol in humans, which accumulates considerably under chronic ketanserin treatment [7]. It has not yet been clarified whether this metabolite contributes pharmacological effects or may be reconverted to its parent compound.

Various methods for the determination of ketanserin have been described. Except for a radioimmunoassay, which allows ketanserin only to be quantitated [8], reversed-phase liquid chromatography was preferentially employed. In order to detect the slow terminal elimination phase recently described [6], it is necessary to measure concentrations of less than 1 ng/ml ketanserin. Using ultraviolet (UV) absorbance [9—12] the required detection limit could not be achieved. A sensitive method recently published [7] includes reversed-phase chromatography of ketanserin as the undissociated base at pH 12, and fluorometric detection. However, this procedure rapidly degrades reversed-phase column packing material and is not compatible with common high-performance liquid chromatography (HPLC) equipment.

We report here a selective and sensitive HPLC method, which involves ion-pairing and fluorometric detection at pH 5.2. Biological specimens from clinical

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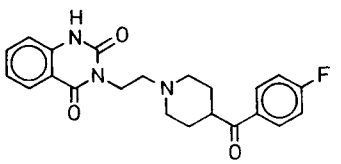
trials were analysed for ketanserin and ketanserinol simultaneously. Pharmacokinetic parameters derived from analytical data lead to improvement of ketanserin preparations, adequate dosage regimens for various patient populations and estimation of therapeutic risks dependent on drug accumulation.

EXPERIMENTAL

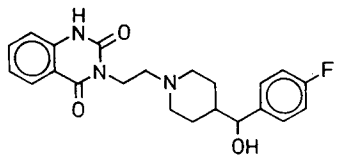
Chemicals and reagents

Ketanserin (R-41,468, 3-{2-[4-(4-fluorobenzoyl)-1-piperidinyl]ethyl}-2,4(1H,3H)-quinazoline dione), ketanserinol (reduced ketanserin, R-46,742, 3-{2-[4-(4-fluorobenzoyl)hydroxymethyl-1-piperidinyl]ethyl}2,4(1H,3H)-quinazoline dione) and the internal standard (R-46,594, 3-{2-[4-(4-chlorobenzoyl)-1-piperidinyl]ethyl}2,4(1H,3H)-quinazoline dione) were provided as reference compounds by Janssen Pharmaceutica, Beerse, Belgium (see Fig. 1 for structures).

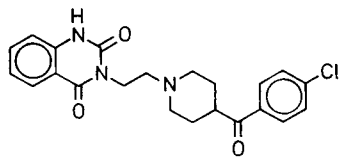
Isoamyl alcohol and pentane in nanograde quality, acetonitrile and methanol in ChromAr quality were purchased from Promochem (Wesel, F.R.G.). Ammonium acetate, acetic acid and all other reagents were obtained from Merck (Darmstadt, F.R.G.). Buffer solutions were prepared in doubly distilled water.



KETANSERIN



KETANSERINOL



INTERNAL STANDARD

Fig. 1. Structures of ketanserin, ketanserinol and internal standard.

Apparatus

UV and fluorescence spectra were recorded on a Varian SuperScan 3 and a Perkin-Elmer 3000 fluorescence spectrometer, respectively. Liquid chromatography was performed on a Spectra-Physics solvent delivery system equipped with a Perkin-Elmer 3000 fluorescence detector and a Spectra-Physics SP 4200

integrator. At pH 5.2 the absorption and emission wavelengths were set at 310 and 370 nm, respectively, at excitation and emission slit widths of 10 μm. The computing integrator linked to the fluorescence detector was operated at a chart speed of 0.25 cm/min. Chromatography columns (25 cm × 4 mm I.D.) packed with Nucleosil 5-μm particles (Macherey, Nagel & Co., Düren, F.R.G.) were kept in an oven compartment at 32°C. A precolumn, 5 cm long, containing the same packing material was purchased from Knauer (Berlin, F.R.G.). The mobile phase consisted of 2% acetic acid—0.17 *M*/l ammonium acetate—acetonitrile (50:10:40). The solvent pump was operated at a flow-rate of 1.2 ml/min and a pressure of 250 atm.

Sample preparation

Standard solutions of ketanserin, ketanserinol and the internal standard in methanol were stored at 6°C. The stability under these conditions was demonstrated for a period of three months.

Patient control plasma was obtained prior to clinical trials. Following disproof of concomitant medication, blank samples were spiked with 1, 2, 5, 10 or 20 ng/ml ketanserin and ketanserinol. A stock solution of 100 μg/l internal standard was prepared, and 300 μl of this solution were added to 1 ml of each plasma sample. Following centrifugation and alkalinization with 1 ml of 0.05 *M* sodium hydroxide solution (pH 12), 4 ml of the extraction mixture (pentane—isoamyl alcohol, 95:5) were added, rotated for 10 min and centrifuged for 5 min. After removal of the organic layer the extraction procedure was repeated. The combined organic layers were evaporated to dryness in conical tubes under pre-cleaned nitrogen in a water bath at 50°C. The residue was dissolved in 100 μl of acetonitrile and injected into the column.

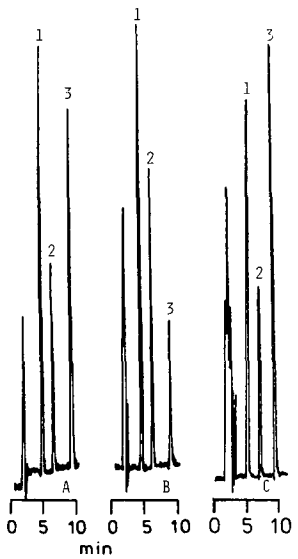
Patient plasma samples containing more than 40 ng/ml ketanserin or ketanserinol were diluted 1:10 with blank plasma. Urine samples of 0.2 ml were treated with a five-fold amount of 0.05 *M* sodium hydroxide solution. The alkalinized urine specimens (pH 12) were extracted twice with 4 ml of pentane—isoamyl alcohol (95:5) and subsequently treated in the same manner as described above.

Peak detection and assignment

Qualitative and quantitative detection of ketanserin, ketanserinol and the internal standard was established by spiking blank plasma with known amounts of these compounds and subsequent extraction using the described procedure. Recovery rates were 92% for ketanserin and 70% for ketanserinol and proved to be concentration-independent. Retention times and retention volumes under these conditions were 4.3 min (5.16 ml) for ketanserinol, 5.8 min (6.96 ml) for ketanserin and 8.9 min (10.68 ml) for the internal standard (Fig. 2).

The detection limits were 200 pg/ml for ketanserin and 100 pg/ml for ketanserinol.

Calibration curves were constructed using spiked plasma containing 1, 2, 5, 10 and 20 ng/ml of each compound. Detector responses calculated from integrated peak areas correlated in a linear fashion with the added amounts up to 40 ng/ml.



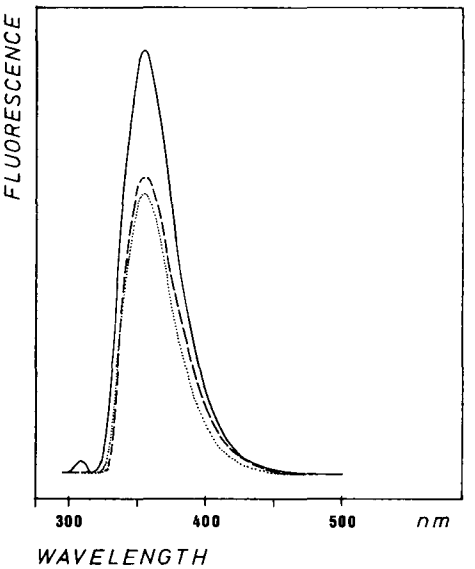


Fig. 2. Representative chromatograms of ketanserin, ketanserinol and internal standard. (A) Standard mixture of 3 ng of ketanserinol, 3 ng of ketanserin and 20 ng of internal standard; (B) human blank plasma sample spiked with 5 ng of ketanserinol, 5 ng of ketanserin and 5 ng of internal standard; (C) plasma sample from subject 10 h after ingestion of 40 mg of ketanserin (internal standard added). Peaks: 1 = ketanserinol; 2 = ketanserin; 3 = internal standard.

Fig. 3. Emission spectra of ketanserin (——), ketanserinol (- - -) and internal standard (· · · ·) recorded in mobile phase (absorption wavelength: 310 nm).

RESULTS AND DISCUSSION

Fluorescence spectra were recorded under the described conditions. Fig. 3 shows emission spectra of ketanserin, ketanserinol and the internal standard with maximum intensity at 370 and 371 nm, respectively. Before employing acetic acid, various ion-pairing reagents with different lipophilicity were tested. However, retention times using these reagents ranged up to 40 min.

At basic pH values of the mobile phase, e.g. according to a method previously described [7], ketanserin and related compounds can be resolved on reversed-phase columns at pH values above 10 as undissociated bases. Applying this method RP-18 chromatography columns survive less than 40 h of operation. Furthermore, the valves and fittings of regular chromatographs are not suitable for continuous alkaline media.

UV absorbance between 210 and 254 nm yields minimum detection limits of 2 ng/ml [8—11]. Following single oral administration of ketanserin a slow terminal elimination phase could be demonstrated requiring measurement of concentrations between 5 and 0.2 ng of the parent compound [12]. The accurate measurement of the low drug concentrations during elimination periods is a prerequisite for estimation of drug accumulation under chronic treatment. In order to detect these levels fluorescence spectroscopy is the most suitable technique for ketanserin and its metabolite. Amounts and detector responses correlated over the range 0.2—40 ng in a linear fasion. The described technique proved to be twice as

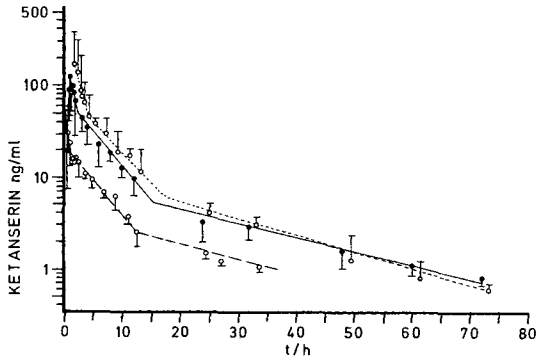


Fig. 4. Ketanserin plasma levels obtained from an elderly subject, who received 40 mg of ketanserin as an oral solution (upper curve), 40 mg of ketanserin as tablets (middle curve) and 10 mg of ketanserin as an intravenous bolus (lower curve).

sensitive for ketanserinol compared to its parent compound. Similar detector responses were found with equal molar concentrations of the internal standard and ketanserin. The following linear equations served as calibration curves: $y = 0.0495x + 0.00424$ (ketanserin) and $y = 0.0748x + 0.0554$ (ketanserinol), where x = amount per ml and y = ratio of detector responses of ketanserin(ol) to internal standard. The regression coefficient of both calibration lines was over 0.999. Reproducibility of the method tested by the intra-assay coefficient of variation at concentrations of 5 ng each amounted to 3.3% for ketanserin and 4.2% for ketanserinol.

The method was applied to plasma and urine specimens obtained from a pharmacokinetic study in elderly subjects (Fig. 4) [5]. All parameters (including materials, column and apparatus, except buffer solutions) remained stable for more than three months (equivalent to over 4000 determinations).

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REFERENCES

1 J. De Cree, J. Leempoels, W. De Cock, H. Geukens and H. Verhaegen, Angiology, 32 (1981) 137.
2 T. Hedner, B. Persson and G. Berglund, Brit. J. Clin. Pharmacol., 16 (1983) 121.
3 G.J. Wenting, A.J. Man In't Veld, A.J. Woittiez, F. Boomsma and M.A.D.H. Schalekamp, Brit. Med. J., 284 (1982) 537.
4 J. De Cree, H. Verhaegen and J. Symoens, Lancet, i (1981) 1161.
5 M. Kurowski, Eur. J. Clin. Pharmacol., 28 (1985) in press.
6 D. Trenk, A. Mosler, W. Kirch, T. Meinertz and E. Jähnchen, J. Cardiovasc. Pharmacol., 5 (1983) 1034.
7 P.O. Okonkwo, I.W. Reimann, R. Woestenborghs and U. Klotz, J. Chromatogr., 272 (1983) 411.
8 V. Simon and P. Somani, J. Chromatogr., 232 (1982) 186.
9 C.L. Davies, J. Chromatogr., 275 (1983) 232.
10 F. Lindelauf, J. Chromatogr., 277 (1983) 396.
11 A.T. Kacprowicz, P.G. Shaw, R.F.W. Moulds and R.W. Bury, J. Chromatogr., 272 (1983) 417.
12 I.W. Reimann, P.O. Okonkwo and U. Kotz, Eru. J. Clin. Pharmacol., 25 (1983) 73.

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Note

Separation of the enantiomers of intact sulfate conjugates of adrenergic drugs by high-performance liquid chromatography after chiral derivatization

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A recent study on the stereochemistry of propranolol metabolism in man [1] suggested that sulfate conjugation of its main active metabolite, 4′-hydroxypropranolol (HOP), to 4′-hydroxypropranolol sulfate (HOPS, Fig. 1A) is stereoselective. This was assessed by isolation and enzymatic hydrolysis of HOPS and determination of the enantiomeric composition of HOP either by gas chromatography—mass spectrometry using a stable isotope approach [1] or by silica gel high-performance liquid chromatography (HPLC), the latter after chiral derivatization with (+)-1-phenylethyl isocyanate (PEI) [2]. Enzymatic hydrolysis of HOPS to the unstable HOP was, however, a tedious and at low levels unpredictable procedure. For studies of the biochemical mechanism(s) of the stereoselective sulfoconjugation of HOP as well as of other adrenergic drugs, separation and quantitation of the enantiomers of intact sulfate conjugates is essential.

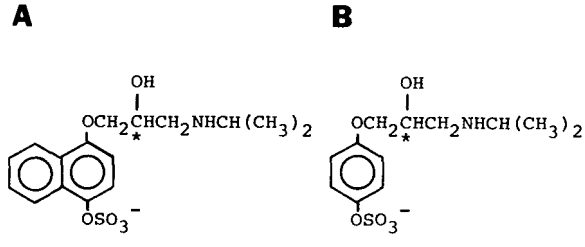

Fig. 1. Chemical structures of (A) 4′-hydroxypropranolol sulfate (HOPS) and (B) prenalterol sulfate.

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Chiral derivatization with PEI failed to give adequate separation of the HOPS enantiomers both on silica gel and reversed-phase HPLC. In this paper we describe the chiral derivatization of intact HOPS with 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) and separation of the resulting diastereomers by reversed-phase HPLC. This reagent has been shown to separate the enantiomers of amino acids [3, 4], catecholamines [5] and several classes of basic drugs [6—8]. This method was shown to be useful for plasma determinations of the enantiomers of HOPS in man during chronic propranolol therapy. Application to prenalterol sulfate (Fig. 1B), a conjugate of a selective β_1-receptor agonist [9], suggests that this derivatization technique may be useful for the separation of the enantiomers of intact sulfate conjugates of many other adrenergic drugs.

MATERIALS AND METHODS

Materials

GITC was synthesized from α-acetobromoglucose (Sigma, St. Louis, MO, U.S.A.) and silver thiocyanate (Pfaltz and Bauer, Stamford, CT, U.S.A.) [3]. The reagent, a white crystalline solid, was stable for months when stored refrigerated in a desiccator. HOP hydrochloride was prepared as previously described [10] as was HOPS [11]. Prenalterol sulfate was a gift from Hässle (Mölndal, Sweden). All solvents were glass-distilled (Burdick & Jackson Labs., Muskegon, MI, U.S.A.). Triethylamine was purchased from Aldrich (Milwaukee, WI, U.S.A.).

Chiral derivatization

Methanol solutions of synthetic HOPS and prenalterol sulfate, 0.1—5 μg, were evaporated to dryness under nitrogen. A 50-μl volume of 0.4% triethylamine in acetonitrile—water (50:50) and 50 μl of 0.06 *M* GITC in acetonitrile were added to the dried samples. After brief mixing, the capped tube was left at room temperature for 5—10 min. Samples of the reaction mixture were injected into the chromatographic column.

Chromatographic conditions

The HPLC column (25 cm × 4.6 mm) was a 5-μm Spherisorb ODS from Alltech Assoc. (Deerfield, IL, U.S.A.). The mobile phase was acetonitrile—methanol—water—acetic acid (35:5:59:1 for HOPS and 30:5:64:1 for prenalterol sulfate) in 0.05 *M* ammonium acetate (pH 4) at a flow-rate of 1 ml/min.

The HPLC system consisted of a Model 6000 high-pressure pump, a Model U6K injector and a Model 440 UV detector with a 313-nm filter from Waters Assoc. (Milford, MA, U.S.A.).

Isolation of HOPS from plasma

Plasma samples (2 ml) from a hypertensive patient, treated with 80 mg propranolol (Inderal) every 6 h, were extracted for HOPS by an ion-pair procedure (chloroform and tetrabutylammonium at pH 12) [12]. HOPS isolated this way (100% extraction) was purified by reversed-phase HPLC using acetonitrile—water (15:85) in 0.01 *M* ammonium acetate (pH 6.5) as

the mobile phase [12]. The peak corresponding to HOPS was collected and after freeze-drying derivatized with GITC as above.

RESULTS AND DISCUSSION

The chiral derivatization of HOPS with GITC was performed in acetonitrile—water (75:25) with 0.2% triethylamine. The presence of both water, to increase the solubility of HOPS, and triethylamine, as a base catalyst, were essential for a rapid and quantitative reaction. These conditions were identical to those used for the derivatization of non-esterified amino acids with GITC [4]. The reaction of GITC with the secondary amino group, resulting in a thiourea derivative, was complete in 5 min with HOPS as a substrate.

A reversed-phase HPLC profile, demonstrating the separation of the (+)- and (—)-enantiomers of synthetic HOPS after chiral derivatization with GITC, is shown in Fig. 2A. It shows complete baseline resolution (R = 2.1) between the enantiomers and an excellent peak symmetry. The order of elution of the enantiomers was established with HOPS isolated from urine of dogs dosed with either (+)- or (—)-propranolol. The peak area ratio (—)-HOPS/(+)-HOPS for the synthetic standard compound was 1.01 ± 0.01 (mean ± S.D.; n = 15). The derivative was stable for at least 24 h.

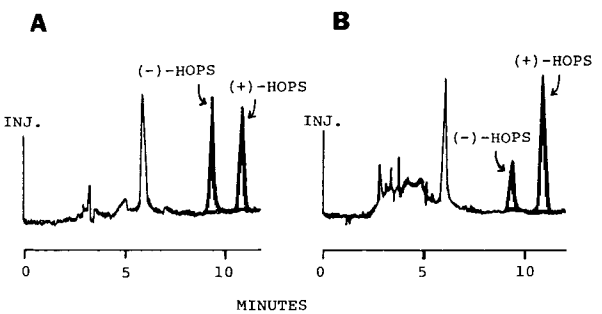

Fig. 2. Reversed-phase HPLC of GITC diastereomer derivatives of (A) synthetic HOPS (250 ng of each enantiomer) and (B) HOPS isolated from human plasma [(—)-HOPS 124 ng/ml and (+)-HOPS 373 ng/ml] at 0.05 a.u.f.s.

The minimum detectable amount of each enantiomer, using 313-nm detection, was about 20 ng. This should, however, be improved by fluorimetric detection. Standard curves (without internal standard) were linear over the range 100—5000 ng derivatized HOPS (correlation coefficients > 0.997). The coefficient of variation at the 1000-ng level was about 5%. The inter-assay variability in slope was less than 7%.

The method was used to determine the plasma concentrations of the enantiomers of HOPS in a patient on chronic therapy with propranolol, 80 mg every 6 h, Fig. 2B. At 2 h after the last propranolol dose the concentration of (+)-HOPS, 373 ng/ml, exceeded that of (—)-HOPS, 124 ng/ml. These concentrations together significantly exceeded the concentration of racemic propranolol measured at the same time, which was 255 ng/ml. The (—)-HOPS/(+)-HOPS ratio of 0.33 is similar to previous findings in urine in man [1, 2]. The method has also been applied to in vitro studies of this conjugation reaction [13].

This chiral derivatization technique was also applied to prenalterol sulfate. The GITC reaction was rapid and quantitative also for this conjugate. The content of acetonitrile in the mobile phase had to be decreased from 35% to 30% to achieve retention volumes similar to those of the HOPS enantiomers, i.e. 8.5 and 10 min. Baseline separation was achieved (R = 2.0). The minimum detectable amount of each enantiomer was 125 ng at 313 nm and 40 ng at 280 nm.

CONCLUSIONS

A method has been described for the separation and quantification of the intact HOPS enantiomers using chiral derivatization with GITC and reversed-phase HPLC. The method is rapid, sensitive and highly reproducible and should be well suited for in vitro studies of the biochemical mechanisms underlying stereoselective sulfation. It should also be suitable for kinetic analyses of the HOPS enantiomers in man, although simplification of the prepurification step would be desirable. Application of this procedure to the separation of the enantiomers of other adrenergic drug sulfates appears feasible.

ACKNOWLEDGEMENTS

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REFERENCES

1 T. Walle, U.K. Walle, M.J. Wilson, T.C. Fagan and T.E. Gaffney, Brit. J. Clin. Pharmacol., 18 (1974) 741.
2 M.J. Wilson and T. Walle, J. Chromatogr., 310 (1984) 424.
3 N. Nimura, H. Ogura and T. Kinoshita, J. Chromatogr., 202 (1980) 375.
4 T. Kinoshita, Y. Kasahara and N. Nimura, J. Chromatogr., 210 (1981) 77.
5 N. Nimura, Y. Kasahara and T. Kinoshita, J. Chromatogr., 213 (1981) 327.
6 A.J. Sedman and J. Gal, J. Chromatogr., 278 (1983) 199.
7 J. Gal, J. Chromatogr., 307 (1984) 220.
8 K.J. Miller, J. Gal and M.M. Ames, J. Chromatogr., 307 (1984) 335.
9 K.-J. Hoffmann, A. Arfwidsson and K.O. Borg, Drug Metab. Dispos., 10 (1982) 173.
10 J.E. Oatis, Jr., M.P. Russell, D.R. Knapp and T. Walle, J. Med. Chem., 24 (1981) 309.
11 J.E. Oatis, Jr., T. Walle, H.B. Daniell, T.E. Gaffney and D.R. Knapp, J. Med. Chem., in press.
12 K.H. Wingstrand and T. Walle, J. Chromatogr., 305 (1984) 250.
13 D.D. Christ and T. Walle, Drug Metab. Dispos., in press.

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Note

High-performance liquid chromatographic method for the determination of bromodeoxyuridine and its major metabolite, bromouracil, in biological fluids

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Administration of the halogenated nucleoside, 5-bromodeoxyuridine (BUDR) to mammalian systems is followed by its incorporation into DNA resulting in an increased susceptibility of mammalian cells to the lethal effects of x-irradiation [1—4]. Because tumor cells generally have higher turnover and therefore a higher DNA synthesis rate than the surrounding normal tissues, this radiosensitization effect of BUDR makes it a useful agent in the treatment of neoplastic disorders.

Several clinical trials have been conducted to evaluate the efficacy of BUDR [5—8]. The initial human study utilized ^{82}Br-radiolabeled BUDR and demonstrated the drug's rapid plasma clearance. Subsequent trials [6, 7] using continuous intracarotid arterial infusions of BUDR in the treatment of gliomas did not include drug level data. In a recent study [8] BUDR was administered by intravenous infusion for 12 h per day for fourteen days. Plasma BUDR

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levels were determined using a high-performance liquid chromatographic (HPLC) method which necessitated washing the column with methanol following each injection.

This paper describes a rapid, accurate and sensitive HPLC procedure for the simultaneous measurement of BUDRand bromouracil (BU) in plasma and urine samples.

MATERIALS AND METHODS

Reagents

Ethyl acetate and methanol were purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.), monobasic ammonium phosphate from J.T. Baker (Phillipsburg, NJ, U.S.A.), potassium hydroxide and ammonium sulfate from Mallinckrodt (St. Louis, MO, U.S.A.), BUDR, bromouracil and iodouracil from Sigma (St. Louis, MO, U.S.A.).

Stock solutions

Bromodeoxyuridine solution (0.5 mg/ml in methanol), bromouracil solution (0.5 mg/ml in methanol) and iodouracil (IU) solution (1.0 mg/ml in methanol) were prepared and stored at −20°C. Saturated ammonium sulfate solution and 0.5 *M* potassium hydroxide solution were prepared and stored at room temperature.

Standards

BUDR and BU stock solutions were mixed 1:1 and diluted with water to yield the first BUDR+BU standard solution (standard I = 100 μg/ml of each). Standard I was further diluted to 10 μg/ml of each to give the second standard solution (standard II). Iodouracil stock solution was diluted with water to yield the internal standard solution containing 10 μg/ml IU.

Quality control samples at three concentrations (200, 800 and 1600 ng/ml each of BUDR and BU) were prepared by spiking blank plasma with the appropriate volumes of the BUDR+BU standards. After mixing, 1.0-ml aliquots were transferred to glass tubes, tightly capped, and stored frozen at −30°C. Two quality control samples of each concentration were included with every group of experimental samples to be analyzed.

Chromatographic conditions

The HPLC unit included a Waters Assoc. Model 6000A pump, a Waters Assoc. Model 440 UV/VIS absorbance detector with a 280-nm filter, a Waters Assoc. Model 710B WISP autosampler and a Hewlett-Packard Model 3390A integrator. The column used was a Dupont Zorbax C_8 reversed-phase column (25 cm × 4.6 mm I.D., spherical, 6 μm particle size). The mobile phase was a 0.05 *M* ammonium phosphate buffer (pH = 7.3)—methanol mixture with a final methanol concentration of 12%. The flow-rate was set at 1.0 ml/min.

Under these conditions the retention times of BU, IU and BUDR were 5.1, 7.0 and 8.0 min, respectively. Fig. 1 depicts the chemical structures of BU, BUDR and the internal standard, IU.

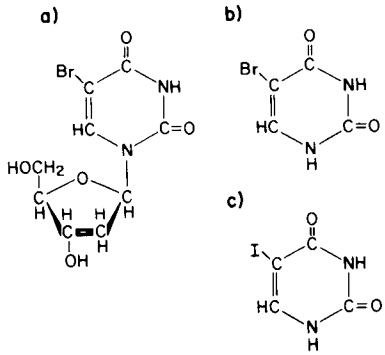

Fig. 1. Chemical structures of (a) 5-bromodeoxyuridine (BUDR), (b) 5-bromouracil (BU) and (c) 5-iodouracil (IU), the internal standard.

Sample preparation

To glass tubes (15 ml capacity) were added 1.0 ml plasma, 100 μl internal standard solution (1000 ng IU), 2.0 ml saturated ammonium sulfate solution, 100 μl ammonium phosphate buffer, pH 6.7, and 8.0 ml ethyl acetate. The tubes were tightly capped and shaken for 15 min at room temperature. In unknown plasma samples where drug concentrations were expected to exceed 2000 ng/ml, a smaller volume was utilized with the difference to 1.0 ml made up with an appropriate volume of blank plasma. After centrifugation (room temperature) for 10 min at 1200 *g*, the ethyl acetate phase was transferred to clean conical-bottomed glass tubes and concentrated to approximately 1.0 ml by evaporation in a waterbath under a stream of air. To each sample were added 400 μl of 0.5 *M* potassium hydroxide and the drugs and internal standard back-extracted by shaking for 15 min at room temperature. After centrifugation (room temperature) at 1000 *g* for 10 min, the ethyl acetate phase was removed by aspiration. A 3.0—30.0 μl aliquot of the alkaline aqueous phase was injected for HPLC analysis. The analysis of urine samples was conducted in an identical fashion.

Standard calibration curve

Blank plasma samples were spiked in duplicate with the appropriate volumes of BUDR+BU standard solutions I or II to concentrations ranging from 0 to 2000 ng/ml and subjected to the sample preparation procedure described above. Calibration curves were run with each set of experimental samples.

Calculations

Calibration curves were constructed by plotting the ratio of the peak height of each drug to that of the internal standard as a function of the plasma drug concentration. The best-fit straight line was determined using the method of least squares. The BU and BUDR concentrations of unknown samples were calculated from the least-squares regression line of the calibration curve.

RESULTS AND DISCUSSION

Under the described conditions, the retention times of BU, internal standard

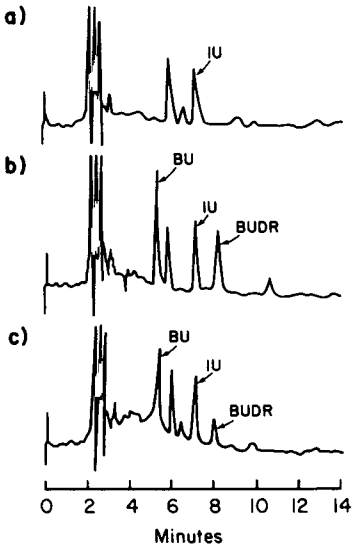

Fig. 2. Typical chromatograms of (a) control plasma, (b) a quality control sample containing 800 ng/ml BU and 800 ng/ml BUDR in plasma and (c) a plasma sample obtained from a dog 15 min post-administration of 4.0 mg/kg BUDR as an intravenous bolus injection. Internal standard (IU) was added to each sample.

and BUDR were 5.1, 7.0 and 8.0 min, respectively. Fig. 2 illustrates typical chromatograms for control plasma, a quality control plasma sample containing 800 ng/ml BU and 800 ng/ml BUDR, and a plasma sample obtained from a dog 15 min post-administration of 4.0 mg/kg BUDR as an intravenous bolus injection. The plasma samples elicit no interfering peaks. The total analysis time required for each run was 15 min. The validity of the assay procedure was established through a careful study of the linearity of response, reproducibility, accuracy and precision.

The peak height ratio was directly proportional to the BU and BUDR concentrations over a range of 50—2000 ng/ml and 100—2000 ng/ml, respectively. The best-fit lines were obtained using linear regression analyses. The results of the regression analyses for BU and BUDR were: $y = 0.00184x + 0.0171$ ($r = 0.9980$) and $y = 0.00105x + 0.094$ ($r = 0.9940$), respectively.

The accuracy and precision of the method were assessed by seeding plasma at BU and BUDR concentrations of 200, 800 and 1600 ng/ml of each. Triplicate quality control samples at each concentration were assayed on each of three consecutive days. Table I gives the results of this experiment. The precision of the assay was found to have coefficients of variation (C.V., %) ranging from 6.2% to 10.6% and 5.8% to 10.3% for BU and BUDR, respectively. The concentration means for the seeded control samples were found to be with −2.2% to +0.4% and −12.2% to −4.8% of the theoretical values for BU and BUDR, respectively.

Application of the method developed was demonstrated by measuring the plasma BU and BUDR levels in a dog following the intravenous bolus administration of a 4.0 mg/kg BUDR dose. Peripheral venous blood samples were withdrawn at regular intervals and the plasma obtained was used for drug

TABLE I

BROMOURACIL AND BROMODEOXYURIDINE CONCENTRATIONS IN SEEDED CONTROL SAMPLES ASSAYED OVER A THREE-DAY PERIOD

Day	Concentration (ng/ml)					
	Bromouracil			Bromodeoxyuridine		
1	213.2	809.8	1511.1	191.6	770.3	1455.3
	197.5	784.7	1517.6	163.1	737.6	1499.7
	204.4	739.6	1406.3	162.1	692.5	1382.8
2	216.2	802.2	1628.8	190.9	702.6	1453.1
	221.3	860.6	1721.5	190.9	929.8	1604.5
	221.3	806.2	1705.4	185.0	699.2	1656.0
3	173.2	820.7	1576.0	163.8	831.4	1618.2
	199.2	718.8	1571.0	183.6	737.8	1510.2
	160.8	699.4	1576.0	149.6	716.1	1534.5
Mean	200.8	782.4	1579.3	175.6	757.5	1523.8
S.D.	21.3	52.5	98.1	15.9	77.8	89.0
C.V. (%)	10.6	6.7	6.2	9.1	10.3	5.8
Percent from theoretical	+0.4	−2.2	−1.3	−12.2	−5.3	−4.8

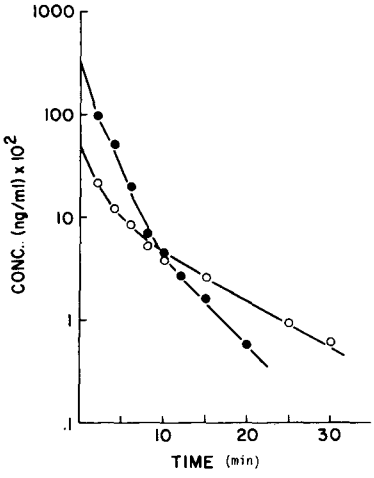

Fig. 3. The plasma BUDR and BU concentration—time profiles in a dog following the intravenous bolus administration of 4.0 mg/kg BUDR. (•) BUDR; (○) BU.

and metabolite analysis. Fig. 3 illustrates the plasma BUDR and BU concentrations versus time plots from this experiment. The BUDR data are well described by the function:

$$C_{BUDR} = 1539e^{-0.164t} + 28728e^{-0.541t}$$

The distribution half-life ($t_{½\alpha}$) of BUDR was 1.28 min and the elimination half-life ($t_{½\beta}$) was 4.23 min. The BU data are also well described by a biexponential function:

$$C_{BU} = 3472e^{-0.560t} + 1336e^{-0.105t}$$

Here, the $t_{½\alpha}$ of BU was 1.24 min and the $t_{½\beta}$ was 6.6 min.

REFERENCES

1 B. Djordjevic and W. Szybalski, J. Exp. Med., 112 (1960) 509.
2 W. Szybalski and B. Djordjevic, Genetics, 44 (1959) 540.
3 M.T. Hakala, J. Biol. Chem., 234 (1958) 3072.
4 W.C. Mahler and M.D. Elkind, Exp. Cell Res., 30 (1963) 481.
5 J.P. Kriss, Y. Maruyama, L.A. Tung, S.B. Bond and L. Revesz, Cancer Res., 23 (1963) 260.
6 K. Sano, T. Hoshino and M. Hagai, J. Neurosurg., 28 (1968) 530.
7 M.A. Bagshaw, R.L.S. Doggett, K.C. Smith, H.S. Kaplan and T.S. Nelsen, Radiology, 99 (1967) 886.
8 A. Russo, L. Gianni, T.J. Kinsella, R.W. Klecker, J. Jenkins, J. Rowland, E. Glatstein, J.B. Mitchell, J. Collins and C. Myers, Cancer Res., 44 (1984) 1702.

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Note

Measurement of meptazinol in plasma by high-performance liquid chromatography with electrochemical detection

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(First received September 5th, 1984; revised manuscript received January 9th, 1985)

Meptazinol, an effective analgesic agent for the treatment of moderate pain, is now available in both oral and parenteral formulations. Previous methods for the measurement of this drug in plasma have used the techniques of gas—liquid chromatography with flame ionisation detection [1] and high-performance liquid chromatography (HPLC) with fluorescence detection [2]. The method of Rosseel et al. [1] was not sufficiently sensitive for the measurement of therapeutic plasma concentrations, with a lower limit of detection of 30 μg/l. The method of Frost [2] improves upon this with a lower limit of detection of 3 μg/l from a 1-ml plasma sample. However, the sensitivity of detection of meptazinol by fluorescence is restricted by the similarity in the excitation (282 nm) and emission (300 nm) wavelengths. Sensitivity is further reduced if a high-resolution detector is not available.

This paper describes a method using the technique of electrochemical detection following HPLC separation with the advantages of increased sensitivity and general availability, a simple sample preparation procedure and a reduced sample volume requirement over previously published methods.

EXPERIMENTAL

Materials and reagents

Meptazinol [*m*-(3-ethyl-1-methylhexahydro-1H-azepin-3-yl] phenol (Fig. 1A) was obtained from Wyeth Labs. (Taplow, U.K.). The internal standard used was a 40 μg/l solution of fenethazine (supplied by Rhone-Poulenc, France) (Fig. 1B) in 2 *M* aqueous tris(hydroxymethyl)aminomethane (analytical-reagent grade) solution. Methanol was HPLC grade and the glacial acetic acid and ammonia solution (SG 0.88) were analytical-reagent grade.

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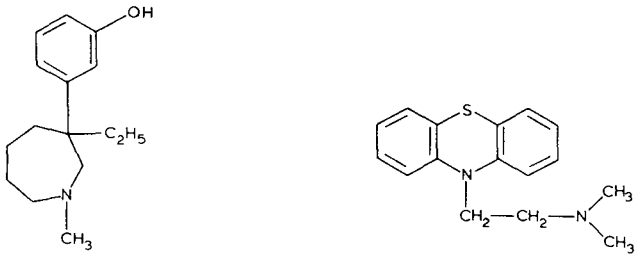

(A) (B)

Fig. 1. Structural formulae of (A) meptazinol and (B) fenethazine, internal standard.

High-performance liquid chromatography

The solvent delivery system was a single, high-speed piston pulseless pump (Applied Chromatography Systems, Model 300/01/02) and sample injection was performed using a Rheodyne Model 7120 syringe-loading valve fitted with a 100-μl sample loop. Stainless-steel tubing (0.25 mm I.D.) was used to connect the outlet of the valve to the analytical column, a stainless-steel tube 125 mm × 5 mm I.D. packed with 5-μm silica (Spherisorb S5W: Hichrom, Woodley, U.K.), which was used at ambient temperature (normally 22°C). Detection was by electrochemical oxidation using a glassy carbon electrode LCA 15 (EDT Research, London, U.K.) in a flow cell and at a potential of +1.2 V applied against a silver/silver chloride reference electrode. The mobile phase was methanol—acetic acid—ammonia solution (996:3:1). The flow-rate was 2 ml/min.

Sample preparation

Plasma (200 μl) was pipetted into a small glass (Dreyer) test tube. Internal standard solution (100 μl) and methyl *tert.*-butyl ether (200 μl) were added using Hamilton repeating mechanisms fitted with 5-ml Hamilton gas-tight Luer fitting glass syringes. The contents of the tube were vortex-mixed for 30 sec and the tube was centrifuged at 9950 *g* for 2 min. A portion (ca. 110 μl) of the organic phase was taken to fill the sample loop of the injection valve.

To increase sensitivity where sufficient sample was available, plasma (1 ml) was pipetted into a 5-ml stoppered polypropylene tube (Elkay Products, Shrewsbury, MA, U.S.A.). Internal standard solution (300 μl) and methyl *tert.*-butyl ether (300 μl) were added. The contents of the capped tube were vortex-mixed (30 sec) after which the tube was centrifuged at 3000 *g* for 5 min. The organic phase was transferred to a Dreyer tube and recentrifuged at 9950 *g* for 2 min. A portion (110 μl) of the extract was taken to fill the sample loop of the injection valve. Analyses were performed in duplicate and mean results taken.

Instrument calibration

Standard solutions containing meptazinol at concentrations of 5, 20, 50 and 100 μg/l were prepared in bovine plasma. On analysis of these solutions the ratio of the peak height of meptazinol to the peak height of the internal standard when plotted against meptazinol concentration was linear and passed through the origin of the graph.

RESULTS AND DISCUSSION

The chromatogram obtained on analysis of an extract of analyte-free human plasma (200 μl) is illustrated in Fig. 2A. Fig. 2B shows the chromatogram obtained on analysis of an extract of a plasma standard (1 ml) containing 1 μg/l meptazinol, and Fig. 2C shows the chromatogram obtained on analysis of an extract (200 μl) of a plasma sample from a patient 6 h after receiving a single 25-mg intramuscular dose of meptazinol.

The recovery of the sample preparation was constant and virtually complete at over 98% (n = 5). The intra-assay coefficients of variation (C.V.) measured

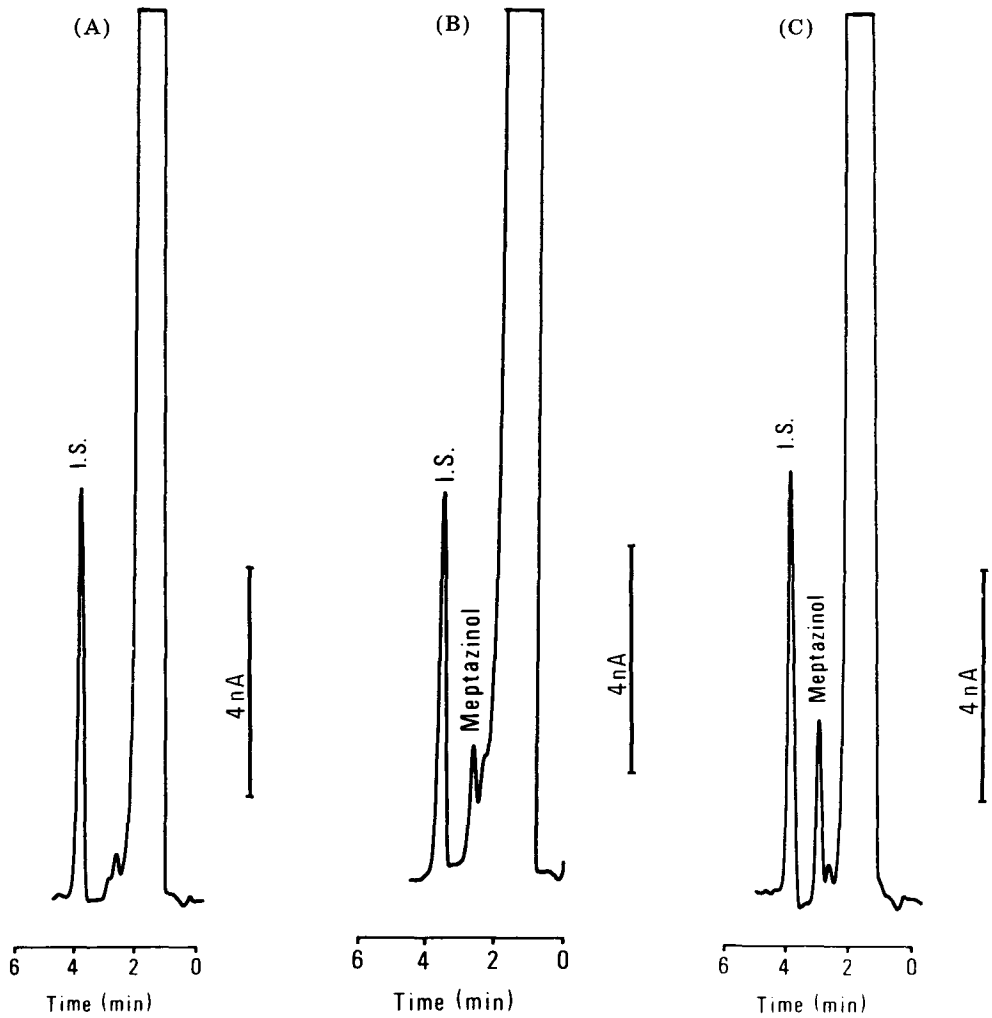

Fig. 2. Chromatograms obtained on analysis of an extract of (A) drug-free human plasma (200 μl); (B) drug-free human plasma (1 ml) containing meptazinol (1 μg/l); and (C) a plasma sample (200 μl) obtained from a patient 6 h after a 25-mg intramuscular dose of meptazinol. The concentration of meptazinol was 7 μg/l. I.S. = internal standard. For chromatographic conditions, see text.

TABLE I

INTRA-ASSAY REPRODUCIBILITY OF THE ASSAY

n = 10 at each concentration.

Meptazinol concentration (μg/l)	C.V. (%)
50	1.9
5	6.8
0.5	11.1

using three solutions prepared in bovine plasma are shown in Table I. The inter-assay C.V. measured from replicate analysis (n = 10) of a spiked solution of meptazinol (23 μg/l) prepared in bovine plasma was 8.0%. The lower limit of detection was 0.5 μg/l for meptazinol using a 1-ml sample.

No endogenous sources of interference have been found. The strong electrochemical response to the drug at a potential of +1.2 V in the organic mobile phase used in this method gives a significant improvement in sensitivity over previous methods. The use of straight-phase HPLC with non-aqueous ionic elements on silica columns for the analysis of basic drugs has been discussed in a previous publication [3]. Similarly, the technique of using highly efficient rapid micro-extraction of small sample volumes developed for HPLC [4] has been adapted and incorporated into this method.

This method has been used to measure the plasma concentration of meptazinol in man after intravenous and intramuscular doses for up to 24 h. Pharmacokinetic studies on meptazinol have shown a short elimination half-life

TABLE II

PLASMA CONCENTRATIONS OF MEPTAZINOL OBTAINED AFTER BOTH INTRAVENOUS (i.v.) AND INTRAMUSCULAR (i.m.) ADMINISTRATION OF 25 mg IN A VOLUNTEER SUBJECT

Time (h)	Concentration (μg/l)	
	25 mg i.v.	25 mg i.m.
0	ND*	ND
0.25	93	71
0.50	56	89
0.75	57	86
1	40	79
2	29	43
3	25	39
4	18	26
5	13	20
6	8	17
8	5	13
12	3	6
24	0.3	3

*ND = Not detected.

(of the order of 2 h) and a low oral bioavailability [5]. The present method has sufficient sensitivity to follow the elimination of meptazinol from plasma over a period of 24 h, as can be seen in Table II.

ACKNOWLEDGEMENTS

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REFERENCES

1 M.T. Rosseel, M.G. Bogaert, F.M. Belpaire and W. Oosterlinck, Curr. Med. Res. Opin., 3 (1975) 181.
2 T. Frost, Analyst, 106 (1981) 999.
3 R.J. Flanagan, G.C.A. Storey, R.K. Bhamra and I. Jane, J. Chromatogr., 247 (1982) 15.
4 R.J. Flanagan, G.C.A. Storey and D.W. Holt, J. Chromatogr., 187 (1980) 391.
5 R.A. Franklin, A. Aldridge and C.B. White, Brit. J. Clin. Pharmacol., 3 (1976) 497.

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Note

High-performance liquid chromatographic determination of tranilast in plasma

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Tranilast [N-(3,4-dimethoxycinnamoyl)anthranilic acid] is a new remedy for allergic diseases, which has been developed in Japan as a result of extensive drug design based on research into the antiallergic properties of some ingredients of nandin (*Nandina domestica*). Unlike the symptomatic drugs used so far, the mechanism of action of tranilast lies in the inhibition of release of chemical mediators and it approaches closer to the basic allergic reaction [1, 2]. Tranilast has been used for the treatment of bronchial asthma and is usually combined with theophylline and other antiasthmatic drugs.

We found that free levels of theophylline in plasma increased when given in combination with tranilast [3]. It is important to investigate the relationship between tranilast plasma levels and the variation in theophylline plasma levels. However, there are very few reports about the determination of tranilast in plasma [1]. In this paper we describe a simple and reproducible high-performance liquid chromatographic (HPLC) method using a reversed-phase column and UV detector.

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EXPERIMENTAL

Materials

Tranilast and N-cinnamoyl anthranilic acid were kindly supplied by Kissei, Matsumoto, Japan. All chemicals were of reagent grade and used without further purification.

Procedures

A 100-μl volume of methanol containing N-cinnamoyl anthranilic acid as an internal standard (25 μg/ml) were added to 100 μl of plasma in a test tube, and agitated with a vortex mixer. After centrifugation at 12 000 *g* for 5 min, 100 μl of the supernatant were centrifuged at 12 000 *g* for 5 min once again; 10 μl of the supernatant were injected into the HPLC system.

Chromatographic conditions

A liquid chromatograph (Hitachi 638-50) equipped with a multi-wavelength UV detector (Hitachi 635 M) was used. A reversed-phase column (Radial Pak C_{18}, 10 μm, 10 cm × 5 mm I.D.; Waters Assoc.) was used at room temperature. The mobile phase consisted of 0.01 *M* dipotassium hydrogen phosphate buffer—acetonitrile (4:3). Before mixing, the buffer was brought to pH 3.5 with phosphoric acid. The flow-rate was 1.5 ml/min. The wavelength was 280 nm at 0.04 a.u.f.s.

Calibration graph

Two plasma standard curves were generated over the range 0.5—10 μg/ml and 5—50 μg/ml tranilast. The ratios of the peak height of tranilast to that of N-cinnamoyl anthranilic acid (internal standard) were used to construct a calibration graph.

Monitoring of plasma concentrations

The experiment was performed on two healthy subjects each aged 32 years, weighing 63 and 80 kg. Tranilast (200 mg in capsule form, Rizaben; Kissei) were administered orally with 100 ml of tap water. Plasma samples were obtained just before the administration and at 0.5, 1, 2, 3, 4, 6, 8, 10 and 24 h after administration. Food and beverages were not restricted after administration.

RESULTS AND DISCUSSION

Selectivity

Fig. 1 shows the chromatogram of blank plasma, plasma sample spiked with 30 μg/ml tranilast, and a plasma sample at 3.0 h after the administration of tranilast to a healthy subject. Tranilast and internal standard were well separated from endogenous substances.

Two calibration curves of peak height ratio were linear with a correlation coefficient of 0.996 (0.5—10 μg/ml) and 0.997 (5—50 μg/ml). The coefficients of variation at 0.5, 5 and 23 μg/ml of plasma were 4.28% (n = 8), 2.43% (n = 10) and 1.31% (n = 15), respectively. The relative recovery of tranilast from

plasma containing 30 μg/ml was estimated by comparing it with the recovery from an aqueous sample (distilled water) and was found to be 99.3 ± 1.8% (mean ± S.D., n = 10). Plasma was spiked with tranilast by the same procedure as described for the calibration graph. The limit of sensitivity for quantitation was 0.5 μg/ml plasma.

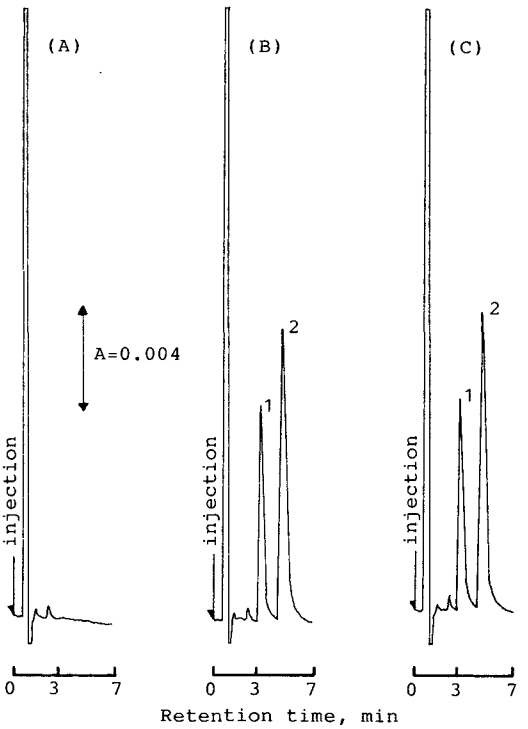

Fig. 1. High-performance liquid chromatograms of (A) blank plasma, (B) plasma sample spiked with 30 μg/ml tranilast and (C) a healthy subject. Peaks: 1 = tranilast, 2 = internal standard.

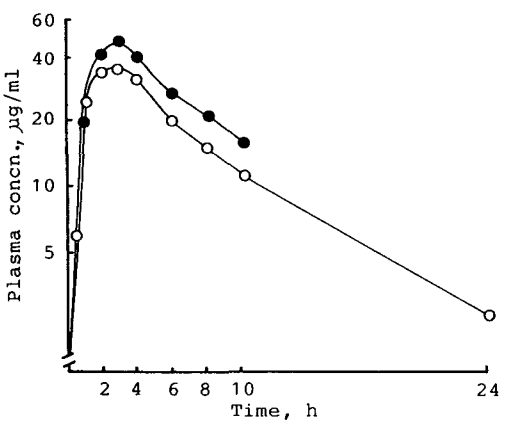

Fig. 2. Plasma concentration profiles of tranilast after oral administration of 200 mg of tranilast to two volunteers.

Plasma concentration profile

Plasma concentrations of tranilast were monitored using the newly developed assay method. The plasma concentration profiles are shown in Fig. 2. Peak plasma concentrations of tranilast were 48.0 and 34.4 μg/ml, and the elimination half-lives were 3.8 and 4.1 h, respectively.

It is possible to determine low plasma concentrations of tranilast rapidly, reproducibly and sensitively by the method described in this report. Our results suggest that the method is useful for both therapeutic drug monitoring and pharmacokinetic studies.

The effect of the concentration of tranilast in plasma on the variation of theophylline plasma levels is currently being examined.

REFERENCES

1 Y. Yui, Y. Yanagihara, H. Mita and T. Shida, Jap. J. Allergol., 28 (1979) 370.
2 M. Daikoku, T. Kondo and H. Motoya, Jap. J. Pediat., 31 (1978) 710.
3 K. Tadano, Y. Yuhki, K. Kokubun, I. Aoki and H. Niwa, Yakuzaigaku, 44 (1984) 121.

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Note

Quantitative analysis of vigabatrin in plasma and urine by reversed-phase high-performance liquid chromatography

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Vigabatrin (4-amino-5-hexenoic acid, GVG) is a catalytic inhibitor of brain γ-aminobutyric acid (GABA) transaminase [1]. Consequently, when GVG is administered to laboratory animals it produces an increase in brain GABA concentrations [2, 3]. This is believed to be effective in the treatment of neurological and psychiatric disorders associated with GABA deficiencies. Preliminary trials have proven this to be particularly true in cases of epilepsy and tardive dyskinesia [4, 5].

In order to carry out pharmacokinetic and bioavailability studies, it was necessary to develop a relatively simple quantitative analytical method for GVG in plasma and urine. A previous method illustrated the possibility of analyzing GVG by using an amino acid analyzer with microcolumns [6]. This is a time-consuming method requiring regeneration of the column after each analysis. Haegele and Schoun [7] have developed a gas chromatographic—mass spectrometric (GC—MS) method which uses a chiral capillary column for the analysis of the *R*- and *S*-enantiomers of GVG in plasma, urine and cerebrospinal fluid (CSF). The method is somewhat complex, requiring double derivatization and does not lend itself to a large number of samples at one time. However, it is highly selective and sensitive. Methods for analysis of GABA, which are potentially applicable to GVG, include ion-exchange high-performance liquid chromatography (HPLC) with post-column derivatization and fluorescence detection [8, 9]. These are sensitive methods, measuring picomoles of GABA in CSF, but requiring dedicated and somewhat complex instrumentation. Griesmann et al. [10] report a reversed-phase HPLC method for detection of Dns-derivatized GABA in brain tissue. The method involves a tedious series of

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reactions and extractions and does not emphasize accurate quantitation.

This paper describes a reversed-phase HPLC method for accurate and precise determination of GVG in plasma and urine. It functions on readily available equipment, permitting the analysis of many samples on a daily basis. The method is statistically evaluated and applied to a preliminary pharmacokinetic study in dogs.

EXPERIMENTAL

Reagents

Glass-distilled acetonitrile, dioxane, diethyl ether and ethyl acetate were purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.) Reagent-grade boric acid, sodium borate and phosphoric acid were purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.). Copper(II) chloride and Dns chloride were purchased from Aldrich (Milwaukee, WI, U.S.A.). Glass-distilled water was used in all aqueous preparations. GVG and γ-phenyl GABA (γ-aminobenzene-butanoic acid), the internal standard, were synthesized by Merrell Dow Research Institute (Cincinnati, OH, U.S.A.).

Instrumentation

Analyses were performed on an HPLC system consisting of Waters Assoc. (Milford, MA, U.S.A.) Model 6000A solvent delivery system and WISP Model 710B auto-injector. The fluorometric detector was a Kratos Model FS 970 (Schoeffel Instrument Division, Westwood, NJ, U.S.A.). The excitation wavelength was 345 nm and a 418-nm cut-off filter was used on the emission side. Chromatography was on a 25 cm × 4.6 mm I.D. DuPont (Wilmington, DE, U.S.A.) Zorbax C_8 column of 6 μm particle size. The mobile phase was acetonitrile—dioxane—0.5 *M* orthophosphoric acid (35:15:50) with a flow-rate of 1.0 ml/min at room temperature.

Standard solutions

A stock standard solution for the analysis of plasma samples was prepared by dissolving GVG in water (1 mg/ml). A 5-ml aliquot of this was diluted to 25 ml with either human or dog control plasma, making a working standard solution (200 μg/ml) from which six other standards ranging from 150 to 5 μg/ml were made by dilution.

The urine standards, varying in concentration from 4 to 0.02 mg/ml were prepared by dilution from a solution of 10 mg/ml of GVG in control urine.

γ-Phenyl GABA, the internal standard (I.S.), was prepared in a water solution at 0.4 mg/ml.

Plasma

Standard and sample plasma (100 μl) were placed in 100 × 13 mm screw-cap test tubes to which were added 20 μl of the internal standard solution. To this were added 200 μl of acetonitrile followed by 100 μl of 0.03 *M* copper chloride solution. After centrifugation at approximately 800 *g* for 15 min, the supernatant was transferred to a 100 × 13 mm screw-cap test tube. To this were added 200 μl of borate buffer (25 ml of 0.2 *M* boric acid plus 20 ml of 0.05 *M*

sodium borate diluted to 100 ml, pH = 8.45 ± 0.05) and 200 μl of Dns chloride solution (2 mg/ml in acetonitrile). The reaction mixture was vortexed for 5 sec and placed in a 50°C water bath for 15 min. After cooling to room temperature the reaction mixture was extracted with 1 ml of diethyl ether which was subsequently discarded. The aqueous phase was then extracted with 1 ml of ethyl acetate. The ethyl acetate was transferred to a 100 × 13 mm screw-cap test tube and washed with 1 ml of water. After transferring to a dry 100 × 13 mm test tube, the ethyl acetate was evaporated to dryness in a 35°C water bath under nitrogen. The residue was dissolved in 2—4 ml of mobile phase and 50—100 μl were injected on the HPLC column.

Urine

The urine analysis was performed on 10 μl of sample to which were added 100 μl of water, 20 μl of internal standard solution (0.4 mg/ml), followed by 200 μl of acetonitrile and 100 μl of 0.015 *M* copper chloride solution. The mixture was vortexed for 5 sec before the addition of 200 μl of borate buffer (50 ml of 0.4 *M* boric acid plus 20 ml of 0.125 *M* sodium borate diluted to 100 ml, pH = 8.05 ± 0.05) and 200 μl of Dns chloride solution (2 mg/ml in acetonitrile). The reaction mixture was heated at 50°C for 15 min in a water bath. The extraction procedure and ensuing sample preparation for HPLC injection were exactly the same as that described for plasma.

Calculation

Data were analyzed to give the peak area ratio of GVG to internal standard. Values for the samples were determined from the daily standard calibration curve which was calculated by linear regression.

RESULTS AND DISCUSSION

The copper(II) ion complexes with the endogenous α-amino acids [11] which prevents their Dns derivatization. This greatly enhances the specificity of the assay as well as simplifying chromatographic conditions, resulting in a relatively clean chromatogram. The Dns derivatization procedure was developed from conditions reported by Bayer et al. [12] and Tapuhi et al. [13].

Fig. 1A is a typical chromatogram of a plasma standard sample containing 40 μg/ml GVG and 80 μg/ml internal standard. The Dns derivative of GVG elutes at 8.8 min and the internal standard at 13.5 min. Fig. 1B shows the chromatogram of a blank plasma sample and indicates that the region of GVG is free from extraneous interference. The chromatograms of urine extracts are essentially identical to that of plasma. The only difference is that the baseline is flatter with fewer small peaks from endogenous material.

The method for plasma is linear over the concentration range 5—200 μg/ml with a minimum detectable limit of ca. 0.5 μg/ml. In urine GVG is linear from 20 to 4000 μg/ml with a minimum detectable limit of ca. 10 μg/ml.

For a validation of the analysis of GVG in plasma, twelve unknown samples at six different concentrations, making a total of seventy-two samples, were prepared. The samples were assayed in groups of twelve on six different days. Each group contained the six different concentrations in duplicate. They

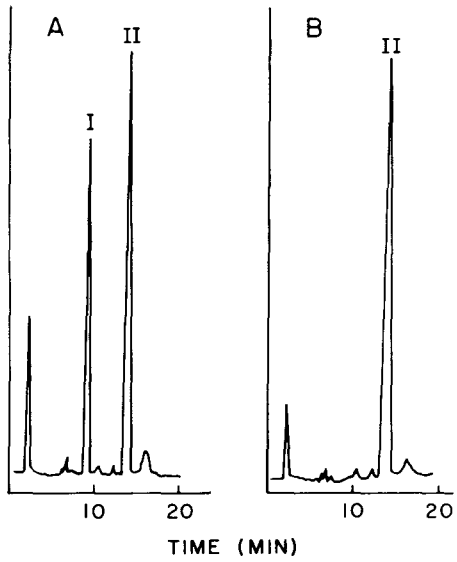

Fig. 1. HPLC profiles obtained from analysis of (A) a plasma sample containing approximately 40 μg/ml GVG and 80 μg/ml internal standard and (B) a blank plasma sample containing internal standard. Peaks: I = GVG; II = internal standard.

TABLE I

RESULTS OF VALIDATION STUDY FOR THE ANALYSIS OF VIGABATRIN IN PLASMA

Theoretical concentration (μg/ml)	Experimental concentration (mean ± S.D., n = 12) (μg/ml)	C.V. (%)
183.5	186.9 ± 5.9*	3.2
116.5	111.6 ± 7.4	6.6
68.0	65.7 ± 3.5	5.3
20.4	20.3 ± 1.5	7.5
4.08	4.1 ± 0.38	9.2
0	0	—

*n = 11.

were compared each day to two standard curves of eight points each including a blank. The mean value of the twelve determinations for each unknown ranged from 95.8% to 101.8% of the theoretical value. The coefficient of variation (C.V.) ranged from 3.2% for the highest concentration of 183.5 μg/ml to 9.2% for the lowest concentration of 4.08 μg/ml. These data are presented in Table I.

Since the urine samples are assayed by essentially the same procedure as the plasma samples, a comprehensive validation of the method was not repeated in urine. However, the mean correlation coefficient of five standard curves in urine run in duplicate was 0.9984 (S.D. = 0.0012, C.V. = 0.12%). There are two differences between the methods for plasma and urine. Because there is much less protein and amino acids in urine, one half the concentration of copper(II) chloride is used for urine as compared to plasma. Because the pH of urine

is usually lower than that of plasma and the salt concentration can be higher, the borate buffer added prior to Dns derivatization is stronger and the pH is 8.05 instead of 8.45. The apparent pH of the urine or plasma sample mixtures prior to the addition of Dns chloride is comparable (urine: pH = 9.0, plasma: pH = 9.1) and the proportion of water to acetonitrile is the same. These are the critical conditions for derivatization and extraction of GVG and internal standard.

It is anticipated that GVG will be excreted rapidly and mostly unchanged; therefore, the urine sample size was reduced compared to plasma and the concentration range of the standard curve was expanded.

Application to preliminary pharmacokinetic study in dogs

A preliminary pharmacokinetic study of GVG was carried out in dogs in order to determine the adequacy of the analytical method regarding plasma levels after dosing.

Two female beagle hounds were given 50 mg/kg GVG in a 5% solution. This was thought to approximate the probable dose that will be administered to man. One dog was dosed intravenously and the other orally. A sample of blood was drawn just prior to dosing and at 0.083, 0.166, 0.25, 0.5, 1, 2, 3, 4, 5, 6, 7 and 8 h after dosing. The blood was drawn by syringe, then added to lithium heparin tubes (Venoject T-200 SLH). It was centrifuged and the plasma was frozen until it was analyzed. Each sample was analyzed in duplicate. The assay results are listed in Table II. The average values were plotted as concentration of GVG (μg/ml) versus time (h) (Fig. 2). The highest plasma concentration of 138 μg/ml for the intravenous dosing was observed at 5 min which was the first sample after dosing. The oral dose peak concentration of 77 μg/ml occurred at

TABLE II

RESULTS OF DUPLICATE ANALYSIS OF GVG IN DOG PLASMA AFTER INTRAVENOUS AND ORAL DOSING OF FEMALE BEAGLE HOUNDS AT 50 mg/kg

Time (h)	Intravenous dosing (dog No. 81-103) (μg/ml)			Oral dosing (dog No. 81-104) (μg/ml)		
	1	2	Average	1	2	Average
Pre-dose	0	0	0	0	0	0
0.083	144.4	133.3	138.3	0	0	0
0.166	126.4	104.5	115.5	7.3	4.0	5.65
0.25	116.3	91.5	103.9	13.9	15.2	14.5
0.5	79.3	69.7	74.5	58.8	66.2	62.5
1	54.0	52.8	53.4	72.7	81.7	77.2
2	23.5	—	23.4	41.4	35.7	38.4
3	17.5	15.5	16.5	19.8	21.9	20.8
4	11.6	11.9	11.7	19.0	19.4	19.2
5	10.4	9.1	9.7	13.2	10.3	11.8
6	6.8	8.4	7.6	12.4	7.2	9.8
7	3.7	5.5	4.6	11.0	3.7	7.4
8	3.0	5.0	4.0	5.9	3.4	4.6
Trapezoidal AUC (μg-min/ml)			10 585			11 644

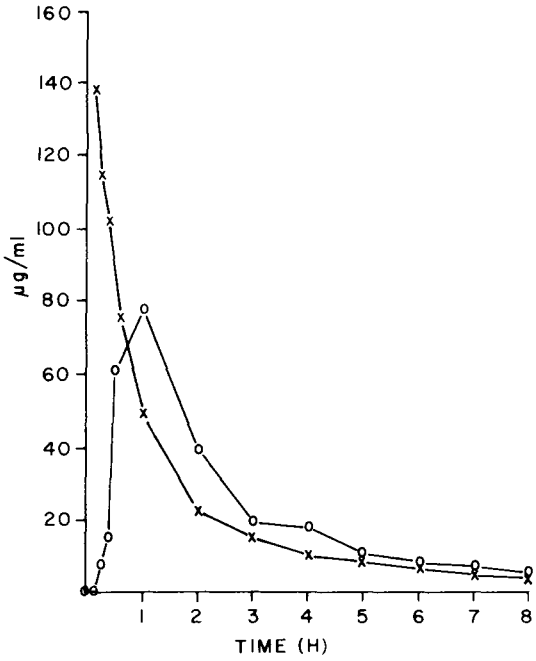

Fig. 2. Concentration of GVG in dog plasma as a function of time after an oral (○) and intravenous (×) dose of 50 mg/kg.

1 h. After 8 h the plasma concentration in both intravenously and orally dosed dogs dropped to approximately 5 µg/ml.

Since the intravenous and oral data (Table II) were obtained from different dogs, no calculations could be done to relate the two routes of dosing. The area under the curve (AUC) values (Table II) indicate, however, that oral absorption was probably substantial if not complete for GVG.

CONCLUSION

The quantitative method described in this report of GVG in plasma and urine is selective and shows a high degree of accuracy and precision. As the preliminary pharmacokinetic study in dogs indicates, it is adequate for full scale bioavailability and pharmacokintic studies.

REFERENCES

1 B. Lippert, M.J. Jung, B.W. Metcalf and P. Cosara, Eur. J. Biochem., 74 (1977) 441.
2 B.W. Metcalf, Biochem. Pharmacol., 28 (1979) 1705.
3 J. Grove, P.J. Schechter, J. Warter, L. Ruanbach, G. Tell, J. Koch-Weser and C. Marescaux, Lancet, ii (1980) 647.
4 K. Gale and M. Iadarcla, Science, 208 (1980) 289.
5 G. Tell, J. Koch-Weser, J.P. Chabannes, P.J. Schechter, P. Cantiniaux and P. Lambert, N. Engl. J. Med., 305 (1981) 581.
6 J. Grove, J.R. Fozard and P.S. Mamont, J. Chromatogr., 223 (1981) 409.
7 K.D. Haegele and J. Schoun, Merrell Dow Research Institute, Strasbourg, personal communication.

8 P. Bohlen, P.J. Schechter, W. Von Domme, G. Coquillat, J.C. Dosch and J. Koch-Weser, Clin. Chem., 24 (1978) 256.
9 T.A. Hare and N.V.B. Manyam, Anal. Biochem., 101 (1980) 349.
10 G.E. Griesmann, W.Y. Chan and O.M. Rennert, J. Chromatogr., 230 (1982) 121.
11 A.C. Kurtz, J. Biol. Chem., 180 (1949) 1253.
12 E. Bayer, E. Grom, B. Kaltenegger and R. Uhmann, Anal. Chem., 48 (1976) 1106.
13 Y. Tapuhi, D.E. Schmidt, W. Lindner and B.L. Karger, Anal. Biochem., 115 (1981) 123.

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Note

Quantitation of levorphanol in plasma using high-performance liquid chromatography with electrochemical detection

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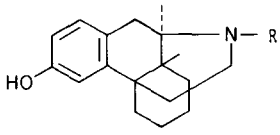
Levorphanol, (—)-3-hydroxy-N-methylmorphinan (Fig. 1), is one of the most widely used oral and parenteral narcotic analgesics in the management of patients with moderate to severe pain associated with cancer and terminal illness. As a congener of morphine, it has similar properties and actions, 2 mg of i.m. levorphanol having equal analgesic potency as 10 mg of i.m. morphine [1]. Levorphanol also has good oral efficacy with an i.m./p.o. ratio of about one half [1].



R = CH_3 LEVORPHANOL

= $CH_2CH = CH_2$ LEVALLORPHAN

Fig. 1. Structures of levorphanol, (—)-3-hydroxy-N-methylmorphinan, and levallorphan, internal standard.

At the present time there are only two reported procedures with the necessary sensitivity for the quantitation of plasma concentrations of the drug encountered in the clinical situation. Dixon et al. [2] reported a radioimmunoassay (RIA) while more recently Min and Garland [3] developed a gas chroma-

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tography—mass spectrometry (GC—MS) procedure and confirmed the selectivity of the original RIA. However, neither of these methods is readily accessible to most investigators; the RIA requires the production of specific antiserum while GC—MS involves costly instrumentation.

To overcome these limitations, the present study describes the development of a simple high-performance liquid chromatographic (HPLC) procedure utilizing electrochemical detection (ED) to determine levorphanol concentrations as low as 1 ng/ml in human plasma.

EXPERIMENTAL

Reagents and materials

All reagents were of analytical reagent grade. Solvents were supplied by Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Levorphanol was supplied by Hoffmann-La Roche (Nutley, NJ, U.S.A.).

High-performance liquid chromatography

The HPLC—ED system was composed of a Waters Assoc. (Milford, MA, U.S.A.) Model 6000A solvent delivery pump, Model U6K universal liquid chromatograph injector equipped with a 2-ml sample loop and a Waters reversed-phase column, 300 mm × 4 mm I.D., containing μBondapak C_{18} (10 μm particle size). A 2-μm precolumn filter (Waters 84560) was placed before the reversed-phase column. Compound detection was achieved with an amperometric detector system (LC-4B, Bioanalytical Systems, Lafayette, IN, U.S.A.). The amperometric detector with a glassy carbon electrode and Ag/AgCl reference electrode was set in the oxidation mode at an applied potential of +1.00 V.

Chromatography was performed at ambient temperature using a mobile phase composed of acetonitrile—0.01 *M* sodium chloride (30:70) containing 0.1 m*M* EDTA. The aqueous phase was adjusted to pH 4.8 with 1 *M* hydrochloric acid before mixing with the acetonitrile. The flow-rate was 1.0 ml/min.

Plasma extraction

To a 1-ml aliquot of plasma containing either known or unknown concentrations of levorphanol, 20 ng of levallorphan (Fig. 1) was added as an internal standard. After mixing, 1 ml of 1 *M* borate buffer (pH 9) was added and the sample extracted by shaking for 10 min with 10 ml of hexane—ethyl acetate (9:1). Following centrifugation (2500 rpm for 10 min), 9 ml of the organic extract was taken and evaporated to dryness at 50°C under nitrogen in a conical tube. The residue was dissolved in 200 μl of the mobile phase of which a 100-μl aliquot was injected for HPLC—ED analysis.

Calculations

Calibration curves were derived by plotting peak height ratios of levorphanol to internal standard response versus concentration and fitting by least-squares regression analysis using a power function. Correlation coefficients were typically 0.999. Calibration curves were run daily. The standards were taken through the extraction procedure and handled in an identical manner to the unknown, thereby avoiding any corrections for recovery in the unknowns.

Based on the response of unextracted standards, the extraction procedure gave a levorphanol recovery of 104%.

RESULTS AND DISCUSSION

Assay characteristics

Under the chromatographic conditions specified, levorphanol and levallorphan, internal standard, eluted at 6.5 and 9.5 min, respectively (Fig. 2). No interfering peaks were noted in the chromatograms in the retention region of levorphanol or the internal standard when plasma extracts were chromatographed. Calibration curves derived from plasma fortified with levorphanol and extracted by the procedure outlined above were constructed over the

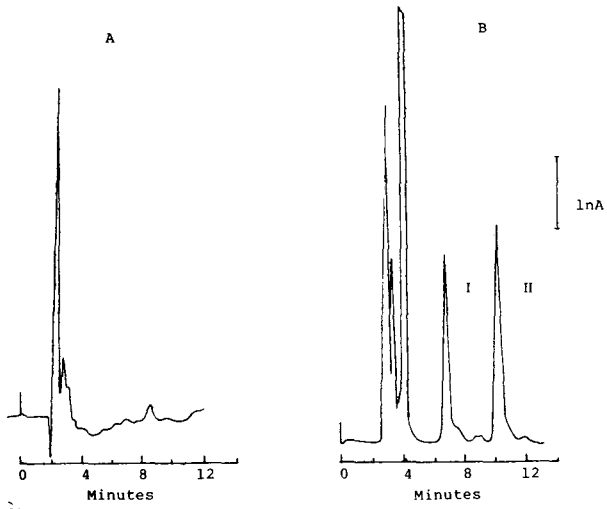

Fig. 2. Chromatograms of plasma extracts: (A) control plasma; (B) 10 min following a 5-mg i.v. dose of levorphanol in man. Column: μBondapak C_{18}, 300 mm × 4 mm, mobile phase: acetonitrile—0.01 *M* sodium chloride, pH 4.8, (30:70); flow-rate 1.0 ml/min; applied potential, +1.00 V. Peaks: I = levorphanol, 15.8 ng/ml; II = levallorphan, internal standard, 20 ng/ml.

TABLE I

RETENTION TIME AND DETECTOR RESPONSE FOR COMMON NARCOTIC ANALGESICS

No detector response was observed for codeine, morphine, hydrocodone, oxycodone, hydromorphine, meperidine, propoxyphene, 1-α-acetylmethodol, acetaminophen or caffeine at concentrations of 125 ng/ml.

	Retention time (min)	Response (mm/ng/ml)
Levorphanol	6.4	1.50
6-Acetylmorphine	4.2	1.08
Oxymorphine	3.2	0.20
Pentazocine	14.4	1.07
Heroin	4	0.22

concentration range 1.25—50 ng/ml. Over the concentration range 1.25—50 ng/ml the intra- and inter-assay coefficients of variation (n = 3) did not exceed 11.8% and 9.5%, respectively.

Selectivity of assay

To determine the possibility of interference from other commonly used drugs and/or their metabolites which may be present in patient's plasma, the assay selectivity was evaluated by assaying plasma samples containing 125 ng/ml of the following compounds: heroin, 6-acetylmorphine, codeine, morphine, hydrocodone, oxycodone, hydromorphine, meperidine, propoxyphene, oxymorphine, 1-α-acetylmethodol, acetaminophen and caffeine. No chromatographic interference was found in the determination of levorphanol or the internal standard levallorphan from any of the compounds tested. No detector response was found for any of the compounds tested except heroin, 6-acetylmorphine, oxymorphine and pentazocine. All of which were well separated from levorphanol (Table I).

Analysis of clinical plasma samples

In order to assess the clinical usefulness of the HPLC—ED procedure, plasma samples from patients who had received levorphanol i.v., i.m. and p.o. were assayed for drug content. Concentrations of levorphanol determined in two patients (A and B) who had received 2-mg and 5-mg i.v. doses of the drug, respectively, are given in Fig. 3. Patient A had been receiving daily 2-mg doses of levorphanol i.m. prior to this present i.v. administration which may account

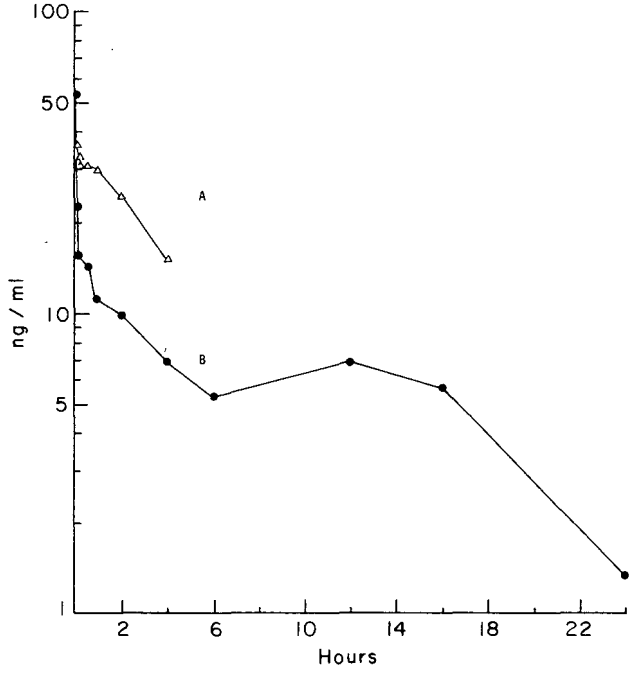

Fig. 3. Plasma concentration—time curves of levorphanol in two patients after receiving levorphanol: (A) 2 mg levorphanol i.v. (preceded by 2 mg levorphanol i.m. daily); (B) single 5-mg i.v. dose of levorphanol.

for the high concentrations of levorphanol found relative to the concentrations observed in patient B who received only a single 5-mg i.v. dose. Due to limited data obtained from these two patients, no pharmacokinetic interpretation was attempted.

Steady-state plasma concentrations of levorphanol were quantitated in a patient who had been receiving 16 mg of drug orally twice daily. The concentrations ranged from 65 to 87 ng/ml up to 3 h after drug administration. When the same patient was switched to 8 mg of levorphanol i.m. with the same dosing schedule, similar plasma concentrations were observed and ranged from 58 to 76 ng/ml. It should be noted that the i.m. and p.o. doses which result in equivalent plasma concentrations of levorphanol have an i.m./p.o. ratio of one half which is equivalent to the i.m./p.o. efficacy ratio reported previously [1].

In conclusion, the HPLC—ED method provides a simple approach for the quantitation of levorphanol at clinical doses.

REFERENCES

1 R.W. Houde, S.L. Wallenstein and W.T. Beaver, in C. De Stevens (Editor), Analgesics, Academic Press, New York, 1975, p. 75.
2 R. Dixon, T. Crews, E. Mohacsi, E. Inturrisi and K. Foley, Res. Comm. Chem. Pathol. Pharmacol., 32 (1981) 545.
3 B.H. Min, W.A. Garland and J. Pao, J. Chromatogr., 231 (1982) 194.

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Note

Quantitative determination of perphenazine and its dealkylated metabolite using high-performance liquid chromatography

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The introduction of neuroleptics into the treatment of psychotic symptoms has demonstrated, that it may be difficult to achieve a satisfactory balance between extrapyramidal side-effects and the antipsychotic effect. Furthermore, cases have been reported where the therapeutic effect has not been observed despite long-term neuroleptic treatment [1, 2].

In 1968, when Curry [3] published his first gas chromatographic method for the determination of chlorpromazine, many thought that therapeutic monitoring of this drug was just around the corner. During the intervening fifteen years a large number of analytical methods for the quantification of many different neuroleptics in human plasma have been published [4, 5].

Among the pharmacokinetic parameters of clinical consequence discovered in these investigations have been the big inter-individual variations in the plasma concentrations reached during a fixed-dose therapy [1, 6]. However, it has been difficult to exploit this knowledge effectively in the clinical treatment, as few investigations have hitherto documented an adequate correlation between the clinical effect and the plasma concentration of the administered neuroleptic [7]. Some possible reasons for this lack of correlation are: first, inhomogeneity of the material investigated; second, unknown time of delay

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between the measurement of the plasma concentration and the occurrence of the clinical effect at the receptors; third, lack of appreciation of the need for careful design of the method, a design that takes into account application to the clinical situation.

The aims of this paper are two-fold. First, to evaluate the advantages and drawbacks of different analytical methods commonly applied to plasma-monitored neuroleptic treatment. Second, to describe a selective high-performance liquid chromatographic (HPLC) method for the determination of perphenazine and its dealkylated metabolite.

REVIEW OF DIFFERENT METHODS

In our opinion, an analytical method must indicate unequivocally to the clinician whether the lack of a therapeutic response is due to non-compliance or to rapid metabolism to inactive metabolites. We will emphasize the necessity for a chromatographic system to proceed the quantification to ensure a separate estimate of parent compound and one or more metabolites.

A review of the literature indicates that the most frequently applied methods for determination of neuroleptics are gas-liquid chromatography (GLC), HPLC, radioimmunoassay (RIA) and radioreceptor assay (RRA). The two chromatographic methods (GLC and HPLC) give a specific quantification of both the parent compound and the metabolites, and may be classifiable as adequate for the clinical situation.

RIA will give a specific estimate of the active compound in only a few situations, because of a substantial similarity between the chemical structures of the parent compound and the metabolite(s). This will result in cross-reaction between added antibody and parent compound/metabolite(s) and consequently cause false increased values. Neither does the RIA method permit an evaluation of changed metabolizing capacity or non-compliance. We therefore conclude that this method is of little help to the psychiatrist in judging whether the actual medical treatment will result in an acceptable therapeutic effect.

RRA determines specifically the amount of dopamine-receptor-active substances in a specimen. The principle is based on an in vitro competition between active substance in the sample and an isotope-labelled neuroleptic for the binding sites in a suspension of dopamine receptors extracted from brain tissue of an animal. The clinical relevance of such biologically specific estimates depends on two things. First; the binding affinity must be similar to that in human receptors, and second, the blocking in vitro must be equivalent to that in vivo. Besides, a total estimate of active compounds in the sample does not give any information about the ratio between the parent compound and the active metabolites. This might explain the lack of correlation between the antipsychotic effect and the measured plasma concentrations of neuroleptics that form active metabolites, as has been found with the RRA method. We therefore doubt that the RRA method will bring any substantial contribution to the improved monitoring of neuroleptics. We disagree with the many authors [8—10] who argue for the use of the RRA method as an adequate tool to make medical treatment with neuroleptics effective. Arguments in favour of RIA and

RRA methods that make reference to high capacity and low cost price are of minor importance in this connection.

EXPERIMENTAL

Reagents

Ethyl acetate, methanol and methylene dichloride (E. Merck, Darmstadt, F.R.G.) were of analytical grade. Hexane (Mallinckrodt, KY, U.S.A.) was of nanograde purity. Sodium hydroxide and hydrochloric acid were prepared in our laboratory.

Reference substances

Stock solutions (1 g/l) in ethanol of perphenazine (PPZ), of dealkylated perphenazine {1-[γ-(2-chloro-10-phenothiazinyl)propyl]piperazine dihydrochloride, DAPPZ}, and of the internal standard {4-[3-(2,8-dichlorophenothiazin-10-yl)propyl]-1-piperazinethanol, CPPZ} were all from Schering (Bloomfield, NJ, U.S.A.). The solutions were stable under refrigeration for one year.

Extraction procedure

To a centrifuge tube containing 2500 μl of plasma, 7.5 ng of the internal standard were added. To the sample were added 100 μl of 1 *M* sodium hydroxide, and this solution was extracted with 6 ml of organic solvents (ethyl acetate—hexane, 4:2) by vigorously shaking the tube for 30 sec. After centrifugation for 3 min, the organic phase was transferred to a centrifuge tube containing 2 ml of 0.1 *M* hydrochloric acid, using a methanol moistened Pasteur pipette.

The compounds were extracted into the aqueous phase by vigorously shaking the tube for 30 sec. After centrifugation (3 min) the organic phase was discarded. To the aqueous phase were added 100 μl of 6 *M* sodium hydroxide and 3 ml of hexane. By shaking vigorously (30 sec) the compounds were re-extracted into the organic phase. After centrifugation a moistened pipette was used to transfer the organic phase to a tapered tube. The hexane was evaporated to dryness under a stream of nitrogen. The residue was dissolved in 75 μl of solvent, and 20 μl of this solution were injected into the chromatograph.

Liquid column chromatography

A liquid chromatograph (Pye Unicam, Cambridge, U.K.) Type LC 3, equipped with a UV detector Type LC 3, was used. The column (2 cm × 4.6 mm I.D.) was filled with C_{18} material (Perkin-Elmer, U.S.A.) with particle size 3 μm. The mobile phase was methanol—water—methylene dichloride—ammonia (200:40:10:3) at a flow-rate of 1.0 ml/min. The detection was carried out at 257 nm.

Calculations

The plasma concentrations were read from standard curves constructed from chromatograms of plasma samples containing different known amounts of PPZ

and DAPPZ. The peak height ratios PPZ/CPPZ and DAPPZ/CPPZ were plotted against the concentrations.

RESULTS

Chromatograms of plasma extracts are illustrated in Fig. 1. Retention times for PPZ, CPPZ and DAPPZ were 2.1, 2.5 and 5.9 min, respectively. The peaks of PPZ and DAPPZ correspond to plasma concentrations of 1.0 and 2.5 nmol/l, respectively (A). The chromatogram of a plasma blank showed no interfering peaks (B). The left-hand chromatogram (C) illustrates the determination of a plasma sample from a patient receiving oral treatment with 8 mg twice daily. The sample was drawn 12 h after the last dose, and the plasma concentrations of PPZ and DAPPZ were estimated to 3.8 and 1.6 nmol/l, respectively.

The lower limit for safe quantification (sensitivity) was defined as a peak height ten times greater than the fluctuation of the baseline. Defined in this way, we found the sensitivity to be below 0.5 nmol/l for PPZ, extracting 2.5 ml of plasma.

The inter- and intra-assay variations for PPZ and DAPPZ present in various

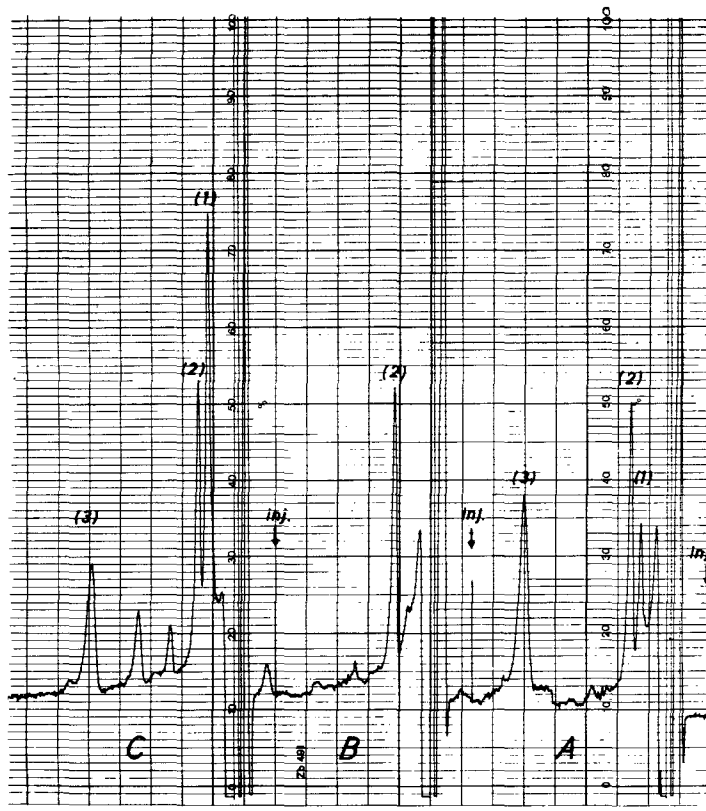

Fig. 1. Chromatograms of three plasma samples. (A) Human plasma with the following additions: 6.25 ng of PPZ (1); 7.50 ng of C-PPZ (2); 15.75 ng of DAPPZ (3). (B) The same human plasma with only 7.50 ng of C-PPZ (2) added. (C) PPZ (1) and DAPPZ (3) in a patient receiving continuous oral PPZ medication.

TABLE I

INTER- AND INTRA-ASSAY TESTS FOR PERPHENAZINE (PPZ) AND DEALKYLATED PERPHENAZINE (DAPPZ) FROM PLASMA

The plasma volume extracted was 2.5 ml in each instance. $n = 10$.

Concentration added (nmol/l)		Calculated concentrations (mean ± S.D., nmol/l)			
		Inter-assay		Intra-assay	
PPZ	DAPPZ	PPZ	DAPPZ	PPZ	DAPPZ
0.50	1.00	0.46 ± 0.03	1.02 ± 0.08	0.49 ± 0.01	1.00 ± 0.04
1.00	2.00	0.98 ± 0.07	2.00 ± 0.11	1.01 ± 0.04	2.01 ± 0.08
2.00	4.00	2.01 ± 0.10	4.07 ± 0.15	2.00 ± 0.06	4.04 ± 0.04
3.00	6.00	2.98 ± 0.12	6.07 ± 0.10	3.02 ± 0.10	6.03 ± 0.10
4.00	8.00	4.01 ± 0.09	8.05 ± 0.16	3.99 ± 0.06	7.95 ± 0.11
6.00	12.00	6.06 ± 0.19	11.90 ± 0.24	6.02 ± 0.05	11.97 ± 0.20
8.00	16.00	8.01 ± 0.17	16.11 ± 0.30	8.07 ± 0.09	15.95 ± 0.24

concentrations were estimated on ten plasma samples within the therapeutic range (Table I).

Recovery tests were performed by comparing the peak heights of PPZ, CPPZ and DAPPZ from plasma extracts with the peak heights that resulted from injections of ethanolic solutions of equivalent amounts. About 70% recovery from plasma samples was attained.

The analytical selectivity was examined by comparing retention times of other psychotropic drugs with those of PPZ and DAPPZ. Other neuroleptics (except levomepromazine) and diazepam should be avoided, whereas anti-parkinsonian drugs (biperidine, orphenadine), antidepressants (amitriptyline, nortriptyline, imipramine and clomipramine), nitrazepam and tetraethyldisulfiram (Antabus®) did not interfere in the chromatogram.

To ensure that identical results were obtained with the GLC method [11] used in our earlier investigations and the new HPLC method, thirty plasma

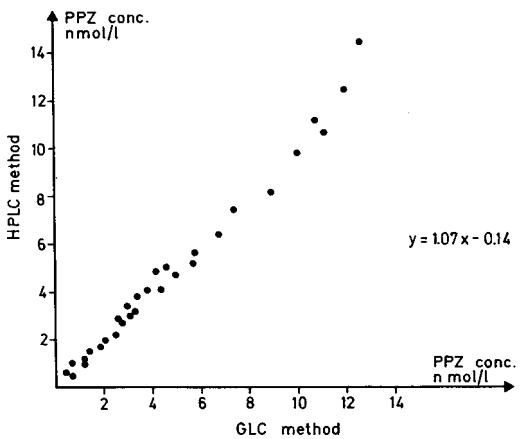

Fig. 2. Correlation of the GC and HPLC methods applied to thirty plasma samples from PPZ-medicated patients.

samples drawn from PPZ-medicated patients were analysed by the two methods. Fig. 2 illustrates the excellent uniformity of the results obtained over a wide concentration range ($r = 0.99$).

DISCUSSION

Previous investigations by our group [12, 13] have revealed that the PPZ plasma concentration should lie in the range 2—6 nmol/l during the entire dose interval to achieve the antipsychotic effect and to reduce the risk of provoking extrapyramidal side-effects. This observation has been confirmed by routine application over a three-year period, so it seems likely that the parent compound (PPZ) is the only active compound at the receptors. However, RRA examinations of the in vitro dopamine receptor activity of different PPZ metabolites indicated that PPZ and the 7-hydroxy metabolite had similar effects. The lipophility of the metabolite is less than that of the parent compound, and so it seems likely that the metabolite does not cross the blood—brain barrier. We therefore consider that quantification of this metabolite is of minor importance.

The metabolite estimated in this method, dealkylated perphenazine (DAPPZ), is different from that selected in our earlier published GLC method. This is for analytical reasons only, because the sulphoxide metabolite estimated in the GLC method has a restricted UV spectrum at 257 nm, which is the optimal UV-absorbing wavelength for PPZ and DAPPZ.

In spite of the biological inactivity of DAPPZ, the simultaneous estimation of this metabolite and of PPZ is essential if the right conclusion is to be reached concerning the reason for poor effect of the treatment. If the PPZ plasma concentration is too low to be detectable, then a metabolite concentration about zero indicates non-compliance, whereas a high metabolite concentration indicates a marked metabolizing capacity. The results in Table I show that the method is sensitive enough to ensure accurate quantification even in the subtherapeutic range. Furthermore, the correlation between detector deflection and concentration values is linear over a wide range for PPZ and DAPPZ, respectively.

Table I illustrates a coefficient of variance not exceeding 7% for both components, a methodological precision which is moderate in relation to biological variations, giving no noise at the steady-state level.

As mentioned earlier, we have monitored PPZ treatment for three years using plasma concentrations as a guide. The results obtained have been so convincing that the capacity of the GLC method was inadequate to comply with the continuously increasing demand. This is the main reason for the development of the HPLC method, which is technologically more robust than the GLC method.

CONCLUSION

A critical review of different analytical methods for quantification of neuroleptics in human plasma is made. The chromatographic methods (GC and HPLC) are classifiable as adequate for the clinical situation, whereas a RIA

and RRA have certain clinical shortcomings because of lack of specificity. A new developed HPLC method for the determination of perphenazine and its dealkylated metabolite in human plasma is described.

The sensitivity, precision and selectivity make the method adequate to estimate, by routine, the concentrations of the parent compound and its dealkylated metabolite in schizophrenic patients. The method has been compared to an earlier published GC method and was found to give almost identical results.

REFERENCES

1 P.L. Morselli, G. Bianchetti and M. Dugas, Ther. Drug Monitor., 4 (1982) 51.
2 R.C. Smith, J. Crayton, H. Dekirmerjian, D. Klass and J.M. Davis, Arch. Gen. Psychiatry, 36 (1979) 579.
3 S.H. Curry, Anal. Chem., 40 (1968) 1251.
4 T.B. Cooper, Clin. Pharmacokinet., 3 (1978) 14.
5 N.-E. Larsen, in E. Usdin (Editor), Clinical Pharmacology in Psychiatry, Macmillan, London, 1981, p. 3.
6 S.G. Dahl, in E. Usdin (Editor), Clinical Pharmacology in Psychiatry, Macmillan, London, 1981, p. 125.
7 P.L. Morselli and E. Zarifian, in Drug Concentrations in Neuropsychiatry, Ciba Foundation Symposia 74, Elsevier, Amsterdam, 1980, p. 115.
8 I. Creese and S.H. Snyder, Nature, 270 (1977) 179.
9 S.R. Lader, J. Immunoassay, 1 (1980) 57.
10 H.Y. Meltzer, J.M. Kane and T. Kolakowska, in J.T. Coyle (Editor), Neuroleptics: Neurochemical, Behavioral and Clinical Perspectives, Raven Press, New York, 1983, p. 255.
11 N.-E. Larsen and J. Næstoft, J. Chromatogr., 109 (1975) 259.
12 L. Bolvig Hansen, N.-E. Larsen and N. Gulmann, Psychopharmacology, 78 (1982) 112.
13 P. Knudsen, L. Bolvig Hansen, K. Højholdt and N.-E. Larsen, Acta Psychiatr. Scand., (1985) in press.

PUBLICATION SCHEDULE FOR 1985

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

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