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CHROMBIO. 747

IDENTIFICATION OF TWO THORMÄHLEN-POSITIVE COMPOUNDS FROM MELANOTIC URINE BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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(Received July 22nd, 1980)

SUMMARY

The isolation of two Thormählen-positive compounds from the urine of a patient with malignant melanoma and the elucidation of their structure by gas chromatography—mass spectrometry is described. The compounds were isolated using a poly-N-vinylpyrrolidone column and separated by preparative thin-layer chromatography. After elution they were analyzed by gas chromatography and gas chromatography—mass spectrometry as their trimethylsilyl derivatives and after hydrolysis also as their *tert.*-butyldimethylsilyl derivatives. The results showed the main Thormählen-positive compound A to be the glucuronide of 5-hydroxy-6-methoxyindole, whereas the minor compound AX appeared to be the glucuronide of its isomer 6-hydroxy-5-methoxyindole.

INTRODUCTION

The suggested biochemical pathway towards the production of eumelanin involves the formation of compounds of an indolic nature [1—3]. Therefore, it is not unreasonable to assume that the overproduction of melanin is the cause of

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an increased excretion of indolic compounds in the urine of patients suffering from pigmented malignant melanoma [4—6].

The group of so-called Thormählen-positive indoles [7] consists of at least five indolic compounds [4] which contain unsubsituted pyrrole rings [8]. The use of paper chromatography or thin-layer chromatography (TLC) for the investigation of their structure has been described by several authors [9—14]. Recently, the structure of one Thormählen-positive compound has been studied by nuclear magnetic resonance spectroscopy [15].

This paper describes the use of gas chromatography—mass spectrometry for the elucidation of the structure of two Thormählen-positive compounds which were isolated from the urine of a patient with melanoma using a poly-N-vinylpyrrolidone (PVP) column and TLC.

EXPERIMENTAL

Chemicals

Ethyl acetate (gas chromatographic (GC)—spectroscopic quality) was purchased from Baker Chemicals (Deventer, The Netherlands), beta-glucuronidase from *Escherichia coli* (100 U/ml) was from Boehringer (Mannheim, G.F.R.), *tert.*-butyldimethylchlorosilane—imidazole reagent was from Applied Science Labs. (State College, PA, U.S.A.), PVP was from Polyclar (Gaf, Austria).

5-Hydroxy-6-methoxyindole (5H6MI) and 6-hydroxy-5-methoxyindole (6H5MI) were prepared by decarboxylation of the corresponding indole-2-carboxylic acids and kindly supplied by Dr. Budĕšínská from Prague. All other chemicals and solvents were purchased from Merck (Darmstadt, G.F.R.). Glass-distilled water was used throughout.

Apparatus

Analyses were performed on Varian 3700 gas chromatographs equipped with capillary columns and flame-ionisation detectors. A capillary column, 25 m × 0.26 mm I.D., coated with SE-54 (Franzen Analysen-Technik, Bremen, G.F.R.) was used for the analysis of *tert.*-butyldimethylsilyl (*t*-BDMS) derivatives. The column temperature was programmed as follows: initial temperature at 120°C was maintained for 5 min and then programmed to 260°C at 4°C/min and maintained at 260°C for 30 min. Helium flow-rate was 0.73 ml/min. Injector and detector temperatures were 280°C. For the analysis of trimethylsilyl (TMS) derivatives a column 25 m × 0.26 mm I.D. coated with SE-30 (Jaeggi, Labor für Chromatographie, Trogen, Switzerland) was used. The oven temperature was programmed from 100 to 250°C at 4°C/min; detector and injector temperatures were set at 250°C. For the analysis of per-TMS derivatives of glucuronide conjugates, the temperature was programmed at 20°C/min.

Gas chromatographic—mass spectrometric (GC—MS) analyses were performed with a Varian Aerograph 1400 gas chromatograph coupled to a Varian MAT 112 mass spectrometer, equipped with a 17.5 m × 0.25 mm I.D. capillary column coated with SE-54 (Franzen Analysen-Technik). Helium flow-rate was 4 ml/min, ionisation energy 70 eV, injector temperature 250°C, source temperature 250°C, interface (all glass) temperature 275°C. Column temperature was programmed from 200 to 250°C at 10°C/min (glucuronide conjugates), or from 150 to 250°C at 4°C/min (unconjugated indoles).

Samples

Urine was obtained from a patient with generalized malignant melanoma. The generalisation was characterized in particular by an enlargement of the liver caused by melanoma metastases. The concentration of Thormählen-positive compounds was extraordinarily high — approx. 400 μg equivalents of indole per millilitre.

Procedure

The isolation of Thormählen-positive compounds from melanotic urine was performed using a 10 × 1 cm column filled with PVP (160—200 mesh). Two millilitres of melanotic urine were applied to the column, after which the compounds were eluted with water. The volume of each fraction was 1 ml. The content of Thormählen-positive compounds was measured using the Thormählen reaction as described previously [16]. The procedure was repeated four times, after which fractions of the second positive peak were pooled and lyophylized. The residue, originating from 8 ml of melanotic urine, was dissolved in 4 ml of methanol. The solution was preparatively applied to TLC plates (Cellulose F, precoated, 20 cm × 20 cm × 0.1 mm; Merck). Purification on TLC was carried out in an *n*-butanol—pyridine—water (1 : 1 : 1) solvent system. The bands were visualized on both edges by performing a Thormählen reaction [11]. Unsprayed bands were scratched off under UV light. The elution of two Thormählen-positive bands designated A and AX was performed overnight using the TLC solvent system. After elution the samples were divided into two parts and freeze-dried.

One part of each Thormählen-positive compound was hydrolyzed by adding 2 ml of sodium acetate buffer (0.5 mol/l, pH 6.5) and 100 μl of beta-glucuronidase solution, and incubating for 1 h at 37°C in a shaking water-bath. After incubation, the hydrolysate was extracted twice with 2 ml of ethyl acetate. Pooled extracts were dried over anhydrous sodium sulphate and evaporated under a stream of nitrogen at 40°C.

t-BDMS derivatives were prepared by treating part of the hydrolyzed samples with 100 μl of *t*-BDMS reagent mixture for 2 h at 80°C. After derivatisation, 0.5 ml of *n*-hexane was added, thoroughly mixed and centrifuged. The hexane layer was transferred to a clean tube and used for GC and GC—MS examination.

Per-TMS derivatives of Thormählen-positive compounds eluted from the TLC plates were prepared by treating the sample with 100 μl of bis(trimethylsilyl)trifluoroacetamide (BSTFA) at 80°C for 30 min. TMS derivatives of extracted hydrolysates were prepared in a similar way; 1-μl aliquots were examined by GC and GC—MS.

RESULTS

A quantitative profile of Thormählen-positive compounds eluted from the PVP column is shown in Fig. 1. The control TLC carried out simultaneously indicated that the first peak was caused by a large amount of impurities forming a turbid solution. The second peak, however, was consistent with the compounds of interest. Fraction No. 5 contained a relatively pure compound that

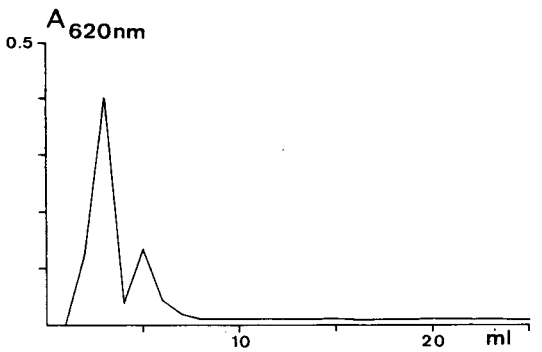

Fig. 1. A profile of Thormählen-positive compounds eluted from PVP. The second peak was found to consist of Thormählen-positive indolic compounds.

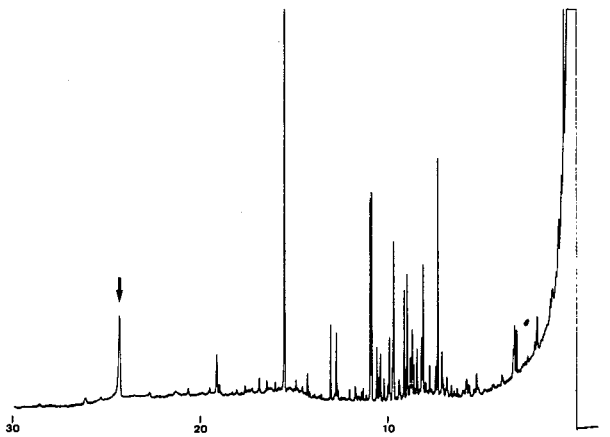

Fig. 2. Gas chromatogram of the per-TMS derivative of Thormählen-positive compound A. The arrow indicates its position in the chromatogram.

is believed to be the main Thormählen-positive indole in melanotic urine [4, 5, 9, 10, 12—14].

A more detailed study showed the presence of a small amount of a second Thormählen-positive compound. In accordance with earlier reports, the main compound was designated A and the minor compound AX. Hence, both compounds were separated on TLC having different R_F values in the solvent system mentioned above (A 0.55, AX 0.47). Derivatisation of these compounds after elution from TLC yielded per-TMS derivatives which formed a peak with a long retention time (Fig. 2). The Kovats retention index (isothermal, 250°C) was 30.67. It was not possible, however, to separate A and AX compounds, presumably because of the high similarity of their per-TMS derivatives. Mass spectra taken of these compounds showed a small molecular ion consistent with that calculated for methoxyhydroxyindole glucuronides (M^+ = 699). In accordance with other authors studying glucurono conjugates [17—19], we were able to assign fragment ions of a glucuronic acid moiety (m/e = 204, 217, and the very characteristic ion of m/e = 375) (Fig. 3).

The indolic moiety was characterized by two fragments. The cleavage of the

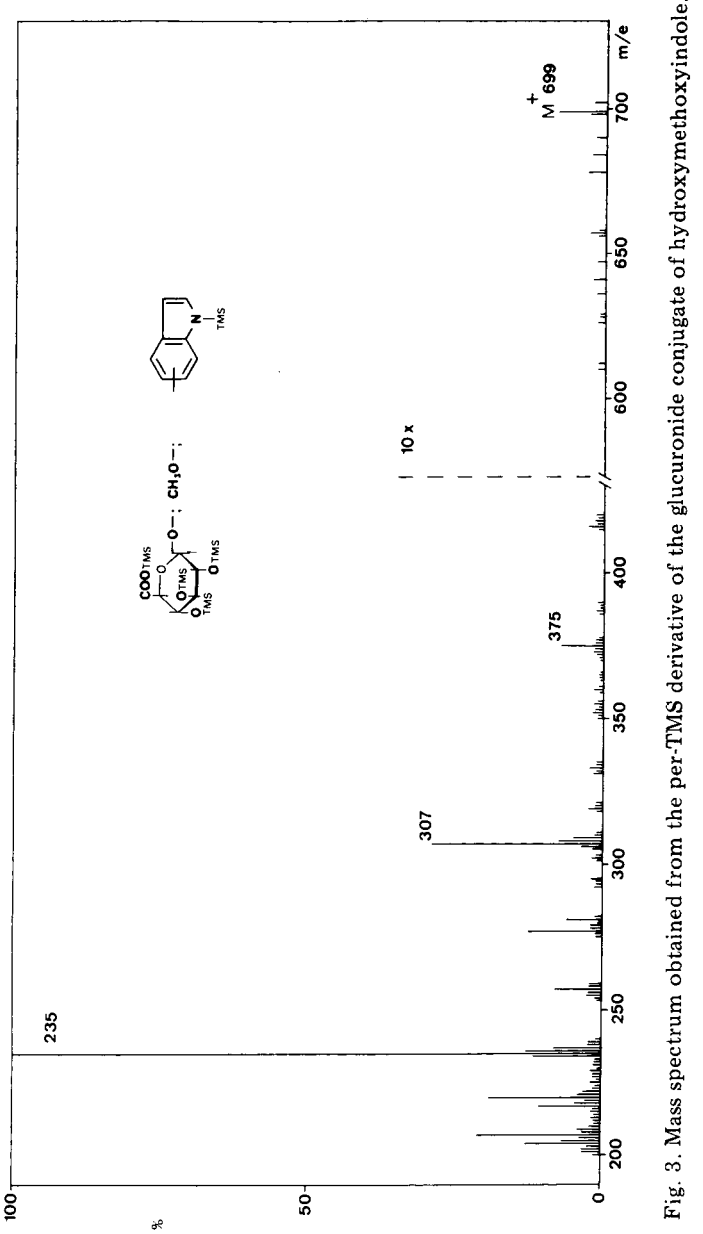

Fig. 3. Mass spectrum obtained from the per-TMS derivative of the glucuronide conjugate of hydroxymethoxyindole.

ether link led to the loss of the glucuronic moiety, resulting in the formation of a fragment ion with m/e = 235. In analogy to similar cases [17, 19], the fragment ion with m/e = 307 is considered to be formed by the elimination of the glucuronic acid moiety and rearrangement of a TMS group onto the hydroxymethoxyindole.

In order to elucidate the position of the substituent in the indolic ring, the hydrolyzed samples of both Thormählen-positive compounds were derivatized and analyzed by GC—MS. Mass spectra of TMS derivatives (Fig. 4) as well as of *t*-BDMS derivatives (Fig. 5) indicated the presence of a methoxyhydroxyindole

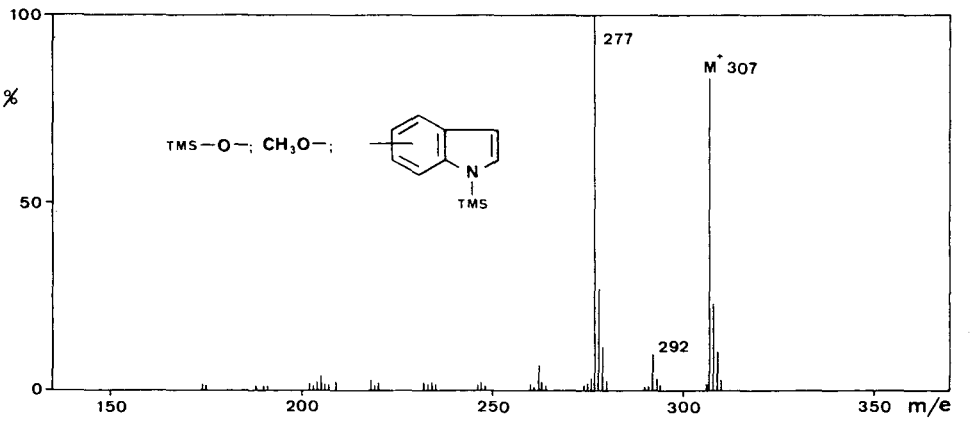

Fig. 4. Mass spectrum of the TMS derivative of hydroxymethoxyindole.

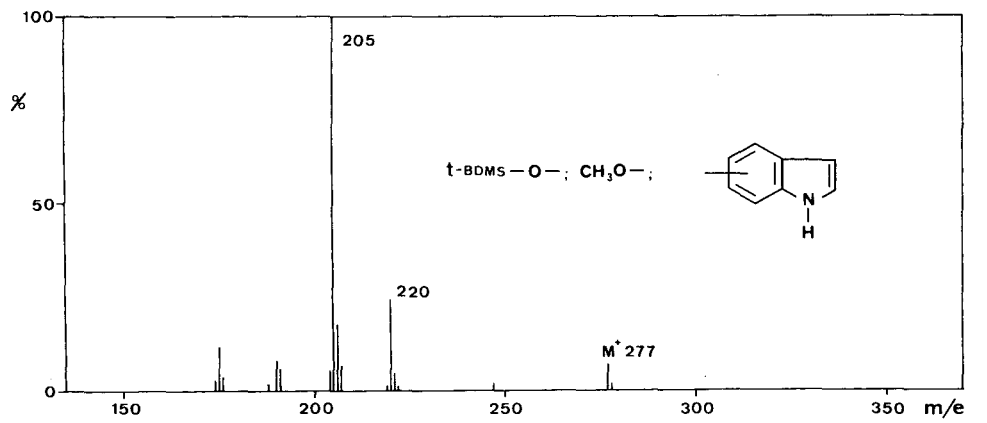

Fig. 5. Mass spectrum of the *t*-BDMS derivative of hydroxymethoxyindole.

TABLE I

METHYLENE UNIT VALUES OF ISOMERS OF METHOXYHYDROXYINDOLE

	TMS	*t*-BDMS
5H6MI	18.72	21.26
6H5MI	18.59	21.13

in the hydrolyzed samples. The comparison of retention times (Table I) with the synthetic 5H6MI and 6H5MI showed that Thormählen-positive compound A contains 5H6MI and minor AX contains isomeric 6H5MI.

DISCUSSION

Attempts to elucidate the structure of Thormählen-positive compounds are numerous. However, few reports have been conclusive until now. Almost half a century ago, Linnel and Raper [20] considered these compounds to be glucuronides or sulphates of 5,6-dihydroxyindole. Leonhardi [9] discovered three Thormählen-positive compounds using paper chromatography and named them A, B and C. He proposed that the structure of compound A was consistent with 5,6-dihydroxyindole bound to a dipeptide from pyrrolidonecarboxylic acid and glutamine [21]. In 1962 Anderson [10] reported the identification of esteric sulphates and the remarkable quantity of glucuronic acid in two different fractions of Thormählen-positive compounds. A reliable proof for the presence of glucuronic acid in Thormählen-positive compound A was provided by Pechan [5, 13]. However, most authors were unable to provide the correct structure of the indolic part of the molecule. Atkinson [12], who also reported glucuronic acid to be part of the molecule of Thormählen-positive compound A, showed the indolic component to be a mixture of 5H6MI and 6H5MI in the approximate ratio of 5 : 1. In the light of our findings, it is most likely that his compound A was a mixture of A and AX. Recently, the structure of compound A was also studied by nuclear magnetic resonance spectroscopy [15]. On the basis of the results obtained, the structure of the glucurono conjugates of 5H6MI has been proposed.

The existence of compound AX has only been described a few times [4, 5, 13, 14]. This compound could be overlapped by the main component A in most cases. In our samples the concentration of A was estimated to be approximately ten times higher than that of AX. Although methylation on position 6 is preferred [22] the ratio between the isomers may be rather variable.

In conclusion, this study shows that GC and/or GC—MS can be very useful for the elucidation of the structures of melanin precursors and their conjugates. In the past, the high instability of unconjugated indolic compounds could have been the main cause of unsuccessful studies. GC and/or GC—MS analysis, besides providing high sensitivity and specificity, offers the possibility of studying volatile derivatives of these compounds that are much less sensitive to oxidation. Quantitative measurements of these compounds using GC and GC—MS are in progress.

REFERENCES

1 H.S. Raper, Biochem. J., 21 (1927) 89.
2 H.S. Mason, J. Biol. Chem., 172 (1948) 83.
3 G.A. Swan, Progr. Chem. Org. Natur. Prod., 31 (1974) 521.
4 J. Duchoň and Z. Pechan, Ann. N.Y. Acad. Sci., 100 (1963) 1048.
5 J. Duchoň, B. Matouš and Z. Pechan, in G. Della Porta and O. Mühlbock (Editors), Structure and Control of the Melanocyte, Springer, Berlin, 1966, p. 175.

6 J. Duchoň and B. Matouš, Clin. Chim. Acta, 16 (1967) 397.
7 J. Duchoň and B. Matouš, Pigment Cell, Vol. 1, Karger, Basel, 1973, pp. 317–322.
8 G. Leonhardi, Naturwissenschaften, 40 (1953) 621.
9 G. Leonhardi, Naturwissenschaften, 41 (1954) 141.
10 A.B. Anderson, Biochem. J., 83 (1962) 10P.
11 J.C. Crawhall, B.J. Hayward and C.A. Lewis, Brit. Med. J., 1 (1966) 1455.
12 M.R. Atkinson, Biochim. Biophys. Acta, 74 (1963) 154.
13 Z. Pechan, Thesis, Purkyne University, Brno, 1963.
14 B. Matouš, Thesis, Charles University, Prague, 1977.
15 B. Matouš, A. Buděšínská, M. Buděšínský, J. Duchoň and S. Pavel, Neoplasma, 28 (1981) in press.
16 J. Horčičko, S. Pavel, J. Borovanský and J. Duchoň, Neoplasma, 26 (1979) 471.
17 H. Miyazaki, M. Ishibashi, M. Itoh, N. Morishita, M. Sudo and T. Nambara, Biomed. Mass Spectrom., 3 (1976) 55.
18 J.E. Bakke, C.E. Price and R.G. Zaylskie, Biomed. Mass Spectrom., 3 (1976) 329.
19 P.W. Aschbacher, J.E. Bakke and R.G. Zaylskie, Biomed. Mass Spectrom., 5 (1978) 409.
20 L. Linnel and H.S. Raper, Biochem. J., 29 (1935) 76.
21 G. Leonhardi, Naturwissenschaften, 42 (1955) 17.
22 J. Axelrod and A.B. Lerner, Biochim. Biophys. Acta, 71 (1963) 650.

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CHROMBIO. 765

IMPROVED PROCEDURE FOR THE ANION-EXCHANGE ISOLATION OF URINARY ORGANIC ACIDS

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(Received August 25th, 1980)

SUMMARY

DEAE-Sephadex equilibrated in 0.5 *M* triethylammonium acetate is suitable for the quantitative isolation of lactonisable organic acids. Mono-, di- and tricarboxylic acids can be eluted sequentially from DEAE-Sephadex by the use of 0.5 *M* triethylamine, 0.5 *M* triethylamine—0.1 *M* acetic acid, and 1.5 *M* pyridinium acetate.

INTRODUCTION

During the last few years, metabolic profiling has been particularly useful in the screening for organic acids in the urine of patients suffering from suspected inborn errors of metabolism and up till 1977, 23 new diseases have been discovered by this route [1]. The establishment of a successful metabolic profiling technique involves the following considerations: efficient and selective extraction of the compounds; rapid and complete derivatization of the compounds; separation and identification of the components of the mixture and interpretation of the data in terms of which compounds are not normally present or are present in abnormal amounts. Techniques of extracting organic acids from urine have been the subject of several studies [2—5]. Two principal methods for the isolation of the acidic constituents of urine, prior to derivatization and gas chromatography (GC), are currently in use [1]. The first is based on solvent extraction, usually with diethyl ether and/or ethyl acetate, the second is based on anion-exchange chromatography. The problem of choosing between the solvent-extraction and anion-exchange methods often arises. It has been the experience of several groups, that if the problem concerns screening for gross metabolic disorders, the detection of which does not require quantitative

*Author deceased.

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separation, then solvent extraction is applicable whilst for quantitative work the more time consuming anion-exchange procedures are essential.

The published ion-exchange procedure [5] involves removal of sulphate and phosphate as barium salts, ion exchange on DEAE-Sephadex, elution of the acids with pyridinium acetate buffer, lyophilization, silylation and GC. We recently applied the above procedure to the quantitative extraction and assay of some abnormal deoxyribose metabolites present in the urine of a child suffering from a new inborn error of metabolism, and found that the main metabolite, a lactonisable organic acid, could not be quantitatively recovered [6]. We now describe a modified anion-exchange procedure which is suitable for the quantitative isolation of lactonisable organic acids.

MATERIALS AND METHODS

Materials

Materials were obtained from the following sources: 2-hydroxy-3-methylbutyric acid, Pfaltz and Bauer (Stamford, CN, U.S.A.); 2-hydroxyisovaleric acid and 2-hydroxy-3-methylvaleric acid, from Sigma (St. Louis, MO, U.S.A.); phenylacetic acid, adipic acid, pimelic acid, glutaric acid and citric acid, Merck (Darmstadt, G.F.R.); DEAE-Sephadex A-25, Pharmacia (Uppsala, Sweden); 3% SE-30 on Gas-Chrom Q, Applied Science Labs. (State College, PA, U.S.A.); bis(trimethylsilyl)trifluoroacetamide (BSTFA), Regis Chemical (Morton Grove, IL, U.S.A.).

Reagents

The 0.5 *M* and 1.5 *M* pyridinium acetate buffers were prepared as described by Thompson and Markey [5]. The 0.5 *M* triethylammonium acetate buffer was prepared by adding 69 ml of triethylamine to 29 ml of glacial acetic acid and diluting the resulting solution to 1 l with distilled water. The pH of the buffer was 5.8. The 0.5 *M* triethylamine buffer was prepared by diluting 69 ml of triethylamine to 1 l with distilled water. The pH of this solution was 12.1.

The 0.5 *M* triethylamine—0.1 *M* acetic acid buffer was prepared by mixing 69 ml of triethylamine and 6 ml of glacial acetic acid and diluting the resulting solution to 1 l with distilled water. The pH of this buffer was 11.5.

Solvent extraction of urinary organic acids

Sodium chloride (0.5 g) was added to 1 ml of urine, followed by sufficient hydrochloric acid to bring the pH to 1. The solution was then extracted twice with ethyl acetate (2 × 2 ml) and once with diethyl ether (2 ml) and the pooled organic phases evaporated to dryness under a gentle stream of nitrogen at room temperature. The dried urinary extract was then silylated with 125 μl of BSTFA (60°C, 30 min) and an aliquot corresponding to 40 nmol creatinine injected onto the GC column. The derivatives were analysed on a 183 × 0.3 cm I.D. glass column of 3% SE-30 on 80—100 mesh Gas-Chrom Q. A helium flow-

rate of 25 ml/min was used and during the analysis the temperature was programmed from 80—220°C at 6°C/min.

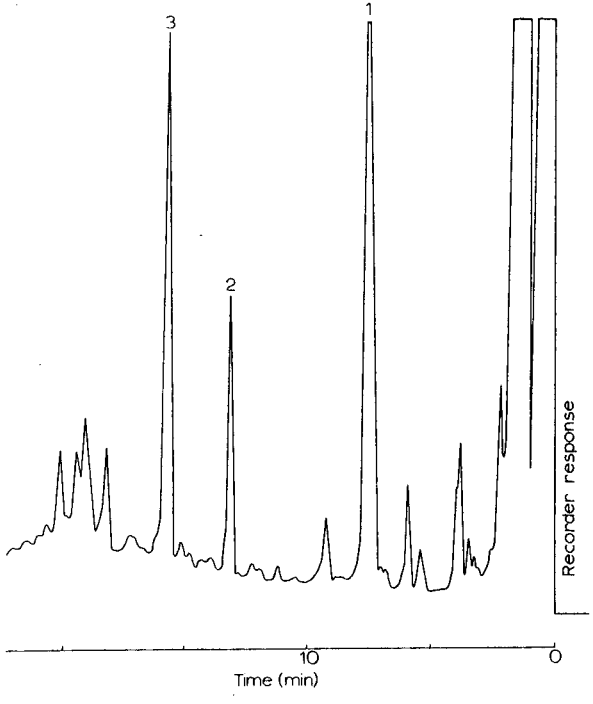
Isolation of urinary organic acids by ion-exchange chromatography

To an aliquot of urine (equivalent to 5 μmol of creatinine) in a centrifuge tube were added 2 ml of 0.1 *M* barium hydroxide. The contents were mixed, then centrifuged for 30 sec. The precipitate was washed with a further 2 ml of barium hydroxide and the supernatants combined. The supernatant was adjusted to pH 10 with 6 *N* hydrochloric acid and loaded on to a column containing 8 ml of DEAE-Sephadex A-25 which had been equilibrated in 0.5 *M* triethylammonium acetate. The column was washed with 40 ml of distilled water and the monocarboxylic acids were eluted with 40 ml of 0.5 *M* triethylamine. Glutaric acid (50 μl, 2000 μg) was added to the triethylamine effluent and the solution freeze-dried. The dried residue was dissolved in 1 ml of water and aliquots of this were redried and derivatized with BSTFA for GC analysis as described above.

Recovery of organic acids from DEAE-Sephadex

Standard solutions of concentration 5 mg/ml of each of the following acids in water or aqueous methanol were prepared: 2-hydroxy-3-methylbutyric, 2-hydroxyisovaleric, 2-methyl-3-methylvaleric, phenylacetic, adipic, pimelic and citric acids. Standard curves for each acid against glutaric acid as the internal standard were constructed by adding 50-μl, 100-μl and 200-μl aliquots of each acid solution, corresponding to 250, 500 and 1000 μg of acid, to separate screw-cap vials containing glutaric acid (100 μl, 500 μg). These solutions were freeze-dried and the dry residue was derivatized with BSTFA (200 μl, 60°C, 30 min) for GC analysis as described above. The ratios of peak heights of organic acids to glutaric acid were then plotted versus μg of each acid. All the calibration curves were linear over the concentration range used.

Aliquots (200 μl) of each of the above acid solutions, corresponding to 1000 μg of each acid, were combined and the resulting solution was adjusted to pH 10—11 with triethylamine. This resulting solution was applied to a column of DEAE-Sephadex (8 ml) equilibrated in 0.5 *M* triethylammonium acetate and the column was washed in turn with water (40 ml), 0.5 *M* triethylamine (40 ml), 0.5 *M* triethylamine—0.1 *M* acetic acid (40 ml) and 1.5 *M* pyridinium acetate. Glutaric acid (100 μl, 500 μg) was added to each fraction, after which the solutions were freeze-dried and derivatized with BSTFA (500 μl, 60°C, 30 min). Similarly, a second sample of the same solution was freeze-dried and derivatized without prior ion-exchange chromatography. The recovery of each organic acid in the triethylamine and triethylamine—acetate fractions were determined from the GC peak height ratios followed by interpolation from the respective calibration curves. The recoveries of the acids obtained in the two fractions have been summarized in Table I. The water wash did not contain any organic acids, whilst the pyridinium acetate eluate contained only the citric acid, confirming that all the other acids had been eluted with the other buffers.



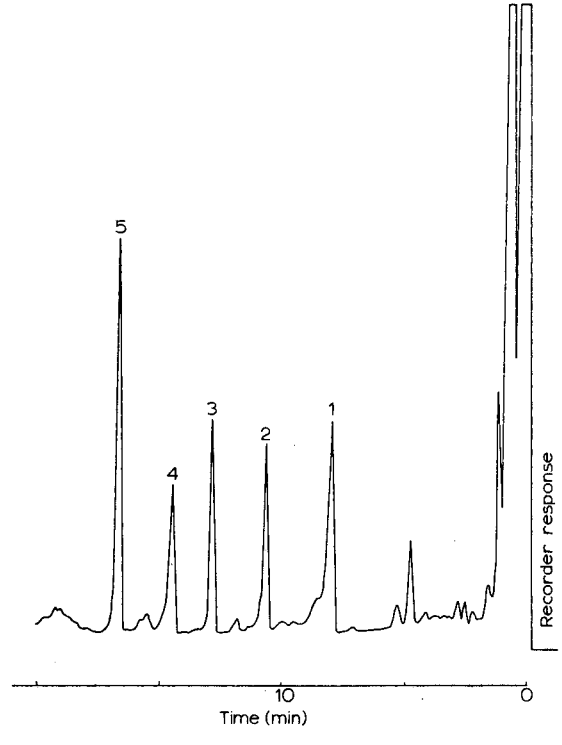


Fig. 1. GC trace of the silylated urinary extract from patient J.S. The organic acids were isolated from urine by solvent extraction. The numbered peaks were: (1) urea; (2) 2-deoxyerythropentono-1,4-lactone; (3) *p*-hydroxyphenylacetic acid.

Fig. 2. GC trace of the silylated urinary extract from patient J.S. The organic acids were isolated from urine by DEAE-Sephadex chromatography in 0.5 *M* pyridinium acetate buffer. The numbered peaks were: (1) phosphoric acid; (2) glutaric acid (internal standard); (3) 2-deoxyerythropentono-1,4-lactone; (4) 2-deoxyerythropentono-1,5-lactone; (5) 2-deoxyerythropentonic acid.

TABLE I

PERCENTAGE RECOVERIES OF ORGANIC ACIDS USING THE MODIFIED ANION-EXCHANGE METHOD ON DEAE-SEPHADEX

Compound	Recovery (%)
2-Hydroxy-3-methylbutyric acid	94
2-Hydroxyisovaleric acid	95
2-Hydroxy-3-methylvaleric acid	98
Phenylacetic acid	85
Adipic acid	97
Pimelic acid	91

Quantitation of 2-deoxyerythropentonic acid in urine

An aliquot of a standard solution (100 μl, 1000 μg) of 2-deoxyerythropentonic acid was added to normal urine corresponding to 10 μmol creatinine. Barium hydroxide (0.1 *M*, 2 ml) was added, centrifuged and the supernatants were removed. The urine solution was adjusted to pH 9—10 and applied to a column of DEAE-Sephadex (8 ml) equilibrated in 0.5 *M* triethylammonium acetate. The column was washed with 40 ml water and the acids were eluted with 40 ml of 0.5 *M* triethylamine. Glutaric acid (400 μl, 2000 μg) was added to the effluent, the solution was freeze-dried and derivatised with BSTFA (200 μl, 60°C, 30 min). The recovery of 2-deoxyerythropentonic acid was calculated from the GC peak height ratio versus the internal standard and interpolation of a standard curve. The recovery was 92—95%.

RESULTS AND DISCUSSION

The new method of isolation of organic acids was applied to a urine sample from a patient suffering from a defect in deoxyribose metabolism. The patient (J.S.) was an 18 months old boy, the only child of unrelated Greek parents. He was admitted to hospital because of poor feeding, vomiting and mild developmental delay. When the silylated ethyl acetate—diethyl ether extract of the patient's urine was analyzed by GC, large amounts of 2-deoxyerythropentono-1,4-lactone were observed (Fig. 1). It seemed likely that a considerable proportion of the lactone in the extract had arisen by lactonisation of 2-deoxyerythropentonic acid under the conditions used for solvent extraction. The rate and extent of lactonisation were not known however and the low extraction efficiencies of such hydrophilic compounds made quantitative studies difficult. In order to overcome this problem, the published ion-exchange procedure [5] was used for the quantitation of the new metabolite.

The GC profile of the silylated urinary extracts obtained by DEAE-Sephadex chromatography in 0.5 *M* pyridinium acetate buffer contained 2-deoxyerythropentonic acid, 2-deoxyerythropentono-1,4-lactone and 2-deoxyerythropentono-1,5-lactone (Fig. 2). The relative ratios of the three compounds obtained in various fractions were not constant, but varied considerably. The reason for the large variation in the extent of lactonisation following elution from DEAE-Sephadex is not known. This problem has already been encountered by other workers when measuring urinary tetronic and deoxy-

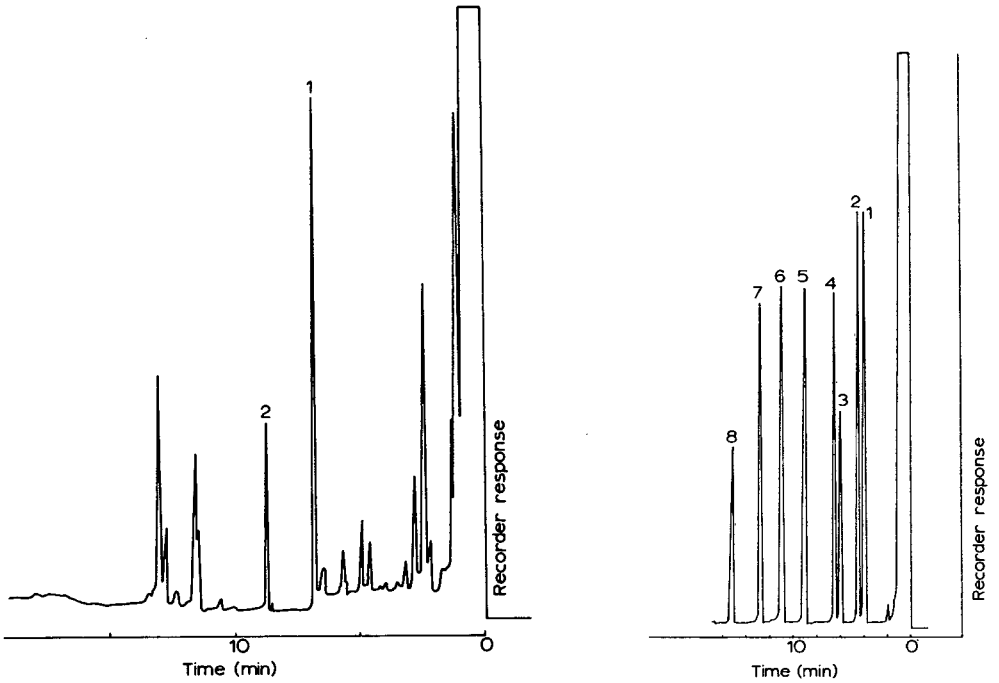
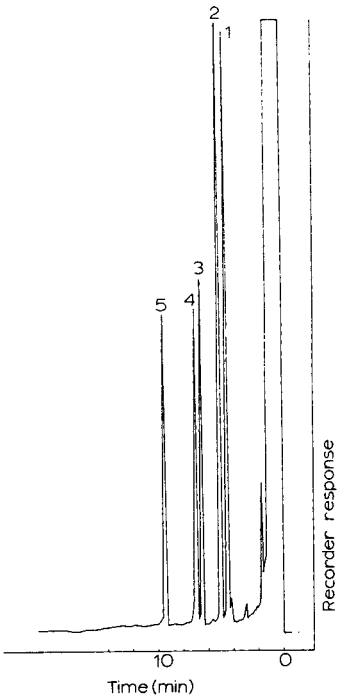


Fig. 3. GC trace of the silylated urinary extract from patient J.S. The organic acids were isolated from urine by the modified anion-exchange chromatography on DEAE-Sephadex. The numbered peaks were: (1) glutaric acid (internal standard); (2) 2-deoxyerythropentonic acid.

Fig. 4. GC trace of the silylated organic acid mixture used prior to DEAE-Sephadex chromatography. Peaks: (1) 2-hydroxy-3-methylbutyric acid; (2) 2-hydroxyisovaleric acid; (3) 2-hydroxy-3-methylvaleric acid; (4) phenylacetic acid; (5) glutaric acid (standard); (6) adipic acid; (7) pimelic acid; (8) citric acid.

tetronic acids [7]. We have now eliminated this problem by chromatographing the organic acids on DEAE-Sephadex equilibrated with 0.5 *M* triethylammonium acetate and eluting the 2-deoxyerythropentonic acid with a higher pH buffer. In this way the acid could be recovered in 92—95% yield, without lactonisation (Fig. 3). The modified procedure was used to analyse a standard mixture of carboxylic acids normally found in urine (Fig. 4) and it was shown that the mono-, di- and tricarboxylic acids could be eluted sequentially from the anion-exchange resin by the sequential use of three buffers, 0.5 *M* triethylamine (Fig. 5), 0.5 *M* triethylamine—0.1 *M* acetic acid (Fig. 6) and 1.5 *M* pyridinium acetate.

As well as providing improved recovery of sugar acids and other compounds which can undergo lactonisation, the use of this ion-exchange method has a number of additional advantages over the previously published procedure. For example in cases where patients are found to excrete novel compounds in the urine, the sequential elution procedure on DEAE-Sephadex when combined with GC, can be used to quickly determine the number of carboxyl groups in an unknown organic acid. The sequential elution process can then be repeated



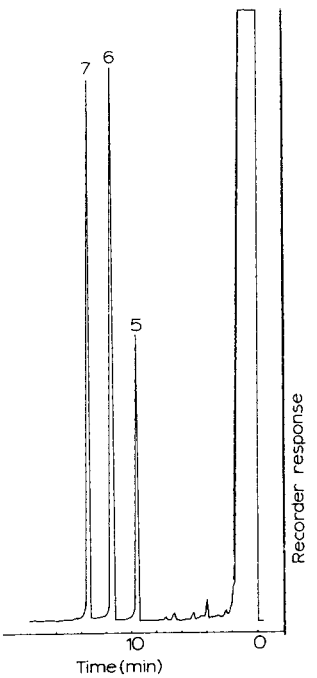


Fig. 5. GC trace of the silylated organic acids eluted from DEAE-Sephadex with 0.5 *M* triethylamine. Peaks: (1) 2-hydroxy-3-methylbutyric acid; (2) 2-hydroxyisovaleric acid; (3) 2-hydroxy-3-methylvaleric acid; (4) phenylacetic acid; (5) glutaric acid (standard).

Fig. 6. GC trace of the silylated organic acids eluted from DEAE-Sephadex with 0.5 *M* triethylamine—0.1 *M* acetic acid subsequent to elution with 0.5 *M* triethylamine. Peaks: (5) glutaric acid (standard); (6) adipic acid; (7) pimelic acid.

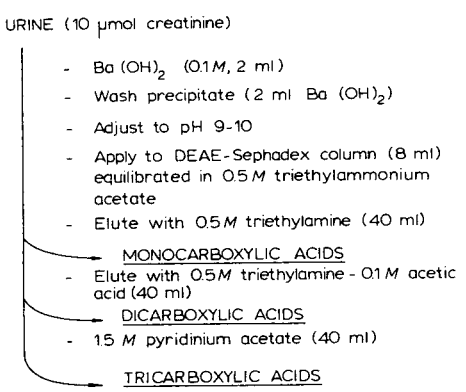


Fig. 7. Flow chart outlining modified anion-exchange chromatography on DEAE-Sephadex.

on a large scale as a step in the purification of such organic acids for subsequent structure determination. The modified ion-exchange procedure (Fig. 7) is particularly valuable for the quantitation of metabolites where the simplification of the urinary profile is of significant benefit.

REFERENCES

1 E. Jellum, J. Chromatogr., 143 (1977) 427.
2 E.C. Horning and M.G. Horning, Clin. Chem., 17 (1971) 802.
3 L. Eldjarn, E. Jellum and O. Stokke, J. Chromatogr., 91 (1974) 353.
4 R.A. Chalmers and R.W.E. Watts, Analyst (London), 97 (1972) 958.
5 J.A. Thompson and S.P. Markey, Anal. Chem., 47 (1975) 1313.
6 R.J.W. Truscott, B. Halpern, J. Hammond, S. Hunt, R.G.H. Cotton, E.A. Haan and D. Danks, Biomed. Mass Spectrom., 6 (1979) 453.
7 J.A. Thompson, S.P. Markey and P.V. Fennessey, Clin. Chem., 21 (1975) 1892.

Journal of Chromatography, 222 (1981) 345—352
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Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 755

GAS CHROMATOGRAPHIC ANALYSIS OF FREE AMINO ACIDS IN THE HYALOPLASM OF THE HYPOPHYSIS, PINEAL GLAND, THYROID GLAND, SPINAL CORD, THYMUS AND LYMPH NODES OF THE COW

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SUMMARY

Studies of the qualitative and quantitative composition of free amino acids in the hyaloplasm of the hypophysis, pineal gland, thyroid gland, spinal cord, thymus and lymph nodes of the cow are described. The following findings are reported: the highest levels of alanine, valine, glycine, isoleucine, histidine, leucine, threonine, serine, phenylalanine, tyrosine and lysine are found in the thyroid gland, methionine and aspartic acid in the spinal cord, tryptophan and hydroxyproline in the pineal gland, and proline and glutamic acid in the thymus gland. The highest level by weight is that of glutamic acid in all tissues. The presence of α-aminobutyric acid combined with sarcosine and 4-aminoisobutyric acid with 2-AOA and citrulline with cystine was demonstrated. α-Aminoisobutyric acid and isovaline were found in the spinal cord.

INTRODUCTION

The present work extends our investigations of the quantitative and qualitative composition of free amino acids* in the hyaloplasm of various tissues. In our previous work we traced the content of amino acids in the hyaloplasm of the liver, kidneys, frontal lobes of the brain, cerebellum, eyeball without muscles, heart muscle and skeletal muscles. This present paper deals with the results obtained from the following organs of the cow: hypophysis, pineal gland, spinal cord, thyroid gland, thymus gland, and lymph nodes.

Analysis of free amino acids by gas—liquid chromatography was performed according to the methods of Gehrke et al. [1,2] and Kaiser et al. [3].

*Abbreviations of amino acids: α-ABA = α-aminobutyric acid; 4-AIBA = 4-aminoisobutyric acid; α-AIBA = α-aminoisobutyric acid; ALLOTHR = allothreonine; 2-AOA = 2-aminooctanoic acid; ARG = arginine; CITR = citrulline; CYS = cystine; CYSH = cysteine; HIS = histidine; IVAL = isovaline; ORN = ornithine; SARC = sacrosine (see also Table I).

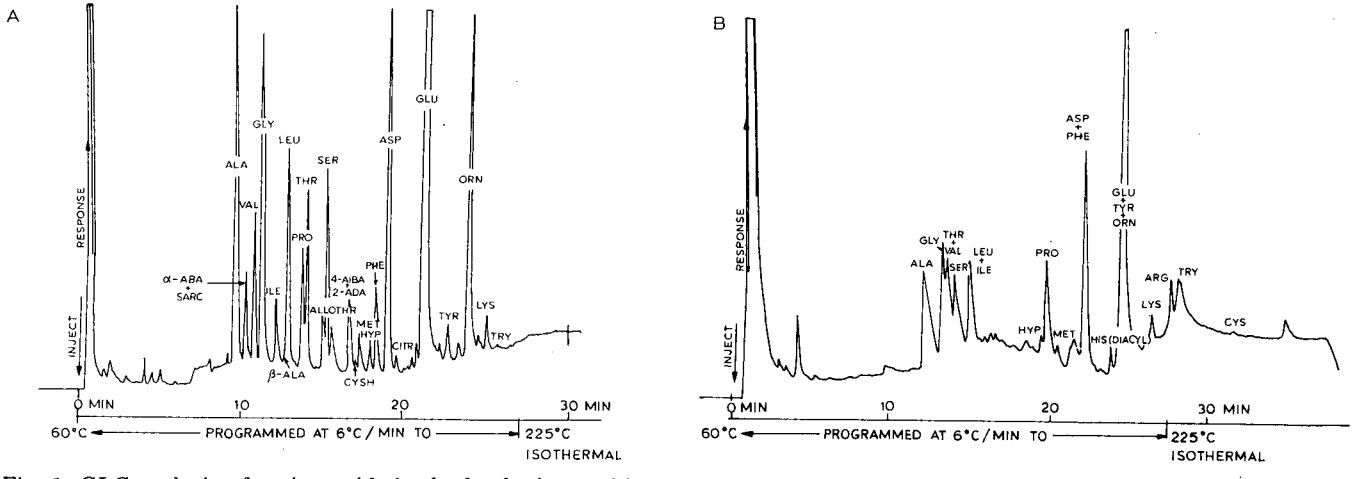

Fig. 1. GLC analysis of amino acids in the hyaloplasm of hypophysis on columns A and B (see Materials and Methods).

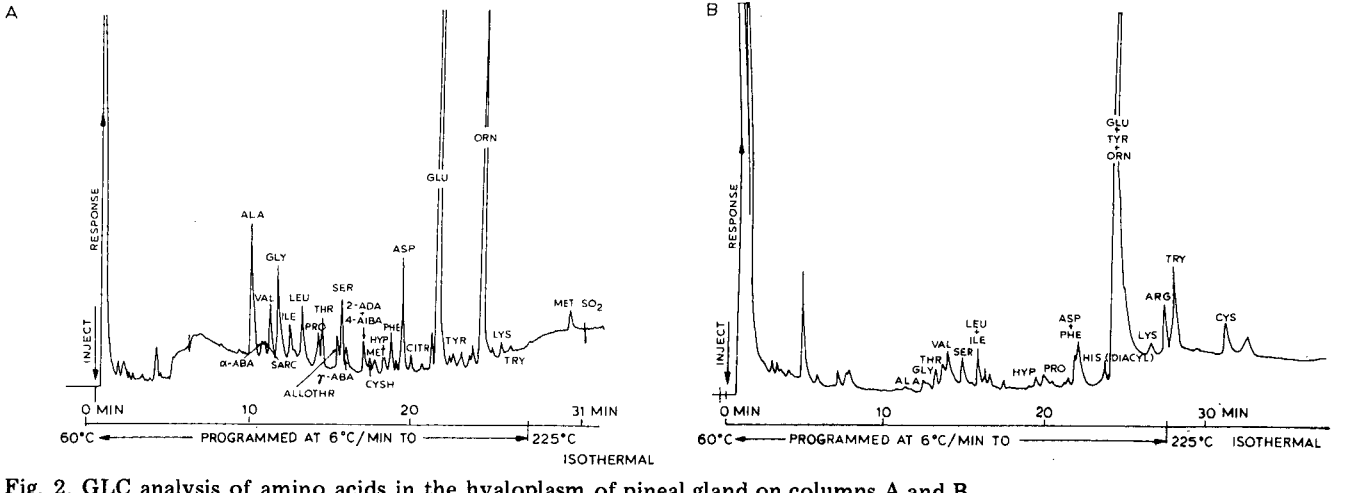

Fig. 2. GLC analysis of amino acids in the hyaloplasm of pineal gland on columns A and B.

Preparation of the hyaloplasm was performed according to the description of Chauveau et al. [4].

MATERIAL AND METHODS

Animals

The material, obtained from the slaughter house, was fixed in saturated picric acid. The tissues obtained were homogenized in picric acid in the ratio 1:5.

Apparatus

The following equipment was used: glass homogenizer (25-ml capacity) with a Teflon piston; K-24 and VAC 601 centrifuges produced by Janetzki Heins, Ilmenau, G.D.R.; ion-exchange columns 12 mm × 150 mm and 15 mm × 300 mm; lyophilizing apparatus produced by VEB MLW Labortechnik, Engelsdorf, G.D.R.; reaction vessels of our own design equipped with Teflon-lined screw-caps; automatic ultrasonic disintegrator UD-11 produced by Techpan, Warsaw, Poland; oil-baths with a thermoregulator; evaporator produced by Büchi, Flawil, Switzerland; gas chromatograph (Varian 3700) with data analyzer CDS 111 C and a recorder A 25 (Varian, Palo Alto, CA, U.S.A.); 10-μl syringes from Hamilton, Reno, NV, U.S.A.; and 1000-μl and 100-μl automatic pipettes produced by Eppendorf, Hamburg, G.F.R.

Reagents

7 *N* pure ammonia solution and Dowex 50W-X8 100—200 mesh (H^+) were products by Fluka, Buchs, Switzerland; pure picric acid was from P.O.CH, Gliwice, Poland. HCl gas was produced by Fluka; *n*-butanol Seq. grade and trifluoroacetic anhydride (TFAA) were from Pierce, Rockford, IL, U.S.A. Standard amino acids were obtained from BDH (Poole, Great Britain), Mann Labs. (New York, NY, U.S.A.), Merck and Pierce. A calibration mixture was prepared by adding the successively estimated amino acids. Column supports were 2% OV-17 + 1% OV-210 on 100—120 Supelcoport (column B) and 0.65% EGA-PS on 80—100 mesh Chromosorb W AW (Supelco, Bellefonte, PA, U.S.A.) (column A).

Preparation of amino acids

Homogenization. The tissues were homogenized in the ratio of 1:5.

Centrifugation and ultracentrifugation. The investigations were performed at +4°C according to the method of Chauveau et al. [4]. The homogenate was centrifuged at 600 *g* for 15 min; the supernatant was then decanted and centrifuged at 105,000 *g* for 60 min. Cell hyaloplasm was obtained in the supernatant, and 1% of picric acid was added in the ratio 1:5. The solution was centrifuged at 3500 *g* for 15 min, the proteins being separated in the sediment. The deproteinized supernatant containing an excess of picric acid was introduced into the ion-exchange column.

Ion exchange. In the following ion-exchange procedure a 60-fold or greater excess of resin capacity to exchangeable ions placed in the column was maintained, i.e. 6 g of Dowex 50W-X8 (100—200 mesh) for 25 ml of the examined

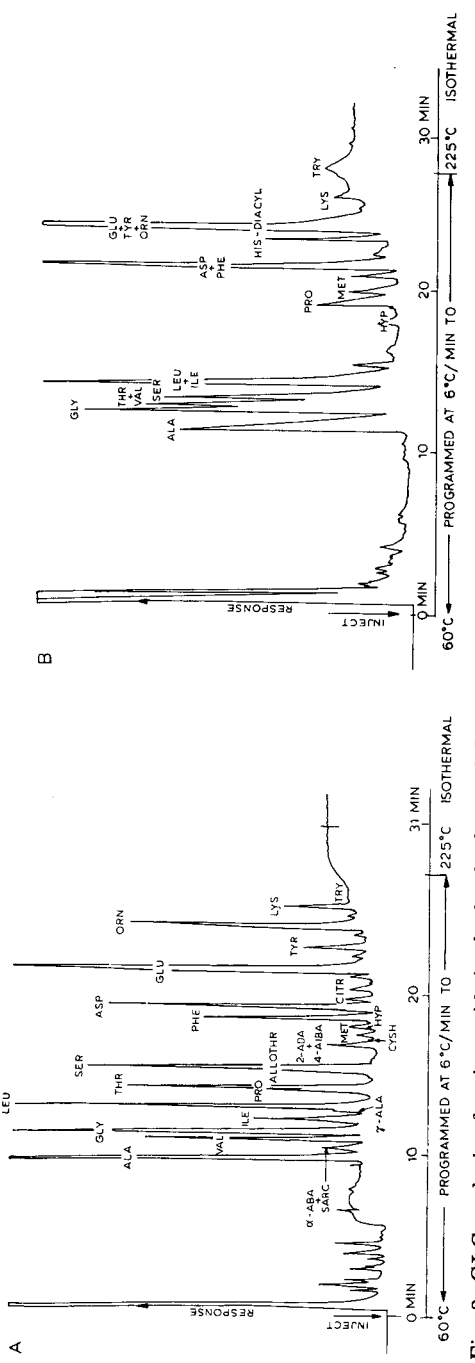

Fig. 3. GLC analysis of amino acids in the hyaloplasm of thyroid gland on columns A and B.

Fig. 4. GLC analysis of amino acids in the hyaloplasm of the spinal cord on columns A and B.

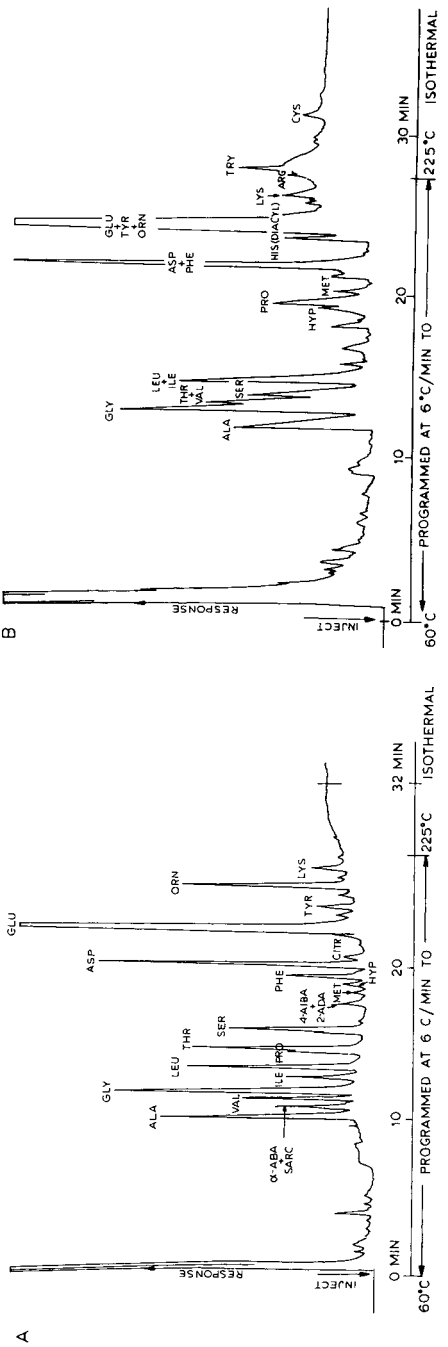

Fig. 5. GLC analysis of amino acids in the hyaloplasm of thymus gland on columns A and B.

Fig. 6. GLC analysis of amino acids in the hyaloplasm of lymph nodes on columns A and B.

TABLE I

COMPARISON OF GAS—LIQUID CHROMATOGRAPHIC ANALYSIS OF HYALOPLASM TISSUES

Values are expressed in μg of amino acid per 110 mg of tissues, and represent the mean ± S.D. of five independent samples.

Amino acid	Abbreviation	Reaction time (min)	Pineal gland	Lymph nodes	Thymus gland	Thyroid gland	Hypophysis	Spinal cord
Alanine	ALA	9.45±0.08	0.69±0.04	0.39±0.05	0.50±0.01	0.89±0.03	0.73±0.02	0.50±0.01
Valine	VAL	10.69±0.05	0.27±0.02	0.28±0.02	0.25±0.00	0.39±0.04	0.25±0.01	0.21±0.00
Glycine	GLY	11.07±0.04	0.59±0.01	0.81±0.07	0.73±0.02	0.85±0.01	0.71±0.02	0.55±0.01
Isoleucine	ILE	11.94±0.04	0.14±0.01	0.23±0.03	0.18±0.01	0.25±0.04	0.11±0.01	0.14±0.01
Leucine	LEU	12.68±0.03	0.31±0.01	0.55±0.05	0.42±0.02	0.85±0.09	0.41±0.01	0.25±0.01
Proline	PRO	13.63±0.05	0.22±0.00	0.23±0.04	0.24±0.00	0.24±0.02	0.24±0.03	0.10±0.03
Threonine	THR	13.89±0.06	0.36±0.02	0.30±0.01	0.35±0.04	0.42±0.04	0.32±0.02	0.33±0.03
Serine	SER	15.10±0.06	0.38±0.07	0.37±0.01	0.33±0.03	0.65±0.04	0.39±0.01	0.43±0.02
Methionine	MET	17.13±0.05	0.07±0.01	0.10±0.01	0.07±0.01	0.19±0.08	0.18±0.00	0.23±0.03
Hydroxyproline	HYP	17.74±0.04	0.13±0.01	0.06±0.00	0.06±0.00	0.07±0.01	0.06±0.00	0.10±0.00
Phenylalanine	PHE	18.17±0.06	0.24±0.01	0.25±0.01	0.22±0.00	0.41±0.01	0.18±0.00	0.21±0.01
Aspartic acid	ASP	18.86±0.09	0.64±0.02	1.20±0.04	0.69±0.00	0.64±0.04	0.78±0.01	1.48±0.02
Glutamic acid	GLU	21.05±0.06	5.22±0.66	4.17±0.13	5.25±0.01	3.16±0.04	4.67±0.09	4.68±0.09
Tyrosine	TYR	22.65±0.04	0.31±0.01	0.50±0.03	0.54±0.01	0.63±0.01	0.38±0.01	0.46±0.02
Lysine	LYS	25.10±0.02	–	0.34±0.04	–	0.45±0.07	0.24±0.02	0.20±0.03
Tryptophan	TRY	25.98±0.21	–	–	0.08±0.01	–		0.12±0.05
Total			9.57	9.78	9.91	10.09	9.65	9.99

supernatant. The 6 g of ion-exchanger were placed in a 500-ml vial, covered with 7 *N* ammonia solution and mixed for 60 min. After sedimentation the fluid was decanted. The procedure was repeated two times. Afterwards the column was washed with bidistilled water until a pH of 7.5 was attained. The ion exchanger was regenerated with 3 *N* HCl (three times), and washed with water until a pH value of 6.2 was attained. Columns 15 mm × 150 mm were filled half-full with the wet resin. The supernatant with the picric acid was passed through the column at a rate of 2 ml/min. The surface was washed with water until the eluate was decolourised. Then 25 ml of 7 *N* ammonia solution and 30 ml of bidistilled and deionized water were passed through the column. The eluate and the washings were collected and mixed, and 55 ml of the mixture were lyophilized. The procedure was carried out according to the description of Zumwalt et al. [5].

Lyophilization. A 55-ml volume of the eluate and washings was collected into a vessel and quickly frozen in liquid nitrogen. The sample was placed in a 1000-ml condenser. After lyophilization the dry sediment was retained and transferred to an esterification vessel.

Derivatization. The reaction was carried out according to the method of Gehrke et al. [1,2].

RESULTS AND DISCUSSION

The quantitative and qualitative content of amino acids and their trace amounts in the cell hyaloplasm (cytosol) of glandular tissues and the spinal cord of cows were studied (see Table I). During the investigation three independent series of analysis of amino acid composition were performed. The following conclusions were drawn: the highest levels of ALA, VAL, GLY, THR, ILE, LEU, HIS, SER, PHE, TYR and LYS were found in the thyroid gland, MET and ASP in the spinal cord and PRO + GLU in the thymus. In the tissues analyzed GLU was found to have the highest concentration of all amino acids. As a result of the separation of amino acid mixture on EGA the presence of α-ABA with SARC and 4-AIBA with 2-AOA was discovered. The works of Raulin et al. [6], Amico et al. [7], and Casagrande et al. [8] were of great help. The peak of α-AIBA with IVAL identified in the spinal cord was the only one found in the material investigated. Quantitative computerized analysis shows a lower weight level of the total bulk of the separated mixture as compared to its amount taken for derivatization purposes. This problem has been also emphasized by Adams et al. [9]. They found ions, lipids and peptides in the investigated material. Attention has been paid to tissue material stored for periods of longer than 14 days. Decreased peak heights for PRO + ALLOTHR, α-ABA, MET, CITR, TYR, ARG, TRY, CYSH and β-ALA were found.

CONCLUSIONS

The highest levels of amino acids were found in thyroid gland, lymph nodes and thymus. TRY, ARG and CYS were the amino acids with the lowest levels. The presence of small peaks indicates a trace content of unidentified compounds.

REFERENCES

1 C.W. Gehrke, D. Roach, R.W. Zumwalt, D. Stalling and L.L. Wall, Quantitative Gas—Liquid Chromatography of Amino Acids in Proteins and Biological Substances. Macro, Semimicro, and Micro Methods, Analytical Biochemistry Laboratories, Inc., Columbia, MO, 1968, p. 1.
2 C.W. Gehrke, R.W. Zumwalt and K. Kuo, J. Agr. Food Chem., 19 (1971) 605—618.
3 F.E. Kaiser, C.W. Gehrke, R.W. Zumwalt and K.C. Kuo, J. Chromatogr., 94 (1974) 113—133.
4 I. Chauveau, I. Moule, C. Bouiller and I. Scheebeli, J. Cell Biol., 12 (1962) 17—29.
5 R.W. Zumwalt, D. Roach and C.W. Gehrke, J. Chromatogr., 53 (1970) 171—193.
6 F. Raulin, P. Shapshak and B.N. Khare, J. Chromatogr., 73 (1972) 35—41.
7 V. Amico, G. Oriente and C. Tringali, J. Chromatogr., 116 (1976) 439—444.
8 D.J. Casagrande, J. Chromatogr., 49 (1970) 537—540.
9 R.F. Adams, J. Chromatogr., 95 (1974) 189—212.

Journal of Chromatography, 222 (1981) 353—362
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 756

DETERMINATION OF DOPAMINE, HOMOVANILLIC ACID AND 3,4-DIHYDROXYPHENYLACETIC ACID IN RAT BRAIN STRIATUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

Two procedures using liquid chromatography with electrochemical detection are described for the determination of dopamine (DA) and its two acidic metabolites, homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC), in subregions of rat striatum and nucleus accumbens. A strong cation-exchange column was used for DA analysis and a C_{18} reversed-phase column was used for the analysis of the metabolites. Effects of pH, temperature and percentage of methanol on the retention time of HVA and DOPAC were studied. Levels of these compounds in the subregions of rat striatum and nucleus accumbens are reported.

INTRODUCTION

High-performance liquid chromatography with electrochemical detection is now a frequently used and well established technique for the determination of catecholamines, related compounds and their metabolites in biological samples. These compounds include dopamine (DA), norepinephrine (NE), epinephrine (EPI) and their acid metabolites homovanillic acid (HVA), dihydroxyphenylacetic acid (DOPAC), and vanillylmandelic acid (VMA). Because of very simple procedures of sample preparation, high sensitivity and selectivity, this method is well suited for the measurement of low levels of these compounds in biolog-

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ical samples. Various methods have been reported for the determination of catecholamines and their metabolites in plasma [1—3], urine [4—8], and tissue [3, 9—12] samples. DA and NE have been determined in whole brain and different parts of the rat brain by analyzing tissues weighing 0.5—500 mg [10, 11]. Wagner et al. [9] determined DA, NE, EPI, and DOPAC in rat brain, heart, kidney, etc., with tissues weighing 40—500 mg. Oke et al. [12] studied the effect of neonatal 6-hydroxydopamine treatment on levels of DA and NE in discrete rat brain regions. Freed and Asmus [3] determined DA, NE, and DOPAC in rat brain samples with approximately 35 mg of the tissue. In the present paper sample size is 1—2 mg.

The sample preparation generally involves two steps: separation of proteins and high-molecular-weight compounds by precipitation followed by the isolation of catecholamines and their metabolites from other organic compounds present in the samples by adsorption or extraction. Isolation of catechols by selective adsorption on alumina is the most frequently used method. The alumina is then washed, compounds are eluted and injected on the liquid chromatography (LC) column. Felice and Kissinger [6] determined HVA in urine samples by combining the techniques of solvent extraction, thin-layer chromatography (TLC) and LC. Sasa and Blank [10] reported a butanol extraction for the determination of DA, NE, and 5-hydroxytryptamine (5-HT) in rat brain samples. Soldin and Hill [7] determined HVA and VMA in urine using anion-exchange resin for the clean-up of the samples.

In the present paper we report simple procedures for the analysis of dopamine and for the analysis of the acid metabolites of dopamine, HVA, and DOPAC, which are easy to perform and require a minimum number of steps. For the determination of DA, the tissue samples, punched from slices of rat brain striatum and weighing 1—2 mg, were homogenized in trichloroacetic acid (TCA) solution and the proteins were precipitated by centrifugation. Other acidic or neutral organic compounds were extracted into isooctane and the organic layer was aspirated. The aqueous layer was directly injected on the LC column. For the DOPAC and HVA analysis, proteins were precipitated similarly and the acids were extracted into ethyl acetate. The organic layer was dried under vacuum, the residue dissolved in the mobile phase and injected on the LC column.

EXPERIMENTAL

Materials

Dopamine hydrochloride and DOPAC were purchased from Sigma (St. Louis, MO, U.S.A.). HVA, 3,4-dihydroxycinnamic acid (DHCA) and 3,4-dihydroxybenzylamine hydrobromide (DHBA) were from Aldrich (Milwaukee, WI, U.S.A.). Other chemicals used were all analytical reagent grade and were used without further purification.

Liquid chromatograph

A Waters Model 6000A solvent delivery system was used with a Chromatronix Model HPSV sampling-valve injector and a thin-layer amperometric detector. The potentiostat/amplifier was built in our laboratory. The detector

consisted of a carbon paste working electrode, Ag/AgCl reference electrode, and a platinum wire as an auxiliary electrode. The carbon paste working electrode was stable for 4—5 weeks in dopamine analysis, where only an aqueous solvent was used. In the HVA and DOPAC analysis, the working electrode was repacked every week. Apparently, the electrode life was shortened by the introduction of methanol into the solvent system.

Dopamine analysis

Chromatographic conditions. A stainless-steel column (1 m × 4.6 mm I.D.) packed with Zipax strong cation-exchange resin (DuPont, Wilmington, DE, U.S.A.), 30—40 μm particle size, was used. The working electrode potential was maintained at +0.61 V vs. the reference electrode. The flow-rate was 0.6 ml/min and the volume injected per sample 10 μl. Analysis was performed at ambient column temperatures and with isocratic elution using citrate—acetate buffer of pH 5.2.

Reagents. Citrate—acetate buffer was prepared by dissolving 8.2 g of anhydrous sodium acetate, 2.1 ml of glacial acetic acid, 4.8 g of sodium hydroxide, 10.5 g of citric acid monohydrate, and 0.37 g of disodium salt of EDTA in 1 l of distilled water. The eluent was degassed by heating the reservoir at 50°C during the analysis.

Stock solutions of dopamine hydrochloride and DHBA were prepared in 0.01 *M* hydrochloric acid containing 0.1% sodium metabisulphite. Standard solutions containing DA free base (0.2—2 ng per 10 μl) and DHBA free base (1.03 ng per 10 μl) were prepared from these stock solutions for standard curves. Hydrochloric acid was deaerated for 15—20 min by passing nitrogen before use. All the solutions were stored at 4°C and stock solutions were prepared fresh every 2 weeks.

For brain tissue analysis a solution of 10% TCA containing 0.1% sodium metabisulphite and 0.95 ng per 10 μl DHBA (free base), as internal standard, was used as the extracting solution.

Sample preparation. Adult male CFE albino rats (Charles River, Wilmington, MA, U.S.A.) were used in all experiments. Animals were killed with a guillotine and the brains quickly removed from the skull and frozen in powdered dry ice. The brains were kept frozen on the stage of a Super Histofreeze (Scientific Products) and five contiguous 1 mm thick sections were cut using a sliding microtome. The landmark for the first section was the nucleus accumbens of AP 10.0 (brain atlas of Pellegrino and Cushman) [13]. As soon as they were cut, the brain slices were transferred to a cold plate and two tissue punches were taken from each slice. These punches were from symmetrical locations in the left and right hemispheres and were combined for further analysis. Fig. 1 illustrates the locations of the samples. Punches were made with stainless-steel tubing of 1.35 mm I.D. The punched samples were transferred to plastic test-tubes containing 250 μl of the extracting solution, immediately homogenized by sonication (Heat Systems Model W-220F) for 5—10 sec and the tubes were placed in ice. These tubes were then centrifuged for 10 min at 2500 *g* to precipitate the protein after which 200 μl of the supernatant were transferred to 1.5-ml micro-test-tubes containing 150 μl of isooctane and shaken for 10 min. The micro-test-tubes were then centrifuged at 15,000 *g* (Eppendorf micro

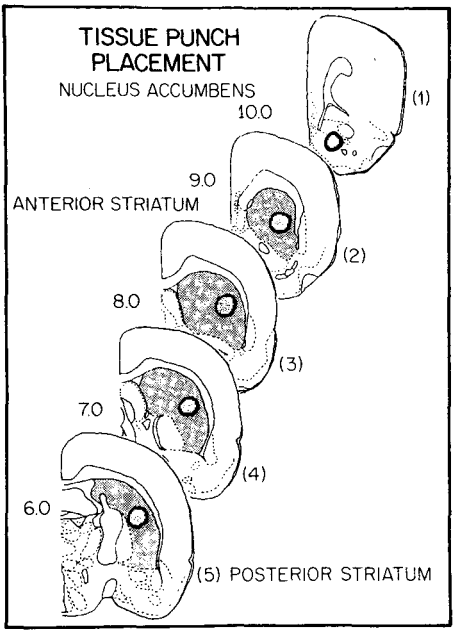


Fig. 1. Tissue punch placement. The first punch was from a 1 mm thick tissue slice containing portions of nucleus accumbens and olfactory tubercle; the next four punches were taken from successive 1 mm thick tissue slices in the striatum (shaded structure). The numbers on the left refer to the anterior—posterior axis coordinates in the brain atlas of Pellegrino and Cushman [13]. Punch diameter, 1.35 mm.

centrifuge) for 4 min and the organic layer was removed by aspiration. Aliquots of 10 μl of the aqueous phase were directly injected into the LC column.

Quantitation. Six standard solutions containing 0.2, 0.4, 0.81, 1.21, 1.61, and 2.02 ng DA per 10 μl with 1.03 ng DHBA per 10 μl were prepared in deaerated 0.01 *M* hydrochloric acid. Standard solutions were run every day before the analysis and curves were prepared by plotting the peak height ratios versus the ratio of the amounts (in ng) of the compound and the internal standard. Dopamine concentrations in the samples as nanogram DA per mg protein are calculated by the following equation:

$$\frac{\text{ng DA}}{\text{mg protein}} = \frac{\dfrac{\text{PH}_{\text{DA}}}{\text{PH}_{\text{DHBA}}} \times \dfrac{0.95 \text{ ng DHBA}}{10\ \mu\text{l}} \times 250\ \mu\text{l}}{\text{slope of standard curve} \times \text{mg protein}}$$

where PH is peak height.

HVA and DOPAC analysis

Chromatographic conditions. A stainless-steel reversed-phase column (25 cm × 2.1 mm I.D.) Zorbax ODS (DuPont), 6—8 μm particle size was used. The potential of the working electrode was set at +0.85 V vs. Ag/AgCl reference electrode. Column temperature was 25°C and the flow-rate 0.4 ml/min. Phos-

phate (0.1 *M*)—citrate (0.05 *M*) buffer pH 4.3 with 10% methanol was used as the mobile phase. The sample loop was 100 μl.

Reagents. For the preparation of phosphate—citrate buffer 14.2 g of disodium hydrogen phosphate and 10.5 g of citric acid monohydrate were dissolved in 850 ml of distilled water and the pH was adjusted to 4.3 by 6 *M* hydrochloric acid. After adjusting the pH 100 ml of methanol were added and the volume was made up to a liter with distilled water. The solvent was degassed by heating the reservoir at 50°C during the analysis.

Stock solutions of HVA, DOPAC, and DHCA were prepared in deaerated phosphate—citrate buffer pH 4.3 (no methanol) which contained 0.1% sodium metabisulphite. Standard solutions containing HVA, DOPAC (0.5—2.5 ng per 100 μl) and DHCA (2.0 ng per 100 μl) were prepared every day for the standard curves. Stock solutions were prepared every two weeks. All the solutions were stored at 4°C.

The extracting solution for brain tissue analysis was prepared as described for dopamine analysis with 2.0 ng per 100 μl of DHCA as internal standard.

Sample preparation. The initial procedure of taking punches was similar to that described for dopamine assay. Tissue punches were homogenized in 250 μl of extracting solution and the tubes were centrifuged for 10 min at 2500 *g*. Aliquots of 200 μl of the supernatant were then transferred to small micro-test-tubes which contained 300 μl of ethyl acetate. These tubes were shaken for 20 min and then 250-μl aliquots of the organic layer were transferred to another micro-test-tube and dried in a vacuum desiccator. The samples were reconstituted in 250 μl of phosphate—citrate buffer pH 4.3 (no methanol) and 100 μl of the sample injected into the LC column.

The precipitate obtained after tissue homogenization and centrifugation was analyzed for protein by the method of Lowry et al. [14] in all analyses.

Quantitation. Five standard solutions containing 0.5, 1.0, 1.5, 2.0, and 2.5 ng HVA and DOPAC each per 100 μl with 2 ng DHCA per 100 μl were prepared every day in the phosphate—citrate buffer. Standard solutions were run before the analysis and the curves were prepared as described for dopamine. The concentration of DOPAC and HVA in each sample is calculated as follows:

$$\frac{\text{ng DOPAC or HVA}}{\text{mg protein}} = \frac{\dfrac{PH_{\text{DOPAC or HVA}}}{PH_{\text{DHCA}}} \times \dfrac{2 \text{ ng DHCA}}{100\ \mu l} \times 250\ \mu l}{\text{slope of standard curve}_{\text{DOPAC or HVA}} \times \text{mg protein}}$$

Recoveries of HVA, DOPAC, and the internal standard were examined as a function of extraction time with ethyl acetate. Samples for extraction were prepared by taking non-striatal tissue samples in ten tubes shown to contain no endogenous HVA and DOPAC, and adding known amounts of HVA, DOPAC and internal standard in 10% TCA containing 0.1% sodium metabisulphite. The samples were extracted for periods of 10, 20, and 60 min. Standard solutions were prepared in buffer containing equal amounts of the three compounds as in the samples. Recoveries were calculated by comparing the peak heights of the standard and the samples. The recoveries with the standard

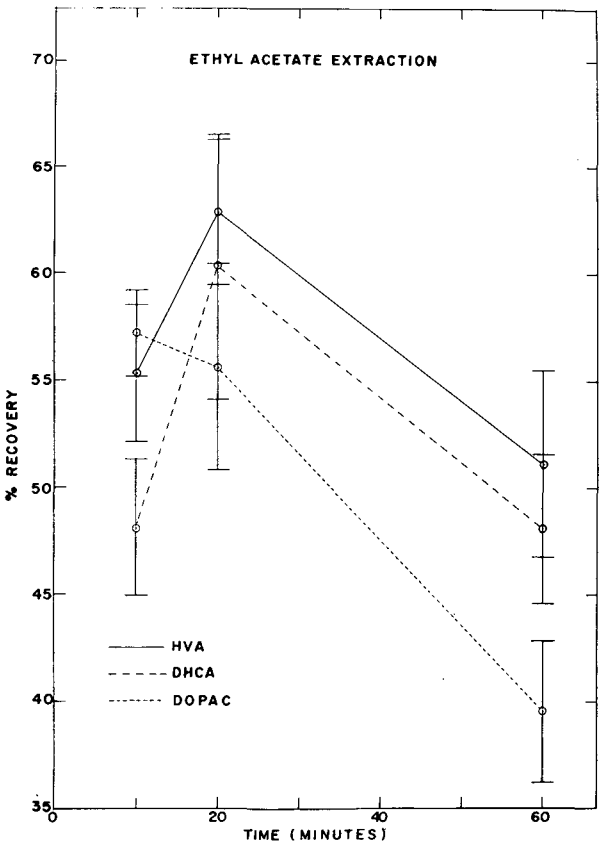


Fig. 2. Recoveries of HVA, DOPAC and the internal standard as a function of extraction time with ethyl acetate (n = 6—9).

deviations are given in Fig. 2. Samples extracted with ethyl acetate for 20 min gave the best recoveries. Longer time periods (1 h) gave low recoveries due to the loss by oxidation. Note that maximum recovery is 67% based on volumes transferred.

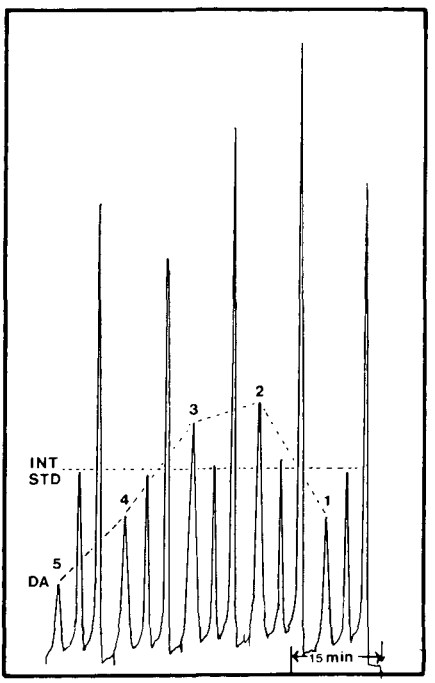
RESULTS AND DISCUSSION

Dopamine

Fig. 3 shows a chromatogram of five samples (from five different regions) for one rat. Retention times for DHBA and DA are 7 min and 10 min, respectively. The total analysis time per sample was 12 min.

HVA and DOPAC

Fig. 4 shows a chromatogram of a brain sample. The first peak after the solvent front is dopamine which is extracted into the ethyl acetate and has a retention time of 4 min. Approximately 2% of the total dopamine is extracted.



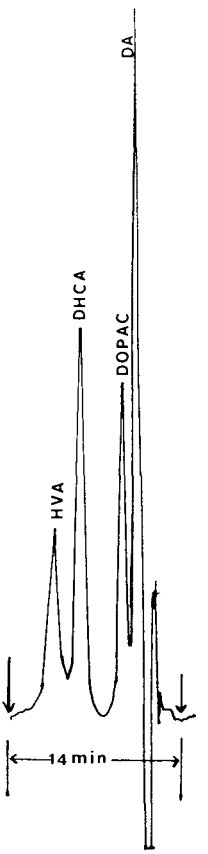


Fig. 3. A chromatogram of five samples (from five different regions) for one rat. Column, Zipax SCX (1 m × 4.6 mm I.D.); mobile phase, citrate—acetate buffer pH 5.2, flow-rate 0.6 ml/min; column temperature, 25°C.

Fig. 4. Chromatogram of a brain sample for HVA and DOPAC analysis. Column, Zorbax ODS (25 cm × 2.1 mm I.D.); mobile phase, phosphate (0.1 *M*)—citrate (0.05 *M*) buffer pH 4.3, 10% methanol, flow-rate, 0.4 ml/min; column temperature, 25°C.

Chromatographic conditions

Effects of temperature, pH and concentration of methanol on the retention time of HVA and DOPAC were studied. Retention time of HVA decreases very significantly with increase in column temperature, concentration of methanol and the pH of the solvent, whereas only slight decrease in retention time is observed in case of DOPAC. Phosphate (0.1 *M*)—citrate (0.05 *M*) buffer pH 4.3 with 10% methanol and column temperature at 25°C were found to be suitable for the analysis of HVA and DOPAC. Under the conditions described here for the analysis the retention times of DOPAC, DHCA, and HVA were 5, 8, and 10 min, respectively. The time for each sample run was 14 min.

Detector stability

The detector was more stable in the aqueous solvent of the DA analysis than in the 10% methanol of the HVA and DOPAC analysis. In the DA analysis, the detector was checked each day by running standard solutions. The standard solutions were stable for two weeks and were used in calibration. Slopes of the standard curves changed less than 7% over a period of one month. In the HVA and DOPAC analysis, the slope changed 15% during a two-week period. Therefore for the data reported, the detector was repacked every week and standard solutions prepared every day.

The least detectable amounts were 250 pg for DA, 100 pg for HVA, and 66 pg for DOPAC. Both analysis procedures gave chromatograms without any interfering peaks from other compounds present in the samples.

Figs. 5 and 6 show levels of DA, HVA, and DOPAC in the five sections of rat brain. The first section is from the nucleus accumbens. The next four sections are from the striatum with sections 2 and 5 being the anterior and the posterior striatum, respectively. The gradient in these sections of striatum is very clear in

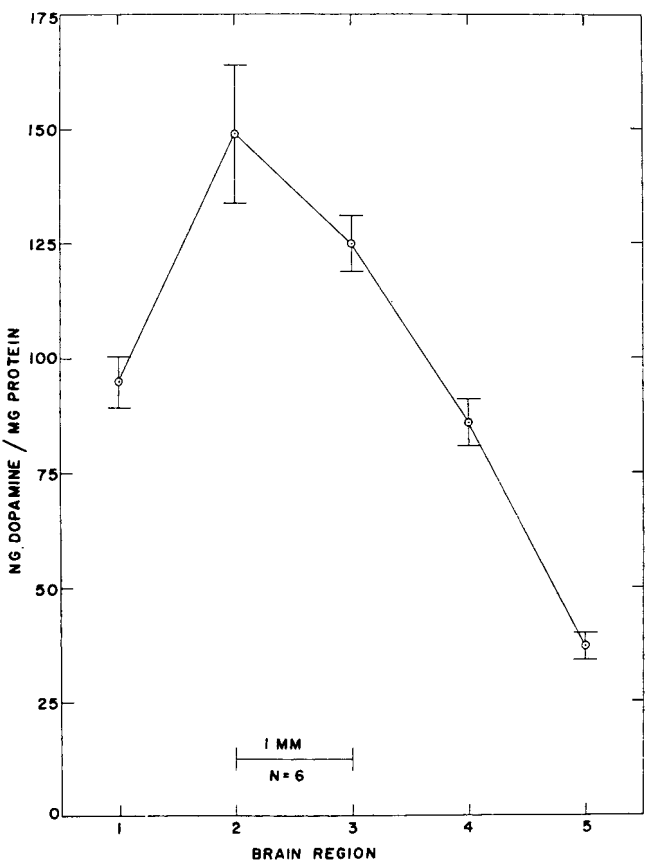


Fig. 5. Average (± S.D.) values of ng dopamine per mg protein in each brain region. Region 1 is the nucleus accumbens, regions 2—5 are the striatum with regions 2 and 5 the anterior and posterior striatal regions, respectively.

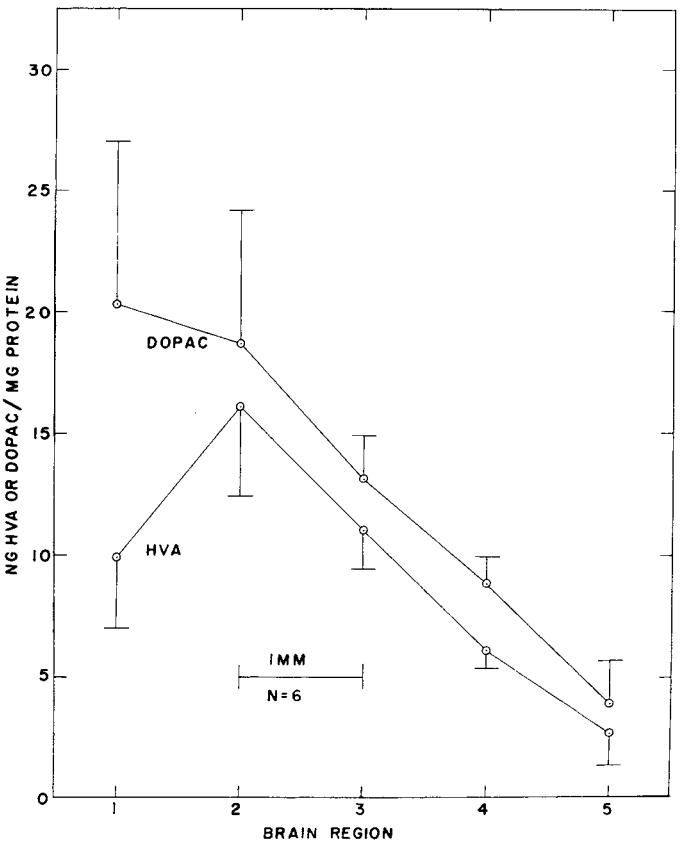


Fig. 6. Average (± S.D.) values of HVA and DOPAC (ng/mg protein) in the regions described in Fig. 5.

all cases. The dopamine content of the nucleus accumbens region 1 of Fig. 1, was found to be 95 ± 6 ng per mg protein. The anterior striatum, region 2, was found to have 149 ± 15 ng per mg protein. Regions 3, 4, and 5 from the striatum had 125 ± 6, 86 ± 5 and 37 ± 3 ng per mg protein respectively (n = 6). HVA and DOPAC were present in much lower concentration. For regions 1—5, HVA content was 9.9 ± 2.9, 16.1 ± 3.7, 11.0 ± 1.6, 6.1 ± 0.8, and 2.7 ± 1.4, respectively (n = 6). DOPAC content was 20.3 ± 6.7, 18.7 ± 5.5, 13.1 ± 1.8, 8.8 ± 1.1, and 3.9 ± 1.8, (n = 6) for the same regions. Tassin et al. [15] observed a similar gradient in dopamine levels (100—40 ng per mg protein) in these regions using a radioenzymatic method. Koslow et al. [16] reported a gas chromatography—mass spectrometry method for dopamine determination and also found an anterior—posterior gradient. Holdiness et al. [17] also showed the gradient in these regions with dopamine levels in the range of 165 to 75 ng per mg protein. The levels of DA reported here are in general agreement with the levels reported earlier.

The procedure described for DA analysis is simple and easy to perform. The method is rapid and 30—40 samples (6—8 rats) can be run in a routine day.

The procedure for HVA and DOPAC analysis also involves only a few simple steps and can be performed rapidly. 20—25 samples (4—5 rats) can be analyzed in a routine day.

The ability to determine levels of DA and its metabolites in small samples of brain tissue by the relatively simple and inexpensive technique described herein should facilitate functional studies of brain dopaminergic systems. Studies which have demonstrated surgically or drug-induced changes in whole striatal DA, HVA, and DOPAC [18—20] can now be extended to subregions of striatum and other areas. For example, we have found [21] that changes in behavior produced by lesions in striatum in rat correlate with resulting DA depletions in striatal subregions.

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REFERENCES

1 P.M. Plotsky, D.M. Gibbs and J.D. Neill, Endocrinology, 102 (1978) 1887—1894.
2 R.M. Riggin, R.L. Alcorn and P.T. Kissinger, Clin. Chem., 22 (1976) 782—784.
3 C.R. Freed and P.A. Asmus, J. Neurochem., 32 (1979) 163—168.
4 P.T. Kissinger, R.M. Riggin, R.L. Alcorn and L.D. Rau, Biochem. Med., 13 (1975) 299—306.
5 R.M. Riggin and P.T. Kissinger, Anal. Chem., 49 (1977) 2109—2111.
6 L.J. Felice and P.T. Kissinger, Anal. Chem., 48 (1976) 794—796.
7 S.J. Soldin and J.G. Hill, Clin. Chem., 26 (1980) 291—294.
8 L.J. Felice, C.S. Bruntlett and P.T. Kissinger, J. Chromatogr., 143 (1977) 407—410.
9 J. Wagner, M. Palfreyman and M. Zraika, J. Chromatogr., 164 (1979) 41—54.
10 S. Sasa and C.L. Blank, Anal. Chim. Acta, 104 (1979) 29—45.
11 R. Keller, A. Oke, I. Mefford and R.N. Adams, Life Sci., 19 (1976) 995—1004.
12 A. Oke, R. Keller and R.N. Adams, Brain Res., 148 (1978) 245—250.
13 L.J. Pellegrino and A.J. Cushman, A Stereotaxic Atlas of the Rat Brain, Appleton-Century-Crofts, New York, 1967.
14 O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem., 193 (1951) 265—275.
15 J.P. Tassin, A. Cheramy, G. Blanc, A.M. Thierry and J. Glowinski, Brain Res., 107 (1976) 291—301.
16 S.H. Koslow, G. Racagni and E. Costa, Neuropharmacology, 13 (1974) 1123—1130.
17 M.R. Holdiness, M.T. Rosen, J.B. Justice and D.B. Neill, J. Chromatogr., 198 (1980) 329—336.
18 A. Argiolas, F. Fadda, E. Stefanini and G.L. Gessa, Naunyn-Schmiedeberg's Arch. Pharmacol. 301, (1978) 171—174.
19 B.H.C. Westerink and J. Korf, Eur. J. Pharmacol., 38 (1976) 281—291.
20 B.H.C. Westerink and J. Korf, Brain Res., 113 (1976) 429—434.
21 D.B. Neill, D. Labiner, L.D. Saraswat, M.R. Holdiness and J.B. Justice, Behav. Brain Res., submitted for publication.

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DETERMINATION OF MONOACETYLDIAMINES AND -POLYAMINES IN URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A procedure is described for the determination of monoacetylputrescine, N^1-acetylspermidine and N^8-acetylspermidine in human urine. The procedure is based on the high-performance liquid chromatographic separation of the 5-dimethylaminonaphthalene-1-sulfonyl (dansyl) derivatives of these amines using two different chromatographic modes. Monoacetyl-1,6-diaminohexane was used as an internal standard. The amines were extracted from urine using a silica gel cartridge. The dansyl monoacetylpolyamines were separated from the mixture of dansyl derivatives of urinary amines on a bonded-phase CN column using a programmed solvent gradient elution. The dansyl acetylpolyamines were rechromatographed on a silica gel column.

This chromatographic procedure was used for the determination of the concentration of N^1-acetylspermidine, N^8-acetylspermidine and monoacetylputrescine in the urine of healthy volunteers and cancer patients.

INTRODUCTION

The polyamines are present in human urine predominantly as conjugates that produce the free amines after hydrolysis [1]. In most studies in which urinary polyamines were utilized as markers of cancer, the urine was hydrolyzed prior to analysis to liberate the polyamines from these conjugates [1, 2]. N^1-Acetylspermidine (N^1-Ac-Spd), N^8-acetylspermidine (N^8-Ac-Spd) and monoacetylputrescine (Ac-Put) were identified as the major excretory form of spermidine (Spd) and putrescine (Put) in the urine of cancer patients and healthy volunteers [3—6]. Furthermore, a majority of the cancer patients studied were found to have a higher ratio of N^1-Ac-Spd to N^8-Ac-Spd in the 24-h urine than did healthy volunteers [5]. These findings suggested that the urinary concentrations of the acetylpolyamines may provide a more precise marker of cancer than the urinary levels of total polyamines obtained after hydrolysis of urine [5].

A variety of procedures are available for the determination of the polyamines in biological samples [7]. However, there are only two procedures which have been successfully used for the quantitation of acetylpolyamines in urine [5, 6]. Both procedures are based on the formation, separation and quantitation of the dansyl derivatives of the acetylpolyamines from urine. A combination of two-dimensional thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) was used for the separation and determination of the dansyl polyamines in previous studies in our laboratory [5]. Recently, Seiler and Knödgen [6] described an elegant procedure for the determination of the naturally occurring monoacetyl derivatives of di- and polyamines in human urine using TLC. These authors obtained results which are in general agreement with some of the previously published findings [5].

In the present paper a procedure for the HPLC determination of the dansyl derivatives of urinary acetylpolyamines is described. This procedure has many advantages over the procedure previously utilized in our laboratory [5]. These advantages include the use of an internal standard for the quantitation of the polyamine, the replacement of the isoamyl alcohol extraction with a purification procedure utilizing Sep-Pak^R silica gel cartridges and the use of a sequential HPLC separation using two different chromatographic modes.

EXPERIMENTAL

Materials

Putrescine dihydrochloride, spermidine trihydrochloride, spermine tetrahydrochloride and 5-dimethylaminonaphthalene-1-sulphonyl chloride (Dns-Cl) were purchased from Sigma (St. Louis, MO, U.S.A.). Monoacetylputrescine hydrochloride was obtained by using a published procedure [8]. N^1-Acetylspermidine, N^8-acetylspermidine and monoacetyl-1,6-diaminohexane (Ac-DAH) were prepared using procedures developed in our laboratory and which will be published elsewhere. Chloroform, isopropanol, hexane and methylene chloride were HPLC grade solvents and were obtained from Fisher (Itasca, IL, U.S.A.) or Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Sep-Pak® silica cartridges were obtained from Waters Assoc. (Milford, MA, U.S.A.).

Urine sample preparation

Twenty-four-hour urine samples were collected in polyethylene bottles and stored under toluene at 4°C. A 6-ml aliquot was pipetted into a plastic cup and a 100-μl portion of 1 m*M* solution of acetyl-1,6-diaminohexane dihydrochloride was added. The sample was adjusted to pH 9.0 with 1.0 *N* sodium hydroxide solution using an automatic titrator (Radiometer, Copenhagen, Denmark). A 2-ml aliquot was pipetted into a syringe and added to a silica gel Sep-Pak which had been previously washed with 5 ml of water. Water (5 ml) was passed through the Sep-Pak and the eluent was discarded. The polyamines were eluted with 10 ml of 0.1 *N* hydrochloric acid and the eluent was collected in a 50-ml centrifuge tube. The eluent was evaporated to dryness at 40°C in vacuo using an evapo-mix (Buchler Instruments, Fort Lee, NJ, U.S.A.). The residue was dissolved in 0.5 ml of 0.6 *N* hydrochloric

acid and mixed with 0.5 ml of a saturated solution of sodium carbonate and 2 ml of a solution of 7.5 mg/ml of Dns-Cl in acetone. The centrifuge tubes were stoppered and placed in an ultrasonic bath for 2.5 h. A 0.2-ml aliquot of a proline solution (5.0 g per 100 ml) was added to each tube and the tubes were placed in the ultrasonic bath for an additional 5 min. The solution was evaporated to dryness at 40°C in vacuo. The residue in each tube was mixed vigorously for 20 sec with a vortex mixer with 1.0 ml of water and 5.0 ml of toluene. The tubes were centrifuged at 900 *g* for 20 min. The toluene layer was transferred to a 12-ml conical centrifuge tube. The toluene extract was evaporated to dryness at 40°C in vacuo. The residue was mixed with 0.5 ml of 0.1 *N* sodium hydroxide and extracted with 2.5 ml of toluene. The toluene extract was evaporated to dryness and the residue was stored at −20°C until analysis.

HPLC analysis

HPLC was performed on a system composed of a U6K injector, two Model 6000 solvent delivery systems and a Model 660 solvent programmer (Waters Assoc.), a LDC Model 1209 FluoroMonitor (Laboratory Data Control, Riviera Beach, FL, U.S.A.) and a strip chart recorder (Linear Instruments, Irvine, CA, U.S.A.). The separation of the dansyl monoacetylpolyamines was carried out on a Micropak CN-10 column (25 × 2.5 mm I.D., particle size 10 μm; Varian, Palo Alto, CA, U.S.A.) with a solvent composed of *n*-hexane—isopropanol (100:3) as solvent A and *n*-hexane—methylene chloride—isopropanol (10:5:1) as solvent B. The residue of dansyl monoacetylpolyamines was dissolved in 125 μl of methylene chloride and a 50-μl aliquot of the resulting solution was injected into the system. The sample was eluted with a programmed solvent gradient using the linear gradient curve No. 6. The gradient changed from 100% of solvent A to 100% of solvent B in 15 min at a flow-rate of 3 ml/min. The elution was continued in the isocratic mode with solvent B for an additional 5 min. The dansyl monoacetylpolyamines eluted after 15—20 min and the column eluent corresponding to these compounds was collected in a conical centrifuge tube. The column was allowed to re-equilibrate with solvent A for 5 min before a second sample was injected.

The column fraction corresponding to the dansyl monoacetylpolyamines was evaporated to dryness. The residue was dissolved in 100 μl of chloroform and 25 μl of the resulting solution were injected into the system. The separation of the dansyl monoacetylpolyamines was carried out on a silica gel column Ultrasphere-Si, 150 × 4.4 mm I.D., packed with 5-μm silica gel (Altex, Berkeley, CA, U.S.A.) or a 250 × 4.6 mm I.D. column packed with 10-μm silica gel, Alltech Assoc. (Deerfield, IL, U.S.A.) with a solvent composed of chloroform—isopropanol (100:6). The sample was eluted in the isocratic mode with a programmed flow-rate using the concave gradient curve No. 9. The flow-rate changed from 1 ml/min to 2 ml/min in 10 min and was maintained at 2 ml/min for an additional 10 min.

Calibration curve

Aliquots (3 ml) from a 24-h urine sample obtained from a healthy male volunteer were pipetted into plastic cups and 100 μl of a 1 m*M* solution of

Ac-DAH were added to each sample. A 3-ml aliquot of either water or one of the three standard solutions of acetylpolyamines, which contained various concentrations of Ac-Put (5—40 nmole/ml), N^1-Ac-Spd (1—8 nmole/ml) and N^8-Ac-Spd (1—8 nmole/ml), was added to each aliquot of urine. The aliquots were treated as described under Urine sample preparation and HPLC analysis. The ratios of the peak heights of each of the dansyl monoacetylpolyamines to that of the internal standard (dansyl monoacetyl-1,6-diaminohexane) were calculated and plotted as shown in Fig. 1. Regression analysis was used to determine lines of best fit to the data points. The slopes of these lines, together with the ratios of the peak heights of the dansyl monoacetylpolyamines to that of the internal standard, were used for the calculation of the concentrations of the acetylpolyamines in the urine samples.

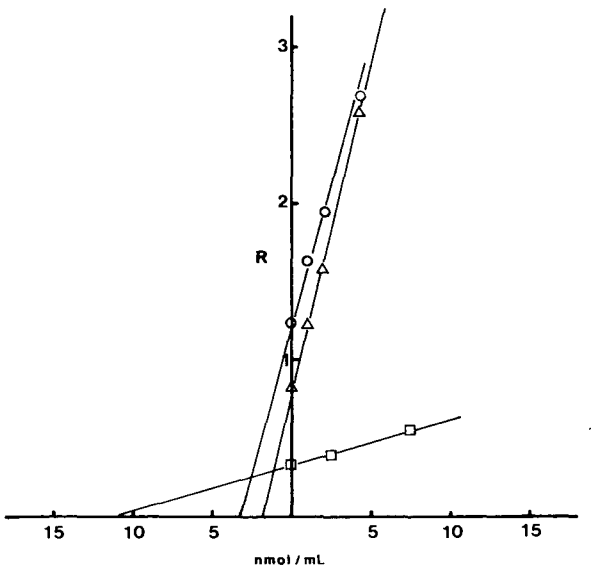

Fig. 1. Correlation graphs for the determination of the monoacetylpolyamines in urine using the method of standard additions. R = ratio of the height of the monoacetylpolyamine peak to the height of the internal standard peak. ○, N^1-Ac-Spd; △, N^8-Ac-Spd; □, Ac-Put. The data on the graph indicate that the urine sample used contained N^1-Ac-Spd, 3.44; N^8-Ac-Spd, 1.91; and Ac-Put, 11.01 nmole/ml.

RESULTS

A number of compounds were evaluated for use as an internal standard in the analysis of urinary acetylpolyamines. Ac-DAH was selected for several reasons. Ac-DAH is chemically similar to the naturally occurring monoacetylpolyamines which resulted in similar recoveries in the extraction and dansylation steps of the analysis. Secondly, the retention volume of this compound on the CN column was similar to those of the natural monoacetylpolyamines. This allowed the collection of only one fraction of the column eluent which contained the natural monoacetylpolyamines and the internal standard. Third-

ly, the retention volume of Ac-DAH on the silica gel column was intermediate between that for N^1-Ac-Spd, N^8-Ac-Spd and Ac-Put. Finally, the Ac-DAH peak was sufficiently separated from the peak due to acetylcadaverine, which was present in small quantities in the urine of some cancer patients.

The acetylpolyamines were extracted from urine, which had been adjusted to pH 9.0, using a silica gel cartridge (Sep-Pak). This procedure is similar in principle to that described by Grettie et al. [9] for the extraction of polyamines from plasma. The use of silica gel for the extraction of polyamines was much more convenient and gave higher recoveries than the extraction procedure used in our laboratory in previous studies [5]. The use of the silica gel cartridge was also more convenient and provided better reproducibility in our hands, than the use of columns prepared according to the procedure described by Grettie et al. [9].

The dansyl derivatives of the extracted polyamines were formed using a standard procedure. The residues of the dansyl polyamines were treated with base to remove interfering substances as suggested by Seiler and Knödgen [6].

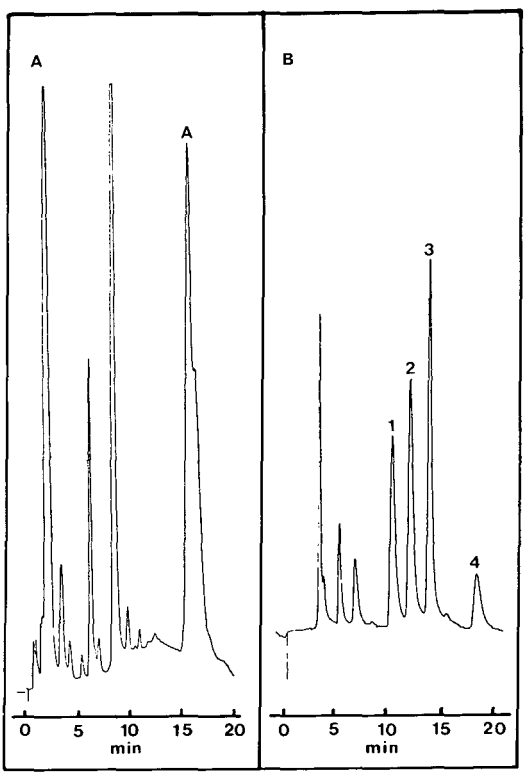

Fig. 2. (A) HPLC separation of the dansyl derivatives of a standard solution of monoacetylpolyamines (Peak A) on a Micropak CN-10 column (250 × 2.5 mm I.D., Varian Aerograph). (B) HPLC separation on a silica gel column (250 × 4.6 mm I.D., 10-μm particle size, Alltech) of the dansyl derivatives obtained after concentration of the Micropak CN-10 column eluent corresponding to peak A. Peaks: 1 = N^1-Ac-Spd; 2 = N^8-Ac-Spd; 3 = Ac-DAH (internal standard); 4 = Ac-Put.

This treatment resulted in a relatively clean preparation of the dansyl derivatives.

The crude mixture of the dansyl derivatives of the urinary polyamines was separated on a bonded-phase CN column using a programmed solvent gradient elution [10]. A typical separation of the dansyl derivatives of a synthetic mixture of N^1-Ac-Spd, N^8-Ac-Spd, Ac-Put and Ac-DAH (internal standard) is shown in Fig. 2. Although partial separation of the monoacetylpolyamines could be obtained using different solvent gradient conditions, we used separation conditions to obtain all the acetylpolyamines in a single peak (peak A in Fig. 2A). The eluent corresponding to peak A was collected and concentrated. The residue was separated on a silica gel column (Fig. 2B). This figure demonstrates the excellent separation between N^1-Ac-Spd and N^8-Ac-Spd.

Fig. 3A represents the chromatogram obtained from the urine of a healthy male volunteer. It is clear from this chromatogram that the acetylpolyamines are present in human urine. Also, a number of fluorescent substances are

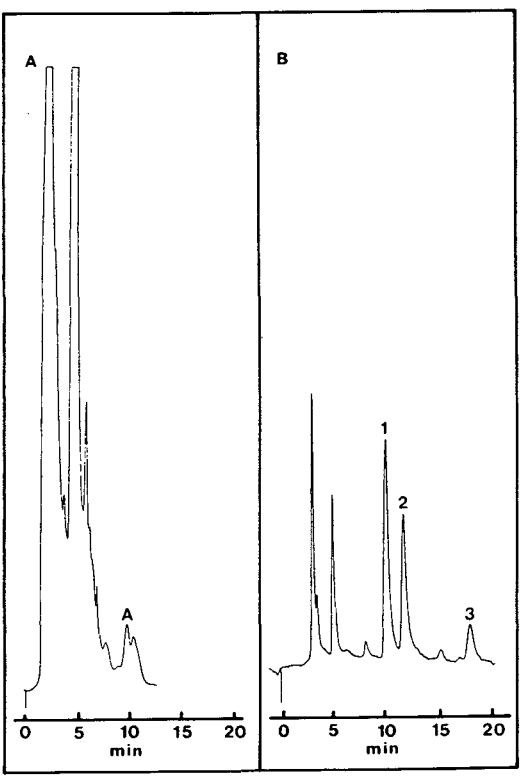

Fig. 3. (A) HPLC separation of the dansyl derivatives obtained from the urine of a healthy male volunteer on a Micropak CN-10 column (250 × 2.5 mm I.D., Varian Aerograph). Peak A corresponds to the naturally occurring monoacetylpolyamines. (B) HPLC separation on a silica gel column (250 × 3.6 mm I.D., 10-μm particle size, Alltech) of the dansyl derivatives obtained after concentration of the fraction of column eluent corresponding to peak A. Peaks: 1 = N^1-Ac-Spd; 2 = N^8-Ac-Spd; 3 = Ac-Put.

present in the sample which have similar retention volumes to the dansyl polyamines. These substances interfere with the dansyl polyamine peaks and make the quantitation of the dansyl polyamines impossible. The dansyl acetylpolyamines peak was rechromatographed on a silica gel column and is shown in Fig. 3B. Fig. 4 illustrates the chromatograms obtained from the urine of a cancer patient.

The working correlation graph for the determination of the acetylpolyamines in urine was prepared using the standard addition method (Fig. 1). This figure illustrates the linear relationship between the amount of acetylpolyamine added and the ratio of its peak height to that of the internal standard. The correlation coefficients (r) were calculated by regression analysis and found to be 0.99 for N^1-Ac-Spd, 0.99 for N^8-Ac-Spd and 0.92 for Ac-Put.

This chromatographic procedure was used to determine the concentration of N^1-Ac-Spd, N^8-Ac-Spd and Ac-Put in the urine of several healthy volunteers and cancer patients. The urinary concentrations of the acetylpolyamines of five representative patients are shown in Table I. The values in Table I are in agreement with those previously published for these compounds.

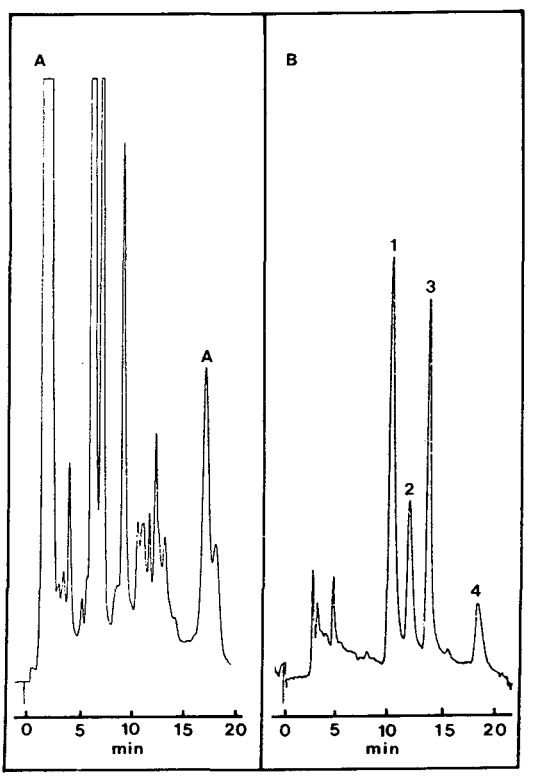

Fig. 4. (A) HPLC separation on a Micropak CN-10 column, of the dansyl derivatives obtained from the urine of a cancer patient. Peak A corresponds to the naturally occurring monoacetylpolyamines. (B) HPLC separation on a silica gel column of the dansyl derivatives obtained after concentration of the fraction of column eluent corresponding to Peak A. Peaks: 1 = N^1-Ac-Spd; 2 = N^8-Ac-Spd; 3 = Ac-DAH (internal standard); 4 = Ac-Put.

TABLE I

PRETREATMENT CONCENTRATIONS OF MONOACETYLPOLYAMINES IN THE 24-h URINE OF CANCER PATIENTS

Patient	Acetylpolyamine conc. (μmole per 24 h)				Ratio*
	N^1-Ac-Spd	N^8-Ac-Spd	Ac-Put	Total	
C.W.	5.2	2.5	22.0	29.7	2.1
R.B.	4.9	1.7	8.0	24.6	2.9
J.C.	9.1	1.7	17.4	28.2	5.4
E.L.	3.5	1.4	19.4	24.3	2.5
C.C.	18.3	7.0	24.7	50.0	2.6

*Ratio N^1-Ac-Spd to N^8-Ac-Spd.

It should be pointed out that the ratio of N^1-Ac-Spd to N^8-Ac-Spd in the urine of the majority of cancer patients examined so far was greater than 2.0. This ratio was found to be 1.0 in the urine of healthy volunteers [5, 6].

ACKNOWLEDGEMENT

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REFERENCES

1 D.H. Russell, Clin. Chem., 23 (1977) 22.
2 U. Bachrach, Ital. J. Biochem., 25 (1976) 77.
3 M. Tsuji, T. Nakajima and I. Sano, Clin. Chim. Acta, 59 (1975) 161.
4 M.M. Abdel-Monem and K. Ohno, J. Pharm. Sci., 66 (1977) 1089.
5 M.M. Abdel-Monem and K. Ohno, J. Pharm. Sci., 67 (1978) 1671.
6 N. Seiler and B. Knödgen, J. Chromatogr., 164 (1979) 155.
7 M.M. Abdel-Monem, Advan. Chromatogr., 16 (1978) 249.
8 H. Tabor, C.W. Tabor and L. DeMeis, Methods Enzymol., 176 (1971) 829.
9 D.P. Grettie, D. Bartos, F. Bartos, R.G. Smith and R.A. Campbell, in R.A. Campbell, D.R. Morris, D. Bartos, G.D. Davis and F. Bartos (Editors), Advances in Polyamine Research, Vol. 2, Raven Press, New York, 1978, p. 13.
10 N.E. Newton, K. Ohno and M.M. Abdel-Monem, J. Chromatogr., 124 (1976) 277.

Journal of Chromatography, 222 (1981) 371—379
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

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CHARACTERIZATION AND DETERMINATION OF NEUROPEPTIDES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND RADIOIMMUNOASSAY

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SUMMARY

A method is described for the separation and analysis of a variety of neuropeptides using reversed-phase high-performance liquid chromatography coupled with radioimmunoassay. The solvent system (an acetonitrile gradient containing 0.08% trifluoroacetic acid) allows UV detection at 206 nm, gives good resolution and, by being volatile, is readily compatible with radioimmunoassay. Three applications of the method are described: (a) thyrotropin releasing hormone immunoreactivity in the rat brain has been characterized; (b) ACTH immunoreactivity in the rat pituitary pars intermedia has been resolved into its component peptides; (c) degradation of luteinizing hormone releasing hormone in vitro has been followed.

INTRODUCTION

The radioimmunoassay (RIA) of biologically important peptides from the central nervous system and other tissues is complicated in many cases by the lack of specific antisera and subsequent cross-reactivity of different peptides. This is a particular problem for peptides which have amino acid sequences in common, for example brain and pituitary peptides derived from the common macromolecular precursor, pro-opiocortin. Unequivocal identification and analysis have often only been possible after chromatographic separation of the cross-reacting components; and high-performance liquid chromatography (HPLC) using reversed-phase systems has provided a rapid and highly resolutive technique in this respect. A number of methods, most of them using organic

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solvent gradients, have been described for isolating various neuropeptides [1—6]. The combination of HPLC and RIA allows a much greater selectivity and speed in the analysis of peptides than has hitherto been available, and has been applied in the analysis of endorphin peptides [7] and substance P [8].

The purpose of this investigation has been to identify a reversed-phase HPLC separation method which is applicable to a wide spectrum of neuropeptides and the smaller pituitary peptides, and which is readily compatible with RIA. The ideal eluting solvents should be volatile, UV transparent below 215 nm to allow detection of peptides without aromatic amino acids and give good resolution with no tailing of peaks. The most thoroughly investigated methods satisfy the third criterion at the expense of the first or second [1—4]. The starting point for this study was the method described by Hancock et al. [9] which employs a linear gradient of acetonitrile in 0.1% phosphoric acid.

MATERIALS AND METHODS

Equipment

The HPLC apparatus consisted of two Model 6000A pumps, a Model 660 gradient programmer, a U6K injector, a Model 450 variable-wavelength detector and a μBondapak C_{18} column (30 cm × 3.9 mm I.D., particle size 10 μm; Waters Assoc., Northwich, Great Britain). In recent work, an LKB Uvicord-S fitted with an HPLC flow-cell has been used for UV detection at 206 nm. The flow-rate was 1 ml/min at ambient temperature. Fractions were collected using an LKB Ultrorac 2070, controlled by a microprocessor (Acorn) to allow collection of any part of the column effluent defined by its elution time. The fractions were evaporated on a Buchler vortex evaporator, and reconstituted in the appropriate buffer for RIA.

Chemicals

Acetonitrile (S-grade), isopropanol and methanol were obtained from Rathburn Chemicals (Peebles, Great Britain) and were filtered and degassed immediately before use. Trifluoroacetic acid (TFA) (spectroscopic grade), acetic acid (chromatography grade), and phosphoric acid (AnalaR) were obtained from BDH (Poole, Great Britain). Water was deionised, distilled and passed through a Porapak Q column.

Peptides

LHRH, TRH, TRH free acid, His-Pro diketopiperazine, substance P, somatostatin, α-MSH, CCK-8, Leu-enkephalin and Met-enkephalin were obtained from Peninsular Labs. (San Carlos, CA, U.S.A.). Human ACTH and ACTH fragments were gifts from Drs. W. Rittel and P.A. Desaulles (Ciba-Geigy, Basle, Switzerland), γ-LPH, β-LPH, β-endorphin and LPH_{88-91} were gifts from Dr. J. Morley (ICI, Alderley Park, Great Britain) and R. Penny (Department of Dermatology, Royal Victoria Infirmary, Newcastle, Great Britain).

Radioimmunoassay

The procedures used were based on those described previously for TRH [10], and LHRH [11] and ACTH [12]. Details of the antibody specificities are provided in the Results section.

RESULTS

Development of a separation

The solvent system described by Hancock et al. [9] gave sharp symmetrical peaks for several peptides (Fig. 1). A 20-min gradient from 5—70% B (i.e. 3.5—49% acetonitrile) in the presence of 0.1% phosphoric acid (pH 2.1) was sufficient to resolve TRH, LHRH, substance P and somatostatin and a variety of other peptides. Slightly inferior resolution was obtained using isopropanol (1.2—40%) in place of acetonitrile. Both solvent systems are transparent at 206 nm but are not volatile and fractions require neutralization before RIA. It was found that the presence of salt interfered with the TRH RIA giving an apparent reduction of 30% in the amount of TRH present.

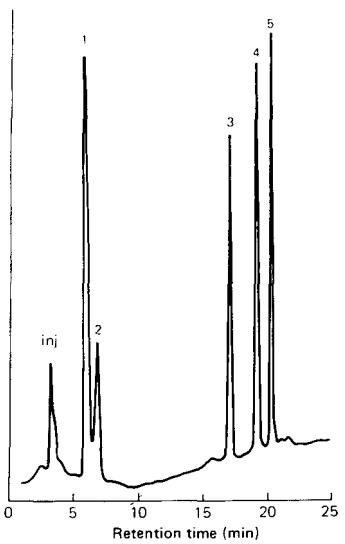

Fig. 1. Separation of peptides using acetonitrile gradient with 0.1% phosphoric acid. Solvent A, 0.1% phosphoric acid; solvent B, 70% acetonitrile in A; 20-min linear gradient from 5—70% B. Peaks: inj = injection; 1-His-Pro-diketopiperazine; 2 = TRH; 3 = LHRH; 4 = substance P; 5 = somatostatin (all 5 μg); 0.4 a.u.f.s. at 206 nm.

Replacing 0.1% phosphoric acid with 0.08% TFA (a volatile acid of the same pH) did not affect the resolution but longer retention times were recorded in the 20-min acetonitrile gradient (Fig. 2a). TFA (1%) has been used in the purification of ACTH peptides from ODS columns [13] and it has been shown that an acetonitrile gradient with 5 m*M* TFA (pH 2.5) was useful for purifying opioid peptides on a μBondapak C_{18} column [14]. We found that a gradient from 3.5—49% acetonitrile containing 0.08% TFA was suitable for the separation of peptides over a wide range of hydrophobicity and molecular weights. Small peptides (e.g. TRH and metabolites such as TRH free acid and His-Pro-diketopiperazine) are resolved in the early part of the chromatogram. β-LPH and several related peptides are also completely resolved (Table I) under these conditions, as were ACTH, α-MSH and CCK-8. No noticeable improvement was detected when sodium chloride (0.9%) was added to the 0.08% TFA (Fig. 2b).

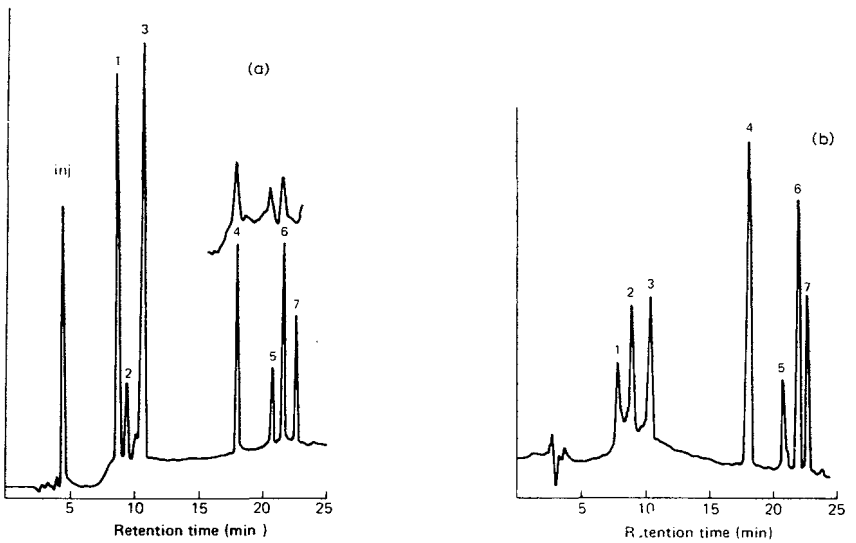

Fig. 2. Separation of peptides using acetonitrile gradient with 0.08% TFA. (a) Solvent A, 0.08% TFA; solvent B, 70% acetonitrile with 0.08% TFA; 20-min linear gradient from 5—70% B. Peaks: inj = injection; 1 = His-Pro-diketopiperazine; 2 = TRH; 3 = TRH-OH; 4 = LHRH; 5 = substance P; 6 = somatostatin; 7 = CCK-8 (all 2.5 μg except 1 and 3 which are 10 μg); 0.2 a.u.f.s. at 206 nm; upper trace, 25 ng using 0.005 a.u.f.s. (b) As (a) except solvent A, 0.09% sodium chloride with 0.08% TFA (all 2.5 μg).

TABLE I

ELUTION TIMES OF β-LPH/ACTH PEPTIDES ON C_{18} COLUMN

3.5—49% acetonitrile with 0.08% TFA over 20 min; flow-rate, 1 ml/min.

Peptide	Elution time (min)
LPH_{88-91}	3.6
Met-enkephalin	16.9
Leu-enkephalin	18.2
α-MSH	18.9
γ-LPH	19.5
hACTH	20.4
β-LPH	21.4
β-endorphin	22.4

The gradient was less suitable for resolving ACTH from C-terminal ACTH-related peptides ($ACTH_{17-39}$, $ACTH_{25-39}$, $ACTH_{18-39}$). However, a 30-min gradient from 21—35% acetonitrile in the presence of 0.08% TFA gave good resolution of these peptides (Fig. 3).

The presence of TFA was essential for good chromatography of peptides. Chromatography of $ACTH_{17-39}$ and LHRH under isocratic elution conditions showed that peak broadening occurred with the former peptide at concentrations of TFA below 0.07%, while the latter showed no loss of resolution down to 0.02% TFA. However, without TFA, the peptides were not eluted from the column.

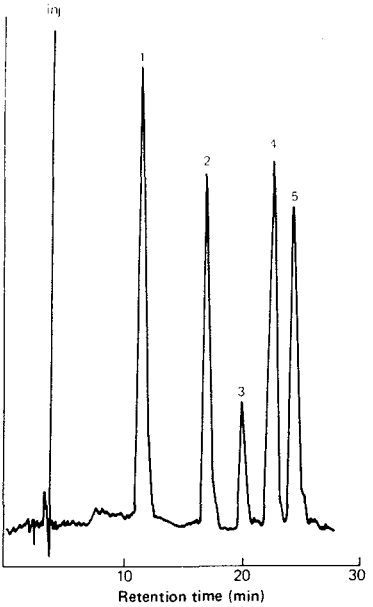

Fig. 3. Separation of ACTH-related peptides on acetonitrile gradient. Solvents A and B as in Fig. 2a. 30-min gradient from 30—50% B. Peaks: inj = injection; 1 = $ACTH_{1-24}$; 2 = α-MSH; 3 = $hACTH_{11-39}$; 4 = $hACTH_{17-39}$; 5 = $hACTH_{1-39}$ (all 2.5 μg); 0.1 a.u.f.s. at 206 nm.

Sensitivity of detection in gradient HPLC is often limited by the sloping baseline caused by the different optical densities of the two gradient components. In the present system we have overcome this problem to some extent by balancing the optical density of solution A with that of solution B by the addition of acetic acid (about 0.02% in solvent A for the reagents in use in our laboratory). The detection limit for LHRH under these conditions at 206 nm is less than 25 ng (see insert, Fig. 2a). Highly reproducible retention times were obtained; in five consecutive runs LHRH was eluted at 17.8, 17.8, 17.9, 17.9 and 17.8 min.

Applications

The acetonitrile—0.08% TFA gradient system has been used in our laboratory for several separations linked to RIA detection in the characterization of brain and pituitary peptides. Three examples are described briefly below.

The identification of extrahypothalamic TRH immunoreactivity. Previous experiments using thin-layer chromatography [15] and gel chromatography [16] have produced conflicting evidence concerning the identity of TRH immunoreactivity (TRH IR) in rat brain. We showed first by RIA that TRH standard (2 ng) could be recovered quantitatively from the column. TRH IR extracted from rat extrahypothalamic brain using methanol was found to have the same retention time as synthetic TRH and TRH from rat hypothalamus on a 0—49% acetonitrile gradient containing 0.08% TFA (Fig. 4) and no other immunoreactive peaks were eluted from the column. This indicates that

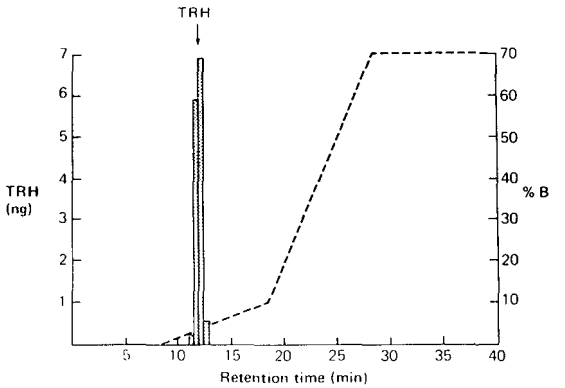

Fig. 4. Elution profile of TRH immunoreactivity from rat extra-hypothalamic brain. Rat brain (less the hypothalamus) was extracted with methanol (10 ml/g tissue). An aliquot was evaporated to dryness, the residue extracted with 1% TFA and the extract applied to the μBondapak C_{18} column. Solvent A = 0.08% TFA; solvent B = 70% acetonitrile with 0.08% TFA; gradients, 0—10% B over 10 min; 10—70% B over 10 min. Fractions were collected, dried and the residue dissolved in buffer (1 ml) for RIA.

TRH IR in rat brain is identical to synthetic TRH and that the antisera used in this study are highly specific for TRH.

The enzymic degradation of LHRH by impurities in bovine albumin preparations. Bovine serum or plasma albumin (BSA or BPA) is used in many radioimmunoassays as a non-specific carrier when the peptide is in low concentra-

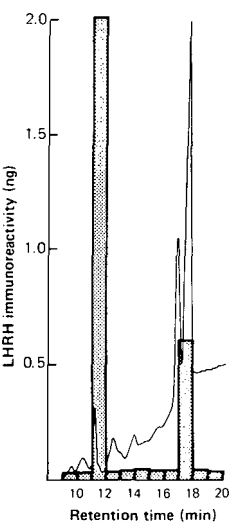

Fig. 5. Elution profile and immunoreactivity of LHRH after incubation with BPA at pH 7. LHRH (1 μg) was incubated at room temperature in Krebs—Ringer solution (1 ml) containing 0.25% BPA for 18 h. Methanol (4 ml) was added, the solution centrifuged and the supernatant evaporated. The residue was dissolved in 0.1% TFA, and applied to the column. Conditions of elution were as in Fig. 2a; UV trace, 0.01 a.u.f.s.; 1-ml fractions were collected and evaporated for RIA (shaded bars).

tion. In experiments to follow the pathway of dopamine-stimulated LHRH degradation by hypothalamic synaptosomes [17] we discovered that LHRH was degraded by BSA (Sigma, St. Louis, MO, U.S.A.) or BPA (Armour, Eastbourne, Great Britain). Although there was no apparent loss of LHRH immunoreactivity in the RIA, little immunoreactive material was found in the HPLC fraction corresponding to LHRH. One major immunoreactive degradation product was seen in the eluate (Fig. 5) and this has been shown to be $LHRH_{6-10}$. The antisera used in this study are known to be directed against the C-terminus of LHRH and this work indicates that the 6—10 sequence cross-reacts on an equimolar basis with $LHRH_{1-10}$.

The separation of carboxy-terminal ACTH fragments from rat pituitary pars intermedia. Corticotropin-like intermediate lobe peptide (CLIP; $ACTH_{18-39}$) and α-MSH ($ACTH_{1-13}$) are thought to be synthesized from ACTH in the pars intermedia. Using an antiserum raised against $ACTH_{17-39}$ we have found three main immunoreactive peaks in acid extracts of rat pars intermedia (Fig. 6). Previous studies using gel filtration have indicated that CLIP was the major C-terminal ACTH-related peptide [18] although recently, there has been evidence to suggest that a glycosylated form of CLIP is present. The antisera used here do not cross-react with glycosylated CLIP [19], being directed against that portion of the molecule where the carbohydrate moiety is attached. The antiserum also fails to recognize porcine ACTH because of the amino-acid substitution of Leu for Ser (position 31) in this region of the molecule. Thus glycosylation is presumably not responsible for the heterogeneity seen on HPLC. The recovery of $hACTH_{1-39}$ and $hACTH_{17-39}$ standards from the column was greater than 90% as demonstrated by RIA (dotted line in Fig. 6).

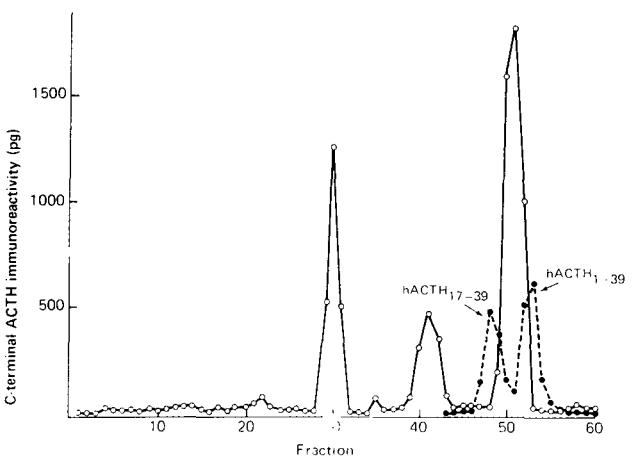

Fig. 6. C-terminal ACTH immunoreactivity in rat pars intermedia. The intermediate lobe of the rat pituitary was extracted with 0.1 *N* hydrochloric acid. The extract was made to 50% methanol, centrifuged and the clear supernatant evaporated. An aliquot (1/20th) was dissolved in 0.1% TFA and applied to the column; 0.5-ml fractions were collected and evaporated for RIA of C-terminal ACTH. $hACTH_{1-39}$ and $hACTH_{17-39}$ (150 ng) were injected in 0.1% TFA (15 μl) and chromatographed as above. Fractions were diluted 1:100 for assay.

DISCUSSION

In this paper we have tried to define the conditions for the reversed-phase HPLC separation of a wide variety of peptides coupled with RIA detection. The most successful and extensively investigated methods for HPLC of peptides have employed an acetonitrile gradient in the presence of ion-pair reagents [1], 0.1 *M* phosphate buffer in pH 2.1 [3], or 0.1% phosphoric acid [2]. An *n*-propanol gradient containing 5% acetic acid has also been described which offers some advantages [4]. While all these methods are potentially compatible with RIA, they suffer from lack of solvent volatility or from not being UV transparent at low wavelengths (below 215 nm). The present method uses volatile solvents (acetonitrile gradient with low concentration of trifluoroacetic acid) and allows the UV detection of peptides at 206 nm. The effect of the gradient on the UV baseline can be overcome by balancing the UV absorbance of solvent A with that of solvent B with acetic acid and this allows UV detection at high sensitivity. In some studies, the presence of salt or buffers has been deemed essential for good resolution [1,3]. However, salt did not improve the resolution obtained with our method.

The potential of HPLC coupled to RIA in neuropeptide analysis has been recognised but applied in only a few specific instances [7,8]. It is particularly useful for the rapid separation of a peptide from the cross-reacting precursors and metabolites often found in tissue extracts and body fluids. The present method is readily compatible with three different radioimmunoassays and is capable of resolving a wide range of peptides. Manipulation of the acetonitrile gradient may prove necessary for optimizing specific separations as we have described for the ACTH-related peptides. An important requirement for the separation of low levels of a number of peptides from a single biological sample (e.g. cerebrospinal fluid) is that elution times are highly reproducible. This is because determinations are made by RIA on specified portions of the column effluent corresponding to the previously determined retention times of peptide standards.

Three examples of the practical application of this method in the characterization of neuropeptides are described briefly and full details will appear elsewhere. The TRH-like immunoreactivity found in extrahypothalamic rat brain has been shown to co-elute with authentic TRH on a highly resolutive HPLC system. This and another study [16] contradict the report that TRH immunoreactivity in rat brain is not due to TRH. We have also confirmed (results not shown) that TRH immunoreactivity in frog (Rana pipiens) brain and skin is identical to synthetic TRH.

The degradation of LHRH by impurities in BSA was discovered unexpectedly and occurred under conditions where the immunoreactivity of the peptide was apparently unimpaired. This result urges caution in the use of serum or plasma albumins as carriers for low concentrations of peptides.

The intermediate lobe is the presumed site of synthesis of α-MSH and CLIP from ACTH. This study demonstrates the presence in significant amounts of other peptides which cross-react in the C-terminal ACTH RIA. The relationship of these peptides to ACTH and CLIP is undergoing further investigation.

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REFERENCES

1 J. Rivier, J. Liquid Chromatogr., 1 (1978) 343.
2 W.S. Hancock, C.A. Bishop, R.L. Prestidge, D.R.K. Harding and M.T.W. Hearn, Science, 200 (1978) 1168.
3 E.C. Nice and M.J. O'Hare, J. Chromatogr., 162 (1979) 401.
4 H.R. Morris, A.T. Etienne, A. Dell and R. Albuquerque, J. Neurochem., 34 (1980) 574.
5 R.V. Lewis, S. Stein and S. Udenfriend, Int. J. Peptide Protein Res., 13 (1979) 493.
6 E. Spindel and R.J. Wurtman, J. Chromatogr., 175 (1979) 198.
7 J.G. Loeber, J. Verhoef, J.P.H. Burbach and A. Witter, Biochem. Biophys. Res. Commun., 86 (1979) 1288.
8 Y. Ben-Air, P. Pradelles, C. Gros and F. Dray, Brain Res., 173 (1979) 360.
9 W.S. Hancock, C.A. Bishop, R.L. Prestidge, D.R.K. Harding and M.T.W. Hearn, J. Chromatogr., 153 (1978) 391.
10 S.L. Jeffcoate, H.M. Fraser, A. Gunn and N. White, J. Endocrinol., 59 (1973) 191.
11 S.L. Jeffcoate, H.M. Fraser, D.T. Holland and A. Gunn, Acta Endocrinol., 75 (1974) 625.
12 L.H. Rees, D.M. Cook, J.W. Kendall, C.F. Allen, R.M. Kramer, J.G. Ratcliffe and R.A. Knight, Endocrinology, 89 (1971) 254.
13 H.P.J. Bennett, A.M. Hudson, C. McMartin and G.E. Purdon, Biochem. J., 168 (1977) 9.
14 C.E. Dunlap, S. Gentleman and L.I. Lowney, J. Chromatogr., 160 (1978) 191.
15 W.W. Youngblood, M.A. Lipton and J.S. Kizer, Brain Res., 151 (1978) 99.
16 M.S. Kreider, A. Winokus and R.D. Utiger, Brain Res., 171 (1979) 161.
17 D. Marcano de Cotte, C.E.L. de Menezes, G.W. Bennett and J.A. Edwardson, Nature (London), 283 (1980) 487.
18 A.P. Scott, P.J. Lowry, J.G. Ratcliffe, L.H. Rees and J. London, J. Endocrinol., 61 (1974) 355.
19 P.J. Lowry, personal communication.

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CHROMBIO. 767

CHARACTERIZATION OF NEUROPEPTIDES BY REVERSED-PHASE, ION-PAIR LIQUID CHROMATOGRAPHY WITH POST-COLUMN DETECTION BY RADIOIMMUNOASSAY

APPLICATION TO THYROTROPIN-RELEASING HORMONE, SUBSTANCE P, AND VASOPRESSIN

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SUMMARY

Neuropeptide contents of rat brain samples were determined by radioimmunoassay (RIA) after fractionation of tissue extracts by high-performance liquid chromatography (HPLC). Solvent systems were composed of acetic acid, acetonitrile and short-chain (5—8 carbons) alkylsulfonic acids. Separate solvent systems were developed for thyrotropin-releasing hormone, substance P, arginine vasopressin and biologic analogs, and the enkephalins. All separation systems tested gave 80—90% recovery of picogram quantities of peptides. When lyophilized, the HPLC solvents did not interfere significantly with the RIAs, allowing quantitation of tissue concentrations of isolated neuropeptides using the lyophilized eluent from the HPLC. The combination of liquid chromatography with RIA should allow for very accurate identification and quantification of peptides in biologic samples containing large numbers of potentially cross-reacting species of molecules.

INTRODUCTION

Although interest in the identification and functions of neuropeptides continues to increase, methods for their quantitation in tissue extracts remain inadequate. Neuropeptides are usually measured by radioimmunoassay (RIA); the accuracy of such measurements depends on the specificity and sensitivity of the RIA [1]. The problem of specificity (i.e., does the RIA antiserum cross-

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react with other than the intended substance in the tissue extract) is often more acute than that of sensitivity because of the presence in tissues of many structurally similar peptides, in widely differing amounts. One strategy for increasing the likelihood that an RIA is actually measuring the intended compound, and not a cross-reacting substance, is to isolate that compound chromatographically prior to its estimation.

Recently, reversed-phase liquid chromatography has become popular as a technique for separating neuropeptides [2, 3] and as a purification step prior to RIA. However, while some peptides are readily isolated by conventional reversed-phase techniques, a number of biologically important peptides are not. For example, substance P (SP) tails when chromatographed reversed-phase, giving poor recovery of picogram quantities [4], and thyrotropin-releasing hormone (TRH) elutes too rapidly for adequate resolution from the solvent front [5]. Such problems in general are amenable to ion-pair chromatography [6—8]. We have explored the possibility as to whether the reversed-phase chromatography of SP and TRH, and of the nonapeptide hormones oxytocin (OXT), arginine vasopressin (AVP), and arginine vasotocin (AVT), can be improved by use of ion-pairing agents. Reversed-phase, ion-pair separations were achieved by complexing positively charged residues of the peptide with the negative charge of an alkylsulfonic acid. The correct choice of the alkylsulfonic acid and of the percentage of organic solvent employed allowed excellent separation and recovery of each of the peptides studied.

EXPERIMENTAL

Materials

Chromatography was performed on a Micromeritics 7000B high-performance liquid chromatograph (Norcross, GA, U.S.A.) or on a Bioanalytic LC-304 high-performance liquid chromatograph (West Lafayette, IN, U.S.A.) equipped with a column heater. Waters Assoc. (Milford, MA, U.S.A.) C_{18} μBondapak reversed-phase columns (30 cm × 3.9 mm I.D., particle size 10 μm) were used throughout. All chromatography was performed at 60°C with a flow-rate of 2 ml/min. All solvent systems used were combinations of acetonitrile and 0.1% 1-pentane-, 1-heptane- or 1-hexanesulfonic acid in 0.02 *N* acetic acid.

Reagents used were acetonitrile (Burdick and Jackson Labs., Muskegon, MI, U.S.A.), acetic acid (Pierce, Rockford, IL, U.S.A.), 1-pentane-, 1-hexane-, and 1-heptanesulfonic acids (Eastman-Kodak, Rochester, NY, U.S.A.), and bovine serum albumin (BSA; ICN Pharmaceuticals, Cleveland, OH, U.S.A.). Distilled water was further purified with a Milli-Q system (Millipore, Bedford, MA, U.S.A.). Arginine vasopressin (AVP) was purchased from Ferring (Malmö, Sweden); arginine vasotocin (AVT) and MK-771* were gifts of Merck, Sharp and Dohme (Rahway, NJ, U.S.A.); oxytocin (OXT) was purchased from Bachem (Torrance, CA, U.S.A.). TRH was a gift of Abbott Labs. (North Chicago, IL, U.S.A.), 11-methionine sulfoxide-substance P (SP sulfoxide) was a gift of Dr. J.L.M. Syrier (Technische Hogeschool, Delft, The Netherlands). All other peptides were purchased from Peninsula Labs. (San Carlos, CA, U.S.A.).

*L-N-(2-oxopiperidin-6-yl-carbonyl)-1-thiazolidine-4-carboxamide.

Detection of peptides

UV absorption. Large quantities (500—3000 ng) of peptides were monitored by their absorption at 206 or 210 nm with either an LKB Uvicord detector or a Gilford 2400 spectrophotometer equipped with a flow-cell.

RIA. Small quantities (10—500 pg) of peptides were detected by RIA of the HPLC eluent. Fractions (0.5—2.0 ml) were collected with an LKB RediRac fraction collector into 12 × 75 mm culture tubes containing 100 μl 1.0% BSA in water. The tubes were placed directly into a Savant Instruments (Hicksville, NY, U.S.A.) Speed-Vac Concentrator for rapid, high recovery lyophilization. Fractions were reconstituted in RIA buffer and assayed for neuropeptides. Equivalent volumes of lyophilized HPLC solvent were also added to all RIA standard curves to prevent solvent artifacts.

TRH. Rat hypothalami were homogenized in 500 μl 2 *N* acetic acid, centrifuged, and the supernatants lyophilized. For injection into the chromatograph, the samples were resuspended in 400 μl HPLC solvent containing 0.1% BSA, centrifuged to remove particulate contamination, and 100 μl injected. The TRH RIA was modified after the method of Bassiri and Utiger [9] as described previously [10]. Maximum sensitivity was 2 pg TRH.

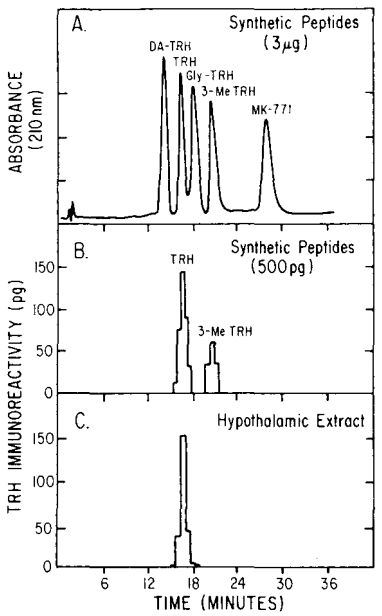
AVP, AVT, OXT. Posterior pituitary lobes were homogenized in 500 μl 10% trichloroacetic acid (TCA), centrifuged, and the pellets washed with 500 μl 0.25% acetic acid. The TCA supernatant and the acetic acid wash were combined, extracted with diethyl ether, and lyophilized. For injection into the chromatograph, the samples were resuspended as above. The RIAs for AVT, AVP and OXT were performed as before [11], except that the final dilution of the AVT antiserum was increased to 1:400,000 to give a maximum sensitivity of 0.6 pg AVT per tube.

SP. Rat striata were homogenized in 400 μl 2*N* acetic acid, centrifuged, and the supernatants lyophilized. For injection into the chromatograph, the samples were diluted 1:20 and resuspended as described above. The SP RIA was based on the method of Mroz and Leeman [12] as described previously [13]. Maximum sensitivity was 5 pg SP per tube.

RESULTS

Optimal separation of TRH from its analogs was achieved with 2.75% acetonitrile plus 0.1% hexanesulfonic acid in 0.02 *N* acetic acid (Fig. 1A). Picogram amounts of TRH (pyroglu-his-proNH_2) and 3-MeTRH (pyroglu-3-methyl-his-proNH_2) were readily separated and recovered from the column for quantitation by RIA (Fig. 1B). Recovery of TRH from tissue extraction, HPLC, and lyophilization was greater than 80%. TRH immunoreactivity in rat hypothalamus was easily measured and corresponded chromatographically only to synthetic TRH (Fig. 1C).

The separation of TRH from its analogs varied with the ion-pairing agent used (Fig. 2). Optimization of separation with 1-heptane-, 1-hexane-, or 1-pentanesulfonic acid required decreasing amounts of acetonitrile. Only with 1-hexanesulfonic acid could all analogs be separated (Fig. 2B). With 1-heptanesulfonic acid, no conditions could be found to separate TRH from Gly-TRH (pyroglu-his-pro-glyNH_2). With 1-pentanesulfonic acid, Gly-TRH could be



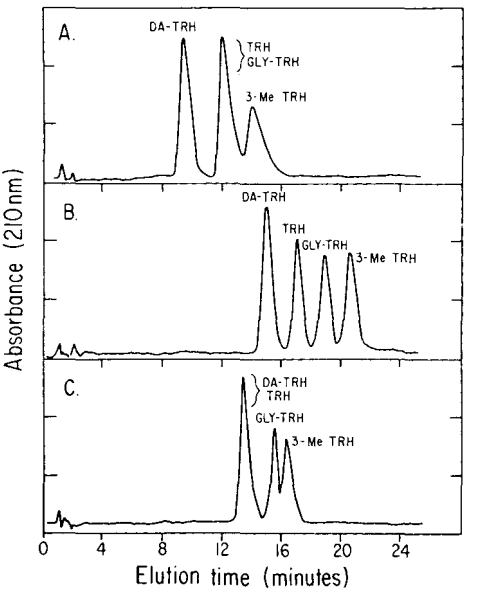


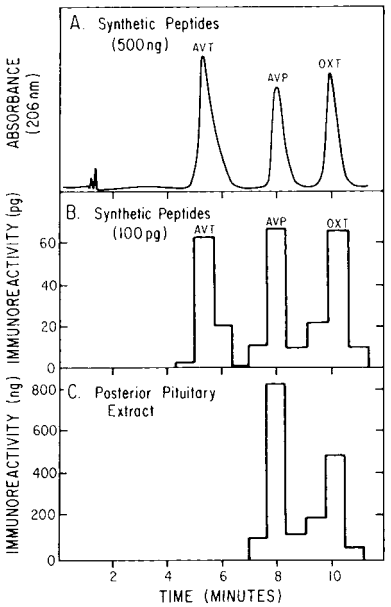
Fig. 1. Separation of TRH and TRH analogs by reversed-phase HPLC. The HPLC solvent was 2.75% acetonitrile plus 0.1% 1-hexanesulfonic acid in 0.02 *N* acetic acid. Chromatograms of (A) standards (3 μg of each peptide); (B) 500 pg TRH and 3-MeTRH; fractions were assayed by RIA; (C) an extract of rat hypothalamus; fractions were assayed by RIA. Peaks: THR = pyroglu-his-proNH$_2$; DA-TRH = pyroglu-his-pro; Gly-TRH = pyroglu-his-proglyNH$_2$; 3-MeTRH = pyroglu-3-methyl-his-proNH$_2$.

Fig. 2. Effect of different ion-pairing reagents on the separation of TRH analogs. (A) 0.1% 1-heptanesulfonic acid plus 6.5% acetonitrile; (B) 0.1% 1-hexanesulfonic acid plus 2.75% acetonitrile; (C) 0.1% 1-pentanesulfonic acid plus 2.0% acetonitrile. Peaks as in Fig. 1.

separated from TRH only by overlapping TRH with deamido-TRH (pyroglu-his-pro). However, when the objective was separation of TRH from deamido-TRH (an important TRH metabolite [14], a solvent with 0.1% 1-heptanesulfonic acid plus 8.5% acetonitrile was most effective, although TRH was no longer clearly separated from 3-MeTRH.

Optimal separation of AVT, AVP, and OXT was achieved with 20% acetonitrile plus 0.1% 1-hexanesulfonic acid in 0.02 *N* acetic acid. AVT eluted in 5.5 min, AVP in 8.1 min and OXT in 10.1 min (Fig. 3A). One hundred picograms each of AVT, AVP, and OXT were readily separated and recovered from the chromatograph for quantitation by RIA (Fig. 3B). Recovery of AVT and AVP was 76—78%; recovery of OXT was 85%. Chromatography of one rat posterior pituitary showed no AVT, 1.1 μg AVP, and 0.76 μg OXT.

Optimal separation of SP from its analogs was achieved with 35% acetonitrile plus 0.1% 1-pentanesulfonic acid (Fig. 4A). Recovery of picogram quantities of SP was greater than 80% (Fig. 4B). The SP immunoreactivity in 1/20 of a single rat striatum was readily quantitated and shown to be almost exclusively authentic SP. A small proportion of SP immunoreactivity was found to correspond to the oxidation product, SP sulfoxide.



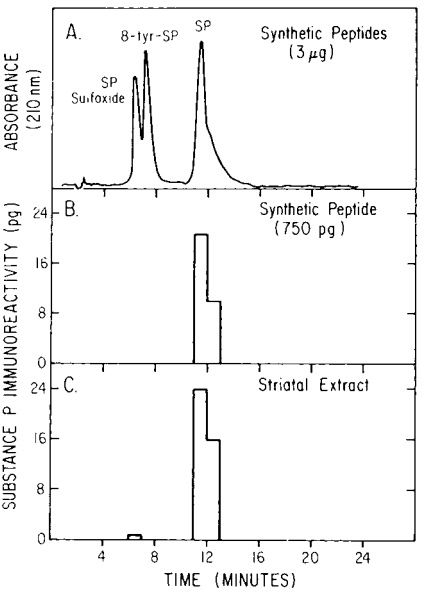


Fig. 3. Separation of AVT, AVP and OXT. The HPLC solvent was 20% acetonitrile plus 0.1% 1-hexanesulfonic acid in 0.02 *N* acetic acid. Chromatograms of (A) standards (500 ng of each peptide); (B) 500 pg of each peptide; fractions were assayed by RIA; (C) an extract of one rat posterior pituitary; fractions were assayed by RIA.

Fig. 4. Separation of substance P and analogs. The HPLC solvent was 35% acetonitrile plus 0.1% 1-pentanesulfonic acid in 0.02 *N* acetic acid. Chromatograms of (A) standards (3 μg of each peptide); (B) 750 pg substance P; fractions diluted 1:20 before RIA; (C) an extract of 1/20 of rat striatum; fractions were assayed by RIA. Peaks: SP sulfoxide = 11-methionine sulfoxide-substance P; 8-tyr-SP = 8-tyrosine substance P; SP = substance P.

The enkephalins could also be separated with a variation of this solvent system. With 0.1% 1-pentanesulfonic acid plus 20% acetonitrile in 0.02 *N* acetic acid, methionine enkephalin eluted in 4.8 min and leucine enkephalin in 9.0 min (chromatogram not shown).

DISCUSSION

Reversed-phase, ion-pair HPLC is a versatile procedure for the high-resolution separation of neuropeptides. By varying the ion-pairing reagent and the percentage of acetonitrile, we have devised separation systems for TRH, AVP, SP, and the enkephalins. This general procedure is applicable to neuropeptides of varying size and relative positive charge.

With 1-hexanesulfonic acid as the ion-pairing reagent, TRH was readily separated from its analogs. This separation was better than that previously achieved with heptanesulfonic acid [5]. 1-Heptanesulfonic acid, however, remains the ion-pairing agent of choice when the objective is separation of TRH from deamido-TRH. The high resolution and high recovery of this technique have allowed us to demonstrate that TRH immunoreactivity in rat brain,

pancreas, and spinal cord does represent the authentic peptide [10].

With 1-hexanesulfonic acid as the ion-pairing reagent, rapid separation of AVT, AVP, and OXT was possible. The high recovery and good separation from the solvent front allowed easy quantitation of the relative amount of each peptide in the rat's posterior pituitary. Using this method, we have also found only very tiny amounts of AVT in the rat's pineal [15], where tissue concentrations of all three peptides are too low for fluorimetric detection [16]. The sequential combination of a non-ion-pairing HPLC isolation of AVP [2, 17] with an ion-pairing separation may provide a rapid isolation procedure for studying AVP biosynthesis.

Substance P could be chromatographed with high recovery by use of ion-pairing. Without ion-pair formation, recovery of picogram quantities of SP from HPLC has been poor, although Ben-Ari et al. [18] have used non-ion-pairing HPLC methods to identify SP in rat brain. Substance P has three positive charges, and requires a short-chain (5 carbon) ion-pairing reagent and a high percentage (35%) of acetonitrile.

The chromatographic methods described here are very flexible, and have been easily adapted to four different classes of neuropeptides. With high resolution, high recovery of applied peptide, and the lack of significant interference from the lyophilized solvent in RIAs, these chromatographic procedures may be ideal for characterizing other neuropeptide immunoreactivities in tissue extracts. Some cautions in the use of ion-pair chromatography are that (1) the ion-pair reagent remains in the solvent residue after lyophilization and might influence some RIAs (although apparently not those for the peptides considered here), and (2) minor variations in the composition of the HPLC solvent can cause marked variation in peptide retention times.

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REFERENCES

1 E. Straus and R.S. Yallow, in H. Gainer (Editor), Peptides in Neurobiology, Plenum Press, New York, 1977, p. 39.
2 R. Burgus and J. Rivier, in A. Loffet (Editor), Peptides, 1976, Éditions de l'Université de Bruxelles, Belgium, 1976, p. 85.
3 R. Burgus and J. Rivier, in M. Goodman and J. Meienhofer (Editors), Peptides -- Proceedings of the Fifth American Peptide Symposium, Wiley, New York, 1977, p. 52.
4 D.J. Pettibone and R.J. Wurtman, unpublished results.
5 E. Spindel and R.J. Wurtman, J. Chromatogr., 175 (1979) 198.
6 W.S. Hancock, C.A. Bishop, R.L. Prestidge, D.R.K. Harding and M.T.W. Hearn, Science, 200 (1978) 1168.
7 W.S. Hancock, C.A. Bishop, L.J. Meyer, D.R.K. Harding and M.T.W. Hearn, J. Chromatogr., 161 (1978) 291.

8 J. Rivier, J. Liquid Chromatogr., 1 (1978) 343.
9 R.M. Bassiri and R.D. Utiger, Endocrinology, 90 (1972) 722.
10 E.R. Spindel and R.J. Wurtman, Brain Res., 201 (1980) 279.
11 J.D. Fernstrom, L.A. Fisher, B.M. Cusack and M.A. Gillis, Endocrinology, 106 (1980) 243.
12 E.A. Mroz and S.E. Leeman, in B.M. Jaffe and H.R. Behrman (Editors), Methods of Hormone Radioimmunoassay, Academic Press, New York, 1978, p. 121.
13 D.J. Pettibone and R.J. Wurtman, Brain Res., 186 (1980) 409.
14 H. Knisatschek and K. Bauer, J. Biol. Chem., 254 (1979) 10936.
15 L.A. Fisher, E.R. Spindel and J.D. Fernstrom, 62nd Annual Meeting of the Endocrine Society, Washington, DC, June 18—20, 1980, Abstr. No. 113.
16 K.A. Gruber, S. Stein, L. Brink, A. Radhakrishnan and S. Udenfriend, Proc. Nat. Acad. Sci. U.S., 73 (1976) 1314.
17 D.D. Blevins, M.F. Burke and V.J. Hruby, Anal. Chem., 52 (1980) 420.
18 Y. Ben-Ari, P. Pradello, C. Gros and F. Dray, Brain Res., 173 (1979) 360.

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CHROMBIO. 741

QUALITATIVE AND QUANTITATIVE ANALYSIS OF SOME SYNTHETIC, CHEMICALLY ACTING LAXATIVES IN URINE BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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SUMMARY

A method for the qualitative and quantitative simultaneous analysis of dioxyanthraquinone, desacetyl-Bisacodyl, phenolphthalein and Oxyphenisatin in human urine using gas chromatography—mass spectrometry (GC—MS) has been developed. The compounds were extracted from urine at pH 7.5 with diethyl ether using Extrelut extraction columns, followed by evaporation and trimethylsilylation.

The method used electron beam ionization GC—MS employing a computer-controlled multiple-ion detector (mass fragmentography). The recovery from urine for the various compounds was between 80% and 100%. The detection limit for these compounds was in the range 0.01—0.05 μg/ml of urine.

The method proved to be suitable for measuring urine concentrations for at least four days after administration of a single oral low therapeutic dose of the laxatives to sixteen healthy volunteers.

INTRODUCTION

It has been demonstrated, that chemically acting laxatives are more dangerous than was expected [1—3]; for example, Oxyphenisatin caused liver damage after use over a prolonged period [4—6] and the number of false diagnoses due to laxative abuse increased [7]. The need for reliable information on laxative abuse is evident in the light of these experiences. It seemed appropriate, therefore, to select dioxyanthraquinone, Bisacodyl, phenolphthalein and Oxyphenisatin as the most frequently used laxatives and investigate the possibilities for their qualitative and quantitative analysis in human urine. The chemical structures of these compounds are given in Fig. 1.

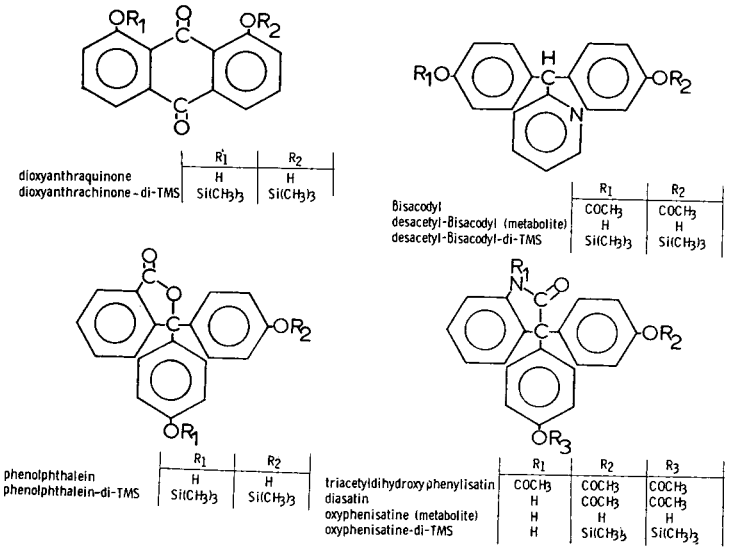


Fig. 1. Chemical structures of the laxatives, some metabolites and their TMS derivatives.

Only a limited amount of work has been done on the qualitative and quantitative analysis of laxatives in human urine. A method for the determination of underivatised dioxyanthraquinone in human urine based on gas chromatography (GC) with flame-ionization detection (FID) has been described [8]. However, dioxyanthraquinone showed unfavourable GC properties, while the other laxatives did not elute at all. This problem can be solved by trimethylsilylation. The method, however, requires extensive clean-up of samples because of many naturally occurring interfering substances.

Some methods for the analysis of laxatives using thin-layer chromatography (TLC) have been developed in various laboratories. A TLC method for the determination of dioxyanthraquinones based on fluorescence densitometry has been described [9]. The method is sensitive, but requires fluorescent laxatives and was not applied to the analysis of human urine. Some qualitative TLC methods [10, 11] based on visualisation of the spots in UV light or using various spray reagents have been developed, but only one method [12] has been used for qualitative analysis of Bisacodyl in human urine and faeces. When this method was used for the simultaneous qualitative analysis of the four selected laxatives in human urine, many problems were encountered with naturally occurring substances, due to lack of selectivity of the spray reagents. Most problems in analysis were caused by Bisacodyl and Oxyphenisatin, which are excreted in low concentrations in urine. In these cases it was necessary to repeat the TLC analysis with the isolated suspected spots from the first TLC separation, but the results were inadequate. Since desacetylation of Bisacodyl is a very important metabolic pathway [13, 14], TLC analysis of desacetyl-Bisacodyl in the urine was also performed, but only slightly better results were obtained.

The GC–mass spectrometric (MS) method reported here quantitates dioxyanthraquinone, desacetyl-Bisacodyl, phenolphthalein and Oxyphenisatin as

their trimethylsilyl derivatives. The sensitivity and specificity of the method fulfil the requirements for its use in investigations into abuse of the selected laxatives. The GC—MS method is able to quantitate 0.1 μg of each compound per milliliter of urine and has a detection limit for the different compounds of about 0.01—0.05 μl/ml.

The method was successfully tested for each investigated laxative on five healthy volunteers who each received a single oral low therapeutic dose. Their 24-h urines were collected over the next four consecutive days [15]. However, Oxyphenisatin was given to only one healthy volunteer because of its dangerous side-effects.

EXPERIMENTAL

Instrumentation and analysis conditions

All analyses were carried out on a Finnigan 3200 mass spectrometer connected by a glass jet-separator to a Finnigan 9500 gas chromatograph. Multiple-ion monitoring (mass fragmentography) was processed by the Finnigan 6000 data system. The mass spectrometric conditions were: electron energy, 70 eV; emission current, about 250 μA; ion source temperature, 80—100°C.

The GC column was a silanized U-shaped column, 180 cm × 3 mm I.D., packed with 3.8% SE-30 on Chromosorb W AW DMCS HP (80—100 mesh), operated isothermally at 280°C with a helium flow-rate of 20 ml/min. Injection port temperature was 280°C, and the glass jet-separator temperature was 270°C.

The derivatization conditions for the trimethylsilyl reagent were investigated using a Hewlett-Packard 5730A gas chromatograph equipped with a flame-ionization detector under the following conditions: injection port temperature, 300°C; detector temperature, 300°C. A silanized spiral-shaped glass column, 180 cm × 3 mm I.D., packed with 3.8% SE-30 on Chromosorb W AW DMCS HP (80—100 mesh), was operated with a temperature program from 230°C to 270°C at a rate of 8°C/min. Helium was used as carrier gas with a flow-rate of 35 ml/min.

Reagents and solvents

The extraction was carried out using Extrelut extraction columns (E. Merck, Darmstadt, G.F.R.) with diethyl ether (pro Analyse, Merck).

The trimethylsilyl reagent was prepared just before use by mixing trimethylchlorosilane, hexamethyldisilazane and pyridine (1:3:6, v/v; pro Analyse, (Merck).

Enzymatic hydrolysis of glucuronides was carried out using Ketodase (5000 Fischmann units of β-glucuronidase per milliliter; Warner Lambert Company, New Jersey, Ireland).

Chloroform (pro Analyse, Merck) was used for the preparation of standard solutions.

Reference compounds

The compounds used were dioxyanthraquinone and phenolphthalein (both from Brocades ACF, Maarssen, The Netherlands), Oxyphenisatin (Winthrop

Laboratories, Fawdon, Great Britain) and desacetyl-Bisacodyl, prepared in our laboratory by acid hydrolysis of Bisacodyl (Brocades ACF).

Standard curves

Known amounts of the pure laxatives were dissolved and diluted in chloroform to give the desired concentration range. For dioxyanthraquinone, desacetyl-Bisacodyl and Oxyphenisatin, standard curves of 25—500 μg/ml were used, covering urine concentrations of 0.17—3.33 μg/ml. For phenolpthalein a standard curve of 40—900 μg/ml was used, covering urine concentrations of 0.27—6.0 μg/ml.

A 100 μl volume of each standard solution was evaporated to dryness and derivatized with 100 μl of trimethylsilyl reagent prior to GC—MS analysis in the same series as the urine extracts.

Hydrolysis of glucuronides

To 15 ml of urine in a 25-ml conical flask was added 1 ml of acetic acid—sodium acetate buffer (pH 4.5, 1 *M*) and if necessary adjusted to pH 4.5. Then 0.5 ml of Ketodase preparation was added and the sample was placed in a thermostatted water-bath at 37°C and allowed to hydrolyse for 1 h.

Extraction

The hydrolysed urine sample was adjusted to pH 7.5 by adding 2 ml of phosphate buffer (pH 7.5, 1 *M*) and if necessary a few drops of 1 *N* NaOH. The sample was then poured into an Extrelut extraction column and allowed to absorb for 10 min. Then the laxatives were extracted by eluting the column first with 40 ml and then with 20 ml of diethyl ether. The combined eluents were dried over anhydrous Na_2SO_4 and collected in a 100-ml evaporating dish. The extract was concentrated on a steam-bath, quantitatively transferred to a 10-ml Teflon-stoppered glass tube and evaporated to dryness in a thermostatted water-bath of 37°C under a gentle stream of dried air.

Derivatization

The residue was dissolved in 100 μl of trimethylsilyl reagent and allowed to react for 15 min at room temperature in a Teflon-stoppered glass tube prior to GC—MS analysis.

Gas chromatography—mass spectrometry

Between 0.5 and 5.0 μl of derivatized urine extract was injected into the GC—MS interspersed with 5-μl injections of derivatized standard solutions to provide a standard curve. The multiple-ion detector measured the abundances of the following selected ions: *m/e* 297, 369 for di-trimethylsilyl-dioxyanthraquinone and *m/e* 343, 421 for di-trimethylsilyl-desacetyl-Bisacodyl during the first 4.5 min; *m/e* 253, 418 for di-trimethylsilyl-phenolphtahlein and *m/e* 268, 432 for di-trimethylsilyl-Oxyphenisatin during the next 9.0 min.

Calculations

For qualitative analysis the area ratio of the two selected ion profiles for each trimethylsilylated laxative as well as the GC retention time of the sub-

stance isolated from the urine sample were compared to the pure trimethylsilylated standard, which was injected in the same series.

For quantitative determination a direct calibration curve was used (ion-peak area vs. concentration). The standards were injected alternately with unknowns. The concentration of the unknowns were calculated from the peak area ratio of the selected ion profile of the unknown and the standard and afterwards corrected for recovery, the aliquot injected and dilutions incurred during the sample processing.

The ions *m/e* 369, 421, 418 and 432 were used for quantitation of dioxyanthraquinone-di-TMS, desacetyl-Bisacodyl-di-TMS, phenolphthalein-di-TMS and Oxyphenisatin-di-TMS, respectively.

Recovery

The overall recovery of dioxyanthraquinone, desacetyl-Bisacodyl, phenolphthalein and Oxyphenisatin was determined by adding known microgram amounts to blank urine, and analysing them by the procedure described above.

To obtain the right concentration of a laxative in urine, 1.0 ml of a standard solution in chloroform at the desired concentration was evaporated to dryness in a water-bath at 37°C under a gentle stream of dried air. The residue was dissolved in 100 µl of ethanol and then in 99.9 ml of urine. In this way two different urine concentrations were prepared for each laxative (exact concentrations are given in Table I). The standard urines were stored at −20°C and defrozen in a water-bath at 37°C prior to analysis.

Test of the method in healthy volunteers

The method was tested in sixteen healthy volunteers. Each time a low therapeutic oral dose was taken of one of the laxatives according to Table II. After intake of the laxative the urine was collected in 24-h volumes for four consecutive days. The 24-h urine samples were homogenized and an aliquot of 100 ml was stored at −20°C until analysis. The urine samples were defrozen at 37°C in a thermostatted water-bath and 15 ml were used for analysis.

RESULTS AND DISCUSSION

Experience in our laboratory over the last five years in the analysis of laxative abuse has indicated a lack in specificity of detection using TLC or GC combined with FID. False positives were readily obtained when a GC method was used without the benefit of a mass spectrometer. The mass spectrometer was used in the multiple-ion detector mode, which offers the required sensitivity to produce reliable quantitative data.

The combination of trimethylsilyl derivatisation with gas chromatography on SE-30 was chosen because of the excellent separation and peak shape characteristics obtained with this system (Fig. 2). The trimethylsilylation of the laxatives proceeded within 5 min at room temperature. Reaction times of up to 1 h at room temperature were investigated. Reaction times longer than 5 min did not increase the peak height of the trimethylsilyl derivatives. A disadvantage, however, was that the trimethylsilyl derivative of dioxyanthraquinone de-

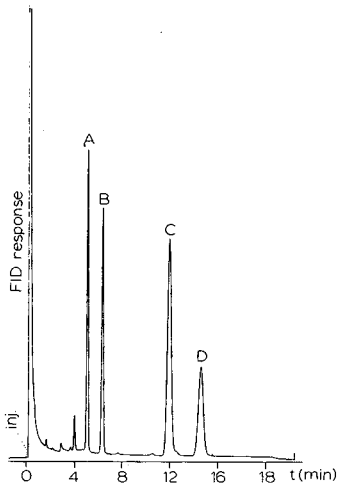


Fig. 2. Temperature-programmed gas chromatogram of the TMS derivatives of the laxatives with FID. (A) di-trimethylsilyl-dioxyanthraquinone; (B) di-trimethylsilyl-desacetyl-Bisacodyl; (C) di-trimethylsilyl-phenolphthalein; (D) di-trimethylsilyl-Oxyphenisatin.

composed slightly under these chromatographic conditions, but this never exceeded 5% (calculated from the peak area ratios of the decomposition product and dioxanthraquinone-di-TMS, with FID).

Mass spectra of the four trimethylsilylated laxatives are shown in Fig. 3.

Linear standard curves (six points), constructed by plotting the peak areas of the selected fragment ions against the concentration of the four trimethylsilylated laxatives, were obtained. Correlation coefficients in the concentration range 25—500 μg/ml of 0.997, 0.999 and 0.998 were found for dioxyanthraquinone-di-TMS, desacetyl-Bisacodyl-di-TMS and Oxyphenisatin-diTMS, respectively. The correlation coefficient for phenolphthalein-di-TMS in the concentration range 40—900 μg/ml was also 0.999.

Extraction of urine at pH 7.5 provided quantitative extraction of the four laxatives investigated and a relative minimum of endogenous substances were co-extracted. Extraction using Extrelut extraction columns instead of separation funnels was less time-consuming and more reproducible because no strong emulsions could form, and cleaner extracts were obtained. The overall recovery and reproducibility of the method for each laxative at two concentration levels in urine are summarized in Table I.

The detection limit was found to be 0.01 μg/ml of urine for dioxyanthraquinone and desacetyl-Bisacodyl, 0.03 μg/ml of urine for phenolphthalein, and 0.05 μg/ml of urine for Oxyphenisatin (signal-to-noise ratio $\geqslant$ 3).

The time required for enzymatic hydrolysis of the glucuronides of the phenolic laxatives was investigated for up to 15 h at 37°C. Incubation times longer than 1 h did not increase the yield of free phenolic laxatives.

The method was tested in sixteen healthy volunteers. Using the method it was possible to measure the administered laxative or its metabolite in the urine of every volunteer for at least four days. Representative mass fragmentograms of the urine extracts are shown in Fig. 4.

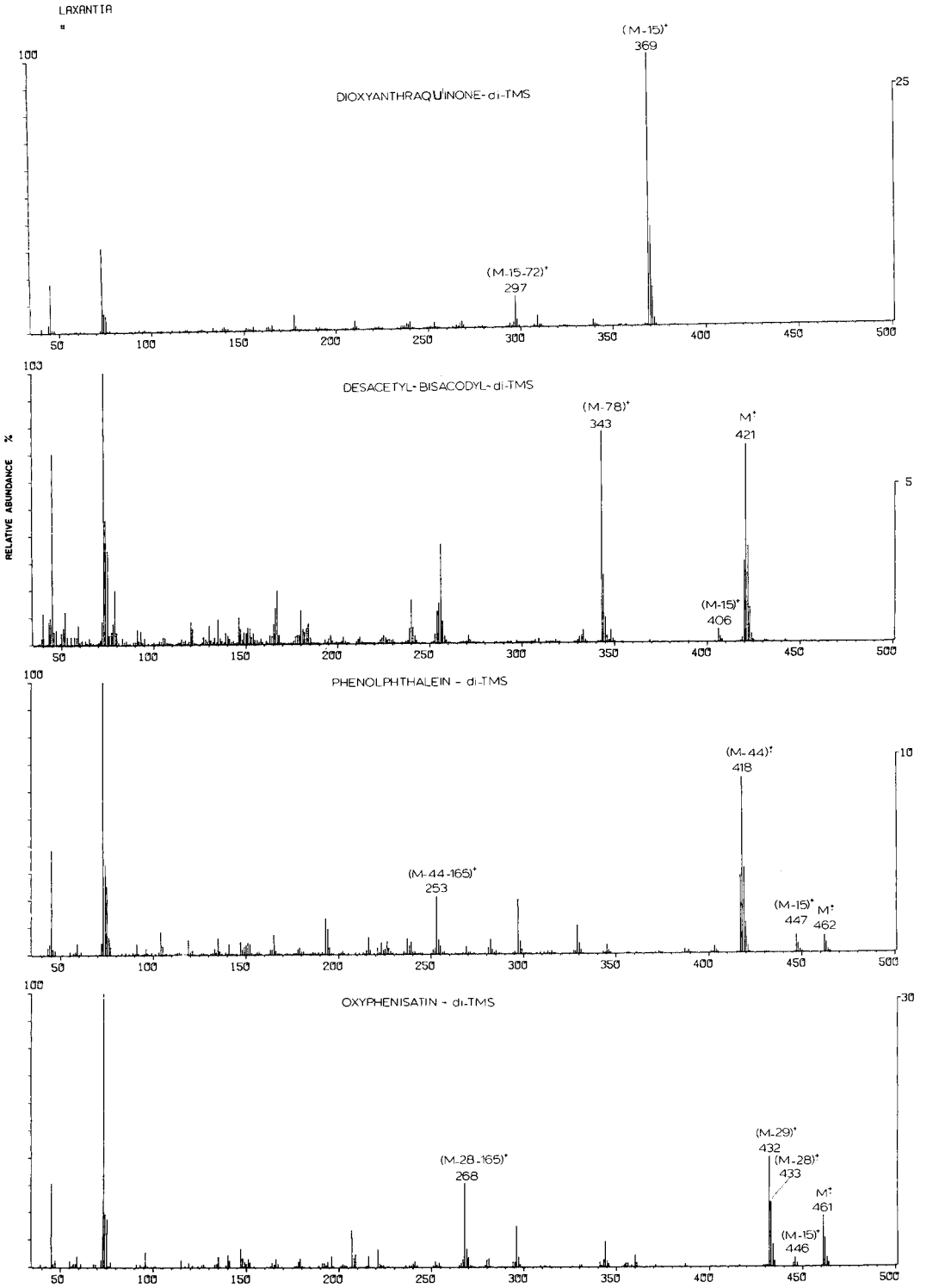
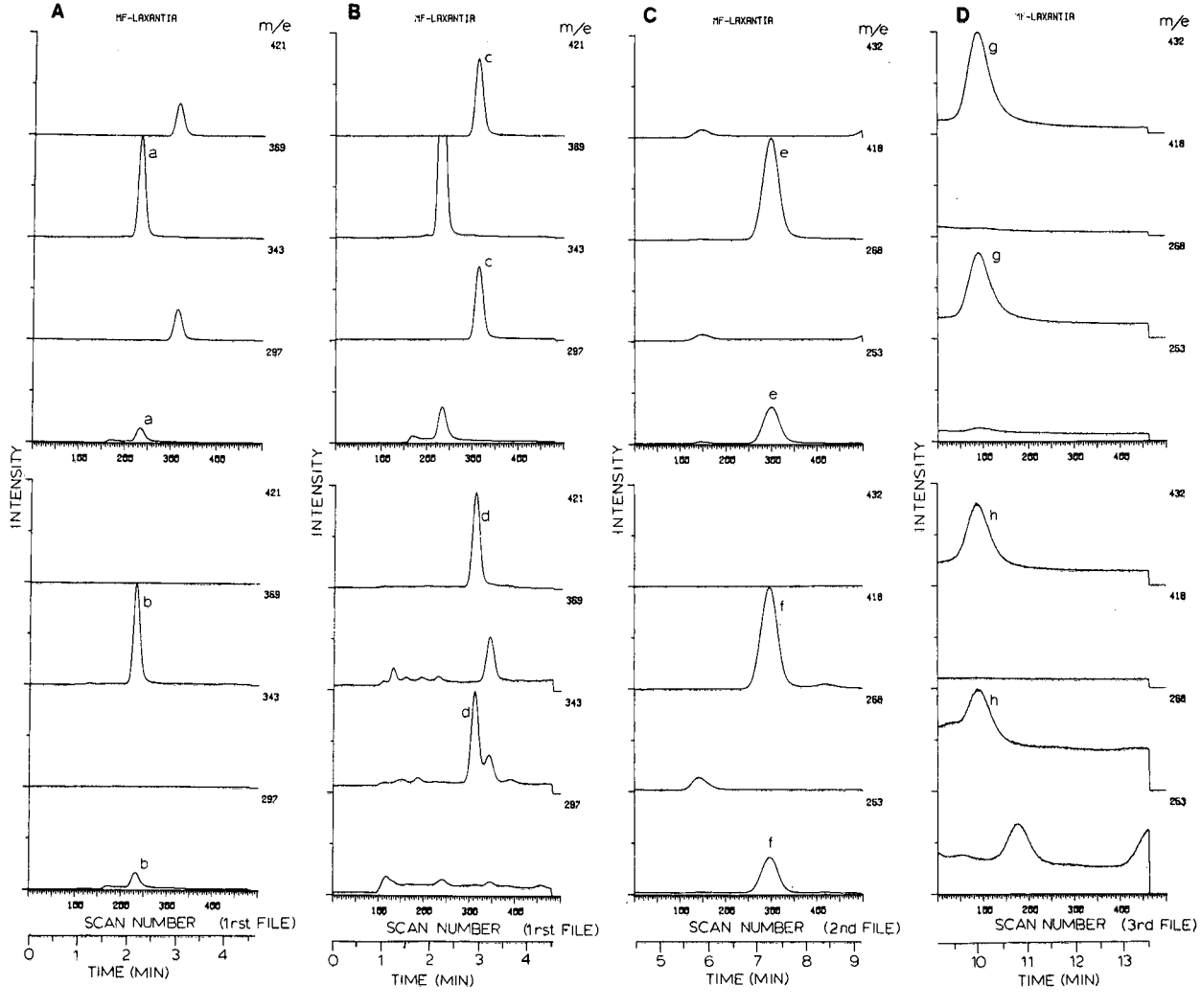


Fig. 3. Mass spectra of the trimethylsilylated laxatives.


A
MF-LAXANTIA
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C
MF-LAXANTIA
432
418
268
253
e
f
SCAN NUMBER (2nd FILE)
D
MF-LAXANTIA
g
h
SCAN NUMBER (3rd FILE)

TABLE I

OVERALL RECOVERY AND REPRODUCIBILITY OF THE METHOD

	Concentration after addition to urine (mg/l)	No. of determinations	Mean concentration found in urine (mg/l)	Standard deviation (mg/l)	Mean recovery (%)
Dioxyanthraquinone	0.254	8	0.261	0.029	103
	2.54	9	2.10	0.092	83
Desacetyl-Bisacodyl	0.218	8	0.203	0.015	93
	2.18	9	2.20	0.129	101
Phenolphthalein	0.434	8	0.427	0.032	98
	4.34	9	3.95	0.214	91
Oxyphenisatin	0.250	8	0.248	0.029	99
	2.51	9	2.539	0.195	101

The concentration range found for each laxative or its metabolite in the urine is summarized in Table II. These calculations resulted in the following quantitative information about excretion rate. Dioxyanthraquinone is mostly excreted in the first 24 h after administration, and ranged from 7% to 23% of the dose in five volunteers, with a mean of 17%. In the following three consecutive days the excretion of dioxyanthraquinone was less than 1% of the dose in each volunteer.

TABLE II

CONCENTRATION RANGE FOR EACH LAXATIVE OR METABOLITE FOUND IN THE URINE AFTER ORAL ADMINISTRATION

No. of volunteers	Laxative tested	Formulation	Oral dose	No. of consecutive days over which analyses were performed	Found concentration range in urine (mg/l)
5	Dioxyanthraquinone	1 × ½ tablet Istizin	75 mg	4	0.5—30
5	Bisacodyl	1 × 1 drageee Dulcolax	5 mg	4	0.1— 1.4
5	Phenolphthalein	1 × 1 tablet Fructine	120 mg	4	1 —16
1	Oxyphenisatin	1 × 1 tablet Diasatine	5 mg	4	—

Bisacodyl was measured in the urine as its metabolite desacetyl-Bisacodyl. Among the five volunteers a large variation in excretion was observed. The total excretion over four days ranged from 8% to 46% of the dose with a mean of

Fig. 4. (A) Representative mass fragmentograms of dioxyanthraquinone-di-TMS analysis of (b) the first 24-h urine from a healthy volunteer after a single oral dose of 75 mg dioxyanthraquinone, and (a) the pure dioxyanthraquinone-di-TMS standard. (B) Representative mass fragmentograms from desacetyl-Bisacodyl-di-TMS analysis of (d) the first 24-h urine from a healthy volunteer after a single oral dose of 5 mg of Bisacodyl, and (c) the pure desacetyl-Bisacodyl-di-TMS standard. (C) Representative mass fragmentograms from phenolphthalein-di-TMS analysis of the (f) first 24-h urine from a healthy volunteer after a single oral dose of 125 mg of phenolphthalein, and (e) the pure phenolphthalein-di-TMS standard. (D) Representative mass fragmentograms from Oxyphenisatin-di-TMS analysis of (h) the first 24-h urine from a healthy volunteer after a single oral dose of 5 mg of Diasatin (diacetyl-Oxyphenisatin) and (g) the pure Oxyphenisatin-di-TMS standard.

23%, while the maximum excretion was reached on the second day. These quantities correlate with earlier published data on the excretion of desacetyl-Bisacodyl as its glucuronide after intake of 5 mg of Bisacodyl [16].

The phenolphthalein excretion over four days ranged from 11% to 31% with a mean of 22%, with maximum excretion in the first two days.

Oxyphenisatin can be administered in several forms, for example as the di- and tri-acetate. Analogous to Bisacodyl, di- and tri-acetyl-Oxyphenisatin are deacetylated in an alkaline reaction in the intestinal tract [17]. One healthy volunteer took 5 mg of Diasatin (diacetyl-Oxyphenisatin) and it was possible to measure Oxyphenisatin for three days, with maximum excretion on the second day. Since Oxyphenisatin was tested in only one volunteer, mean values for excretion are not available.

Finally, it may be concluded that the GC—MS method described had proved to be sufficiently specific and sensitive to analyse laxative abuse for the four investigated laxatives both qualitatively and quantitatively. The method may be helpful in investigating questions dealing with the extent of biotransformation, the dependence of the excretion rate on urinary pH [18] and laxative abuse.

REFERENCES

1 L. Meyer and A. Herxheimer, Side Effects of Drugs, Vol. VI, Excerpta Medica, Amsterdam, 1968, p. 561.
2 Martindale, The Extra Pharmacopoeia, The Pharmaceutical Press, London, 1972, p. 2320.
3 Geneesmiddelenbulletin, 5 (1971) 17—21.
4 T.B. Reynolds, R.L. Peters and S. Yamada, N. Engl. J. Med., 285 (1971) 813—820.
5 G. McHardy and L.A. Balart, J. Amer. Med. Assoc., 211 (1970) 83—85.
6 T.B. Reynolds, A. Lapin, R.L. Peters and H.S. Yamahiro, J. Amer. Med. Assoc., 211 (1970) 86—90.
7 E.C. Klinkenberg-Knol, G.N. Tytgat and L. Rutgeers, Ned. Tijdschr. Geneesk., 121 (1977) 1166.
8 A.J. Baars, R.J. Vermeulen and D.D. Breimer, J. Chromatogr., 120 (1976) 217—220.
9 L. Lemmens, J. Chromatogr., 132 (1977) 363—365.
10 R.P. Labadie, J.J. Scheffer and A. Baerheim Svendsen, Pharm. Weekbl. 107 (1972) 535—539.
11 P.R. Bhandari and H. Walker, J. Chromatogr., 45 (1969) 324—325.
12 J.W. Jansen and P.E. Kamp, Pharm. Weekbl., 11 (1976) 164—166.
13 G. Ferlemann and W. Vogt, Naunyn-Schmiedebergs Arch. Pharmakol. Exp. Pathol., 250 (1965) 479—487.
14 W. Vogt, G. Schimd and T. Dakhil, Naunyn-Schmiedebergs Arch. Pharmakol. Exp. Pathol., 250 (1965) 488—495.
15 D.B. Faber and R.M. Kok, Proceedings of the 20th Federal Meeting of Medical Biological Societies, Groningen, The Netherlands, April 1979.
16 Dulcolax, Boehringer Ingelheim, Middlesex, 3rd ed., 1965.
17 B. Wiegers Hansen and P. Kretschmer, Pharm. Prax., 1968.
18 D.B. Faber and R.M. Kok, in preparation.

Journal of chromatography, 222 (1981) 399—408
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Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 752

SIMULTANEOUS MEASUREMENT OF DOTHIEPIN AND ITS MAJOR METABOLITES IN PLASMA AND WHOLE BLOOD BY GAS CHROMATOGRAPHY—MASS FRAGMENTOGRAPHY

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SUMMARY

A method for the simultaneous measurement of dothiepin and two of its major metabolites, northiaden and dothiepin S-oxide in both plasma and whole blood is described. The method involves the use of gas chromatography—mass fragmentography. It is selective, sensitive (1 μg/l) and reproducible.

It has been used to analyse both plasma and blood samples following single oral doses of 75 mg dothiepin in seven volunteers.

INTRODUCTION

Dothiepin [11-(3-dimethylaminopropylidene)-6,11-dihydrodibenz[*b*,*e*]-thiepin hydrochloride] is one of the tricyclic antidepressant drugs. It is metabolised in man to three active metabolites, northiaden, dothiepin S-oxide and northiaden S-oxide [1, 2]. One procedure has been published for the simultaneous measurement of dothiepin and northiaden in plasma [3]. The two drugs were analysed by high-performance liquid chromatography with a sensitivity limit of 20 μg/l. Although suitable for analysis of steady-state concentrations, this would not be adequate for measuring plasma levels following single oral doses of 75 mg. A more sensitive technique is gas chromatography—mass fragmentography (GC—MF). One GC—MF method has been investigated involving

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chemical ionization but was applied to dothiepin measurement only [4]. A GC—MF method using electron ionization has been developed for the simultaneous analysis of dothiepin and northiaden and dothiepin S-oxide in blood and plasma samples and will be described in this paper.

EXPERIMENTAL

Drug standards

Deuterodothiepin [11-(3-N-trideuteromethyl-N-methylaminopropylidene)-6, 11-dihydrodibenz[*b*,*e*]thiepin hydrochloride] was used as the internal standard for dothiepin and dothiepin S-oxide. This was synthesized and supplied by Boots (Nottingham, Great Britain).

Protriptyline hydrochloride was used as the internal standard for northiaden and was obtained from Merck Sharp & Dohme (Sydney, Australia).

Dothiepin, northiaden, dothiepin S-oxide, and northiaden S-oxide hydrochlorides were supplied by Boots. The structures of the compounds are shown in Fig. 1.

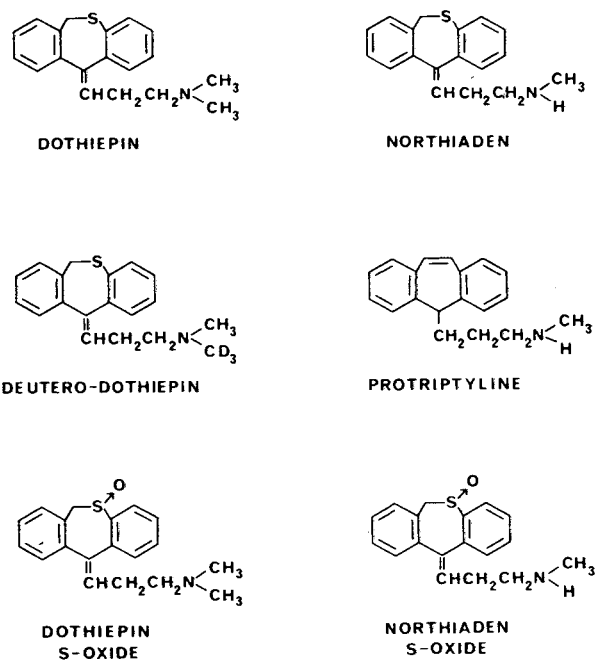

Fig. 1. The structures of dothiepin, deuterodothiepin, northiaden, protriptyline, dothiepin S-oxide and northiaden S-oxide.

Solvents

Nanograde quality *n*-hexane was obtained from Mallinckrodt (St. Louis, MO, U.S.A.). Trifluoroacetic anhydride (TFA) was purchased from Pierce (Rockford, IL, U.S.A.) and absolute ethanol was obtained from E. Merck (Darmstadt, G.F.R.).

Glassware

All glassware (tubes, stoppers, pipettes) was soaked overnight in chromic acid, thoroughly rinsed in tap water, followed by rinsing with glass-distilled water.

Extraction procedure

Samples of either 1 or 2 ml of plasma (or blood) were diluted to 5 ml with glass-distilled water in 20-ml glass tubes. Unknown samples, four plasma (or blood) standards and a quality control were included in each extraction. The internal standard solution, containing deuterodothiepin and protriptyline in ethanol, was added to give a concentration of 50 μg deuterodothiepin and 20 μg protriptyline per liter of sample. After mixing, the samples were alkalinized with 0.5 ml of 5 *M* sodium hydroxide, and 5 ml of *n*-hexane added. The samples were shaken for 10—15 min on a horizontal shaker and centrifuged for 5 min at 1400 *g*. A maximum aliquot of the solvent phase was transferred to clean tubes containing 1 ml 1 *M* hydrochloric acid. The plasma phase was re-extracted with a further 5 ml of *n*-hexane and a maximum aliquot of the solvent added to the first extract. The combined solvent phases were extracted with the acid by shaking for 10—15 min followed by centrifugation as before. The acid phase was removed to clean 5-ml glass tubes, alkalinized with 0.3 ml of 5 *M* sodium hydroxide, and extracted by gentle rotation (10—15 min) with 2 ml *n*-hexane. After centrifugation the solvent phase was transferred to 5-ml V-shaped glass tubes and evaporated under air at 37°C. A second extraction of the acid phase was carried out and the solvent added to the same V-shaped tubes. A 100-μl volume of TFA was added and the samples vortexed for 30 sec. The solvents were evaporated under air at 37°C and the samples were stored at −4°C. Prior to analysis, samples were reconstituted in 7 μl of ethanol. Aliquots of 4—5 μl were injected into the gas chromatograph—mass spectrometer.

Gas chromatography—mass fragmentography

The analysis was carried out on a Hewlett-Packard Model 5992A gas chromatograph—mass spectrometer. Gas chromatographic separation was carried out on a silanised glass column (2 m × 2 mm I.D.) packed with a 3% OV-101 on Gas-Chrom W HP (80—100 mesh). Helium was used as carrier gas at a flow-rate of 20 ml/min. The injection port and jet separator were maintained at 260°C and the oven was programmed from 210°C to 230°C at 4°C/min and kept at 230°C for the remainder of the run. The mass spectrometer was focused on *m/e* 58 (50 msec), 61 (50 msec), 191 (50 msec), and 217 (150 msec). The ionization energy was 70 eV, and the electron multiplier energy ranged from 2 to 3 kV.

Quantitation

Peak areas were integrated using the gas chromatograph—mass spectrometer controller unit (Hewlett-Packard 9825A desktop computer). The ratio of peak area of *m/e* 58 at t_R 3.6 min (dothiepin) to the area of *m/e* 61 at t_R 3.6 min (deuterodothiepin), the ratio of *m/e* 58 at t_R 4.0 min (dothiepin S-oxide) to the area of *m/e* 61 at t_R 3.6 min (deuterodothiepin), and the ratio of the area of 217 at t_R 5.8 min (northiaden TFA) to the area of *m/e* 191 at t_R 4.1 min

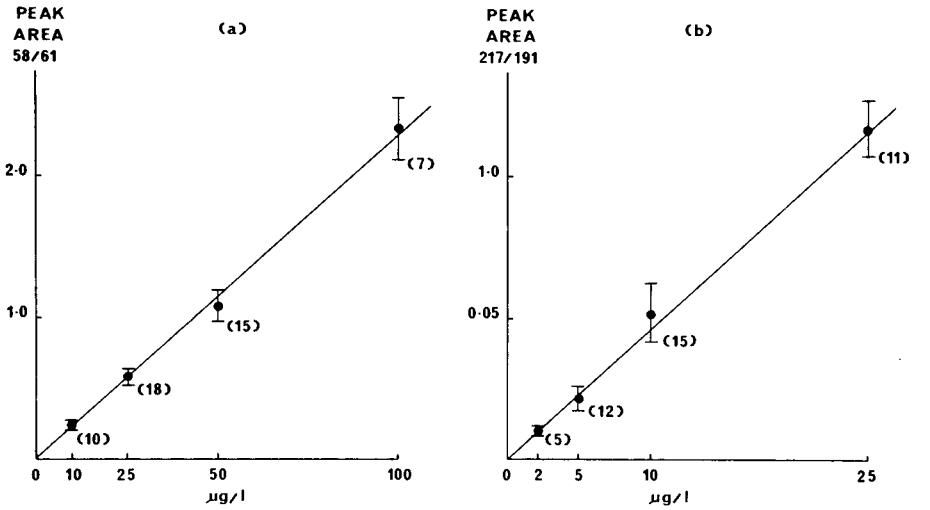

Fig. 2. Plasma standard curves for (a) dothiepin and (b) northiaden. Calculated linear regression lines were (a) $y = 0.0231x + 0.0010$, $r = 0.9992$; (b) $y = 0.0047x + 0.0009$, $r = 0.9978$. The error bars represent ± 1 S.D., the number in parentheses equals the number of determinations at each concentration.

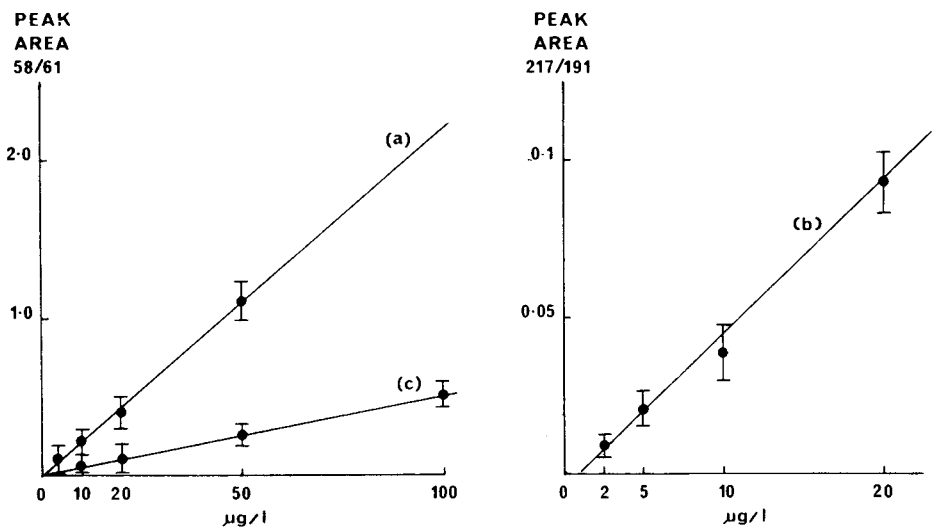

Fig. 3. Blood standard curves for (a) dothiepin, (b) northiaden and (c) dothiepin S-oxide. Calculated linear regression lines were (a) $y = 0.0226x - 0.0078$, $r = 0.9997$; (b) $y = 0.0049x - 0.0042$, $r = 0.9939$; and (c) $y = 0.0053x + 0.0047$, $r = 0.9999$. The error bars represent ± 1 S.D., the number of determinations at each point was six.

(protriptyline TFA) were calculated for each sample. Standard curves were constructed by linear regression analysis of the calculated ratios versus amount of drug added (Figs. 2 and 3). Unknown samples were calculated using the regression equations.

Precision studies

Drug-free whole blood or plasma (Blood Bank) was used to prepare standards to which known amounts of both dothiepin and northiaden were added. Dothiepin S-oxide was also added to the blood standards. These were used each run to prepare standard curves and to evaluate the day-to-day precision of the assay.

Plasma and erythrocyte distribution of dothiepin

Blood samples were taken by venipuncture from six healthy volunteers. Dothiepin (100 ng/0.1 ml in isotonic saline, pH 7.4) was added to 2 ml of whole blood which was then incubated at 37°C for 15—20 min. One duplicate of each blood sample was centrifuged to obtain the plasma fraction. Both blood and plasma were analysed for dothiepin content.

Single-dose experiments

Seven healthy volunteers were given 75 mg of dothiepin in the form of three 25-mg capsules. Samples were taken over the first 8 h via an indwelling heparinized cannula, and by venipuncture over the following 2 days. Half of each blood sample was frozen immediately, the other half centrifuged to obtain plasma. The plasma was then separated and stored frozen till analysed.

RESULTS AND DISCUSSION

Deuterodothiepin provides an ideal internal standard for dothiepin as the recovery of both compounds through the procedure is identical. Protriptyline was chosen as the internal standard for northiaden as it is also a secondary amine tricyclic antidepressant and has similar extraction and derivatization characteristics to northiaden. Derivatization was necessary to prevent the secondary amines adsorbing to the column packing. TFA was found to be the most satisfactory derivatizing reagent compared to acetic anhydride and heptafluorobutyric anhydride, in terms of ease of derivatization and chromatographic characteristics of the derivatives.

The mass spectra of dothiepin, deuterodothiepin, northiaden-TFA and protriptyline-TFA are shown in Figs. 4—7. The most abundant ions were *m/e* 58,

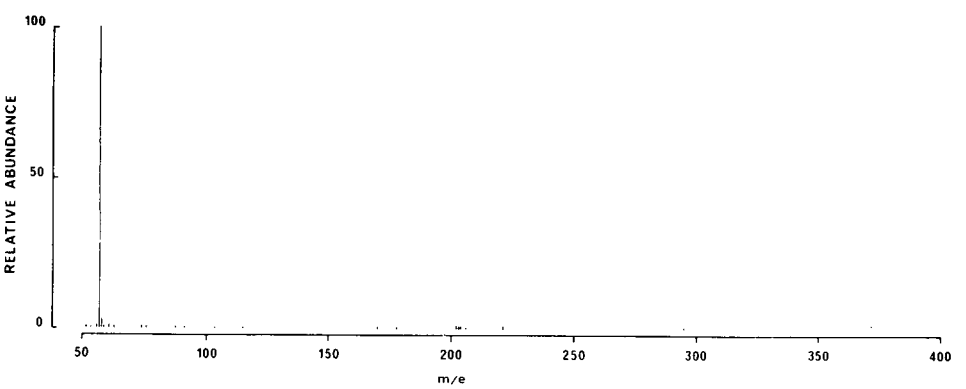

Fig. 4. Mass spectrum of dothiepin, base peak *m/e* 58.

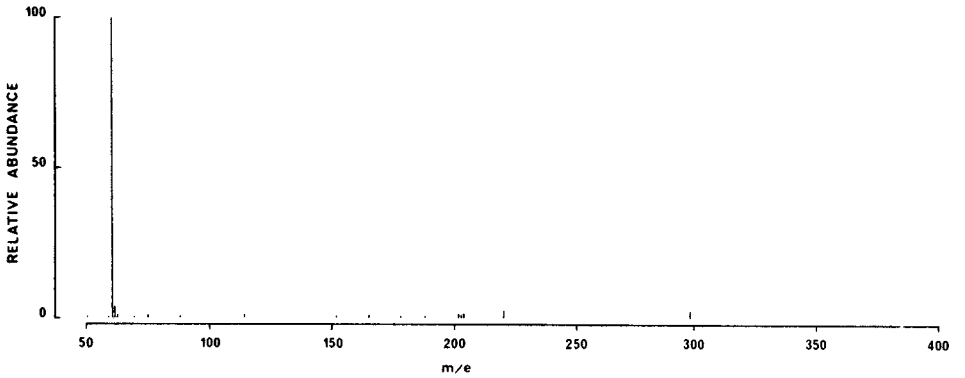

Fig. 5. Mass spectrum of deutero dothiepin, base peak *m/e* 61.

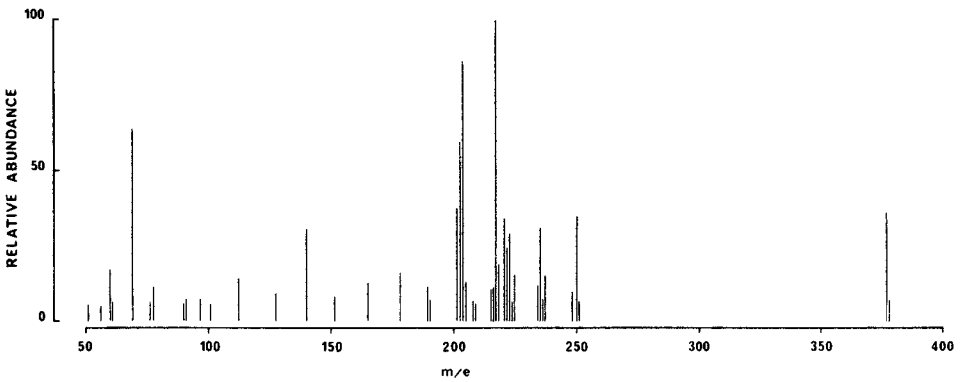

Fig. 6. Mass spectrum of northiaden-TFA, base peak *m/e* 217.

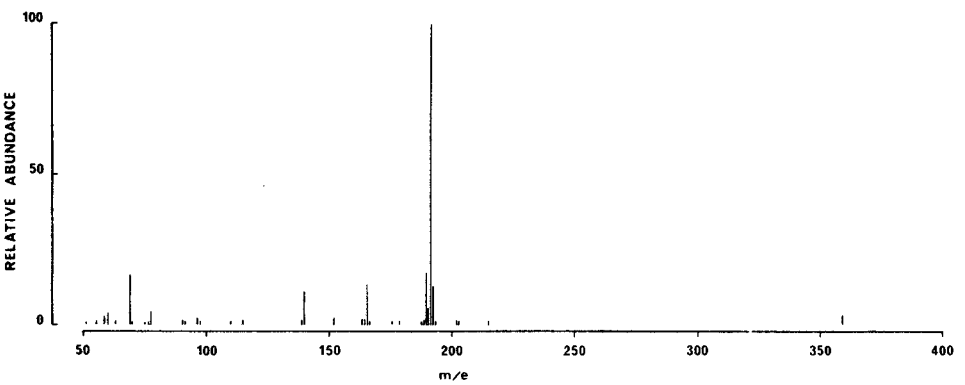

Fig. 7. Mass spectrum of protriptyline-TFA, base peak *m/e* 191.

61, 217, and 191 respectively and these were chosen for simultaneous ion monitoring. A longer dwell time was chosen for 217 due to the weaker abundance of this ion. The fragmentation of dothiepin (and deuterodothiepin) is similar to the other tricyclic antidepressants, amitriptyline and doxepin and *m/e* 58 (61) arises due to loss of part of the side-chain [5]. Similarly, the ion at *m/e* 191 of protriptyline-TFA arises from loss of the complete side-chain [5].

The major ion *m/e* 217 of northiaden-TFA is not so easily explained as loss of part of the side-chain gives rise to *m/e* 250. Subsequent rearrangement and elimination may be the mechanism by which *m/e* 217 occurs. The fragmentation pattern of northiaden-TFA appears quite complex as judged by the number of relatively abundant ions.

The three-step extraction procedure was found necessary to provide clean extracts for analysis. A single solvent extraction of blank plasma gave rise to interfering peaks coinciding with both dothiepin and northiaden. Even with the rigorous extraction procedure as described, occasional interference with the dothiepin peak was observed. This limited the lowest measurable concentration to 1 μg/l. The sensitivity of the method for northiaden was similar, due mainly to the relatively low abundance of *m/e* 217. Extracts of drug-free whole blood were similar to those of plasma.

After completion of the evaluation of the assay for dothiepin and northiaden in plasma, and on analysis of the single-dose samples, a major peak was observed in the single ion trace *m/e* 58 at t_R 4.0 min. A sample of dothiepin S-oxide was then obtained which was found to account for this peak. Dothiepin S-oxide had a slightly longer retention time than dothiepin itself, but the major ion fragment was the same for both compounds (*m/e* 58). The use of deuterodothiepin as the internal standard for measuring dothiepin S-oxide is not ideal since the S-oxide does not extract as well. However, since reproducible results were obtained (see Table I) it was decided to use deuterodothiepin rather than adding a third internal standard which would then necessitate re-evaluating the

TABLE I

REPRODUCIBILITY OF THE ASSAY FOR DOTHIEPIN AND NORTHIADEN LEVELS IN PLASMA AND BLOOD, AND DOTHIEPIN S-OXIDE IN BLOOD

n = 10 at all concentrations.

Drug	Expected concentration (μg/l)	Plasma		Blood	
		Found (μg/l)	C.V. (%)	Found (μg/l)	C.V. (%)
Dothiepin	5	4.9	± 10	4.8	± 11
	10	10.3	± 6	10.6	± 7
	25/20*	25.8	± 5	20.1	± 7
	50	50.7	± 6	51.1	± 5
Northiaden	2	2.1	± 16	2.5	± 20
	5	5.0	± 14	4.9	± 10
	10	10.1	± 8	10.1	± 13
	25/20*	23.4	± 7	20.9	± 4
Dothiepin S-oxide	10			10.7	± 10
	20			22.8	± 13
	50			50.5	± 13
	100			105.0	± 9

*25 μg/l for plasma standards, 20 μg/l for blood standards.

complete procedure. Consequently, evaluation of the precision of the assay for whole blood concentrations was done for dothiepin S-oxide in addition to dothiepin and northiaden.

One other metabolite of dothiepin, northiaden S-oxide was available for testing the specificity of the assay. This metabolite on derivatization gave a compound identical in retention time and major ion fragment to that of

TABLE II

DISTRIBUTION OF DOTHIEPIN

No.	Sex	Age	Blood level (μg/l)	Plasma level (μg/l)	Ratio blood to plasma
1	F	28	109.4	110.0	0.99
2	M	26	109.4	110.6	0.99
3	M	23	113.2	107.7	1.05
4	M	24	109.6	121.4	0.90
5	F	38	107.8	86.2	1.25
6	M	33	110.3	93.1	1.18

northiaden-TFA. However, when added to blank plasma in concentrations of up to 250 μg/l, a response equal to 1—2 μg/l of northiaden resulted. It appears that the S-oxide does not extract under the conditions used.

The precision of the assay has been investigated over a six-month period. The results obtained for day-to-day reproducibility over the concentration ranges 5—50 μg/l for dothiepin and 2—25 μg/l for northiaden in plasma are presented in Table I. Acceptable precision was achieved even at the lowest concentration. The day-to-day reproducibility for dothiepin, northiaden and dothiepin S-oxide in whole blood is also shown in Table I. Again, acceptable precision was obtained, even for the S-oxide.

In general, there was little difference between the extracts obtained from blood or plasma with the exception that the recovery from blood was slightly lower than that from plasma.

The distribution of dothiepin between plasma and erythrocytes was examined under in vitro conditions (Table II). Dothiepin was equally distributed between plasma and erythrocytes, thus whole blood and plasma levels are more or less identical, and either could be used for pharmacokinetic studies. To check this in vivo both plasma and blood samples were analysed from the single-dose studies. Dothiepin blood concentrations were again more or less identical to that found in plasma whereas northiaden plasma concentrations were greater than blood concentrations. Dothiepin S-oxide was only measured in blood.

The concentrations of the three compounds following the single oral doses are shown in Fig. 8. The mean dothiepin peak level was 43 μg/l at 3 h, the mean northiaden peak level was 9 μg/l at 8 h, and dothiepin S-oxide reached the highest peak concentration (78 μg/l) at 4 h post-dose. A full description of the study and the pharmacokinetic parameters calculated from it will be published separately [6].

In six out of the seven volunteers, dothiepin S-oxide reached higher concen-

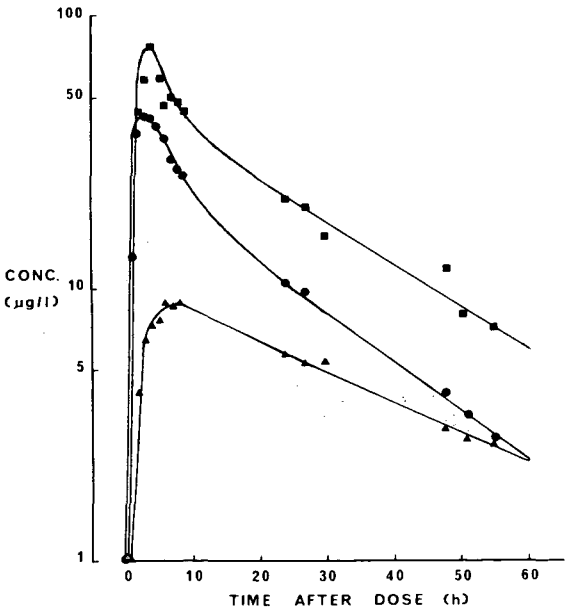

Fig. 8. Mean plasma concentrations of dothiepin and northiaden and the mean blood concentration of dothiepin S-oxide following a single oral dose of 75 mg dothiepin in seven healthy volunteers. ■, Dothiepin S-oxide; ●, dothiepin; ▲, northiaden.

trations than dothiepin itself. Recent studies from our laboratory have shown that both dothiepin and northiaden S-oxides inhibit platelet uptake of [^{14}C]-serotonin [2]. These metabolites should thus be analysed in addition to dothiepin and northiaden in studies of clinical response and drug concentrations. A different extraction procedure would be required to enable quantitation of northiaden S-oxide but the derivatization, chromatography and mass spectrometric conditions would not require alteration.

In conclusion, the GC—MF method as described has been shown to be specific, sensitive and reproducible for dothiepin and two of its major metabolites. It appears more than satisfactory for pharmacokinetic studies of single oral doses of dothiepin.

ACKNOWLEDGEMENTS

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REFERENCES

1 E.L. Crampton, W. Dickinson, G. Haran, B. Marchant and P.C. Risdall, Br. J. Pharmacol., 64 (1978) 405P.
2 T.R. Norman and H. Cheng, in E. Usdin, S. Dahl, L.F. Gram and O. Lingjaerde (Editors), Proceedings of the 2nd International meeting on Clinical Pharmacology in Psychiatry, Macmillan, New York, London, in press.

3 R.R. Brodie, L.F. Chasseud, E.L. Crampton, D.R. Hawkins and P.C. Ridsall, J. Int. Med. Res., 5 (1977) 387.
4 E.L. Crampton, R.C. Glass, B. Marchant and J.A. Rees, J. Chromatogr., 183 (1980) 141.
5 J.T. Biggs, W.H. Holland, S. Chang, P.P. Hipps and W.R. Sherman, J. Pharm. Sci., 65 (1976) 261.
6 K.P. Maguire, G.D. Burrows, T.R. Norman and B.A. Scoggins, Br. J. Clin. Pharmacol., submitted for publication.

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CHROMBIO. 759

DETERMINATION OF 1,4- AND 1,5-BENZODIAZEPINES IN URINE USING A COMPUTERIZED GAS CHROMATOGRAPHIC—MASS SPECTROMETRIC TECHNIQUE*

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SUMMARY

A method for the determination of benzodiazepines and their main metabolites in urine after acid hydrolysis is described. The extract is analyzed by computerized gas chromatography—mass spectrometry. An on-line computer allows rapid detection using mass fragmentography with the masses *m/e* 211, 230, 241, 244, 249, 262, 276, and 285. The mass fragmentogram and the underlying mass spectra of the hydrolysis products (benzophenones and analogues) are documented.

INTRODUCTION

In the last few years ever more benzodiazepines have been put on to the market. Because of their immense application these drugs are encountered very frequently in clinical and forensic toxicology. Screening for these drugs by their determination in the urine after acid hydrolysis to decompose conjugates is recommended. Thereby the benzodiazepine molecules are also decomposed [1]. Decomposition of the conjugates by enzymatic hydrolysis has the advantage that it leaves the benzodiazepine molecules intact, but typically takes 12 h and so does not lend itself to a rapid screening procedure.

Identification of some of the acid hydrolysis products (benzophenones and analogues) employing thin-layer chromatography [2], gas—liquid chromatography [2,3] or high-performance liquid chromatography [4] has been described. In the review article of Hailey [5] several detection methods and

*This work is part of the thesis of H. Maurer at the Universität des Saarlandes, Saarbrücken, G.F.R. In part sis was reported at the symposium "Psychopharmaka und Suchstoffe" of the Gesellschaft für toxikologische und forensische Chemie (GTFCH) in Mosbach, G.F.R., April 25th and 26th, 1980.

further references are given. However, none of these methods allows the rapid and exact differentiation that is important in clinical or forensic estimation because the various benzodiazepines have very different pharmacological potencies. These demands are met by the computerized gas chromatographic—mass spectrometric (GC—MS) technique described below.

EXPERIMENTAL

Apparatus

A Varian Aerograph gas chromatograph series 1400 combined with a Varian mass spectrometer Type 331 A, a Varian data system 111 MS and a Tektronix storage display unit Type 611 was used. The GC conditions were as follows. Column: nickel-capillary 60 cm × 1 mm I.D., packed with Chromosorb W AW DMCS 80—100 mesh re-silanized with dichlorodimethylsilane and coated with 20% UCC-W (Hewlett-Packard). (OV-1 can also be used.) Column temperature: programmed from 100 to 310°C at 20°C/min. Injector port temperature: 270°C. Carrier gas: helium, flow-rate 7 ml/min.

The MS conditions were as follows: ionization energy, 90 eV; ion-source temperature, 200°C. The technique of open coupling was used. About 2 ml/min of gas were dosed by an SGE micro-needle-valve and an SGE shut-off valve (Scientific Glass Engineering, Ringwood, Australia) and introduced into the ion source by a nickel capillary (0.25 mm I.D. heated at 270°C). Details will be published later [6].

For the exact measurement of retention indices a Varian Aerograph gas chromatograph series 3700 was used. The column effluent went to a flame-ionization detector (FID) and a nitrogen-sensitive FID (N-FID) after a 1:1 split by a splitter made from nickel tubing. The column was a nickel tube, 60 cm × 2 mm I.D., packed as for GC—MS but with only 10% stationary phase. The column and injector temperatures were as for GC—MS, the temperature of the detectors was 270°C. Carrier gas was nitrogen at a flow-rate of 30 ml/min.

Hydrolysis and extraction procedure

Ten millilitres of urine are refluxed with 3 ml of hydrochloric acid (37%) for 15 min, then made basic with about 3 g of potassium hydroxide pellets and mixed with 10 ml of 30% aqueous ammonium sulfate to obtain a pH of between 8 and 9. The sample is then extracted twice with 10 ml of diethyl ether. After phase separation by centrifugation the combined ether extracts are evaporated to dryness under vacuum. The residue is redissolved in 0.1 ml of methanol and 1—4 μl of this solution are injected into the gas chromatograph.

Gas chromatographic—mass spectrometric analysis

Mass spectra are recorded at a speed of 6 sec/decade and stored on computer tape during the temperature-programmed GC analysis. Scanning at this relatively slow rate ensures at least two spectra for each GC peak whilst avoiding excessive data accumulation. The identity of positive signals in the reconstructed mass fragmentogram is established by a comparison of the entire mass spectra with those of standards.

RESULTS AND DISCUSSION

The results of our investigations are shown in Table I. The mass fragmentogram with the eight proposed masses shown in Table I allows the detection of the hydrolysis products (benzophenones and analogues) of sixteen important benzodiazepines as themselves or their metabolites. But not all metabolites are listed; only those detectable by the mass fragmentogram and necessary for the identification of the drugs.

TABLE I

MONITORING PROGRAM FOR BENZODIAZEPINES AFTER HYDROLYSIS

Mass spectrum No.	Hydrolyzed drugs and metabolites	*m/e* (relative intensities in %)								Retention index
		211	230	241	244	249	262	276	285	
01	Bromazepam					100		28		2450
02	Camazepam		18		83					2100
02	Methyl-oxazepam		18		83					2100
03	Oxazepam		100							2050
03	Chlorazepate		100							2050
03	Chlordiazepoxide		100							2050
04	Clobazam		10				4	17		2225
05	Nor-			44	35					2210
06	Clonazepam	7	4	100				67		2470
07	(Acet-)amino-	72				4				2287
02	Diazepam		18		83					2100
03	Nor-/Oxazepam		100							2050
08	Flunitrazepam	22								2374
09	(Acet-)amino-	10	3		100					2796
10	(Acet-)amino-nor-	70	100							2167
11	Flurazepam									2554
12	Didesethyl-	100				4		16		2294
13	Hydroxyethyl-						100			2383
14	N-Desalkyl-		20			100				2031
15	Lorazepam		100							2180
16	Lormetazepam				100		17			2220
15	Nor-		100							2180
17	Medazepam				32					2236
18	Nor-		23							2278
03	Nor-diazepam		100							2050
19	Nitrazepam	4		88						2363
20	(Acet-)amino-	92								2225
03	Oxazepam		100							2050
21	Prazepam		17		14				65	2411
03	N-Desalkyl-		100							2050
22	Tetrazepam					55				2200
23	(Androsterone)		5	2	50			2		2476

The entire mass spectra of the benzophenones are shown in Fig. 1 for the precise identification of the compounds. The retention indices were determined using a gas chromatograph combined with FID and N-FID with a temperature program. We found that the retention indices obtained with tempera-

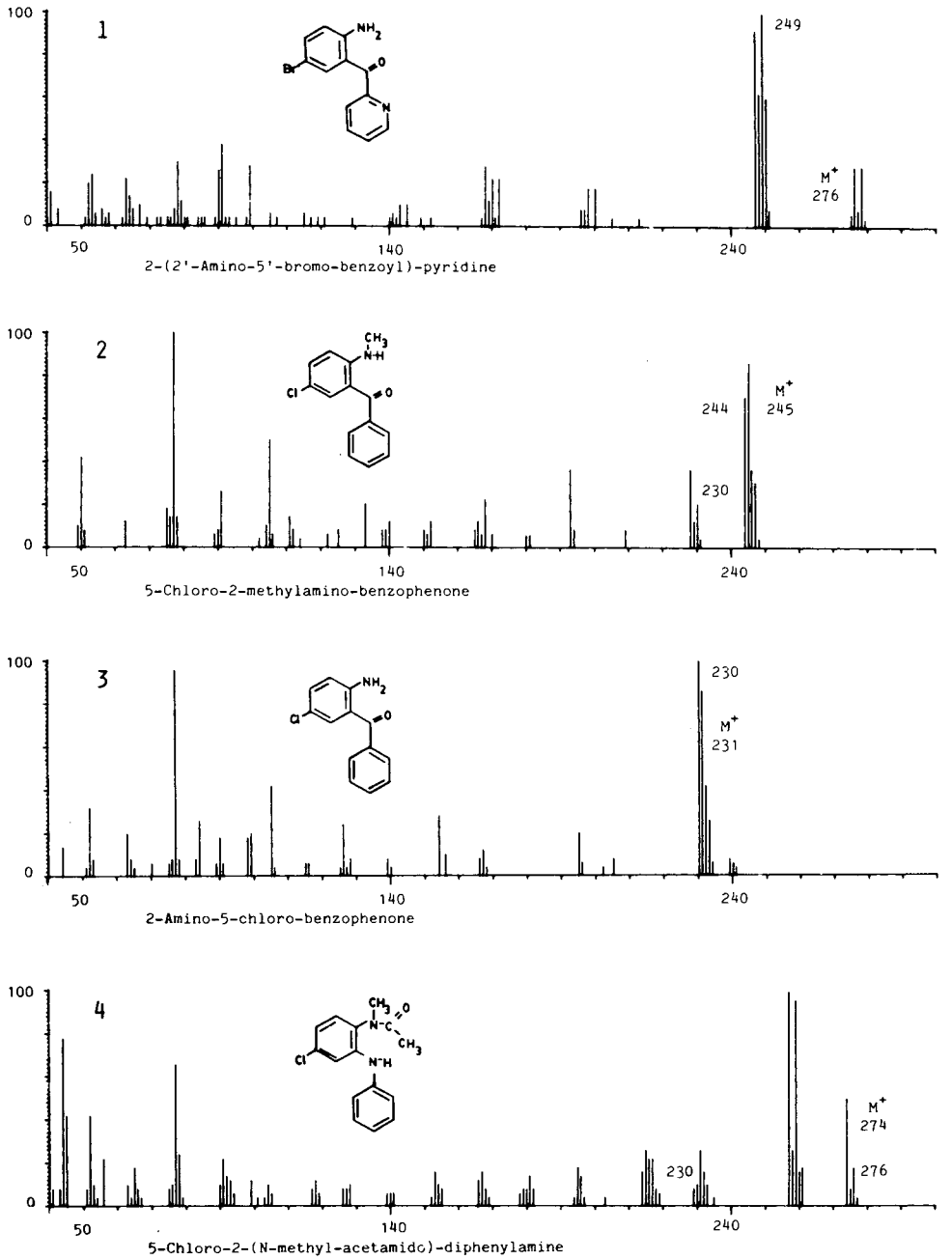
100
1
249
M+
276
0
50
140
240
2-(2'-Amino-5'-bromo-benzoyl)-pyridine
100
2
244
M+
245
230
0
50
140
240
5-Chloro-2-methylamino-benzophenone
100
3
230
M+
231
0
50
140
240
2-Amino-5-chloro-benzophenone
100
4
M+
274
230
276
0
50
140
240
5-Chloro-2-(N-methyl-acetamido)-diphenylamine

Fig. 1.

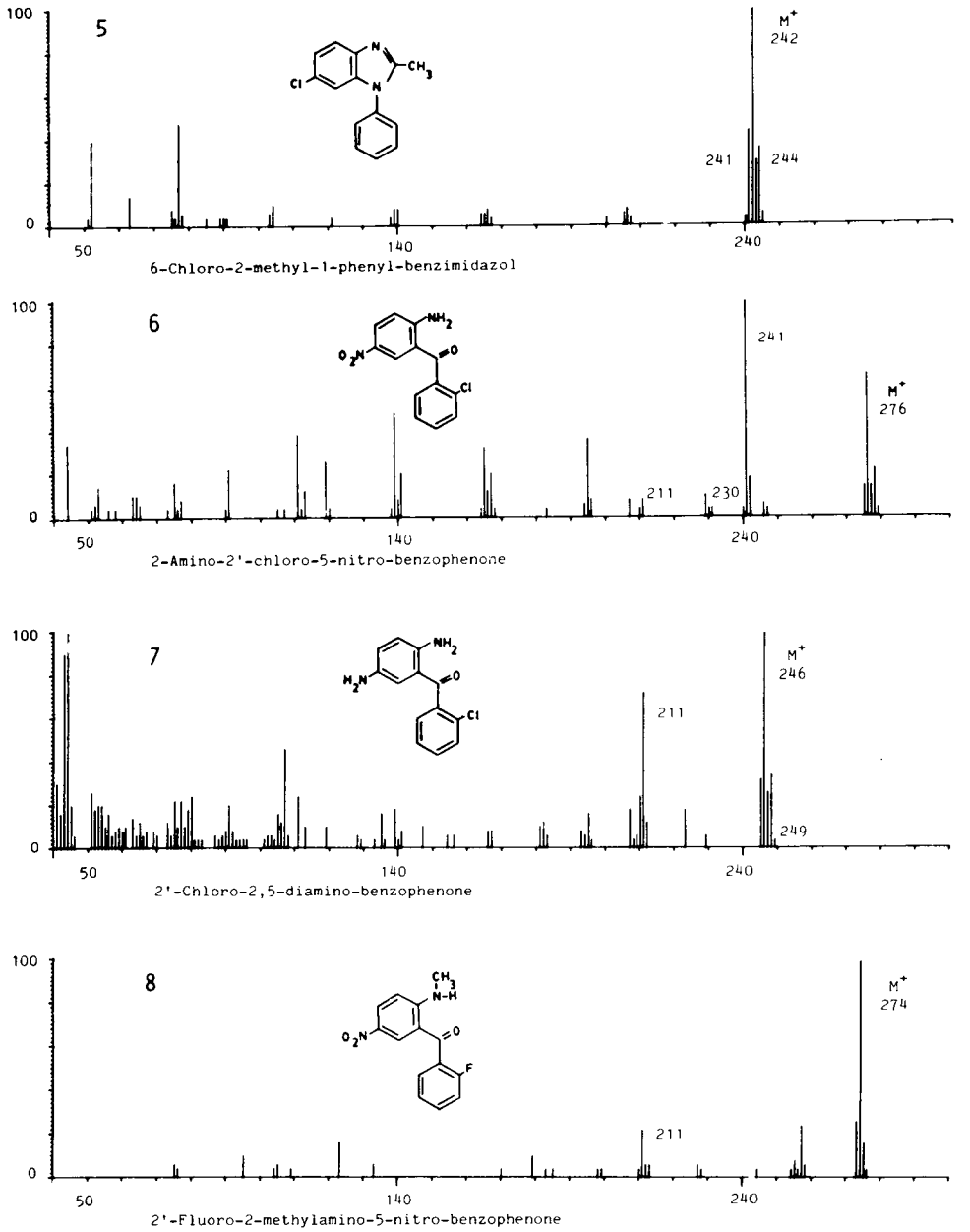

Fig. 1.

(Continued on p. 414)

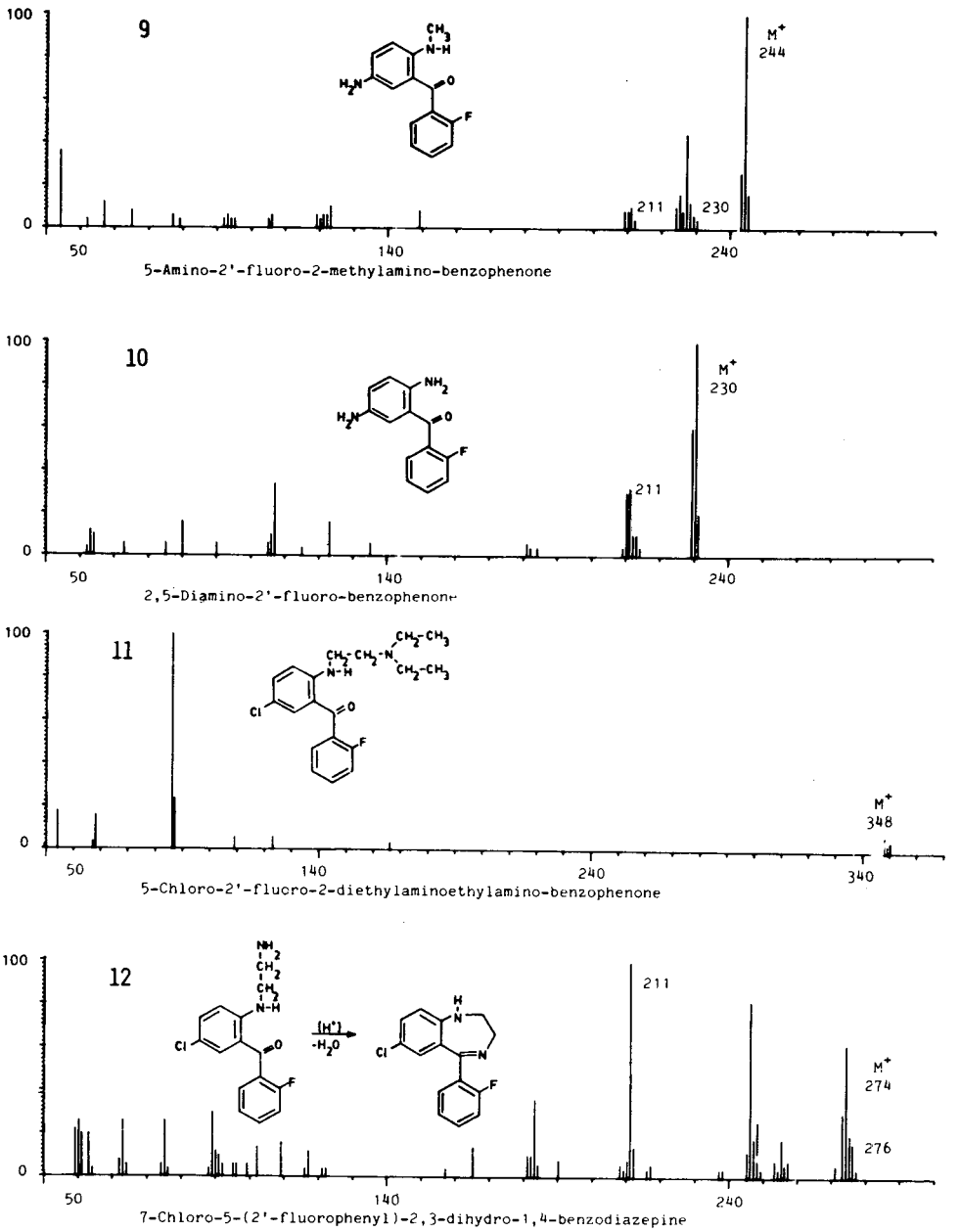
9
100
0
50
140
240
M+
244
211
230
5-Amino-2'-fluoro-2-methylamino-benzophenone
10
100
0
50
140
240
M+
230
211
2,5-Diamino-2'-fluoro-benzophenone
11
100
0
50
140
240
340
M+
348
5-Chloro-2'-flucro-2-diethylaminoethylamino-benzophenone
12
100
0
50
140
240
211
M+
274
276
7-Chloro-5-(2'-fluorophenyl)-2,3-dihydro-1,4-benzodiazepine

Fig. 1.

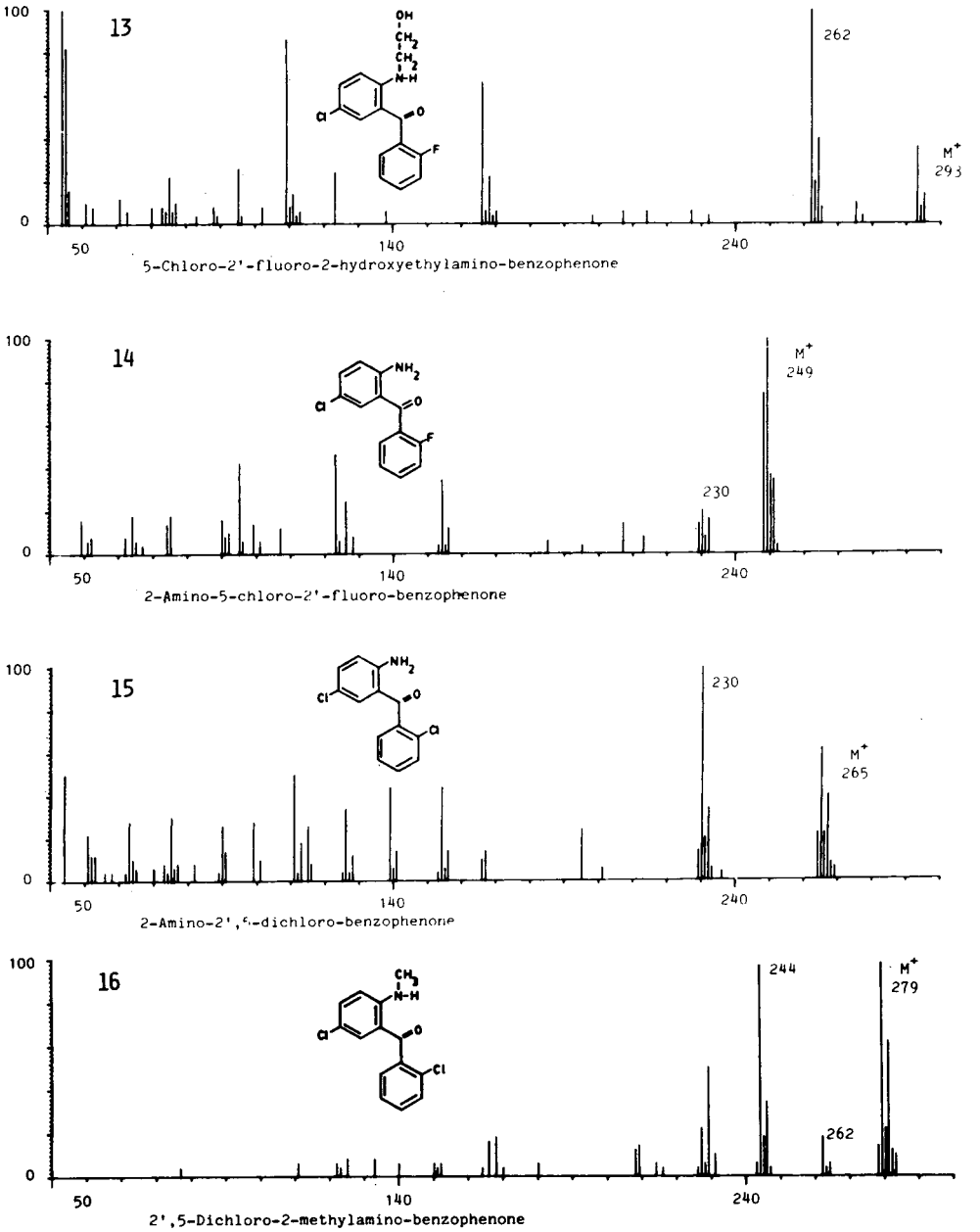

Fig. 1.

(Continued on p. 416)

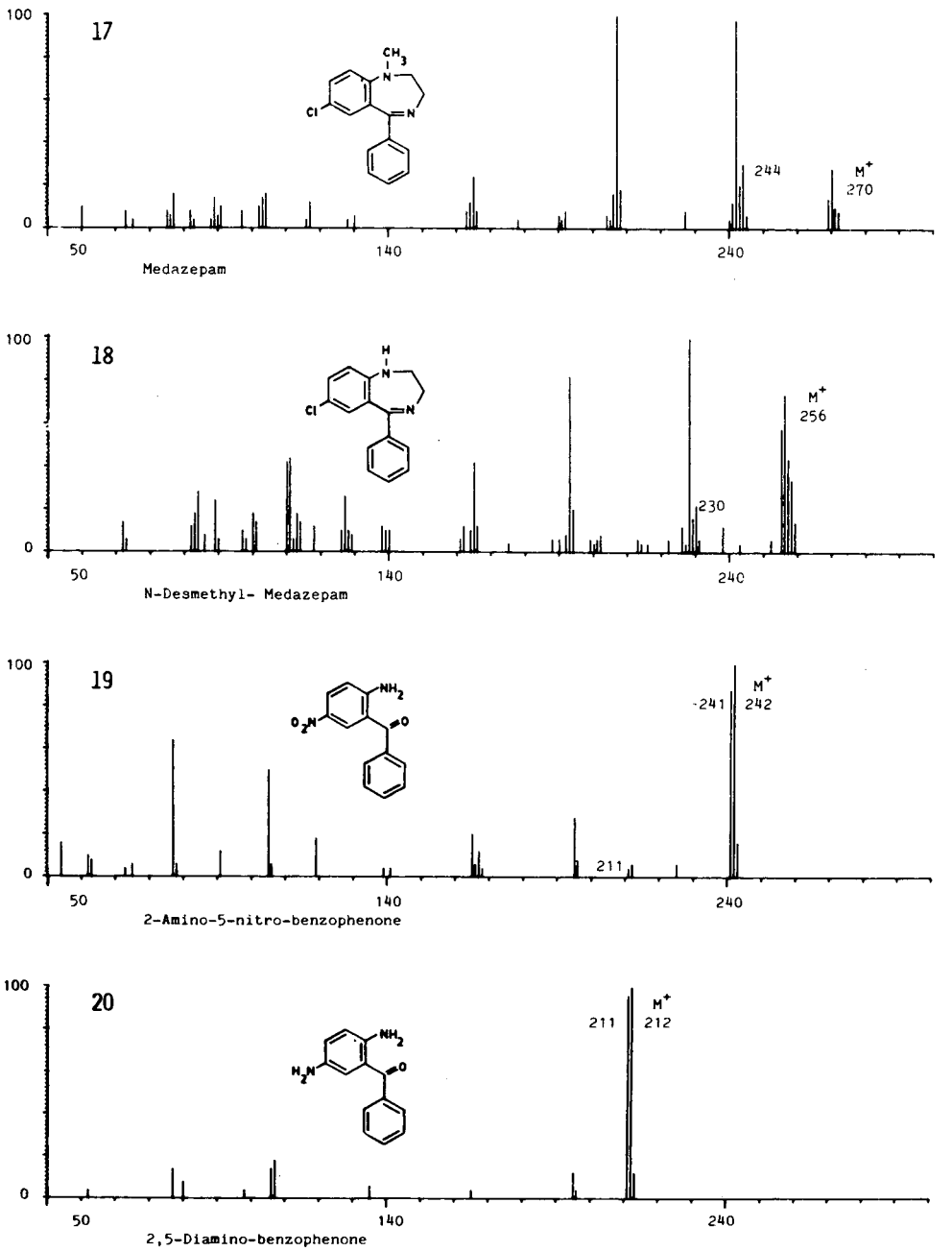
17
100
0
50
140
240
244
M+
270
Medazepam
18
230
M+
256
N-Desmethyl- Medazepam
19
241
M+
242
211
2-Amino-5-nitro-benzophenone
20
211
M+
212
2,5-Diamino-benzophenone

Fig. 1.

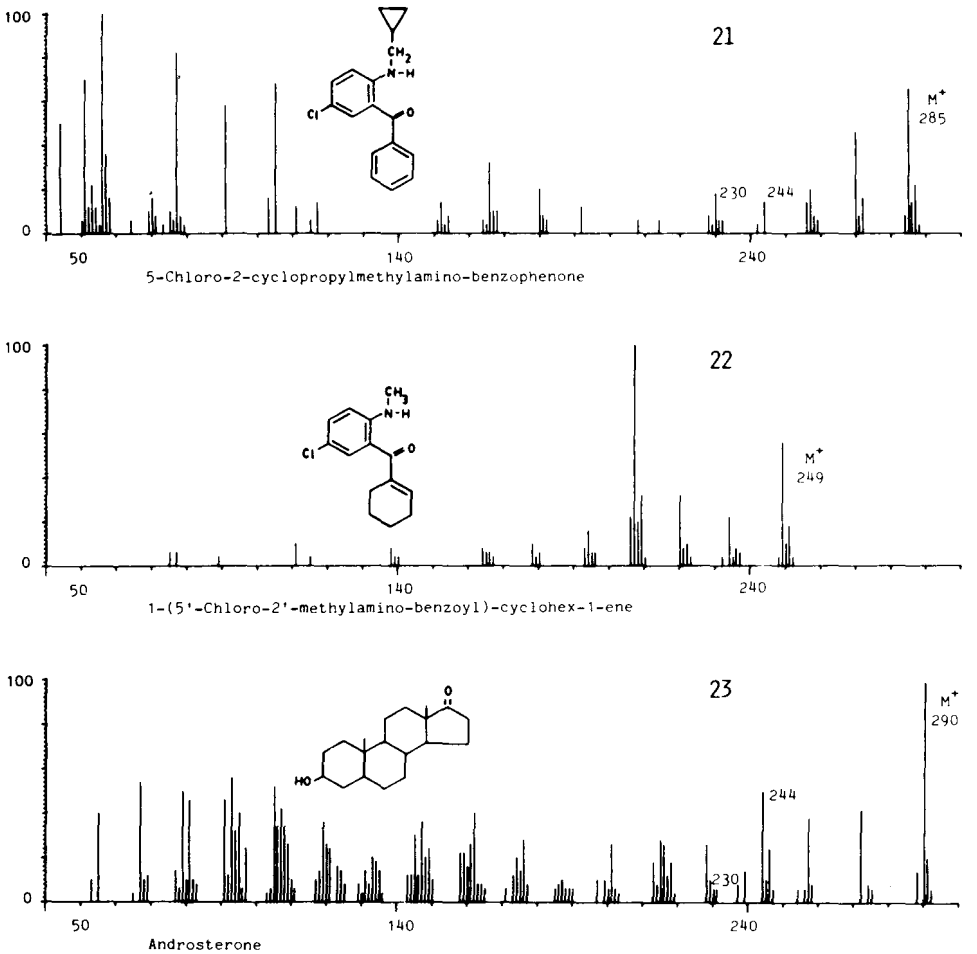

Fig. 1. Mass spectra of the hydrolysis products of benzodiazepines and androsterone.

ture programming were the same as those obtained using an isothermal procedure [6]. In our experience retention indices are not necessary when employing the GC—MS technique but may be useful to gas chromatographers without the latter facility and so they are given here. All investigations were carried out using the urine of man after therapeutic dosage with the exception of clonazepam, flunitrazepam and lormetazepam which were detected — in the absence of human samples — in the urine of rats.

Camazepam, clobazam, clonazepam, flurazepam and medazepam are almost completely excreted in the urine as their metabolites. Medazepam and its desmethyl metabolite are not hydrolyzable, because they contain no lactam ring.

In our experience androsterone is the only endogenous physiological substance that appears in the mass fragmentogram.

Although camazepam and diazepam and their metabolites are hydrolyzed to the same two benzophenones, ingestion of the two drugs can be differentiated

by the relative proportions of the two hydrolysis products. Thus, camazepam leads to more chloromethylaminobenzophenone (CMAB, from methyl-oxazepam) than aminochlorobenzophenone (ACB, from oxazepam), whilst diazepam leads to more ACB (from desmethyl-diazepam and oxazepam) than CMAB (from diazepam).

Only chlorazepate, chlordiazepoxide and oxazepam cannot be differentiated by the hydrolysis products because they and their metabolites are all hydrolyzed to ACB alone. If necessary, they can be identified afterwards in a second urine sample after enzymatic hydrolysis of the conjugates or in the blood.

For an illustration of the method a mass fragmentogram of a sample from a psychiatric clinic is shown in Fig. 2. The first peak at *m/e* 230 indicates ACB (mass spectrum No. 15) from lorazepam, and the peaks at *m/e* 230, 241 and and oxazepam, the peak at *m/e* 244 indicates CMAB (mass spectrum No. 2) from diazepam, the second peak at *m/e* 230 is aminodichlorobenzophenone (mass spectrum No. 15) from lorazepam, and the peaks at *m/e* 230, 241 and 244 indicate the physiological hormone androsterone (mass spectrum No. 23). This example shows that diazepam (in this case given therapeutically), its metabolites, and lorazepam (taken in abuse) can be precisely differentiated. This is impossible in such a short time with any method previously described in the literature.

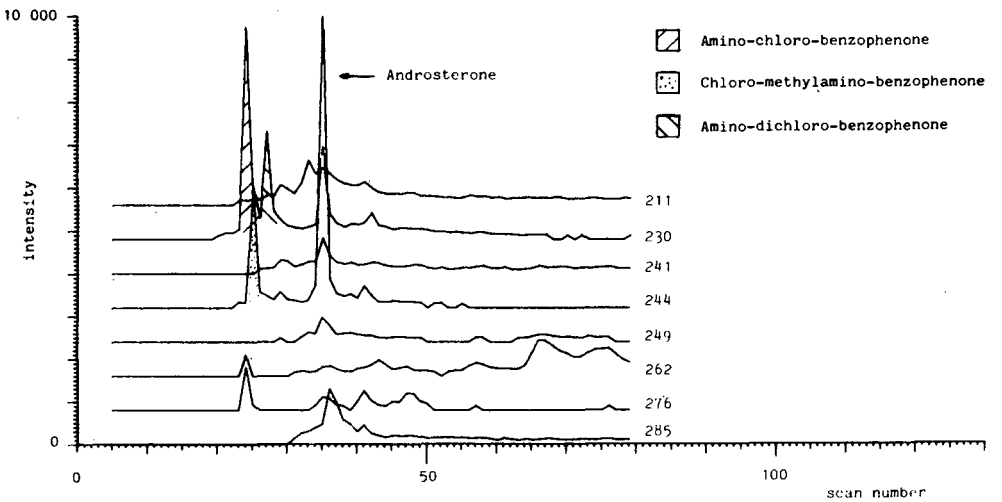

Fig. 2. Mass fragmentogram indicating different benzophenones from the urine of a benzodiazepine addict.

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REFERENCES

1 J. Bäumler and S. Rippstein, Helv. Chim. Acta, 44 (1961) 2208.
2 H.J. Battista, H. Udermann, G. Henning and W. Vycudilik, Beitr. Gerichtl. Med., 37 (1979) 5.
3 H. Schütz and V. Westenberger, Z. Rechtsmed., 82 (1978) 43.
4 K. Harzer and R. Barchet, J. Chromatogr., 132 (1977) 83.
5 D.M. Hailey, J. Chromatogr., 98 (1974) 527.
6 H. Maurer, A. Weber and K. Pfleger, in preparation.

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CHROMBIO. 749

DETERMINATION OF CARBIMIDE IN PLASMA BY GAS—LIQUID CHROMATOGRAPHY

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SUMMARY

A sensitive and selective method for the measurement of carbimide, the hydrolytic product of calcium carbimide, in plasma is described. The procedure involves extraction with ethyl acetate, derivatization with heptafluorobutyric anhydride and analysis by gas—liquid chromatography with electron-capture detection. The lower limit of sensitivity of the assay is 5.0 ng/ml carbimide in plasma. The overall accuracy of the procedure is 96.1% with a coefficient of variation not exceeding 8.7%. This assay has been used to investigate the time-course of plasma carbimide concentration in the rat following oral administration of calcium carbimide.

INTRODUCTION

Calcium carbimide (CC) is used as a pharmacological adjunct in the treatment of alcoholism [1]. In recent years, detailed studies of the CC—ethanol interaction in man [2—5] and in animals [6, 7] have been conducted. CC inhibits aldehyde dehydrogenase (EC 1.2.1.3), one of the enzymes involved in ethanol metabolism [8, 9]. During the CC—ethanol interaction, blood acetaldehyde concentration is increased, which results in a number of untoward effects (e.g. tachycardia, hypotension), thereby deterring further drinking. One of the shortcomings of these studies on CC has been the inability to measure plasma drug levels due to the lack of a selective and sensitive assay. As a result, it has not been possible to assess the extent of variation in the absorption of CC following oral administration or to determine if there is a correlation between plasma drug concentration and the extent of aldehyde dehydrogenase inhibition, especially during the CC—ethanol interaction.

This paper describes a sensitive and selective method for the measurement of carbimide, the hydrolytic product of CC, in plasma. The procedure involves ethyl acetate extraction, derivatization with heptafluorobutyric anhydride (HFBA) and analysis by gas—liquid chromatography with electron-capture detection (GLC—ECD).

0378-4347/81/0000—0000/$02.50

EXPERIMENTAL

Reagents

Ethyl acetate was certified GC-spectrophotometric grade (J.T. Baker, Phillipsburg, NJ, U.S.A.). Stock solutions of carbimide (Aldrich, Milwaukee, WI, U.S.A.) were prepared daily in glass-distilled water. Calcium carbimide and citrated calcium carbimide (Temposil®) were obtained from Lederle (Montreal, Canada). [^{14}C]Carbimide was purchased from ICN Pharmaceuticals (Irvine, CA, U.S.A.). Heptafluorobutyric anhydride was purchased from Supelco (Bellefonte, PA, U.S.A.).

pK_a determination of carbimide

A 0.1 *M* standardized sodium hydroxide solution (Fisher Chemicals, Fair Lawn, NJ, U.S.A.) was used. Carbimide (2.0 mmoles) was dissolved in 100 ml of reverse-osmosis, deionized water. The carbimide solution was acidified with 20 ml of 0.1 *M* hydrochloric acid and then titrated with 0.02-ml aliquots of the sodium hydroxide solution at 21°C. The pK_a of carbimide was determined from the inflection point of the titration curve.

Extraction

A 1.0-ml plasma sample was placed in a FEP-stoppered centrifuge tube (silanized) and the pH was adjusted to 10 with 0.1 *M* sodium hydroxide. After 0.5 g of sodium chloride and 2.0 ml of ethyl acetate were added, the tube was shaken for 2 min on a flat-bed shaker followed by centrifugation at 150 *g* for 3 min. The organic phase was then transferred to a 5-ml reacti-vial (Chromatographic Specialties, Brockville, Canada). The pH of the remaining aqueous layer was readjusted to 10 with 0.1 *M* sodium hydroxide and extracted as previously described with 2.0 ml of ethyl acetate. The organic phase was then transferred to the 5-ml reacti-vial. The combined organic extract was concentrated to dryness with a stream of nitrogen at 50°C and the residue was redissolved in 100 μl of acetonitrile for subsequent derivatization with HFBA and analysis by GLC–ECD.

The efficiency of the procedure for extracting carbimide from plasma using ethyl acetate was determined with plasma standards containing known amounts of [^{14}C]carbimide. A 20-μl aliquot of 0.5 μCi/ml [^{14}C]carbimide (50 μCi/μmole) containing approximately 20,000 dpm was added to each plasma standard. The radioactivity of [^{14}C]carbimide was determined in the ethyl acetate extract by adding 100 μl of the extract to 10.0 ml of ACS scintillation cocktail (Amersham, Arlington Heights, IL, U.S.A.) and liquid scintillation counting in a Searle Mark III counter (Des Plaines, IL, U.S.A.) for 10 min.

Derivatization

The 5-ml reacti-vial, containing the residue of the ethyl acetate extraction dissolved in 100 μl of acetonitrile, was sealed with a mininert valve (Chromatographic Specialties). A 5.0-μl aliquot of HFBA was added through the valve and the contents were mixed for 5 sec using a vortex mixer. The reaction solution was allowed to sit at room temperature (21°C) for 30 min.

The reaction solution was then evaporated to dryness at 45°C with a stream of nitrogen and the residue was redissolved in 100 μl of benzene. A 1.0-μl aliquot of the benzene solution was injected onto the GLC column.

GLC—ECD analysis

A Hewlett-Packard Model 5710A gas chromatograph equipped with a ^{63}Ni electron-capture detector and a 1-mV potentiometric recorder was used. A coiled glass column (1.8 m × 2 mm I.D.) was washed in sequence with distilled water, methanol, acetone, hexane, dried with nitrogen and then silanized with a 10% solution of Dri-Film SC-87 (Chromatographic Specialties) in toluene for 2 h. The column was then rinsed with methanol, dried with nitrogen and heated at 100°C for 1 h. The silanized column was packed with 3% OV-1 on 80—100 mesh Chromosorb W HP (Chromatographic Specialties) and conditioned for 18 h at 220°C with a carrier gas [argon—methane (95:5)] flow-rate of 2 ml/min. For carbimide analysis, the instrumental operating conditions were: injection port temperature, 250°C; column temperature, 185°C; detector temperature, 300°C; carrier gas flow-rate, 20 ml/min.

Gas chromatographic—mass spectrometric analysis

A Biospect (Searle-Scientific Instrument Division, Baltimore, MD, U.S.A.) gas chromatograph—mass spectrometer was used. The mass spectrometer was operated in the chemical ionization mode with methane as the reagent gas and the electron ionization mode, scanning the mass range 70—300 a.m.u. For the gas chromatographic separation, a glass column (1.8 m × 2 mm I.D.) containing 3% SE-30 on 80—100 mesh Chromosorb W (Chromatographic Specialties) was used with a helium carrier gas flow-rate of 15 ml/min. The instrumental operating conditions were: gas chromatograph injection port temperature, 255°C; column temperature, 190°C; mass spectrometer ion source temperature, 210°C; ion source current, 0.2 mA; methane reagent gas pressure, 1 mm Hg.

Aqueous standard curves

Aqueous standards in the concentration range of 5.0—1000 ng/ml were prepared by dissolving known concentrations of carbimide in distilled water; these standards were extracted and analyzed by the procedure outlined above. The peak height of the carbimide signal for each aqueous standard was plotted against the respective carbimide concentration. The concentration of carbimide in a plasma sample was determined by measuring the peak height of the carbimide chromatographic signal and interpolating on the standard curve. The slope and y-intercept of the line of best fit, determined by regression analysis, were used for this calculation.

Recovery study

To evaluate the accuracy and precision of the assay, samples were prepared containing 500, 100 and 5.0 ng/ml carbimide in plasma and were quantitated by the GLC—ECD procedure using aqueous standards. The concentration of carbimide measured was divided by the concentration added and a per cent recovery value was determined for each plasma sample. Precision was

assessed by calculating the coefficient of variation for each plasma carbimide concentration.

Hydrolysis of calcium carbimide to carbimide

To test if there is quantitative hydrolysis of CC to carbimide under simulated gastric conditions, 50 mg of CC, a Temposil tablet containing 50 mg of CC and 100 mg of citric acid, and a pulverized Temposil tablet placed in a gelatinous capsule were individually incubated in 50 ml of 0.1 *M* hydrochloric acid (pH 1.14—1.16) at 37°C with gentle mixing. These conditions were selected to mimic the volume and pH of the stomach contents in man. After 1 h, 1.0 ml of the acidic incubate was extracted and the carbimide concentration determined using GLC—ECD to measure the conversion of CC to carbimide.

RESULTS AND DISCUSSION

In the investigation of the CC—ethanol interaction conducted in this laboratory [2—6], it was deemed necessary to determine the variability in the absorption of CC following oral administration and the correlation between plasma drug concentration and the inhibition of hepatic aldehyde dehydrogenase. This required the development of a sensitive and selective analytical procedure. Since the clinically recommended dose of CC in man is 50 mg (0.7 mg/kg for a 70-kg man), plasma concentrations of the drug would probably be low (< 1.0 μg/ml). Therefore the minimum sensitivity of this assay should be in the 1.0—10 ng/ml plasma concentration range.

The compound CC [Ca^{2+} $(N—C \equiv N)^{2-}$] is insoluble in aqueous and organic material. No known solvent will bring about solution of CC without at least partial hydrolysis to calcium hydrogen carbimide [Ca^{2+} $(HN—C \equiv N)_2^-$], or complete hydrolysis to carbimide ($H_2N—C \equiv N$) [10]. Under simulated gastric conditions, it was determined that for 50 mg CC, the Temposil tablet containing 50 mg CC, and the pulverized Temposil tablet containing 50 mg CC, there was conversion to carbimide to the extent of 92%, 57% and 100% in 1.0 h, respectively. For the Temposil tablet, hydrolysis of CC to carbimide was 100% after 10 h.

The assay described involves the quantitative determination of carbimide in plasma as the HFBA derivative using GLC—ECD. A 5.0-μl aliquot of HFBA, representing a molar ratio of HFBA to carbimide of approximately 850: 1 for 1.0 μg carbimide, with acetonitrile as a catalyst has been found to provide optimal derivatization of the most concentrated carbimide samples. With these derivatization conditions, the lower limit of sensitivity of the assay is 5.0 ng/ml in plasma. Larger amounts of HFBA did not improve the sensitivity of the assay nor did HFBA produce any interfering chromatographic signals. The GLC analysis of derivatized carbimide revealed one signal with a retention time of 1.8 min (Fig. 1). There was no interference from endogenous materials in plasma with retention times similar to that of HFBA-carbimide.

Gas chromatographic—mass spectrometric analysis confirmed that this GLC signal represented an HFBA derivative of carbimide. Chemical ioniza-

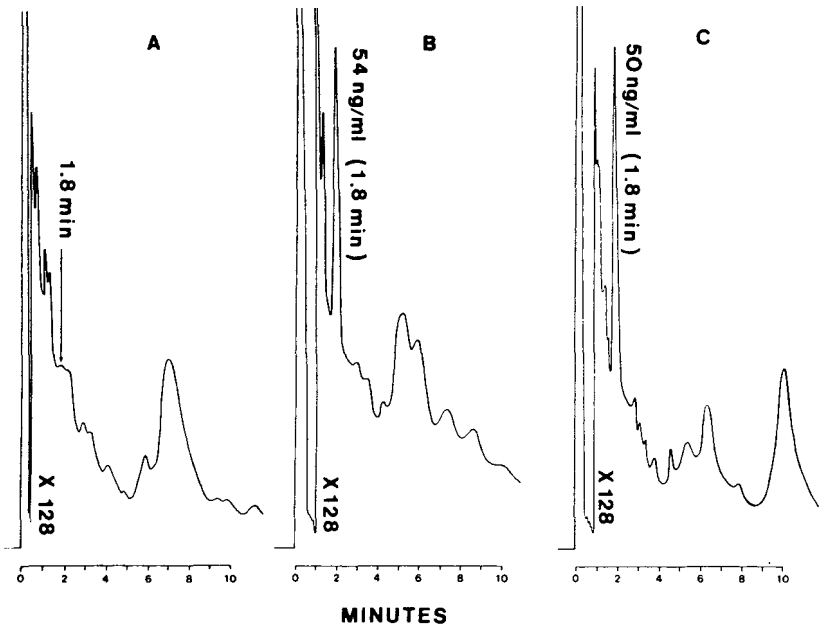

Fig. 1. Chromatograms from carbimide analysis. (A) Blank plasma sample; (B) plasma sample from a rat administered 7.0 mg/kg calcium carbimide orally, containing 54 ng/ml carbimide; (C) aqueous standard containing 50 ng/ml carbimide. The detector sensitivity setting was × 128.

tion produced a quasi-molecular (M + 1) ion at 257 a.m.u.; electron ionization produced the ions 256, 236, 208, 150 and 119 a.m.u. The mass spectral data indicated that the HFBA derivative of carbimide was unicomponent and corresponded to the structure shown in Fig. 2, in which the fragment ions are also tentatively identified. This derivative of carbimide was not expected since the formation of the heptafluorobutyramide would have been predicted. However, the presence of the cyano group on the amino function of carbimide may have compromised the basicity of the amino group due to its negative inductive effect. This could prevent the expected nucleophilic attack of the nitrogen of the amino group on the carboxyl carbon of HFBA, thereby leading to the addition of the heptafluorobutyroxy group to the carbon of the cyano function of carbimide. This carbimide derivative was not heptafluorobutyryl-substituted urea since the retention time of the HFBA-derivatized urea (1.6 min) was different from that of derivatized carbimide (1.8 min) and the molar response with ECD was less for derivatized urea (9 mm peak height per nmole) compared with derivatized carbimide (414 mm peak height per nmole).

For the extraction of carbimide from biological fluids, it was necessary to determine its pK_a value. Using a potentiometric titration procedure [11], the pK_a of the amino function of carbimide was found to be 7.4. At pH 10, the ratio of unionized to ionized carbimide should be in excess of 100:1, thereby facilitating extraction into the organic phase. However, since unionized carbimide is soluble in water and organic solvent, sodium chloride was added to saturate the aqueous phase thereby enhancing the extraction

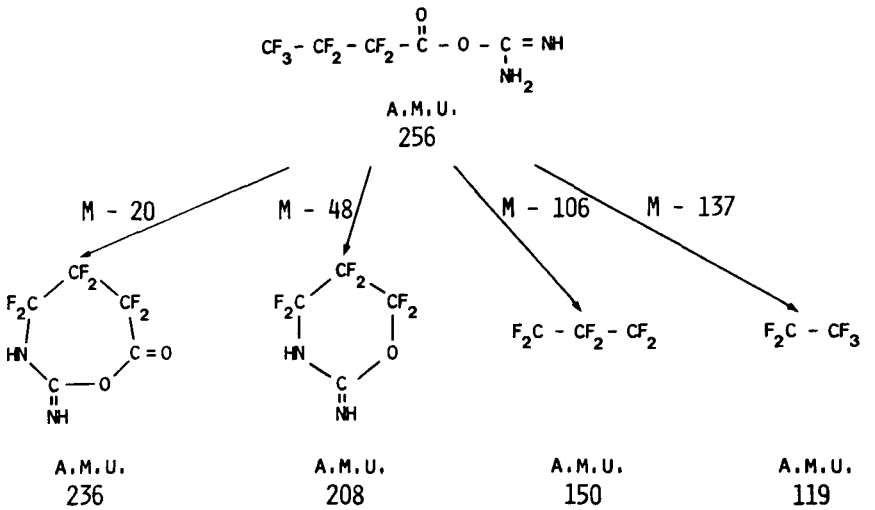

Fig. 2. Chemical structures of HFBA-derivatized carbimide [molecular ion (M) 256 a.m.u.] and fragment ions (236, 208, 150, 119 a.m.u.) as determined by electron ionization mass spectrometry.

of unionized carbimide into ethyl acetate. In order to determine the efficiency of the ethyl acetate extraction step in the assay, [^{14}C]carbimide was used. The absolute efficiency of the ethyl acetate extraction of carbimide was found to be 69.7 ± 3.42 (S.D.) % (n = 102) from plasma and 70.1 ± 4.82% (n = 102) from aqueous solution. The ratio of the efficiency of the ethyl acetate extraction of carbimide from plasma relative to that from aqueous solution is virtually unity.

The ECD response to HFBA-carbimide was linear in the concentration range 5.0—10,000 ng/ml for the GLC analysis of aqueous standards of carbimide. Aqueous standard curves were prepared daily in the range of 5.0—1000 ng/ml for plasma analysis. The data of the recovery study (Table I) indicate that the accuracy of the GLC—ECD procedure for the quantitation of carbimide in plasma, relative to aqueous standards containing known concentrations of carbimide, ranges from 90.8% (5.0 ng/ml) to 105.7% (500 ng/ml) with an overall recovery of 96.1%. The precision of the assay is reflected by the coefficient of variation which did not exceed 8.7% (Table I).

This GLC—ECD assay for carbimide does not include an internal standard. Several compounds were investigated including n-amylamine, n-octylamine, diethylamine, thiourea, β-aminopropionitrile and aminoacetonitrile. However, no compound has yet been found with appropriate chromatographic characteristics. On the basis of the S.D. of the [^{14}C]carbimide extraction efficiency data (3.42%) and the coefficient of variation data for the recovery studies (Table I), there appears to be little within-day and between-day variability in the assay. The aqueous standard curves on different days were also similar, as indicated by the slope values (e.g. range of slope: 0.151—0.168 mm/ng/ml), suggesting little between-day variability in the HFBA derivatization procedure and detector response for HFBA-carbimide.

This GLC—ECD assay has been used to investigate the time-course of plasma carbimide concentration in the rat following oral administration of 7.0 mg/kg

TABLE I

RECOVERY OF CARBIMIDE FROM PLASMA

Carbimide recovery from plasma was determined relative to aqueous standards containing known concentrations of carbimide. Recovery values were the means of five determinations at each plasma concentration.

Carbimide concentration (ng/ml)	Mean recovery (%)	Standard deviation (%)	Coefficient of variation (%)
500	105.7	6.03	5.96
100	91.7	7.97	8.69
5.0	90.8	7.82	8.61

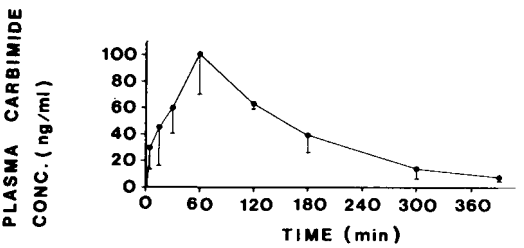

Fig. 3. Time-course of mean plasma carbimide concentration in the rat after oral administration of 7.0 mg/kg calcium carbimide. Each point represents the mean ± S.E.M. of four determinations.

CC (Fig. 3). The procedure allows quantitation of carbimide for at least 6.5 h after drug administration. The method provides a reliable, selective and sensitive procedure for the measurement of carbimide in plasma with a lower limit of sensitivity of 5.0 ng/ml and should permit the evaluation of the pharmacokinetic parameters of carbimide following CC administration to both man and experimental animals.

ACKNOWLEDGEMENTS

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REFERENCES

1 A. Wade (Editor), Martindale. The Extra Pharmacopoeia, The Pharmaceutical Press, London, 27th ed., 1977, pp. 539—540.
2 J.F. Brien, J.E. Peachey, B.J. Rogers and C.W. Loomis, Eur. J. Clin. Pharmacol., 14 (1978) 133.

3 J.F. Brien, J.E. Peachey, C.W. Loomis and B.J. Rogers, Clin. Pharmacol. Ther., 25 (1979) 454.
4 J.F. Brien, J.E. Peachey and C.W. Loomis, Clin. Pharmacol. Ther., 27 (1980) 426.
5 J.F. Brien, J.E. Peachey and C.W. Loomis, Eur. J. Clin. Pharmacol., 18 (1980) 199.
6 D.J. Hoover and J.F. Brien, Can. J. Physiol. Pharmacol., (1981) in press.
7 H. Marchner and O. Tottmar, Acta Pharmacol. Toxicol., 43 (1978) 219.
8 E.B. Truitt, Jr. and M.J. Walsh, in B. Kissin and H. Begleiter (Editors), The Biology of Alcoholism, Vol. 1, Biochemistry, Plenum Press, New York, 1971, Ch. 5, p. 161.
9 R.A. Deitrich, P.A. Troxell, W.S. Worth and V.G. Erwin, Biochem. Pharmacol., 25 (1976) 2733.
10 M. Windholtz (Editor), The Merck Index, E. Merck, Rahway, NJ, 9th ed., 1976, p. 211.
11 D.J. Pietrzyk and C.W. Frank, Analytical Chemistry, An Introduction, Academic Press, New York, 1974, p. 431.

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CHROMBIO. 750

GAS CHROMATOGRAPHIC DETERMINATION OF PHENTOLAMINE (REGITINE®) IN HUMAN PLASMA AND URINE

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SUMMARY

A method for the determination of unconjugated phentolamine at concentrations down to 5 ng/ml in human plasma, and of free and total (free plus conjugated) phentolamine down to 25 ng/ml in urine is described. After addition of 2-[N-(*p*-chlorophenyl)-N-(*m*-hydroxyphenyl)-aminomethyl]-2-imidazoline as internal standard, both compounds are extracted into benzene—ethyl acetate (1:1, v/v) at pH 10, transferred into an acidic aqueous solution and back-extracted at pH 10 into benzene—ethyl acetate. They are then derivatized with N-heptafluorobutyrylimidazole. The derivatives are determined by gas chromatography using a ^{63}Ni electron-capture detector. In urine, total (free plus conjugated) phentolamine is determined after enzymatic hydrolysis. The technique was applied for the study of the plasma concentrations and urinary elimination after oral administration to man.

INTRODUCTION

Phentolamine (Regitine®, Ciba-Geigy) (Fig. 1, I), 2-[N-(*p*-tolyl)-N-(*m*-hydroxyphenyl)-aminomethyl]-2-imidazoline is a sympathicolytic agent. Its determination in blood and urine was described by De Bros and Wolshin [1]. They used a high-performance liquid chromatographic (HPLC) method on a reversed-phase column with an ion-pairing reagent. Pfister and Imhof [2] estimated the plasma concentration of phentolamine on the basis of its inhibitory effect on adrenaline-induced platelet aggregation.

This paper describes a gas chromatographic determination of free and conjugated phentolamine in human plasma and urine, which has been in use in our laboratory since 1974.

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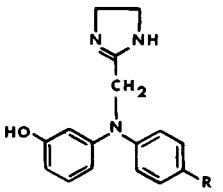

R = -CH_3 (I)
= -Cl (II)
= -COOH (III)

Fig. 1. Chemical structures of phentolamine (I), internal standard (II) and main phentolamine metabolite (III).

EXPERIMENTAL

Chemicals and reagents

Phentolamine and the internal standard (Fig. 1, II) were supplied by Ciba-Geigy (Basle, Switzerland). The solvents and reagents used are all of analytical grade: sulphuric acid (Titrisol, No. 9984, Merck, Darmstadt, G.F.R.), *n*-heptane (Merck 4366), N-heptafluorobutyrylimidazole, HFBI (Pierce, Rockford, IL, U.S.A.), benzene (No. 1043, Mallinckrodt, St. Louis, MO, U.S.A.) and ethyl acetate (Mallinckrodt 3427).

The extraction solvent is benzene—ethyl acetate (1:1, v/v). The acetate buffer, pH 5.5 is prepared with 4.8 ml of 0.2 *M* acetic acid solution and 35.2 ml of 0.2 *M* sodium acetate solution.

The pH 10 buffer (Titrisol, Merck 9890, boric acid—potassium chloride—sodium hydroxide) is prepared by diluting the contents of eight vials with water to a volume of 1000 ml.

The enzyme solution (β-glucuronidase—arylsulphatase) [Calbiochem (Los Angeles, CA, U.S.A.) B grade, 6.66 I.U./ml β-glucuronidase, 3.41 I.U./ml arylsulphatase] is diluted to one-tenth with pH 5.5 buffer.

The two methanolic solutions of internal standard contain 200 ng/ml and 500 ng/ml, respectively.

Equipment

The glassware is washed for 30 min in an ultrasonic bath, first with water and then with methanol.

A Hewlett-Packard Model 5713A gas chromatograph equipped with a Hewlett-Packard Model 18713A electron-capture detector is used. The peak areas are given by a Hewlett-Packard Model 3380A electronic integrator.

The column is operated at 220°C, the injector temperature is 250°C and the detector is set at 300°C with argon—methane (90:10) at a flow-rate of 60 ml/min. The glass column (1 m × 3 mm I.D.) is washed [3] and packed with 3% OV-17 on Chromosorb W HP (80—100 mesh) (Applied Science Labs., State College, PA, U.S.A.). The conditioning procedure was described previously [3].

Enzymatic hydrolysis in urine

Aliquots (1 ml) of urine (diluted with water if necessary), 1 ml water and

500 μl of β-glucuronidase—arylsulphatase mixture, diluted with pH 5.5 acetate buffer (1:10, v/v), are heated for 15 h at 38°C. The extraction is then performed as described below.

Extraction

One ml of the internal standard solution is measured into a stoppered glass tube and dried under a nitrogen stream in a water bath at 37°C. Then 1 ml of the sample (plasma or urine), 2 ml of pH 10 buffer and 5 ml of benzene—ethyl acetate are added. The tube is shaken mechanically (Infors shaker) for 20 min at 180 rpm and centrifuged at 4800 *g* for 10 min. An aliquot of the organic phase is transferred into another tube, and 3 ml of 0.05 *M* sulphuric acid are added. The tube is shaken mechanically for 20 min at 180 rpm and centrifuged. The organic phase is discarded, 3 ml pH 10 buffer and 5 ml benzene—ethyl acetate are added to the acidic phase. The tube is shaken mechanically for 20 min at 180 rpm and centrifuged. An aliquot of the organic phase is transferred into another tube and dried under a nitrogen stream in a water bath at 37°C.

Derivatization and chromatography

To the dry residue are added 100 μl heptane and 10 μl of N-heptafluorobutyrylimidazole. The medium is thoroughly mixed (Vortex mixer) for 30 sec and allowed to stand for 10 min at room temperature. Then, 3 ml of water and 2 ml of heptane are added and the tube is shaken for 30 sec and centrifuged. The aqueous phase is frozen by immersing the tube in a methanol bath containing dry ice. An aliquot of heptane is transferred into another tube and a 3-μl portion is injected into the chromatograph.

The phentolamine content is calculated from the peak area ratio by reference to a calibration curve prepared from a series of methanolic phentolamine solutions between 5 and 100 ng/ml (with 200 ng internal standard), and between 25 and 2000 ng/ml (with 500 ng internal standard). These ranges are used to determine unconjugated phentolamine in plasma, and free and total (free plus conjugated) phentolamine in urine, respectively.

Human study

Three healthy male subjects, who had been advised to take no drugs during the week preceding the experiment and none besides phentolamine throughout the duration of the study, received 20 mg of phentolamine as one tablet of Regitine. Blood samples were collected before and 0.25, 0.50, 0.75, 1 and 2 h after the administration of the drug and centrifuged. Plasma was removed and stored at −20°C until analysis.

Urine was collected at the following intervals: 0—1, 1—2, 2—3, 3—4, 4—5, 5—6, 6—7, 7—8, 8—9, 9—10 and 10—24 h. The volume was measured and an aliquot was stored at −20°C.

RESULTS AND DISCUSSION

Extraction

The extraction yields of [^{14}C]-phentolamine from aqueous solutions at various pH values (Fig. 2) indicate that the drug can be best recovered at pH 10 with benzene—ethyl acetate.

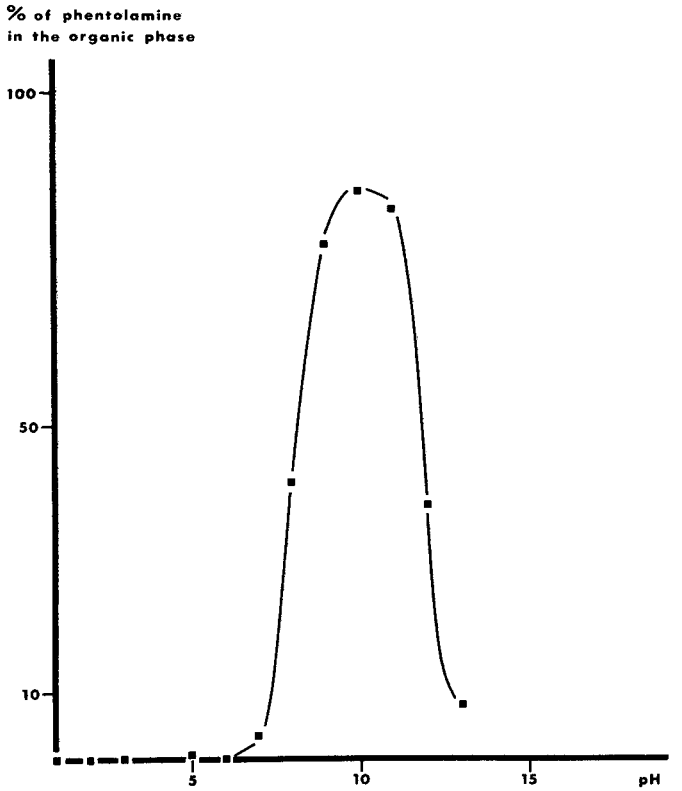

Fig. 2. Partition of phentolamine between the aqueous solution and the extraction solvent benzene—ethyl acetate (1:1, v/v) at different pH values.

The dissociation constants are: pK_a I = 9.5, pK_a II = 11.5 (in 75% methanol by titration).

Derivatization procedure

The derivative obtained with phentolamine and HFBI was heptafluorobutyryl-phentolamine, the phenolic hydroxyl being acylated. Phenolamines are very often derivatized with HFBI [4].

Precision and recovery

Tables I and II give the results obtained when the described procedure was applied to spiked human plasma and urine samples.

These tables demonstrate the good reproducibility of the assay down to concentrations of 5 ng phentolamine per ml plasma and 25 ng phentolamine per ml urine.

Plasma and urine interference

Fig. 3 shows the chromatograms of an extract of human plasma and of the same extract spiked with 100 ng of phentolamine and 200 ng of internal standard. No interference from the normal plasma components was recorded. Urine contains more detectable substances, but phentolamine and the internal stan-

TABLE I

PRECISION AND RECOVERY IN THE DETERMINATION OF UNCONJUGATED PHENTOLAMINE IN SPIKED HUMAN PLASMA

Amount added (ng/ml)	Mean amount found (ng/ml) (n = 6)	Precision (C.V. %)	Recovery (%)
5	5.7	7.5	114.7
10	9.9	6.2	99.5
25	24.6	5.8	98.4
50	50.2	3.6	100.5
100	100.5	2.6	100.5
		Mean	102.7 ± 6.7

TABLE II

PRECISION AND RECOVERY IN THE DETERMINATION OF FREE PHENTOLAMINE IN SPIKED HUMAN URINE

Amount added (ng/ml)	Mean amount found (ng/ml) (n = 6)	Precision (C.V. %)	Recovery (%)
25	23	2.9	92.0
50	50	2.6	100.0
100	99	5.8	98.8
250	264	5.7	105.4
500	522	3.2	104.4
1000	1010	1.7	101.0
2000	1880	4.4	94.0
		Mean	97.4 ± 5.0

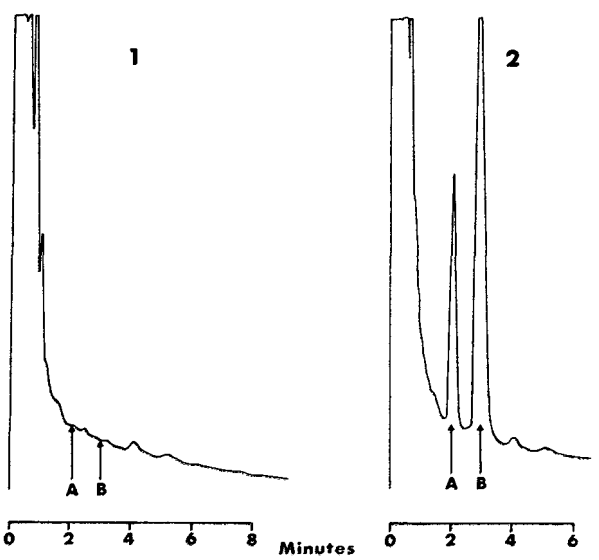

Fig. 3. Chromatograms of (1) human plasma blank (1 ml plasma); (2) same plasma spiked with 100 ng/ml of phentolamine (A), and 200 ng/ml of internal standard (B).

dard are well separated from the normal components of the urine extract. Enzymatic hydrolysis did not change the chromatogram.

Hexane could replace benzene when plasma and urine blanks are suitable.

Stability of calibration curve samples of phentolamine in plasma and urine

For routine analysis, a calibration curve in plasma and two calibration curves in urine (one for free and one for total phentolamine) are used. The calibration curve samples can be stored for up to three days in a refrigerator at +4°C, but a significant decrease in the peak area ratio of phentolamine:internal standard is observed after four days.

Specificity

The main metabolite of phentolamine, 2-[N-(*p*-carboxyphenyl)-N-(*m*-hydroxyphenyl)-aminomethyl]-2-imidazoline (Fig. 1, III), is present in plasma and urine. This compound is amphoteric (in fact it is an amino acid) and therefore difficult to extract using conventional solvent extraction. Amino acids can be extracted if ion-pair principles are used. This compound does not interfere when the described technique is applied. Two HPLC methods have been developed using an aqueous mobile phase to determine this metabolite in plasma and urine [5].

Application

The technique was applied in a study of the elimination of phentolamine after oral administration to three healthy subjects. Very low plasma levels were measured at all sampling times. Measurable concentrations of free and conjugated phentolamine were found in the urine. Fig. 4 shows the average (n = 3) curves of free and total (free plus conjugated) phentolamine urinary excretions.

On average, 1.80% of the administered dose was recovered in the 24-h urine as free phentolamine and 7.95% as total phentolamine.

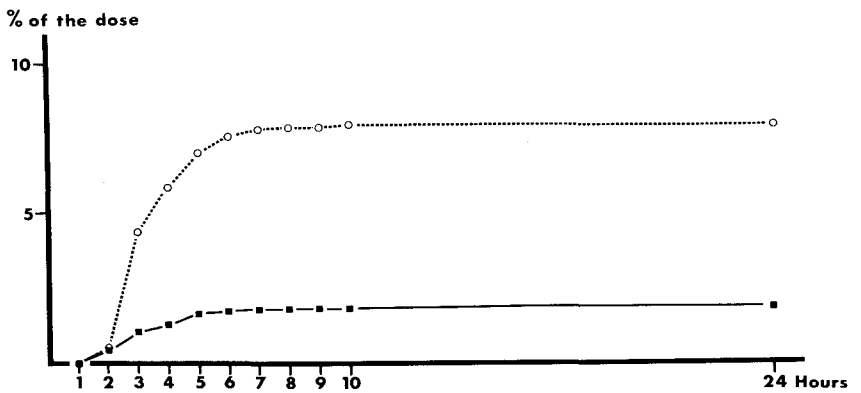

Fig. 4. Average cumulative urinary excretions in three healthy subjects after oral administration of 20 mg of phentolamine. (○), Total phentolamine; (■), free phentolamine.

REFERENCES

1 F. de Bros and E.M. Wolshin, Anal. Chem., 50 (1978) 521.
2 B. Pfister and P. Imhof, Brit. J. Clin. Pharmacol., 5 (1978) 175.
3 A. Sioufi and F. Pommier, J. Chromatogr., 183 (1980) 33.
4 S.D. Seeley and L.E. Powell, Anal. Biochem., 58 (1974) 39.
5 J. Godbillon and G. Carnis, J. Chromatogr., 222 (1981) 461.

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CHROMBIO. 754

DETERMINATION OF PRENALTEROL IN PLASMA AND IN URINE BY GAS—LIQUID CHROMATOGRAPHY

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SUMMARY

A description is given of a gas—liquid chromatographic method for the quantitative determination of unchanged prenalterol in plasma and for the total (free and conjugated) prenalterol in urine. After addition of an adequate internal standard, prenalterol together with the internal standard, is extracted with diethyl ether and derivatized with heptafluorobutyric anhydride—pyridine to form a tri-heptafluorobutyric derivative. This derivative has favourable properties for its estimation by gas—liquid chromatography using electron-capture detection. A large percentage of prenalterol is excreted as sulfate conjugate in man. Thus a hydrolysis step is added to the urine assay. The sensitivity of the method is about 2 ng/ml.

INTRODUCTION

Prenalterol, S-(−)-(4-hydroxyphenoxy)-3-isopropylamino-2-propanol hydrochloride, is a new potent β_1-stimulator [1]. Due to low oral doses, a sensitive assay procedure for the quantitative determination of prenalterol is required. The method described here is based on the gas—liquid chromatographic (GLC) assay as briefly outlined by Rönn et al. [2]. The assay procedure was optimized for high sensitivity and in addition a method for the determination of free prenalterol and prenalterol sulfate in human urine is given.

Hydrolysis of the sulfate conjugate of prenalterol, identified as the major metabolite by Hoffmann and Arfwidsson [3], may be achieved by enzymatic or chemical hydrolysis. The highly hydrophilic free prenalterol was extracted from plasma or urine with diethyl ether at alkaline pH.

Derivatization was carried out by pyridine-catalyzed acylation with hepta-

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fluorobutyric anhydride (HFBA) in diethyl ether. The resulting tri-HFB derivative has favourable properties for its GLC determination by electron-capture detection.

EXPERIMENTAL

Reagents

Prenalterol hydrochloride was obtained from Ciba-Geigy (Basle, Switzerland) and H 155/46, the internal standard from Hässle (Mölndal, Sweden). The buffer solution, pH 10.8 was made up from 2 mole Na_2CO_3 per litre and adjusted to pH 10.8 with 1 *N* hydrochloric acid. Heptafluorobutyric anhydride (HFBA), distilled, was purchased from Fluka (Buchs, Switzerland) and β-glucuronidase—arylsulfatase from Boehringer (Mannheim, G.F.R.). The column packing used was 3% OV-17 on Chromosorb W HP obtained from Supelco (Bellefonte, PA, U.S.A.).

Procedures

Unchanged prenalterol in plasma and urine. Plasma (1 ml) or urine (5—50 μl) (for calibration curves, addition of prenalterol as aqueous solution), 0.1 ml aqueous solution of internal standard (50 ng for range 2—50 ng/ml and 150 ng for range 20—200 ng/ml), 0.1 ml pH 10.8 buffer and 10 ml diethyl ether were shaken for 10 min at 200 rpm (on a mechanical horizontal shaker) and briefly centrifuged.

The organic phase and 0.5 ml 0.02 *N* sulfuric acid were shaken for 10 min at 200 rpm, briefly centrifuged and the organic phase was discarded by aspiration with a water pump. The aqueous phase, 0.1 ml pH 10.8 buffer and 10 ml diethyl ether were shaken for 10 min at 200 rpm, briefly centrifuged and then the organic phase was separated and evaporated to dryness under a stream of nitrogen at 40°C. To the dry residue were added 0.3 ml pyridine (1% solution in diethyl ether) and 0.1 ml HFBA, stoppered and left at room temperature (22°C) for 30 min. The reaction mixture was evaporated to dryness under a stream of nitrogen (ca. 20—30 min). The dry residue was dissolved in 2 ml toluene and washed with 1 ml water by shaking for 2 min followed by brief centrifugation. Aliquots of the organic phase (1—3 μl) were injected into the gas chromatograph.

Prenalterol sulfate conjugate in urine. Urine, 5—50 μl, diluted with water to 0.1 ml, and 0.1 ml 5 *N* hydrochloric acid were stoppered and heated for 90 min at 60°C in a water bath. The reaction mixture was evaporated to dryness under a stream of nitrogen at 40°C. To the dry residue were added 0.1 ml aqueous solution of internal standard (150 ng), 0.9 ml pH 10.8 buffer and 10 ml diethyl ether; the mixture was shaken for 10 minutes at 200 rpm, then the procedure as for plasma was carried out.

Gas chromatography

A Pye 204 instrument, equipped with a ^{63}Ni electron-capture detector, was used. Sample injection was carried out using an S8 Autojector.

A 2 m × 4 mm I.D. pyrex glass column, packed with 3% OV-17 on Chromosorb W HP (100—120 mesh), was used. The carrier gas was nitrogen at a flow-rate of 40 ml/min.

Temperatures were: column 195°C; injector 195°C; and detector 350°C. The retention times with these conditions were 2 min and 2.9 min for the derivatives of prenalterol and the internal standard, respectively.

RESULTS AND DISCUSSION

Extraction

The pH dependence of the extractability of prenalterol and internal standard (H 155/46) was evaluated by adjusting aqueous solutions to pH values ranging from 7—13, using dilute hydrochloric acid and sodium hydroxide. The samples were extracted with diethyl ether and derivatized with HFBA. The percentage extraction was estimated by comparison with pure HFB derivatives of both compounds. It was found that for both prenalterol and internal standard an extraction pH of 11 is optimal (Fig. 1).

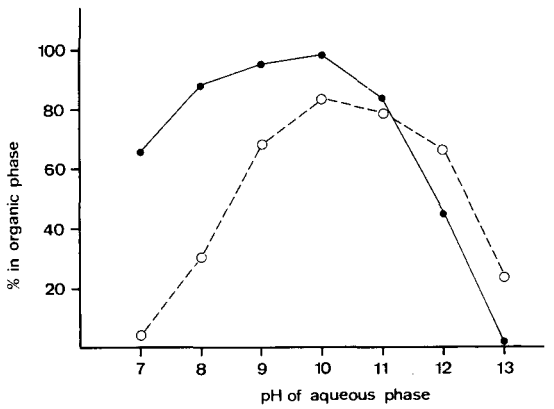
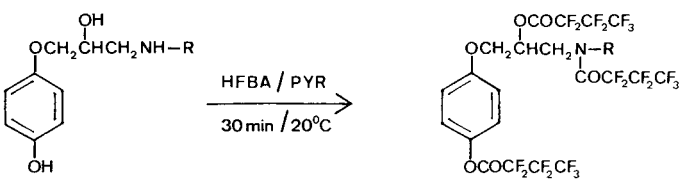


Fig. 1. Dependence of the extractability of prenalterol (○) and internal standard (●) on the pH values of the aqueous solution. Aqueous phase adjusted with 0.1 *N* hydrochloric acid or sodium hydroxide.

Derivatization

The acylation of prenalterol with HFBA results in satisfactory yields only if a catalyst (i.e. pyridine) is used (Fig. 2). After 15 min at room temperature the acylation reaction is already completed and remains unchanged for at least 3 h. There is no benefit if the reaction is carried out at a higher temperature. The



R : $CH(CH_3)_2$ = Prenalterol

R : $CH(CH_2CH_3)_2$ = int.standard (H 155/46)

Fig. 2. Structure of prenalterol, H 155/46 and their HFB derivatives.

yields of the derivatives are the same, but interfering background peaks increase dramatically. Thus an optimal reaction time of 30 min at room temperature was chosen. The structures of the HFB derivatives were verified by mass spectrometry.

Hydrolysis

For the determination of the sum of free and conjugated prenalterol in human urine, a hydrolysis step must precede the standard assay. About 50% of the prenalterol dose is excreted in the urine as a sulfate conjugate and ca. 20% as unchanged drug. Hydrolysis of the sulfate may be achieved either enzymatic-

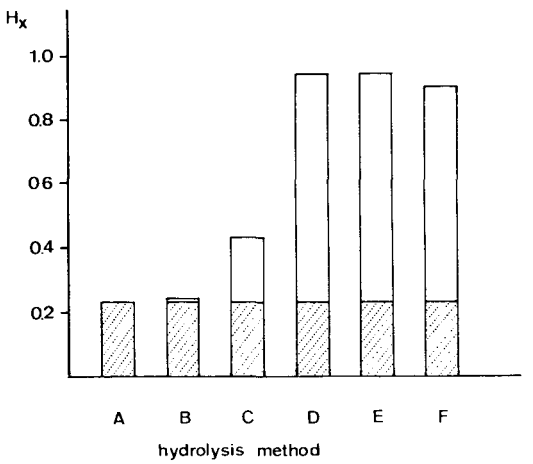


Fig. 3. Hydrolysis of the sulfate conjugate in a human urine sample (0—2 h after single oral dose of 10 mg prenalterol). (A) Free prenalterol (no hydrolysis); (B) total prenalterol after hydrolysis in 2.5 *N* hydrochloric acid for 1 h at 20°C; (C) total prenalterol after hydrolysis in 2.5 *N* hydrochloric acid for 16 h at 20°C; (D) total prenalterol after hydrolysis in 2.5 *N* hydrochloric acid for 1 h at 60°C; (E) total prenalterol after hydrolysis in 5 *N* hydrochloric acid for 1 h at 60°C; (F) total prenalterol after enzymatic hydrolysis with β-glucuronidase—arylsulfatase for 16 h at 37°C.

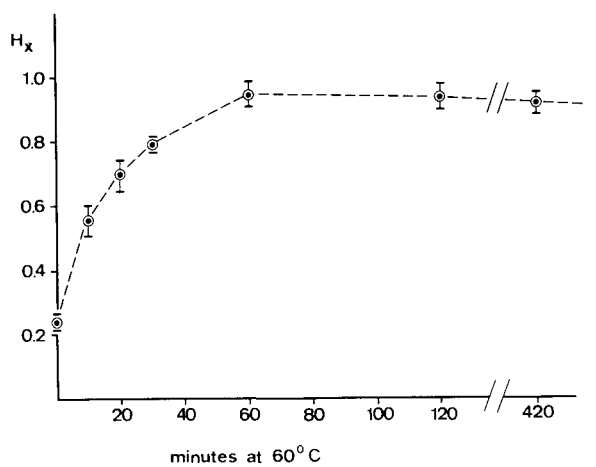


Fig. 4. Optimization of the acid hydrolysis. Human urine sample (0—2 h after a 10-mg dose of prenalterol) hydrolysed in 2.5 *N* hydrochloric acid at 60°C for up to 7 h.

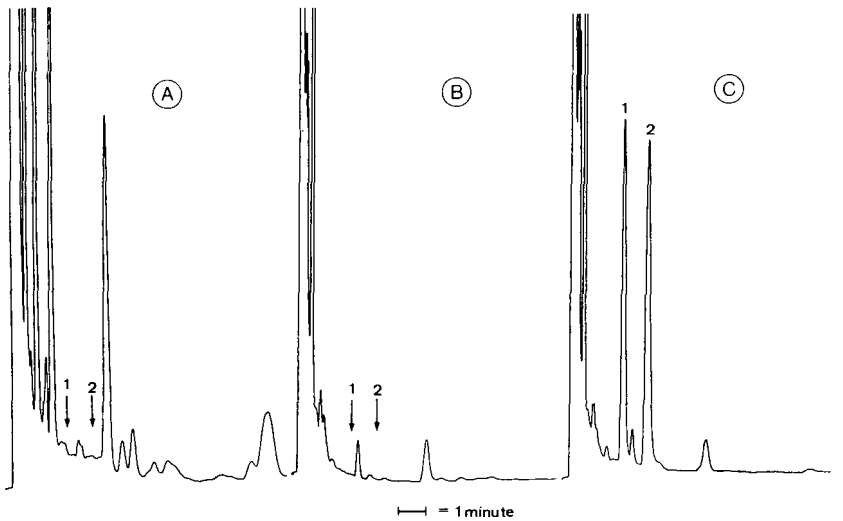


Fig. 5. Chromatograms of blank extract of 5 μl urine after (A) enzymatic hydrolysis; (B) acid hydrolysis; (C) extract of 5 μl urine from a volunteer (who had received a 10-mg dose of prenalterol), after acid hydrolysis, 150 ng internal standard added *after* hydrolysis. Peaks: 1 = prenalterol, 2 = internal standard.

ally or chemically by hydrochloric acid. A number of different hydrolysis conditions were tested using urine samples from a human bioavailability study. It was found that either the enzymatic method, using β-glucuronidase—arylsulfatase, or the chemical method, using 5 *N* hydrochloric acid, gave good results (Fig.3). Both methods were optimized and it was found that enzymatic hydrolysis was completed after 16 h at 37°C. Acid hydrolysis was completed after 1 h at 60°C (Fig.4). The two methods, compared by analysis of a human urine sample, gave the same results. However, the acid hydrolysis was the method of choice because a large number of background peaks were present in the chromatograms of the samples which had been treated with enzyme (Fig.5). Typical chromatograms of plasma extracts are shown in Fig.6.

Over-all yield

The absolute over-all yield (extraction from plasma and derivatization) was estimated to be about 55—60%. However, the use of an internal standard allows for recoveries of between 93.7 and 106.1%.

Calibration graphs

Calibration graphs for two concentration ranges in plasma were made. The low concentration range (5—50 ng/ml) is analysed with 50 ng internal standard and the higher concentration range (20—200 ng/ml) with 150 ng internal standard. The peak height ratios (H_x) as calculated by dividing the peak height of the prenalterol signal by the peak height of the internal standard signal are plotted in Fig.7.

Calibration graphs for urine are constructed with 150 ng internal standard because concentrations in urine are high. The urine graph has the same slope as the plasma graph for the higher range.

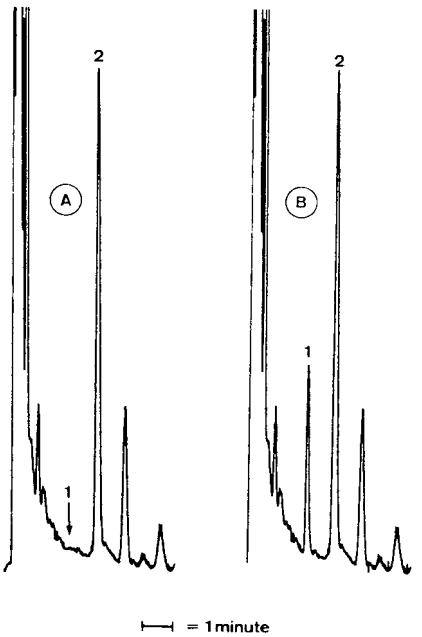


Fig. 6. Chromatograms of an extract of 1 ml plasma (A) containing 150 ng of internal standard; (B) spiked with 50 ng prenalterol and 150 ng internal standard. Peaks: 1 = prenalterol, 2 = internal standard.

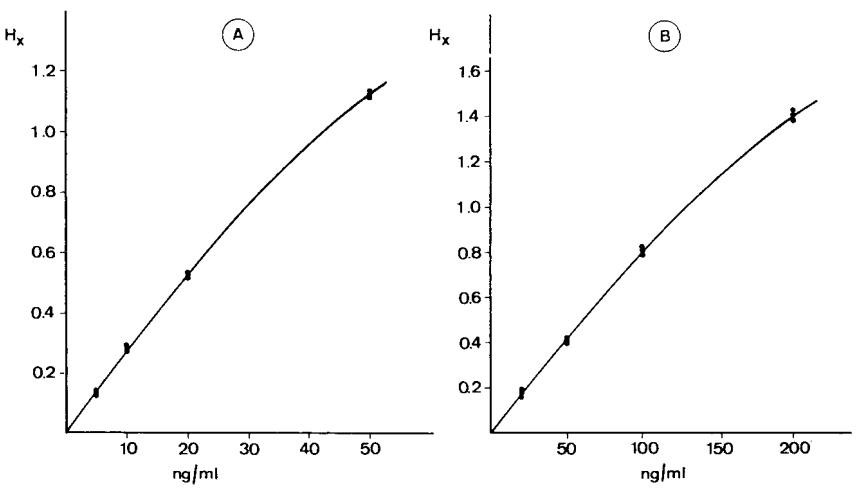


Fig. 7. Calibration graphs for the entire analytical procedure. (A) Low concentration range, with 50 ng internal standard; (B) high concentration range, with 150 ng internal standard.

Accuracy and precision

The accuracy and precision were evaluated by analysing spiked plasma and urine samples. The plasma samples contained 5.0—100 ng prenalterol per gram and the urine samples 0.175—3.735 μg prenalterol per gram.

Between ten and sixteen independent determinations were carried out with

TABLE I

TEST SAMPLES FOR METHOD VALIDATION

The spiked plasma and urine samples were analysed for prenalterol.

Initial	Found		*n*	% deviation of mean from initial
	$\overline{X}$	C.V. (%)		
Prenalterol in plasma (ng/g)				
5.0	5.0	20	16	0
45.0	44.0	5.5	16	−1.3
15.0	15.3	7.2	10	+2.2
100.0	97.0	2.8	10	−3.0
Prenalterol in urine (μg/g)				
0.175	0.175	9.1	3	0
0.555	0.575	2.6	3	+3.5
0.950	1.012	2.5	3	+6.1
2.082	1.979	1.6	3	−4.9
2.974	2.786	3.1	3	−6.3
3.735	3.571	1.1	3	−4.4

the plasma samples. The urine samples were analysed in triplicate. The coefficient of variation values ranged from 1.1 to 9.1% for all concentrations except the lowest (5 ng/g) where the coefficient of variation was 20%.

The deviation of the mean found from the given concentrations was between −6.3% and +6.1% (Table I).

Application

The applicability of these methods was tested by analysing plasma samples

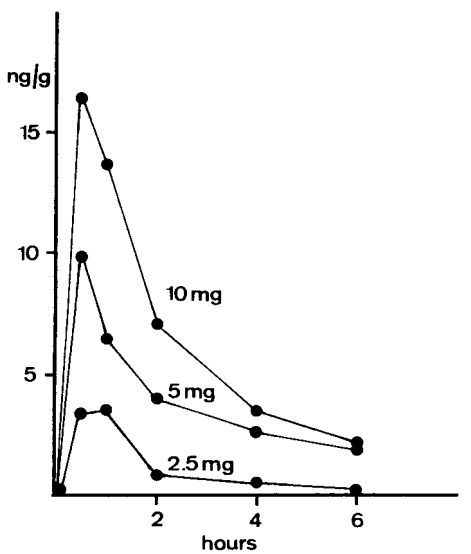


Fig. 8. Mean plasma levels of unchanged prenalterol in healthy volunteers (n = 6) following single oral doses of 2.5, 5 and 10 mg prenalterol.

from a dose—response study in healthy volunteers. Six volunteers received 2.5-, 5- and 10-mg single, oral doses of prenalterol (tablets) in a cross-over experiment. The mean plasma profiles are illustrated in Fig.8. The mean areas under the plasma curves were 6.9, 23.0 and 38.3 $ng \cdot g^{-1} \cdot h$ for the three dosages, respectively.

In a separate study, urine was collected quantitatively for 24 h following oral administration of 10 mg prenalterol (sustained release tablet) to one healthy volunteer. The free as well as the total (free and conjugated) prenalterol were measured using the described methods. The following amounts were excreted: A, free prenalterol, 2.16 mg/24 h (21.6% of the dose); B, total prenalterol, 7.47 mg/24 h (74.7% of the dose); C, (B − A) conjugated prenalterol, 5.31 mg/24 h (53.1% of the dose).

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REFERENCES

1 D.H.T. Scott, G.R. Arthur, R.N. Boyes and D.B. Scott, Brit. J. Clin. Pharmacol., 7 (1979) 365—370.
2 O. Rönn, C. Graffner, G. Johnson, L. Jordö, P. Lundberg and J. Wikstrand, Eur. J. Clin. Pharmacol., 15 (1979) 9—13.
3 K.-J. Hoffmann and A. Arfwidsson, to be published.

Journal of Chromatography, 222 (1981) 445—452
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF PENICILLAMINE IN WHOLE BLOOD, PLASMA, AND URINE

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SUMMARY

A high-performance liquid chromatographic method for the determination of penicillamine in plasma, whole blood, and urine samples is described. The method uses a commercially available electrochemical detector at a potential of +0.1 V versus the Ag/AgCl reference electrode. This method is selective and sensitive for sulfhydryl compounds. The chromatography separates penicillamine from other endogenous sulfhydryl compounds with a limit of detection for penicillamine in biological samples of ca. 10^{-7} *M*.

INTRODUCTION

D-Penicillamine has been used as a medicinal agent since 1954 when it was first used for the treatment of hepatolenticular degeneration [1]. More recently it has also been used in the treatment of cystinuria [2] and rheumatoid arthritis [3]. A recent review of penicillamine [4] lists many other possible uses for this compound.

Since penicillamine has been shown to be effective for the treatment of rheumatoid arthritis [5], a great interest in research on this therapeutic agent has been stimulated due to the high incidence and debilitating nature of this diease. However, accurate pharmacokinetic studies of penicillamine and other areas of penicillamine research have been restrained due to the lack of a sufficiently sensitive and specific assay for penicillamine. Most of the pharmacokinetic studies done in the past have employed radiolabeled penicillamine

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[6—11]. The results of these studies are inappropriate for the description of penicillamine pharmacokinetics since the results represent both metabolized and parent drug.

A number of analytical methods including colorimetry [12, 13], gas chromatography [14] and radioimmunoassay [15, 16], have been developed for penicillamine. These methods either require complex sample manipulation during which penicillamine degradation may occur or are not specific for penicillamine. Thus, these methods are inappropriate for pharmacokinetic sample analysis.

The recent development of a high-performance liquid chromatographic (HPLC) method for the determination of penicillamine [17, 18] using electrochemical detection has provided a simple, specific, and sensitive assay for the investigation of penicillamine. We have modified this HPLC method and are using it in our laboratory for the analysis of penicillamine pharmacokinetic samples. The details of the modified method are reported in this paper.

MATERIALS AND METHODS

HPLC apparatus

Cationic ion-exchange chromatography was used to separate penicillamine from the other electroactive components in the analytical samples. Two columns (5 × 0.41 cm and 30 × 0.41 cm) packed with Zipax SCX (strong cation-exchange, silica microbead coated, spherical glass beads, 30 μm; DuPont; Wilmington, DE, U.S.A.) were used as guard and analytical column, respectively. A citric acid—dibasic sodium phosphate buffer (0.03 *M* and 0.01 *M*, respectively) was pumped through the columns using a Milton Roy Model 396 minipump (Riviera Beach, FL, U.S.A.) at a flow-rate of 2.5 ml/min. Prior to use, the citric acid—phosphate buffer was deoxygenated by vigorously bubbling nitrogen through the buffer solution for 20 min. Then during use, nitrogen was continuously passed over the top of the buffer solution and the PTFE tubing leading from the buffer reservoir to the pump. Deoxygenation of the buffer was necessary to prevent excessively high background currents caused by the presence of dissolved oxygen. The analytical samples and standards of penicillamine were injected onto the column using a 20-μl loop valve (Model 7010; Rheodyne, Berkeley, CA, U.S.A.).

The electrochemical detector used for this analysis was the LC-4 Amperometric Controller (Bioanalytical Systems, West Lafayette, IN, U.S.A.). The detector used was a thin-layer electrochemical cell (Model TL-6A; Bioanalytical Systems) which consists of a mercury/gold amalgam working electrode, a glassy carbon auxiliary electrode, and a Ag/AgCl reference electrode. A Faraday cage was used to enclose the electrochemical cell and waste reservoir. The potential of the working electrode was maintained at +0.1 V versus the Ag/AgCl reference electrode. The background current was generally less than ca. 10 nA.

Sample preparation

Because of the rapid loss of penicillamine in plasma [19] and urine [17], the biological samples must be treated immediately upon collection to stabilize the amount of penicillamine present in the sample. This is accomplished by

decreasing the pH and/or precipitating the proteins in the samples.

For the treatment of plasma samples, 0.2 ml of trichloroacetic acid (20%, w/v) was added per ml of plasma. The precipitated samples were centrifuged and the supernatants were decanted. Whole blood samples were first hemolyzed by the addition of an EDTA solution (1 g/l) (1 ml per ml of whole blood sample) and then a metaphosphoric acid solution (500 g/l) was added to decrease the pH and precipitate the proteins. The urine samples were diluted (1:5 or 1:10) using a 0.4 *M* citric acid solution to buffer the pH to acidic conditions.

The resulting analytical samples from the above procedures should be refrigerated (1—4°C) until analyzed and the assays should be completed as soon as possible (1—4 days) after the collection of the samples to avoid a change in the concentration of penicillamine. The diluted urine samples, and the plasma and whole blood supernatants are injected directly into the HPLC system for analysis without further treatment.

Assay standards and procedures

Analytical standards of penicillamine were prepared at nine concentrations (75, 30, 15, 7.5, 3.0, 1.5, 0.75, 0.30, and 0.15 μg/ml) to be used in the assay procedure. The concentrations of these nine standards were chosen so that each standard will produce approximately a 3/4 full scale response when injected into the HPLC system with the sensitivity setting of the electrochemical detector set at one of the nine possible sensitivity settings used (500, 200, 100, 50, 20, 5, 2, and 1 nA/V). To make these standards, an accurate quantity of D-penicillamine (99%+ purity; Aldrich, Milwaukee, WI, U.S.A.) was weighed out (Cahn microbalance, Paramount, CA, U.S.A.) and was dissolved in an EDTA (1 g/l) solution to produce the most concentrated standard. From this concentrated standard all other standards were prepared by appropriate dilution using the EDTA (1 g/l) solution. The standards were filled into glass ampoules, purged with nitrogen, and flame sealed and refrigerated (1—4°C) until used. After opening the glass-sealed standards for use, the standards were stored in air-tight test-tubes, used for one week, and then discarded. Excellent stability for penicillamine solutions prepared and stored as above has been reported by others [17, 20]. Our results [19] and experience also confirm that no significant change in the concentrations of the standards occurred over a storage period of 3—6 months.

A 20-μl aliquot of each unknown analytical sample is injected via the loop valve into the HPLC system followed immediately by a 20-μl aliquot of a penicillamine standard. After the unknown analytical sample is injected and a suitable response is recorded with the electrochemical detector set at one of the nine sensitivity settings, the corresponding standards for that sensitivity setting are injected. The concentration of the unknown analytical sample is determined by multiplying the ratio of the observed peak heights (unknown sample:standard) times the concentration of the standard used. The concentration of the unknown analytical sample must then be corrected for dilution caused by the sample preparation procedure to give an estimate of the concentration of the actual biological sample. To minimize errors, the ratio of the peak heights of the unknown analytical sample to that of the penicillamine standard should be close to unity.

RESULTS

The chromatography of penicillamine and other similar sulfhydryl compounds using Zipax SCX has been well described by Rabenstein and coworkers [17, 18, 21—24]. As shown by these workers, the retention of penicillamine can be increased or decreased by decreasing or increasing, respectively, the pH of the eluting buffer. Table I shows the different compositions of citric acid—phosphate buffer which may be used for the chromatography of penicillamine. The approximate pH of the buffer and the relative effect of the buffers on the retention times of penicillamine are also indicated. Considerable differences between individual columns have been noted during our experience. Maintaining a constant flow-rate, it was necessary to use buffers 5 and 7 to achieve comparable retention times on two different columns.

Fig. 1 shows chromatograms of the separation of penicillamine in whole blood, plasma and urine samples. Each unknown sample that is injected into the HPLC system is followed by an injection of a penicillamine standard. Fig. 2 shows a chromatogram of penicillamine in an EDTA solution. This chromatogram demonstrates the potential sensitivity of this method as the peak shown represents 10^{-9} *M* penicillamine.

The recoveries of penicillamine added to plasma and urine samples of a normal volunteer are shown in Table II. Each of the different concentrations studied were done in triplicate. The theoretical concentrations of penicillamine were based upon the amount of penicillamine added in a concentrated solution form to the normal human plasma or diluted urine and the total volume of the biological sample after this addition. The volume of concentrated penicillamine solution was always less than 3.5% of the total sample volume.

For the recovery studies, the urine was diluted 1:5 with 0.4 *M* citric acid solution prior to the addition of the concentrated penicillamine solution. The plasma samples were treated immediately after the addition of the concen-

TABLE I

RESULTING pH AND RELATIVE PENCILLAMINE RETENTION OF CITRIC ACID—SODIUM DIBASIC PHOSPHATE BUFFERS OF VARIOUS COMPOSITION

Buffer No.	Buffer Composition (*M*)		pH	Relative* penicillamine retention
	Citric acid	Na_2HPO_3		
1	0.0400	0.0000	2.66	
2	0.0375	0.0025	2.72	
3	0.0350	0.0050	2.84	
4	0.0325	0.0075	2.95	
5	0.0300	0.0100	3.09	
6	0.0275	0.0125	3.25	decrease
7	0.0250	0.0150	3.37	
8	0.0225	0.0175	3.58	
9	0.0200	0.0200	3.87	↓

*The relative retention of penicillamine when penicillamine is chromatographed using the indicated buffer system and a Zipax SCX column.

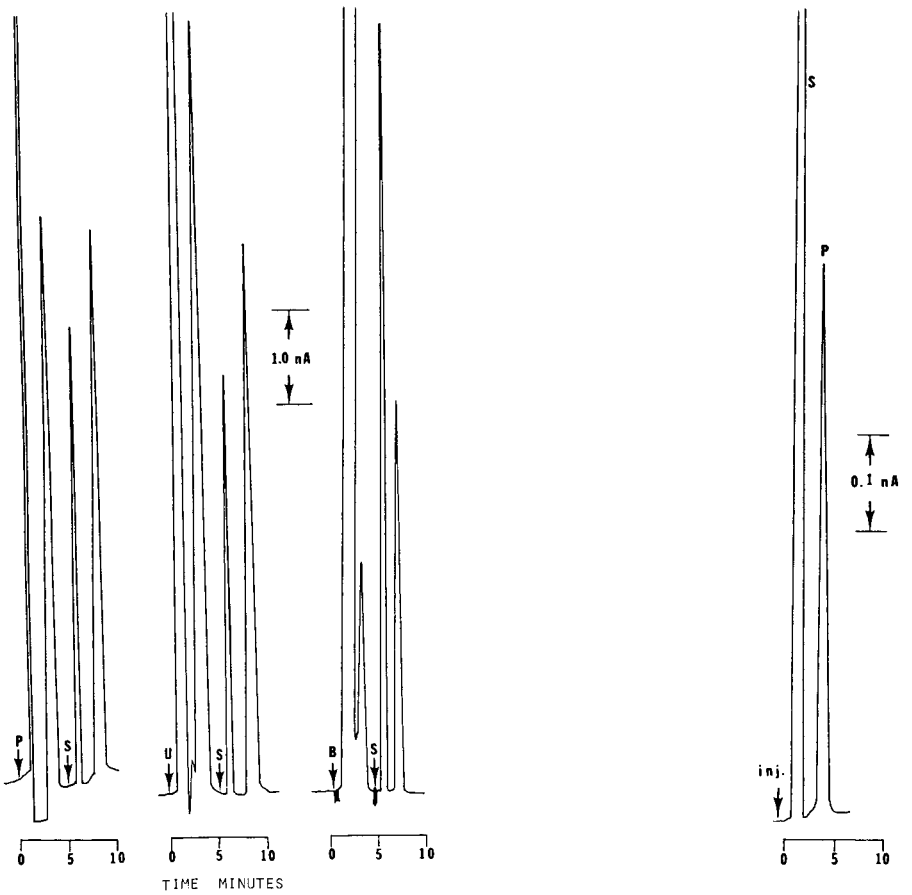

Fig. 1. Separation of penicillamine in whole blood, plasma, and urine samples. Injection points: P = plasma; U = urine; B = whole blood and S = penicillamine standard.

Fig. 2. A chromatogram of 10^{-9} *M* penicillamine in a solution of EDTA (1 g/l). S = solvent front, P = penicillamine peak, and inj. = sample injection.

trated penicillamine solution by the procedures outlined in Materials and methods section. The poor recovery of penicillamine from the plasma samples at the two lowest concentrations is due to the initial rate of loss of penicillamine as observed in the results of our recent publication [19]. Thus for statistical analysis these data were omitted.

The loss of penicillamine associated with the presence of plasma proteins probably also occurs in vivo. Therefore at the time of sampling the reaction rate will be much less than the initial rate observed in vitro as is the case in these recovery studies. Our preliminary results indicate that very little change in the penicillamine concentration occurs if the samples are treated immediately upon collection by the procedures outlined in Materials and methods section.

TABLE II

RECOVERY OF PENICILLAMINE FROM PLASMA AND URINE

Theoretical Conc. (μg/ml)	Normal plasma		Normal urine (diluted)	
	Mean percent recovered*	C.V.** (%)	Mean percent recovered*	C.V.** (%)
72.4	100	5.25	108	2.23
36.8	103	2.25	107	2.69
18.6	107	3.19	112	1.60
7.24	99.4	2.96	108	1.21
3.68	101	2.20	110	1.93
1.86	102	5.94	113	2.28
0.724	94.8	4.32	106	1.37
0.368	82.5***	2.55	98.9	2.14
0.186	77.4***	7.30	103	0.70
Grand§	101.0	3.68	107.0	4.25

*The mean recovery from three samples for each concentration using individually prepared plasma or diluted urine specimens and reported as a percentage of the theoretical concentration.

**The coefficient of variation of the three observed samples.

***These data were omitted from the grand mean because of a significant effect of the initial rate of loss of penicillamine (see text).

§ The grand mean and coefficient of variation of all the observed samples.

DISCUSSION

The HPLC method described in this paper utilized commercially available equipment unlike the previously published method [17] which required a homemade thin-layer electrochemical cell. Our method differs also in that a pre-established calibration curve for pencillamine is not necessary; rather the unknowns are quantitated by injecting a standard of penicillamine of similar concentration immediately after the unknown. By a comparison of the peak heights of the unknown and standard, the concentration of the unknown sample can be calculated.

The above method for quantitation of the unknown samples was used instead of the usual internal standard technique because a suitable internal standard for penicillamine could not be found. Due to the specificity of the detector, the choice of an internal standard is primarily limited to other sulfhydryl compounds, many of which are endogenous.

However, many of the classical reasons for using an internal standard were not necessary for this assay. The sample preparation does not require an extraction procedure and only a dilution of the actual biological sample is necessary. The samples are injected onto the HPLC column using a precise loop valve which reduces the injection volume error to a level below that of the error of the overall assay [25]. Thus for this assay, an internal standard is not necessary to quantitate the efficiency of an extraction process or the amount of sample injected.

The use of the standard injection after the sample vastly reduced the error in the estimation of the amount of penicillamine in an unknown sample because the response of the detector was not always consistent. Due to a deterioration of the surface of the mercury/gold amalgam working electrode, the response of the electrode changes over a period of time and the electrode must be resurfaced frequently. The frequency of resurfacing the working electrode's surface is dependent upon the types of samples analyzed and the conditions used. A newly resurfaced electrode may operate efficiently for only several minutes or for several days. The surface of the electrode should be resurfaced at a minimum of once every five days, however, following continual usage to ensure an adequate response. During the course of a particular HPLC run, the response of the electrode is consistent for a period of time, however, the response slowly changes with continued use. Thus, comparing the results of an unknown sample to a calibration curve made earlier in the same run or during another run would lead to erroneous results. But a comparison of the sample to a standard injected at the same time leads to a minimal error resulting from the changes in the response of the detector.

The assay reported in this paper is specific for reduced penicillamine. The metabolites of penicillamine as shown in Fig. 3 include penicillamine disulfide, penicillamine cysteine disulfide, and S-methyl-pencillamine [26]. These metabolites are not detected in this assay because of the specificity of the electrochemical detector. At the conditions employed by the detector, only reduced sulfhydryl compounds will be detected. The endogenous sulfhydryl compounds, cysteine, glutathione, homocysteine, and ergothionine, are separated from penicillamine by chromatography, which has been described by other investigators [17, 21—24].

Plasma and urine samples from five normal volunteers and five rheumatoid arthritis patients were injected into the HPLC system before and after the addition of exogenous penicillamine to check for any endogenous peaks that would interfere with the peak for pencillamine. The rheumatoid arthritis patients were not taking penicillamine but were using other medications including aurothioglucose, aurothiomalate, aspirin, prednisone, sulindac, hydroxychloroquine sulfate, aminophylline, iron, and multiple vitamin products. None of the samples from the volunteers or rheumatoid arthritis

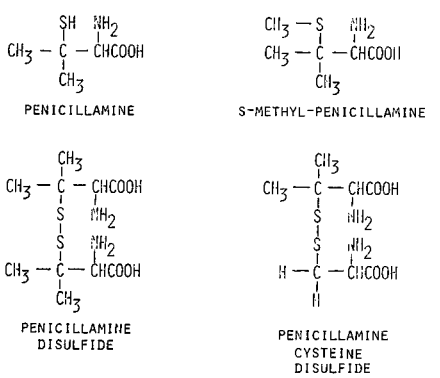

Fig. 3. Structure of penicillamine and related compounds.

patients displayed any peaks that would interfere with the analysis of penicillamine.

The HPLC method reported in this paper is suitable for the determination of unchanged penicillamine in biological samples for pharmacokinetic analysis or other research applications. The results of the application of this method to the samples of a pharmacokinetic study of penicillamine in animals and man in the authors' laboratory are being prepared for publication. The limits of detection of penicillamine in biological samples are $5 \cdot 10^{-7}$ *M* for plasma and undiluted urine samples and $3 \cdot 10^{-6}$ *M* for whole blood samples due to the additional dilution of whole blood samples during processing.

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REFERENCES

1 J.M. Walshe, Amer. J. Med., 21 (1956) 487—495.
2 J.C. Crawhall, E.F. Scowen and R.W.E. Watts, Brit. Med. J., 1 (1963) 588—590.
3 I.J. Jaffe, Arthritis Rheum., 8 (1965) 1064—1079.
4 W.H. Lyle, Clinics Rheum. Dis., 5 (1979) 569—601.
5 Multicentre Trial Group, Lancet, i (1973) 275.
6 F. Planas-Bohne, Arzneim.-Forsch., 22 (1972) 1426—1433.
7 E. Polig and F. Planas-Bohne, Biophysik, 10 (1973) 321—336.
8 A. Ruiz-Torres, Arzneim.-Forsch., 24 (1974) 914—917.
9 A. Ruiz-Torres, Arzneim.-Forsch., 24 (1974) 1043—1046.
10 A. Ruiz-Torres and I. Kürten, Arzneim.-Forsch., 24 (1974) 1258—1261.
11 K. Patzschke and L.A. Wegner, Arzneim.-Forsch., 27 (1977) 1152—1158.
12 P.R. Pal, J. Biol. Chem., 234 (1959) 618—619.
13 P. Vermeit, Pharm. Weekbl., 112 (1977) 130—132.
14 E. Jelleum, V.A. Bacon, W. Patton, W. Pereira, Jr. and B. Halpern, Anal. Biochem., 31 (1969) 339—347.
15 E.S.K. Assem, Curr. Med. Res. Opin., 2 (1974) 568—572.
16 E.S.K. Assem and M.R. Vickers, Post-Grad. Med. J., 50 (1974) 10—14.
17 R. Saetre and D.L. Rabenstein, Anal. Chem., 50 (1978) 276—280.
18 A.S.Russell, R. Saetre, P. Davis and D.L. Rabenstein, J. Rheum., 6 (1979) 15—19.
19 R.F. Bergstrom, D.R. Kay and J.G. Wagner, Life Sci., 27 (1980) 189—198.
20 J.R.B.J. Browers, P. Vermeit and J. Wermer, Pharm. Weekbl., 112 (1977) 121—129.
21 D.L. Rabenstein and R. Saetre, Anal. Chem., 49 (1977) 1036—1039.
22 R. Saetre and D.L. Rabenstein, Anal. Biochem., 90 (1978) 684—692.
23 R. Saetre and D.L. Rabenstein, Agr. Food Chem., 26 (1978) 982—983.
24 D.L. Rabenstein and R. Saetre, Clin. Chem., 24 (1978) 1140—1143.
25 Technical Notes 1, Rheodyne, Berkeley, CA, September 1979.
26 D. Perrett, W. Sneddon and A.D. Stephens, Biochem. Pharm., 25 (1976) 259—264.

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Biomedical Applications
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CHROMBIO. 742

QUANTITATIVE ANALYSIS OF MELPHALAN AND ITS MAJOR HYDROLYSATE IN PATIENTS AND ANIMALS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

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SUMMARY

The detection of 4-bis-(2-hydroxyethyl)amino-1-phenylalanine (L-DOH) in blood samples taken from patients after treatment with melphalan [4-bis-(2-chloroethyl)amino-1-phenylalanine, L-PAM] suggests that the quantification of this major hydrolysate of L-PAM can be of considerable importance in L-PAM chemotherapy. A reversed-phase high-performance liquid chromatographic procedure has been developed for the quantitative analysis of both L-PAM and L-DOH in biological samples, with a detection sensitivity of 0.1 ppm. This method provides a distinct separation of L-PAM (retention time 12 min) and L-DOH (retention time 6.5 min), with no interference from the biological background (retention time 1.4—3 min).

INTRODUCTION

Melphalan [4-bis-(2-chloroethyl)amino-1-phenylalanine, L-PAM] is an antineoplastic alkylating agent used in the clinical treatment of multiple myeloma, ovarian carcinoma, and breast cancer [2—6]. Recent reports [7—12] indicate that the phenomenon of L-PAM degradation has been studied in whole blood, blood plasma and serum, in bile, in distilled water, and in buffer solutions at various pH values. Because of the rapid degradation of L-PAM, noted in most of these reports, the development of a technique for the distinct separation and quantification of its major hydrolysate, 4-bis-(2-hydroxyethyl)amino-1-phenylalanine (L-DOH), assumes a corresponding importance. While the measurement of L-PAM itself in various media has been achieved by gas—liquid chromatographic (GLC) [13] and high-performance liquid chromatographic (HPLC)

*A preliminary report has been presented at the FASEB meeting, Dallas, TX, 1979 [1].

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methods [8, 10, 12] in the analysis of biological fluid samples, the quantification of L-DOH has been largely neglected, in part as a consequence of difficulties in analytical procedures. The GLC procedure of Goras et al. [13] requires the time-consuming and laborious derivatization of L-PAM and its hydrolytic products, and in the HPLC analyses [8, 10, 12] the biological background (t_R = 1.4—3 min) interferes with L-DOH determination (t_R = 2 min). The role of L-DOH in the mechanism by which L-PAM exerts its antineoplastic effect is not known. In order to verify such a role a quantitative HPLC analysis of both L-PAM and L-DOH in biological samples has been developed and is described in this report.

MATERIALS AND METHODS

Reagents

Melphalan (L-PAM), pharmaceutical grade, was obtained from Burroughs Wellcome (Research Triangle Park, NC, U.S.A.) through the courtesy of Dr. W.D. Brinkman. Dihydroxylated melphalan derivative (L-DOH) was synthesized according to the procedure of Furner et al. [12]. Acetonitrile, HPLC grade, was obtained from Fisher Scientific (Fairlawn, NJ, U.S.A.) and was used as received. High-purity glacial acetic acid (Fisher Scientific) was diluted to 0.0175 *M* with double-distilled water. Other chemicals were of the highest purity available and were obtained from various commercial sources.

Instruments

A Perkin-Elmer Model 601 high-pressure liquid chromatograph (Perkin-Elmer, Norwalk, CT, U.S.A.), equipped with two pumps and a gradient programmer, delivered the eluting solvents at a constant rate to a high-capacity reversed-phase column (10 μm HCODS/SIL X-C_{18} (Perkin-Elmer), 25 × 0.26 cm I.D.) with a variable-wavelength ultraviolet detector (Perkin-Elmer Model LC 55) attached to its outlet. The detector was interfaced with a laboratory data processing computer (Hewlett-Packard 3352-C) for instantaneous on-line quantification of the chromatographic data. Output signals were also recorded by a strip chart recorder (Perkin-Elmer Model 023).

Analytical procedures

Standard L-PAM solution. The solution of L-PAM (5 μg/ml) was prepared in 5% trichloroacetic acid to simulate the solution in which the drug was extracted from biological samples. To minimize L-PAM hydrolysis, the solution was prepared immediately before use. A 10-μl aliquot of the solution was injected on to the column maintained at 50°C and solvent programming was begun at once with a flow-rate of 1.5 ml/min and chart speed of 0.5 cm/min. Within 14 min the composition of the solvent, initially 12% acetonitrile and 88% 0.0175 *M* acetic acid, was converted by a concave gradient programmer to 80% acetonitrile and 20% 0.0175 *M* acetic acid. Detector sensitivity was varied from 0.002 to 0.2 absorbance units full scale. L-PAM and its hydrolysate L-DOH were detected at 263 nm. This procedure allowed reproducible measurement of L-PAM levels as low as 0.1 ppm.

Standard curve of L-PAM and L-DOH in human blood. Aliquots of L-PAM

or L-DOH solution (1 mg/ml) were added to human blood, obtained from the blood blank, to produce concentrations ranging from 0.1 to 1.0 μg/ml of blood. Each blood sample was centrifuged and the plasma collected. A 1-ml aliquot of plasma was treated with 0.5 ml of 5% trichloroacetic acid solution, vortexed and centrifuged. The supernatant was filtered through a Pillicon molecular filter (0.45 μm, Millipore). A 10-μl aliquot of the clear filtrate from each concentration was injected on to the column and standard curves obtained by plotting absorbance against concentration (μg/ml) as shown in Fig. 2 (see under Results).

Clinical application of the analytical technique. Blood samples from patients receiving L-PAM chemotherapy were obtained under the supervision of John Costanzi, Director of the Oncology Division, University of Texas Medical Branch at Galveston. In L-PAM chemotherapy [Southwest Oncology Group (SWOG) protocols], patients receive the drug orally for five days (5 mg/m^2 per day), then rest for three weeks before medication is administered again. Day 0 is the last day of the resting period before a new cycle of medication; day 6 is 24 h after the last dose of the drug. The blood of patients at days 0 and 6 of a treatment cycle was analyzed.

All blood samples were prepared for chromatographic analysis by the same procedures used to prepare samples for determination of the L-PAM standard curve as previously described.

Time course of L-PAM and L-DOH in rats. Male Sprague-Dawley rats (Charles River, Wilmington, MA, U.S.A.), ranging in weight from 190 to 200 g, were used. The animals were fed standard lab chow and tap water ad libitum. A fresh stock solution of L-PAM (4 mg/ml) was prepared in 0.12 *N* HCl. The dose of L-PAM (20 mg/kg) was administered orally through an esophageal tube. Groups of three rats were killed by decapitation to collect blood samples at intervals of 0, 0.5, 1, 1.5, 2, 4, 8, 12, 24, 48, and 72 h. The samples were prepared for analysis by the procedures previously described.

RESULTS

Fig. 1 illustrates the HPLC analysis of L-PAM (5 μg/ml) in 5% trichloroacetic acid (A) and of L-PAM plus L-DOH "spiked" in human blood samples (B). The separation of L-PAM from L-DOH is distinct, allowing individual quantification. The peak corresponding to L-DOH (t_R = 6.5 min) is separated both from the L-PAM peak (t_R = 12 min) and from background interference caused by the biological molecules (t_R = 1.4—3 min).

The standard curves for L-PAM and L-DOH in human blood (Fig. 2) demonstrate the sensitivity and reproducibility of the new method. Each point on the graph represents the mean of at least three separate analyses, and linearity is evident for both compounds from 0.1 to 1.0 μg/ml (10 μl injection volume); linearity actually persisted to levels as high as 20 μg/ml (not shown in the graphs). None of the amino acids such as phenylalanine, tyrosine, or dihydroxyphenylalanine (DOPA) was found to interfere with the L-PAM or L-DOH assay. This technique allows quantification with a high degree of accuracy: in ten separate analyses of a standard concentration of L-PAM or L-DOH (1 μg/ml), for instance, the range of difference in area under the L-PAM or L-DOH peak is only ± 10% of the mean.

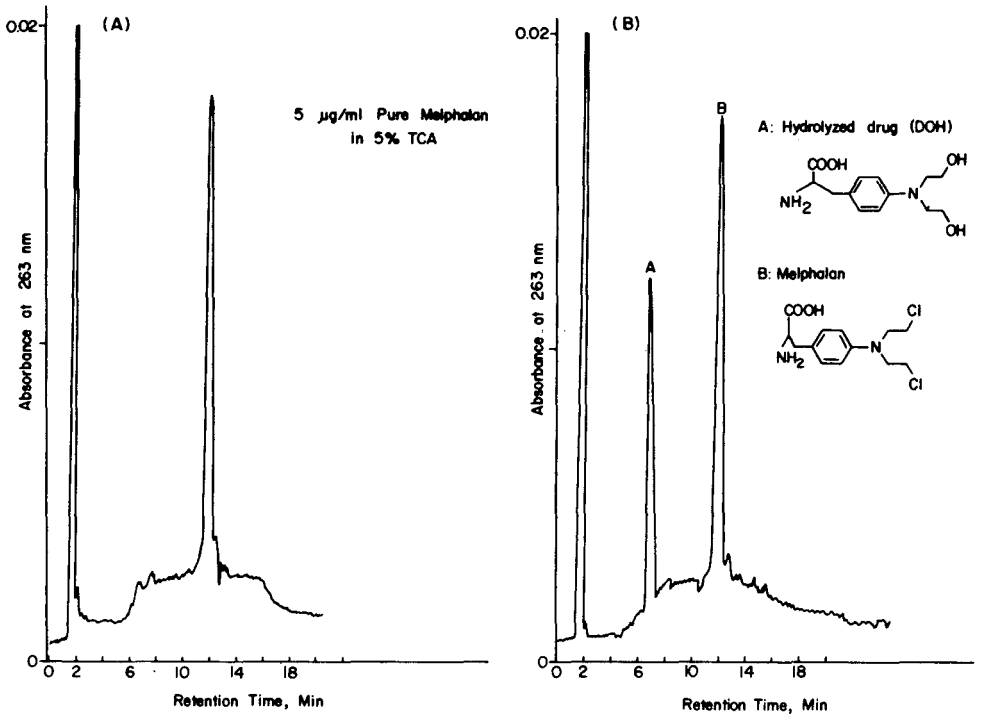

Fig. 1. HPLC analysis, using UV detection, of L-PAM and L-DOH "spiked" in human blood serum. Blood samples were extracted with 5% trichloroacetic acid (TCA) and 10 μl of the extract were injected on to the chromatograph (see Materials and methods). (A) L-PAM; (B) L-PAM plus L-DOH.

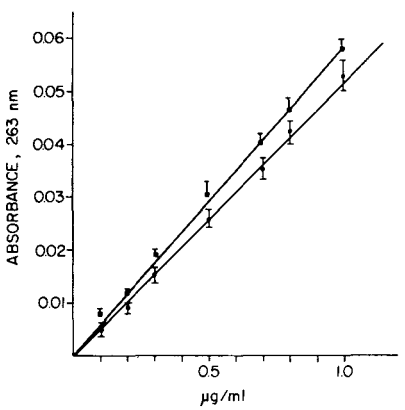

Fig. 2. Standard curves for L-PAM (•) and L-DOH (▪) in human blood samples. Points are the mean of three determinations ± S.D. (100-μl aliquots were injected on to the HPLC column at 0.002 a.u.f.s.).

Fig. 3 represents three chromatograms of human blood by HPLC analysis. The first chromatogram (Fig. 3A) is of normal blood supplied by a blood bank. The second and third (Fig. 3B and C) are typical chromatograms of blood samples from patients, at days 0 and 6, respectively, undergoing treatment un-

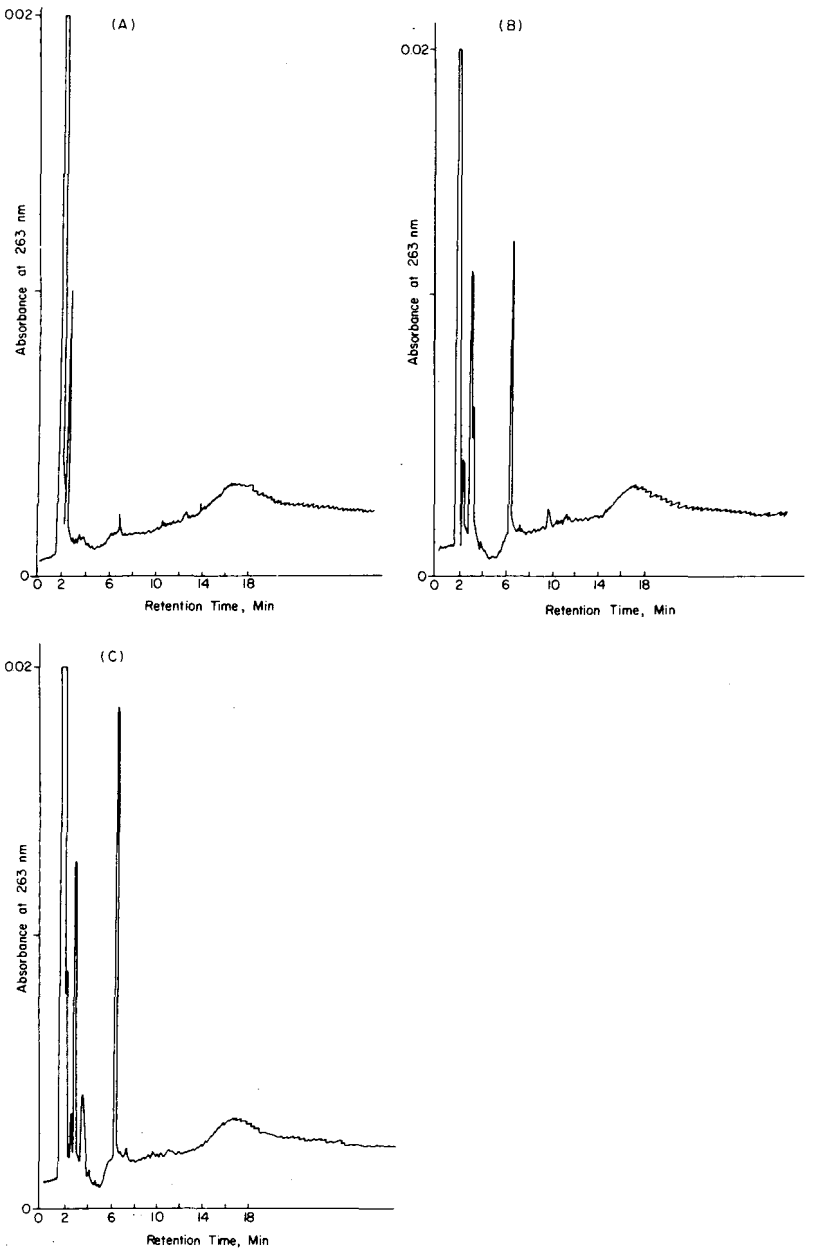

Fig. 3. Chromatograms of human blood samples. Typical chromatograms of: (A) normal, human blood; (B) from a patient at day 0 of L-PAM treatment; and (C) from a patient at day 6 of treatment.

der the SWOG L-PAM regimen. Identical preparation techniques were used for blood samples in all analyses so that the chromatograms could be compared exactly. In the day 0 chromatogram (Fig. 3C) no L-PAM peak appears, but the presence of L-DOH (t_R = 6.5 min) is clearly detected. In the day 6 chromato-

gram (Fig. 3C) the L-PAM peak is again absent, but the peak corresponding to L-DOH is considerably larger than in the day 0 chromatogram. These findings are consistent with the results of previous investigations which demonstrated that L-PAM disappears within a few hours following oral administration [14]. The present investigation, however, indicates that L-DOH remains in the blood for considerably longer periods of time than L-PAM; in fact, the L-DOH peak is detectable in blood samples collected more than three weeks after the last L-PAM ingestion.

Further identification of the L-DOH peak was achieved through chemical ionization (CI) mass spectrometry. HPLC fractions corresponding to the L-DOH peak were collected, evaporated to dryness, dissolved in acetonitrile, and analyzed by direct-probe ammonia CI process, using a Finnigan 3200 quadruple gas chromatograph—mass spectrometer. The M + H ion peak (taken as the base peak) and the peaks of other ions characteristic in CI spectra of L-DOH were identical to those of an L-DOH authentic sample.

The detection of L-DOH in patients' blood long after L-PAM administration triggered the study of comparative levels of L-PAM and L-DOH in rats following a single oral dose. Fig. 4 illustrates the results of analyses of animal blood samples over a period of 72 h following L-PAM administration. The concentration of L-PAM reaches its peak in about 2 h, decreases sharply within 4 h, then drops below 1 μg/ml. The concentration of L-DOH, however, ranges from 2 to 5 μg/ml and does not vary significantly over a 72-h period.

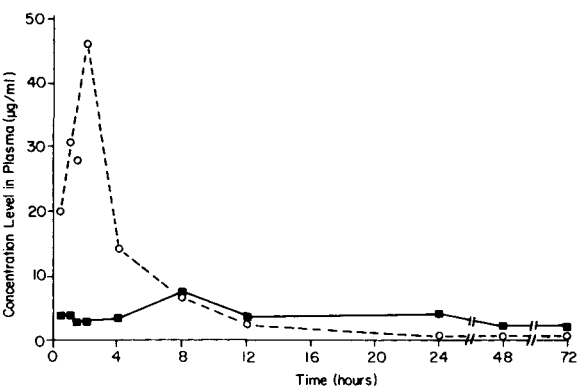

Fig. 4. Levels of L-PAM (○) and L-DOH (■) in blood samples over a period of 72 h. Each point represents the mean of three animals.

DISCUSSION

The determination of drug metabolites is essential for the understanding of the mechanism of action of the parent molecule. Previous studies on L-PAM, however, have neglected the determination of melphalan metabolites and their roles in the mechanism of action of L-PAM. The quantification of L-PAM metabolites has been impeded by difficulties in the analytical procedure. Chang et al. [8] reported that quantitative analysis of L-PAM hydrolytic products in biological samples is impossible by their HPLC method because of interference

from the biological matrix background. The HPLC methods of Furner et al. [12] and Flora et al. [10] present similar problems of interference.

There are several significant advantages of the HPLC method reported here; it accomplishes, by comparatively simple means, the distinct separation and quantification of L-PAM and L-DOH in biological samples with no background interference, and with a high degree of sensitivity accuracy, and reproducibility. The sensitivity of detection is consistently as low as 0.1 ppm, and the linearity of the standard curve in human blood serum persists at concentrations as high as 20 μg/ml.

This method allows the detection and quantification of L-DOH in patients receiving L-PAM chemotherapy. The observation that L-PAM was not detected in blood of patients 24 h after the last dose is in agreement with previous pharmacokinetic studies [14]. These studies suggested that, in humans following an oral administration, L-PAM quickly disappeared with a half-life of 67 min. Although the fate of L-DOH was not reported in their studies, Tattersall et al. [14] indicated that there is a prolonged terminal phase in the disappearance of label from the plasma of patients receiving an oral dose of L-[^{14}C]PAM. The delayed detection of L-DOH observed in our studies agrees with this observation and suggests that L-DOH is the major component of the label. Albert et al. [15] reported that no L-DOH was detected 24 h following an oral administration of L-[^{14}C]PAM. In their studies, however, they utilized HPLC methods described by Chang et al. [8] and Furner et al. [12], where the quantitation of L-DOH seems to be impossible due to the interference from biological materials.

Our animal studies also indicated a delayed elimination of L-DOH where 2—3 μg/ml was detected 72 h following the administration of a single oral dose. Vistica et al. [16] reported that L-PAM is transported across the cellular membrane by an amino-acid carrier transport system. Therefore delayed elimination of L-DOH in humans and rats may be due to its reabsorption in the kidney tubules by the same mechanism responsible for the active transport of amino acids. Although the role of L-DOH in L-PAM chemotherapy is not yet known, the rapid degradation of L-PAM compared with the persistence of L-DOH suggests that its role, which needs to be clarified, may be an important one.

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REFERENCES

1 A.E. Ahmed, P.J. Bour and K. Patel, Fed. Proc., Fed. Amer. Soc. Exp. Biol., 38 (1979) 31.
2 R. Alexanian, D.E. Bergsagel, P.J. Migliore, W.K. Vaughn and C.D. Howe, Blood, 31 (1968) 1—10.
3 B. Clarkson, in A.C. Sartorelli and D.G. Johns (Editors), Antineoplastic and Immunosuppressive Agents, Vol. I, Springer-Verlag, New York, 1974, p. 172.
4 B. Fisher, P. Carbone, S.G. Economon, R. Frelick, A. Glass, H. Lerner, C. Redmond, M. Zelen, P. Band, D.L. Katrych, N. Wolmark and E.R. Fisher, N. Engl. J. Med., 292 (1975) 117—122.

5 R.B. Livingston and S.K. Carter, Single Agents in Cancer Chemotherapy, Plenum Press, New York, 1970, pp. 99—111.
6 T.H. Wasserman, R.L. Comis, M. Goldsmith, H. Handelsman, J.S. Penta, M. Slavik, W.R. Soper and S.K. Carter, Cancer Chemother. Rep., 6 (Part 3) (1975) 399—419.
7 S.Y. Chang, D.S. Alberts, D. Farquhar, L.R. Melnick, P.D. Walson and S.E. Salmon, J. Pharm. Sci., 67 (1978) 682—684.
8 S.Y. Chang, D.S. Alberts, L.R. Melnick, P.D. Walson and S.E. Salmon, J. Pharm. Sci., 67 (1978) 679—682.
9 S.Y. Chang, T.L. Evans, D.S. Alberts and I.G. Sipes, Life Sci., 23 (1978) 1697—1701.
10 K.P. Flora, S.L. Smith and J.C. Cradock, J. Chromatogr., 177 (1979) 91—97.
11 R.L. Furner, R.K. Brown and G. Duncan, Cancer Treat. Rep., 61 (1977) 1637—1646.
12 R.L. Furner, L.B. Mellet, R.K. Brown and G. Duncan, Drug Metab. Dispos., 4 (1976) 577—583.
13 J.T. Goras, J.B. Knight, R.H. Iwamoto and P. Lim, J. Pharm. Sci., 59 (1970) 561—563.
14 M.H.N. Tattersall, M. Jarman, E.S. Newlands, L. Holyhead, R.A.V. Milstead and A. Weinberg, Eur. J. Cancer, 14 (1978) 507—513.
15 D.S. Alberts, S.Y. Chang, H.S.G. Chen, T.L. Evan and T.E. Moon, Clin. Pharmacol. Ther., 26 (1979) 737—745.
16 D.T. Vistica, J.N. Toal and M. Robinobitz, Biochem. Pharmacol., 27 (1978) 2865—2870.

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CHROMBIO. 751

DETERMINATION OF THE MAJOR METABOLITE OF PHENTOLAMINE IN HUMAN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A reversed-phase, high-performance liquid chromatographic method using UV detection is described for the assay of the major metabolite of phentolamine in plasma and urine before or after enzymatic hydrolysis. Plasma is deproteinized with methanol. The sensitivity limit is 200 ng/ml using 150-μl samples. Urine is either diluted with water or purified after enzymatic hydrolysis. Concentrations down to 2—3 μg/ml could be quantified with acceptable precision. This method was applied to plasma and urine samples from subjects given phentolamine.

INTRODUCTION

Phentolamine (Regitine®, Ciba-Geigy) or 2-[N-(*p*-tolyl)-N-(*m*-hydroxyphenyl)-aminomethyl]-2-imidazoline (Fig. 1) is a sympathicolytic agent. Very low plasma levels are reached after oral administration and less than 2% of the dose is recovered unchanged in urine over 24 h [1]. The metabolites in the urine accounted for about 60% of the administered dose [2]. The main one was identified as compound I, 2-[N-(*p*-carboxyphenyl)-N-(*m*-hydroxyphenyl)-aminomethyl]-2-imidazoline (Fig. 1). This compound is amphoteric and therefore difficult to extract from biological materials using conventional solvent extraction. A high-performance liquid chromatographic (HPLC) technique using an aqueous mobile phase and bonded silica gel packing material was developed for its assay in urine and plasma. This technique permitted the possible conjugation of I to be investigated.

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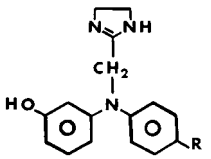

R = CH_3 : PHENTOLAMINE

R = COOH : I

Fig. 1. Phentolamine and its major metabolite.

EXPERIMENTAL

Chemicals

Phentolamine and its metabolite were supplied by Ciba-Geigy (Basle, Switzerland).

β-Glucuronidase—arylsulphatase solution (Helix pomatia) was purchased from Calbiochem (Los Angeles, CA, U.S.A.). One ml of the enzyme solution is diluted with 10 ml of pH 5.5 buffer (sodium acetate—acetic acid).

Apparatus

Chromatography is performed on a Hewlett-Packard Model 1082A instrument, equipped with a fixed-wavelength detector (254 nm) and a loop injector (Rheodyne, Model 7120, Berkeley, CA, U.S.A.).

Column

Stainless-steel columns are used.

For urine, the column (25 cm × 4.7 mm I.D.) is filled with LiChrosorb RP-18 or RP-8, 10 μm (Merck, Darmstadt, G.F.R.), using the balanced-density-slurry packing technique.

For plasma, the column (10 cm × 4.7 mm I.D.) is filled with LiChrosorb RP-8, 5 μm. A precolumn (10 cm × 4.7 mm I.D.) filled with Permaphase ODS (DuPont, Wilmington, DE, U.S.A.) is used to protect the analytical column.

Chromatography

The mobile phase and the column are at room temperature.

Urine. The degassed mobile phase $2.6 \cdot 10^{-3}$ *M* orthophosphoric acid—acetonitrile (60:40, v/v) is used at a flow-rate of 3 ml/min.

Plasma. The degassed mobile phase pH 4 buffer (hydrochloric acid—sodium citrate)—acetonitrile (85:15, v/v) is used at a flow-rate of 2 ml/min.

Sample preparation

Precipitation of proteins from plasma. Plasma, 150 μl, 50 μl of distilled water, and 700 μl of methanol, are mixed into a 10-ml conical glass tube. The tube is cooled and then centrifuged for 10 min at 2000 *g*. A 400-μl aliquot of the supernatant is transferred into a 5-ml glass ampoule and evaporated to dryness under nitrogen at room temperature. Distilled water (300 μl) is added to

the residue (method 1). The ampoule is shaken for a few seconds on a Vortex mixer and 50 μl are injected into the column. For concentrations of metabolite lower than 0.50 μg/ml, 600 μl of supernatant are evaporated and 200 μl of distilled water are added to the residue (method 2) and 50 μl are injected.

Urine dilution without enzymatic hydrolysis. Urine, 100 μl, and 900 μl of distilled water are mixed into a 10-ml glass tube; 40 μl are injected.

Urine dilution with enzymatic hydrolysis. Urine, 1 ml, 1 ml of water and 500 μl of enzyme solution are mixed into a 10-ml glass tube and heated for 15 h at 38°C. Hydrolysate, 500 μl, and 1500 μl of methanol are mixed into a 10-ml conical glass tube. The tube is cooled and centrifuged at 2000 *g* for 10 min. A 1-ml aliquot of supernatant is transferred into a 2-ml glass ampoule and evaporated to dryness under nitrogen at room temperature. The dry residue is dissolved in 1 ml of pH 10.7 buffer (sodium carbonate—hydrogen carbonate) and extracted with 1 ml of chloroform. A 500-μl aliquot of the aqueous phase and 500 μl of $36 \cdot 10^{-3}$ *M* phosphoric acid are mixed into a 2-ml glass ampoule. The aqueous phase is washed with 1 ml of dichloroethane; then 40 μl of the upper aqueous phase are injected.

Calibration curves

Calibration samples are prepared by adding aliquots of different aqueous solutions of the metabolite to plasma and urine. A calibration curve is established every day.

RESULTS AND DISCUSSION

Recovery and precision

Spiked plasma and urine samples were prepared and analysed several times. The results summarized in Tables I and II show that the described method permits the accurate and precise determination of the metabolite of phentolamine at concentrations down to 0.2 μg/ml in plasma and 2—3 μg/ml in urine.

TABLE I

PRECISION AND RECOVERY IN SPIKED PLASMA SAMPLES

Concentration added (μg/ml)	Within-day reproducibility			Day-to-day reproducibility		
	Concentration found (μg/ml) (mean of *n* replicates)	Coefficient of variation (%)	Mean recovery (%)	Concentration found (μg/ml) (mean of *n* replicates)	Coefficient of variation (%)	Mean recovery (%)
0.20	0.20 (9)	8.8	101			
0.54	0.60 (8)	7.35	112	0.55 (8)	9.2	101
1.1	1.0 (10)	4.8	91	1.1 (10)	6.6	98

TABLE II

PRECISION AND RECOVERY IN SPIKED URINE SAMPLES

	Concentration added (μg/ml)	Within-day reproducibility			Day-to-day reproducibility		
		Concentration found (μg/ml) (mean of *n* replicates)	C.V. (%)	Mean recovery (%)	Concentration found (μg/ml) (mean of *n* replicates)	C.V. (%)	Mean recovery (%)
Without hydrolysis	2.2	2.15 (8)	6.25	96			
	4.7	5.0 (9)	6.4	106	4.8 (12)	4.6	101
	18.9	18.9 (9)	2.7	100	19.0 (12)	2.0	100
With hydrolysis*	3.0	3.2 (10)	9.5	105			
	9.1	9.2 (9)	6.0	101	9.2 (8)	7.9	100
	12.2	12.2 (9)	5.3	100	12.0 (7)	5.4	99

*Urine spiked with the unconjugated compound.

Specificity

Plasma. The metabolite of phentolamine is well separated from the plasma components (Fig. 2). The parent drug, phentolamine, which is eluted as a large peak within 50 min, did not interfere since the concentrations reached after administration are in the nanogram range [1].

Urine. The metabolite of phentolamine is well separated from the urine components with or without hydrolysis. No interference was observed using urines from different volunteers. The parent drug is eluted within 20 min and does not interfere.

Stability

The metabolite of phentolamine remains stable in frozen urine samples for at least three months.

Application

The present method was used to determine the urinary excretion of the metabolite of phentolamine after the administration of single intravenous and oral doses of phentolamine to volunteers. The corresponding urinary excretion of the parent drug has been also determined [1]. The results summarized in Table III indicate that phentolamine is metabolized to a greater extent after oral than after intravenous administration. About 40% of the given dose was eliminated in the urine in the form of metabolite I within 24 h after oral administration (Table III).

The plasma concentrations and the urinary excretion of the metabolite were also determined in a patient receiving chronic treatment with phentolamine (oral doses of 400 mg daily). The data in Table IV indicate that the metabolite

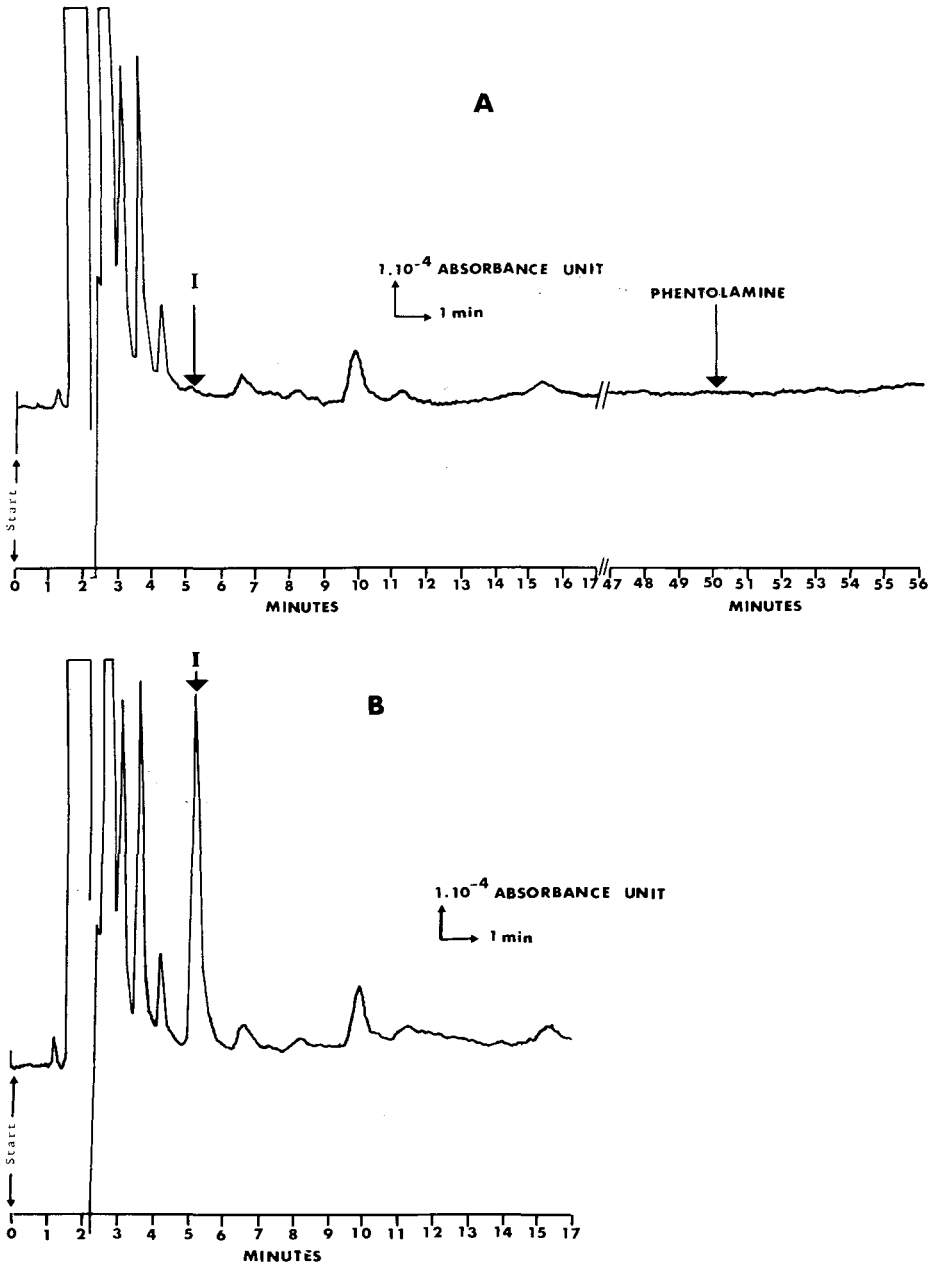

Fig. 2. Chromatograms of (A) blank human plasma and (B) spiked with the metabolite (method 1, see Sample preparation).

TABLE III

URINARY EXCRETION OF PHENTOLAMINE AND ITS MAJOR METABOLITE AFTER SINGLE INTRAVENOUS AND ORAL ADMINISTRATIONS

Mean values from 3 volunteers

	Dose			
	10 mg intravenous		20 mg oral	
	Phentolamine	I	Phentolamine	I
Total urinary excretion* (% of the dose)	15.3	16.8	8.0	41.6
Unconjugated fraction (% of total)	85	100	22.5	96.5

*Free + conjugated compound.

TABLE IV

PLASMA CONCENTRATIONS AND URINARY EXCRETION OF PHENTOLAMINE METABOLITE DURING REPETITIVE ADMINISTRATION

Plasma		Urine		
Time after administration (h)	Concentration (μg/ml)	Time interval	Amount excreted (mg)	Per cent of the dose
0	4.6	6 p.m.—6 a.m.	79.0	17.8
1	5.3	6 a.m.—6 p.m.	96.2	21.7
4	4.6	24 h	175.2	39.5

seems to accumulate in the plasma during such treatment. As after the administration of a single oral dose, about 40% of the dose was eliminated in the urine over 24 h in the form of the unconjugated metabolite (Table IV).

REFERENCES

1 A. Sioufi, F. Pommier, P. Mangoni, S. Gauron and J.P. Metayer, J. Chromatogr., 222 (1981) 429.
2 F. Früh and W. Theobald, personal communication.

Journal of Chromatography, 222 (1981) 467—474
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DETERMINATION OF PLASMA THEOPHYLLINE BY STRAIGHT-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY: ELIMINATION OF INTERFERING CAFFEINE METABOLITES

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SUMMARY

Several authors have recently reported interference in theophylline analysis by paraxanthine (1,7-dimethylxanthine), an important metabolite of caffeine. A method for the determination of theophylline in plasma is described, eliminating caffeine and related compounds by means of straight-phase high-performance liquid chromatography. The resulting procedure is sufficiently rapid, accurate and sensitive to be applied in routine monitoring of therapeutic levels in patients as well as for pharmacokinetic purposes. Although only 0.1 ml of sample is required, concentrations as low as 0.2 mg/l can be measured with acceptable precision. A brief comparative evaluation of this procedure with a radioimmunoassay is made.

INTRODUCTION

Theophylline and its derivatives are frequently used for treatment of acute and chronic bronchial asthma and apnea of the newborn. The low therapeutic index of the drug and the poor relationship between dosage regimens and

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plasma concentrations have in several cases caused severe intoxication, sometimes resulting in fatal accidents [1]. However, the significant correlation between plasma levels and clinical response allows, by careful monitoring of plasma drug concentrations, an adequate, individualized dosage schedule to be arranged [2].

A great variety of techniques for theophylline determination in biological fluids has been published, ranging from spectrophotometry [3,4], fluorimetry [5], gas chromatography [6—8] and thin-layer chromatography [9,10] to more recent methods such as radioimmunoassay [11], EMIT [12] and isotachophoresis [13].

Several publications emphasize the advantages of high-performance liquid chromatography (HPLC) with respect to sensitivity, specificity and the possibility of using microsamples. Most HPLC techniques for the determination of theophylline and related compounds differ from each other in sample preparation or in the chromatographic system used. The biological sample can be injected directly into the chromatograph [14,15], or after elimination of proteins by trichloroacetic acid [16], by solvent denaturation [17—21] or by molecular filtration [22]. These methods often involve expensive material, large sample volumes, or special care to maintain the characteristics of the analytical system.

Sample extraction prior to injection [23—31] offers, by optimising the pH and extracting solvent, the advantage of a higher specificity while evaporation and dissolution of the residue in a minimal amount of a suitable solvent adds to the sensitivity of the procedure. Most analyses are developed on bonded octadecyl reversed-phase systems with aqueous eluents containing acetate or phosphate salts to obtain a pH varying between 3.5 and 7 [18—27]. Good separation is also achieved on ion-exchange materials [15,28] or straight-phase systems [29,30].

Numerous workers have succeeded in eliminating interferences caused by compounds related to theophylline, originating from dietary or metabolic

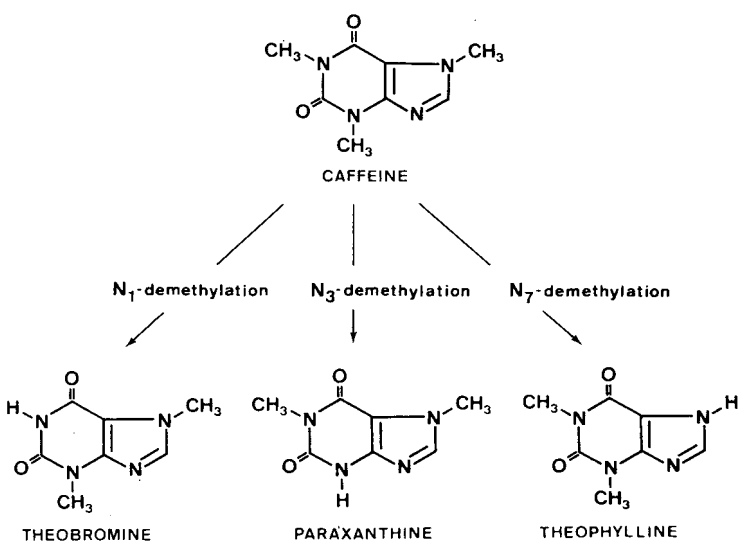

Fig. 1. Metabolic N-demethylation routes of caffeine in man [35].

sources. Nevertheless, the potential interference of paraxanthine (1,7-dimethylxanthine), an important metabolite of caffeine (Fig. 1), has been mostly overlooked or underestimated [32]. Observed by Thompson in 1974 [33], by Sved in 1976 [34] and identified mass spectrometrically by Midha [35], paraxanthine has a similar UV absorption and a retention time identical to that of theophylline in most reversed-phase systems. This often results in misleading elevations of serum theophylline values up to 3 mg/l. Assuming a therapeutic range of 5—20 mg/l theophylline serum concentration, the deviation from the true value can consequently reach 50% or more. Only a few investigators have proposed a suitable chromatographic procedure [36—41] to solve this problem.

In our laboratory we have developed a fairly rapid HPLC method using a straight-phase isocratic system on which the above-mentioned dimethylxanthines can be distinguished. Although only 100 μl of plasma is required, the limit of detection is as low as 0.2 mg/l with acceptable precision.

MATERIALS AND METHODS

Reagents

Chloroform was obtained from Riedel-De Haën (Seelze, Hannover, G.F.R.); formic acid was from Noury-Baker (Deventer, The Netherlands); theophylline was from Serva Feinbiochemica (Heidelberg, G.F.R.); 3-isobutyl-1-methylxanthine from Aldrich-Europe (Beerse, Belgium); 1,7-dimethylxanthine and theobromine from Sigma (St. Louis, MO, U.S.A.); and caffeine was from Knoll (Ludwigshafen, G.F.R.). Dioxane, 2-propanol and ammonium sulphate were purchased from E. Merck (Darmstadt, G.F.R.). All reagents were used as received.

Apparatus

HPLC analyses were carried out with a Waters Assoc. Model 6000 chromatography pump in conjunction with a U6K injector (Waters Assoc., Milford, MA, U.S.A.) and an LC—UV variable-wavelength absorbance detector (Pye Unicam, Cambridge, Great Britain). An SP 4000 Chromatography Data System connected with an SP 4050 printer/plotter (Spectra Physics, Santa Clara, CA, U.S.A.) was used for quantitation of the results.

Chromatographic conditions

A 10 cm × 2.8 mm I.D. stainless-steel column (RSL, Eke, Belgium) was slurry-packed in a carbon tetrachloride—methanol mixture (70:30, v/v) with silica gel (RSil 5 μm; RSL). The slurry was compressed with light petroleum (b.p. 40—60°C) at 315 bar by means of an air-driven fluid pump (Haskel, Burbank, CA, U.S.A.). The solvent system consisted of chloroform—dioxane—formic acid (95.5:4.5:0.01, v/v), degassed in an ultrasonic bath and kept capped and stirred while in use. The flow-rate was 1.4 ml/min, corresponding to a pressure of 84 bar at the head of the column. All chromatograms were run at ambient temperature. The eluent was monitored continuously at 273 nm with a detector sensitivity setting of 0.04 a.u.f.s.

Extraction procedure

Equal volumes (100 μl) of plasma, saturated ammonium sulphate solution and internal standard solution (3-isobutyl-1-methylxanthine, 25 mg/l) are pipetted into a 18 × 100 mm polypropylene centrifuge tube and extracted by vortexing for 20 sec with 10 ml of a chloroform—2-propanol mixture (95:5, v/v). Following centrifugation (5 min at 800 *g* RCF*), the upper aqueous layer is removed by aspiration and 8 ml of the organic layer are transferred to a conical glass test-tube and evaporated to dryness at 60°C under a stream of dry nitrogen. The residue is stored at low temperature until analysis. After reconstitution in 50 μl of the mobile phase, 20 μl are injected into the chromatograph.

Preparation of standard curves

A 100-μl volume of a 4 mg/ml theophylline solution in 0.01 *N* hydrochloric acid was added to 19.9 ml of drug-free pooled blood-bank plasma, resulting in a stock solution of 20 mg/l. Theophylline concentrations ranging from 0.2—20 mg/l were prepared by further diluting with plasma. After incubation at 37°C for 14 h, all standards were extracted and analysed in duplicate by the described procedure.

Peak area drug/internal standard ratio was plotted against concentration and a straight-line fit of the data was made by linear regression analysis.

RESULTS AND DISCUSSION

Fig. 2 shows typical chromatograms of serum samples collected before and after theophylline administration to a patient not withdrawn from caffeine-containing beverages. All constituents eluted in the order of increasing polarity, the retention times of the internal standard and theophylline being 4 and 8 min, respectively. The flow-rate and the percentage of dioxane in the mobile phase were dictated by a compromise between baseline resolution and chromatographic analysis time.

With the exception of one case, in which the nature of the interfering compound could not be elucidated, no drugs or naturally occurring endogenous substances interfered during the 5-month period in which we analysed a large number of samples collected from numerous patients. Considering the potential interference of theophylline metabolites, we used 5% 2-propanol in chloroform as the extraction solvent, which separated the drug from the methyluric acids but not from 3-methylxanthine [42]. The latter compound, however, as well as 1-methylxanthine, does not seem to be present in detectable amounts in plasma [43]. Nevertheless, no chromatographic response was obtained in vitro, presumably due to retention on the column.

Using the extraction solvent—salt combination [44] good extraction efficiency could be achieved (91.03% for theophylline and 99.8% for the internal standard). At a neutral pH, strongly basic and acidic material remains in the aqueous phase and most lipid and co-extractable material is excluded by the establishment of a lipid—protein interphase between the organic and the aqueous layer. Furthermore, the problem of a preliminary pH fluctuation connected with the use of an acetate buffer [45] is avoided.

*Relative centrifugal force.

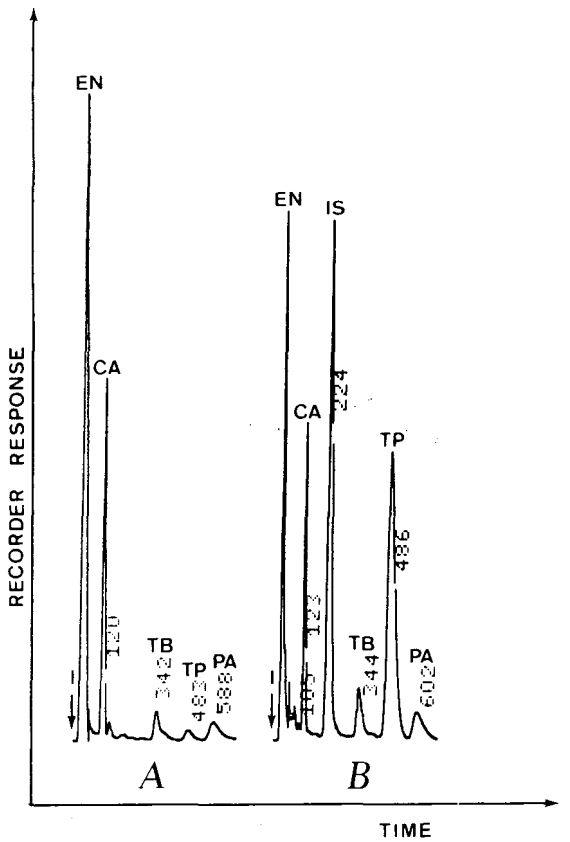

Fig. 2. Typical chromatogram of a blank serum from a non-fasting patient (A) and a serum sample after administration of theophylline (B). I = injection; EN = endogenous compounds; CA = caffeine; IS = internal standard; TB = theobromine; TP = theophylline; PA = paraxanthine. Retention times (sec) are printed at each peak.

Sensitivity and selectivity were increased by measuring at 273 nm, the optimum detection wavelength for theophylline. We found 10 ng to be the detection limit.

Excellent linearity ($r = 0.9999$) was noted in the relationship peak area drug/internal standard versus plasma theophylline concentrations ranging from 0.25 to 20 mg/l.

Small fluctuations in solvent strength, changes in adsorbant water content or ambient temperature variations cause shifting of the peaks. This can seriously affect the accuracy of the method when measuring peak height ratios. Therefore we preferred to use an automatic data-processing equipment from which direct digital read-out of peak areas was obtained.

As illustrated in Table I, systematically repeated calibration was not necessary. Kept in a tightly closed glass container at room temperature, the aqueous 3-isobutyl-1-methylxanthine solution proved to be stable for several months. Yet standard theophylline solutions were run periodically to evaluate the precision of the assay under routine conditions.

TABLE I

STATISTICAL EVALUATION OF THREE CALIBRATION CURVES CARRIED OUT AT 1-MONTH INTERVALS

	Intercept	Slope
Calibration curve 1	0.0008945	0.045249
Calibration curve 2	0.0003188	0.044660
Calibration curve 3	−0.008156	0.045267
Statistically expected coefficients	0.00012809	0.045059
Variation	0.127×10^{-5}	0.192×10^{-7}

An estimation of "within-run" variation was carried out by analysing spiked plasma samples containing 1 mg/l (C.V. = 2.5%, n = 5), 8 mg/l (C.V. = 2.2%, n = 5) and 15 mg/l (C.V. = 2.7%, n = 5). The accuracy of the method was further evaluated by directly comparing the results of 100 theophylline determinations, performed by the described procedure and by a radioimmunologic assay (RIA) in patient plasma specimens.

All RIA measurements were carried out with a Gammadab [^{125}I]Theophylline Radioimmunoassay Kit (Clinical Assays, Travenol Lab., Lessines, Belgium),

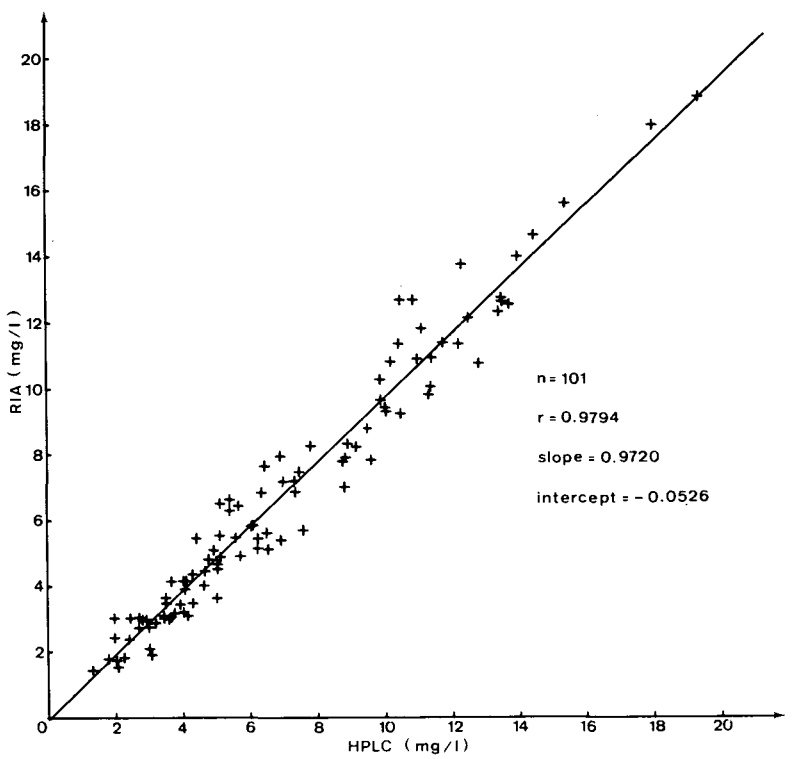

Fig. 3. Correlation of patient plasma readings as determined by the HPLC procedure and by RIA.

TABLE II

"BETWEEN-RUN" PRECISION (2-MONTH PERIOD): COMPARISON OF THE HPLC PROCEDURE AND RIA

Procedure	Theophylline plasma conc. (mg/l)	No. of samples (n)	Mean recovery (%)	Standard deviation (mg/l)	Coefficient of variation (%)
HPLC	8.0	16	101.0	0.27	3.3
RIA	15.0	54	97.3	0.73	5.0

following the directions provided by the supplying company. A calibration curve was established by linear regression analysis after logit/log transformation of the data ($r > 0.996$), and the final results were automatically calculated by a programmed desk-top calculator (Hewlett-Packard HP 9810A). As illustrated in Fig. 3, good correlation was found between the methods.

Although RIA offers the advantages of speed and specificity, its precision (Table II) and sensitivity (minimal detectable concentration = 2 mg/l) may be acceptable in routine monitoring of therapeutic plasma levels, but are hardly sufficient for pharmacokinetic purposes. Furthermore, the high cost and the need for consistent reliability of the reagents have to be taken into consideration as compared to the HPLC technique, which, on the other hand, involves more technician time and investment in rather expensive and complex chromatographic equipment.

CONCLUSIONS

By the HPLC method described in this paper, good separation is achieved of theophylline and its isomers, resulting in higher accuracy in routine monitoring plasma drug concentrations. Chromatographic conditions remained stable for a long period of time without special cleaning or maintenance procedures of the system. The small sample size, coupled with high sensitivity, allows plasma collection by capillary puncture, making the method attractive for pediatric and pharmacokinetic investigation where multiple sampling is mostly required.

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REFERENCES

1 M. Lagas and J.H. Glerum, Pharm. Weekbl., 112 (1977) 97.
2 L. Hendeles, M. Weinberger and G. Johnson, Clin. Pharmacokinet., 3 (1978) 294.
3 J.A. Schack and S.H. Waxler, J. Pharmacol. Exp. Ther., 97 (1949) 283.
4 A.J. Fellenberg and A.C. Pollard, Clin. Chim. Acta, 92 (1979) 267.
5 J.M. Meola, H.H. Brown and T. Swift, Clin. Chem., 25 (1979) 1835.
6 V.P. Shah and S. Riegelman, J. Pharm. Sci., 63 (1974) 1283.
7 A.G. de Boer and D.D. Breimer, Pharm. Weekbl., 112 (1977) 764.

8 R.E. Chambers, J. Chromatogr., 171 (1979) 473.
9 B. Wesley-Hadzija and A.M. Mattocks, J. Chromatogr., 115 (1975) 501.
10 H. Roseboom, H. Lingeman and G. Wiese, Z. Anal. Chem., 292 (1978) 239.
11 T. Nishikawa, M. Saito and H. Kubo, Chem. Pharm. Bull., 27 (1979) 893.
12 H.M.C. Heick, A. Mohammed, C. Golas and E.M. Patterson, Clin. Biochem., 12 (1979) 68.
13 U. Moberg, S.G. Hjalmarsson and T. Mellstrand, J. Chromatogr., 181 (1980) 147.
14 B.R. Manno, J.E. Manno and B.C. Hilman, J. Anal. Toxicol., 3 (1979) 81.
15 M. Weinberger and C. Chidsey, Clin. Chem., 21 (1975) 834.
16 W.J. Jusko and A. Poliszczuk, Amer. J. Hosp. Pharm., 33 (1976) 1193.
17 O.H. Weddle and W.D. Mason, J. Pharm. Sci., 65 (1976) 865.
18 J.J. Orcutt, P.P. Kozak, S.A. Gillman and L.H. Cummins, Clin. Chem., 23 (1977) 599.
19 J.W. Nelson, A.L. Cordry, C.G. Aron and R.A. Bartell, Clin. Chem., 23 (1977) 124.
20 G.P. Butrimovitz and V.A. Raisys, Clin. Chem., 25 (1979) 1461.
21 G. Morgant, F. Veinberg, M.L. Bouthenet and P. Aymard, Feuill. Biol., 20 (1979) 117.
22 L.C. Franconi, G.L. Hawk, B.J. Sandmann and W.G. Haney, Anal. Chem., 48 (1976) 372.
23 M.A. Peat, T.A. Jennison and D.M. Chinn, J. Anal. Toxicol., 1 (1977) 204.
24 R.E. Hill, J. Chromatogr., 135 (1977) 419.
25 S.J. Soldin and J.G. Hill, Clin. Biochem., 10 (1977) 74.
26 C. Gonnet, M. Porthault, C. Bory and P. Baltassat, Analusis, 6 (1978) 406.
27 P.J. Naish, M. Cooke and R.E. Chambers, J. Chromatogr., 163 (1979) 363.
28 G.W. Peng, V. Smith, A. Peng and W.L. Chiou, Res. Commun. Chem. Pathol. Pharmacol., 15 (1976) 341.
29 D.S. Sitar, K.M. Piafsky, R.E. Rango and R.I. Ogilvie, Clin. Chem., 21 (1975) 1774.
30 A.G. Maijub and D.T. Stafford, J. Chromatogr. Sci., 14 (1976) 521.
31 C.V. Manion, D.W. Shoeman and D.L. Azarnoff, J. Chromatogr., 101 (1974) 169.
32 E.C. Lewis and D.C. Johnson, Clin. Chem., 24 (1978) 1711.
33 R.D. Thompson, H.T. Nagasawa and J.W. Jenne, J. Lab. Clin. Med., 84 (1974) 584.
34 S. Sved, R.D. Hossie and I.J. Mc Gilveray, Res. Commun. Chem. Pathol. Pharmacol., 13 (1976) 185.
35 K.K. Midha, S. Sved, R.D. Hossie and I.J. McGilveray, Biomed. Mass Spectrom., 4 (1977) 172.
36 S. Sved and D.L. Wilson, Res. Commun. Chem. Pathol. Pharmacol., 17 (1977) 319.
37 M. Danhof, B.M. Loomans and D.D. Breimer, Pharm. Weekbl., 113 (1978) 672.
38 J.R. Micsic and B. Hodes, J. Chromatogr. Sci., 68 (1979) 1200.
39 J.H. Jonkman, R. Schoenmaker, J.E. Greving and R.A. de Zeeuw, Pharm. Weekbl., 115 (1980) 557.
40 A.A. van den Bemd, M.J. van Gorp and M.C. Verhoof, Pharm. Weekbl., 113 (1978) 424.
41 H.H. Farrish and W.A. Wargin, Clin. Chem., 26 (1980) 524.
42 J.W. Jenne, E. Wyze, F.S. Rood and F.M. MacDonald, Clin. Pharmacol. Ther., 13 (1972) 349.
43 R.K. Desiraju, E.T. Sugita and R.L. Mayock, J. Chromatogr. Sci., 15 (1977) 563.
44 H.A. Schwertner, T.M. Ludden and J.E. Wallace, Anal. Chem., 48 (1976) 1875.
45 J. Zuidema and A.M. IJsselstijn, Pharm. Weekbl., 111 (1976) 1326.

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CHROMBIO. 745

Note

Natural products chemistry
81*. Analysis of rat thymus steroids by liquid-gel chromatography and gas chromatography—mass spectrometry**

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The presence of unidentified corticosteroids in rat thymus has been reported [1, 2]. In vivo and in vitro studies also showed the presence of α,β-unsaturated ketosteroids [3]. In the course of a gas chromatographic—mass spectrometric (GC—MS) study of steroids in thymus lipid extracts we identified 4-pregnene-3, 20-dione and 21-hydroxy-4-pregnene-3,20-dione in calf thymus [4].

In connection with studies of steroids in tymus tissues, a group isolation procedure has been developed [4, 5]. This is based on a combination of reversed- and straight-phase liquid-gel chromatography on hydroxyalkoxyalkyl Sephadex. The mixture of purified steroids was analysed by combined GC—MS methods.

MATERIALS AND METHODS

Thymus glands were obtained from 4-week-old male Wistar-strain rats. The total lipids were extracted from the tissues with chloroform—methanol (2:1) and purified as described before [6]. The extract was subjected to reversed-phase gel chromatography on a column of 18 g Lipidex-5000 (Packard-Becker, Groningen, The Netherlands) in chloroform—methanol—water (2:9:1) [4, 5]. The steroid-containing fraction was purified on a column of 7 g Lipidex-5000 in *n*-hexane—chloroform (9:1) [4, 5].

The steroids were analysed as O-methyloxime—trimethylsilyl ether (MO—TMS) derivatives [7]. Acid contaminants from the reaction with methoxy-

*For part 80, see J. Reisch, I. Mester, S.K. Kapoor, Zs. Rôzsa and K. Szendrei, Justus Liebigs Ann. Chem., (1980) in press.
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amine · HCl were removed by chromatography through anion exchanger Amberlyst A-26 (Serva, Heidelberg, G.F.R.) [5].

For the gas—liquid chromatographic (GLC) analysis, the derivatized steroids were run on a 70 m × 0.25 mm I.D. glass capillary column coated with OV-101 programmed at a temperature of 180—280°C at a rate of 1°C/min. The methylene unit (MU) values were obtained with *n*-alkanes of even carbon numbers from C_{24} to C_{36}.

Mass spectra were obtained using a Varian MAT 44S gas chromatograph—mass spectrometer interfaced to a Spectra System-MAT 188 with spectrometer conditions: ion source temperature 200°C, separator temperature 250°C and an electron energy of 70 eV. The samples were run using the GC inlet.

For GC—MS analysis, all samples were run on a 25 m × 0.25 mm I.D. glass capillary column coated with SE-30 programmed at a temperature of 60—250°C at a rate of 10°C/min.

RESULTS AND DISCUSSION

The gas chromatogram of the isolated fraction showed two peaks with MU values and mass spectra consistent with those of authentic 4-pregnene-3,20-dione [4]. This steroid was recently reported to be present in calf thymus [4].

A second steroid was detected on a gas—liquid chromatogram with a MU value of 30.42 identical with that of authentic 11β-hydroxy-4-pregnene-3,20-dione. The MS investigation of the derivatized fraction on a GC—MS instrument using the GC inlet showed a substance with the parent ion M^+ = 460. The presence of the 20-oxo structure was indicated by an intense ion at *m/e* 100 (base peak) composed of C_{16}/C_{17} with substituents and additional hydrogen atom [8]. The presence of the TMS group was indicated by the fragment ion at *m/e* 370 due to loss of trimethylsilanol, $(CH_3)_3SiOH$. The fragment ions at *m/e* 143 and 240 are characteristic of 11β-hydroxy-steroid derivatives [9]. The identity of the component with authentic 11β-hydroxy-4-pregnene-3,20-dione was confirmed by direct comparison of their mass spectra (Fig. 1).

The detection of ketosteroids of unknown structures in calf [10] and rat thymus [1—3] has been reported. We found 4-pregnene-3,20-dione and 21-hydroxy-4-pregnene-3,20-dione in calf thymus [4]. The present report shows the presence of 4-pregnene-3,20-dione and 11β-hydroxy-4-pregnene-3,20-dione in rat thymus. The question regarding its source of biogenesis cannot be answered yet. The presence of the latter compound was, however, previously reported to be formed mainly by the adrenal tissues of different animals [11—13].

The binding of steroids to different thymus cell fractions was studied and it was found that 4-pregnene-3,20-dione binds to the greatest extent while 11β, 17-21-trihydroxy-4-pregnene-3,20-dione binds to the least extent [14]. This could indicate that the origin of the former is extra-thymic.

It is not known whether active sites for 11β-hydroxylation of 4-pregnene-3,20-dione to 11β-hydroxy-4-pregnene-3,20-dione are present, which could explain the presence of the latter compound in rat thymus. It was shown that the corticoid content of rat thymus decreased in adrenalectomized animals [1, 2]. This could indicate also an extra-thymic origin of 11β-hydroxy-4-pregnene-3,20-dione.

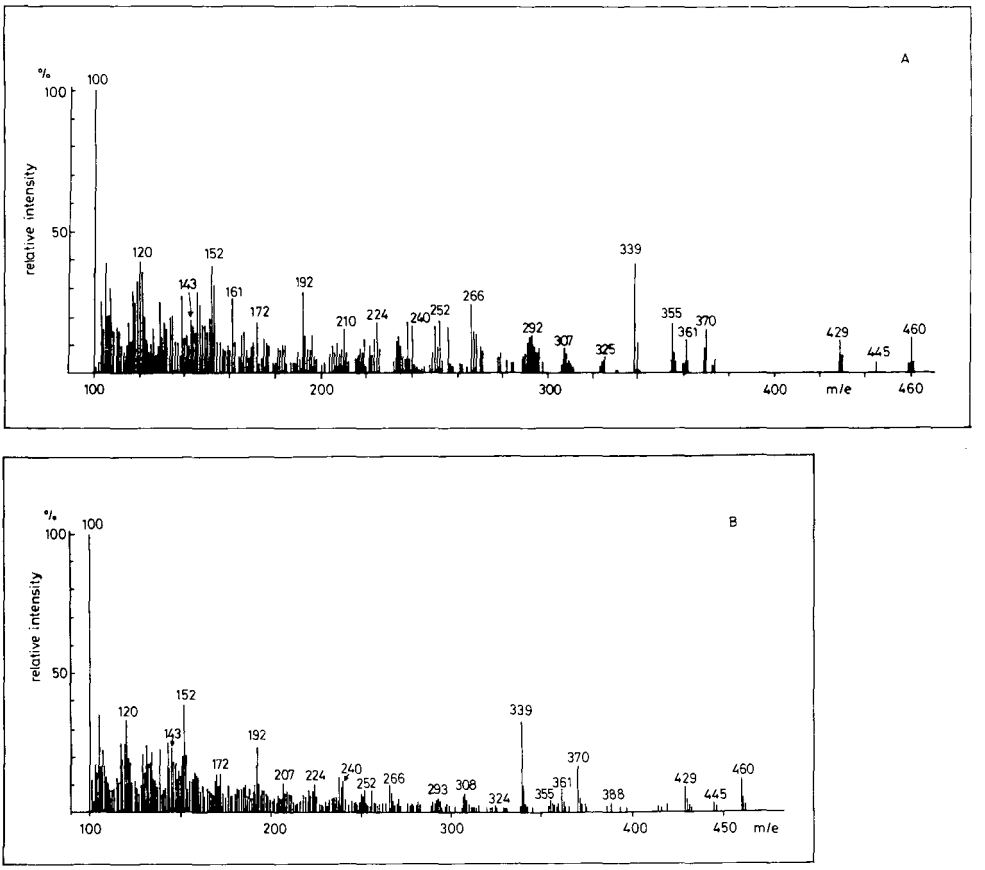

Fig. 1. Mass spectra of the MO—TMS derivatives of 11β-hydroxy-4-pregnene-3,20-dione from rat thymus (A) and authentic compound (B).

REFERENCES

1 M. Negeon-Dureuil, M. Rabinowicz and A.R. Ratsimamanga, C.R. Soc. Biol., 146 (1952) 1929.
2 T. Rahandraha and A.R. Ratsimamanga, Bull. Soc. Chim. Biol., 35 (1953) 301.
3 A.D. Abraham, Rev. Roum. Endocrinol., 8 (1971) 83.
4 J. Reisch and A.S.El-Sharaky, Hoppe-Seyler's Z. Physiol. Chem., 361 (1980) 791.
5 M. Axelson, G. Schumacher and J. Sjövall, J. Chromatogr. Sci., 12 (1974) 535.
6 J. Folch, M. Lees and G.H. Sloane Stanley, J. Biol. Chem., 226 (1957) 497.
7 W.L. Gardiener and E.C. Horning, Biochim. Biophys. Acta, 115 (1966) 524.
8 C.J.W. Brooks and D.J. Harvey, Steroids, 15 (1970) 283.
9 P. Vouros and D.J. Harvey, J. Chem. Soc., Perkin Trans., 1 (1973) 727.
10 W. Henning, H. Möllmann, J. Reisch, H. Kindler and H. Alfes, Z. Zellforsch., 142 (1973) 465.
11 M. Holzbauer and H.M. Newport, J. Physiol. (London), 200 (1965) 821.
12 N.A. Yudaev and S.A. Afinogenova, Endocrinol. Exp., 9 (1975) 114.
13 M. Weiss and V.L. Ford, Comp. Biochem. Physiol., B57 (1977) 15.
14 W.K. Brunkhorst and E.I. Hess, Biochim. Biophys. Acta, 82 (1964) 385.

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CHROMBIO. 762

Note

Reliability of the estimation of serum cortisol by high-performance liquid chromatography

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Because of its non-radioactive feature, the determination of serum cortisol by high-performance liquid chromatography (HPLC) is promoted as a useful alternative to radioimmunoassay (RIA) techniques in routine laboratory analyses [1—11]. In almost all of the HPLC methods hitherto published, the UV absorbance of cortisol is used for quantitation [1—10]. The interest of the present study focused on the question, as to whether UV detection is indeed sufficiently selective for cortisol assessment in serum. Therefore, we estimated cortisol by HPLC in a series of 195 serum samples arising in our routine laboratory work. The HPLC results were correlated with the values obtained by the specific RIA method.

EXPERIMENTAL

Materials

Solvents, reagents, extraction devices, non-labelled steroids and labelled cortisol were as previously described [12]. The solid phase ^{125}I-RIA kit was obtained from Clinical Assays (Cambridge, MA, U.S.A.). The liquid phase ^{3}H-RIA and the characteristics of the corresponding antiserum used have also been described previously [12].

Instrumentation

The high-performance liquid chromatograph equipped with a UV detector at a fixed wavelength of 254 nm, an automatic sampler and fraction collector was from Hewlett-Packard (Model 1084B). For HPLC, a polar coated silica, Diol® (particle size 5 μm; Knauer, Berlin, G.F.R.), as stationary phase, *n*-hexane and isopropanol as eluent and gradient mode were used. Details of the HPLC system applied have been reported recently [12].

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Serum samples

Parameters measured in the batch of serum samples studied included cortisol base levels and levels before and after adrenal stimulation (ACTH, insulin) or suppression (dexamethasone). Serum was stored at −20°C until analysis.

Analytical procedures

HPLC (method I). Serum (1 ml) traced with 100 ng of prednisone, was extracted with diethyl ether using a solid phase extraction technique [13]. The dried residue was dissolved in the eluent and automatically chromatographed. The ratios of peak heights of cortisol and prednisone were evaluated by a standard curve established from cortisol standards ranging from 27.6—1380 nmol/l. The standard curve itself was linear up to 540 nmol/l.

Solid phase RIA (method II). The protocol followed the instructions of the manufacturer using aliquots of 10 μl of serum.

HPLC—RIA (method III). The cortisol-containing fractions eluted by HPLC (see method I) were automatically collected, evaporated, redissolved in 5 ml of water, and finally quantitated by liquid phase ^{3}H-RIA [12]. In this technique, [^{3}H]cortisol added to the serum sample prior to the assay, was used for the recovery measurement.

RESULTS AND DISCUSSION

Fig. 1a demonstrates the UV chromatogram of steroidal drugs commonly used in steroid therapy. Fig. 1b shows the corresponding chromatogram of

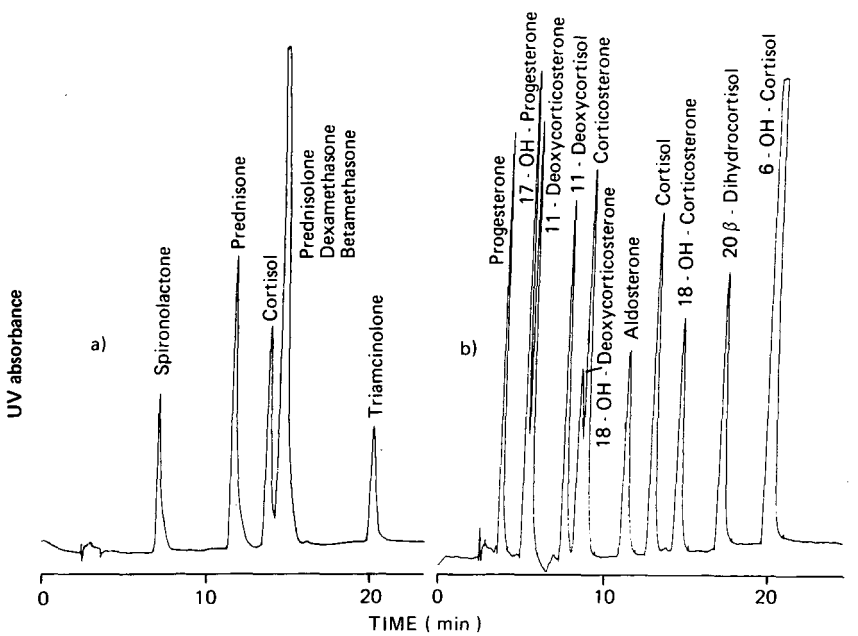

Fig. 1. UV chromatograms of steroidal drugs (a) and of biogenic steroids (b). HPLC system used: column, Diol, 250 × 4.5 mm I.D.; solvent A: *n*-hexane; solvent B: *n*-hexane—isopropanol (75:25); flow-rate: 1.3 ml/min; sample volume injected: 150 μl; temperature of column, 30°C; detection at 254 nm.

the adrenal steroids naturally prevailing in human serum. A distinct separation of cortisol from both drugs and biogenic steroids is apparent. The degree of resolution is equivalent to or better than that of the HPLC systems described hitherto [1—10].

The UV chromatograms of normal serum samples are shown in Fig. 2. While the chromatogram in Fig. 2a was well suited for peak height evaluation, there was a strong UV-absorbing background in the area of the prednisone reference peak (Fig. 2b), which made a reliable assessment of serum cortisol exclusively through UV detection impossible. In a series of 195 routine samples, there were 145, which exhibited a chromatographic profile similar to that in Fig. 2a. The analytical variables under these conditions were as follows:

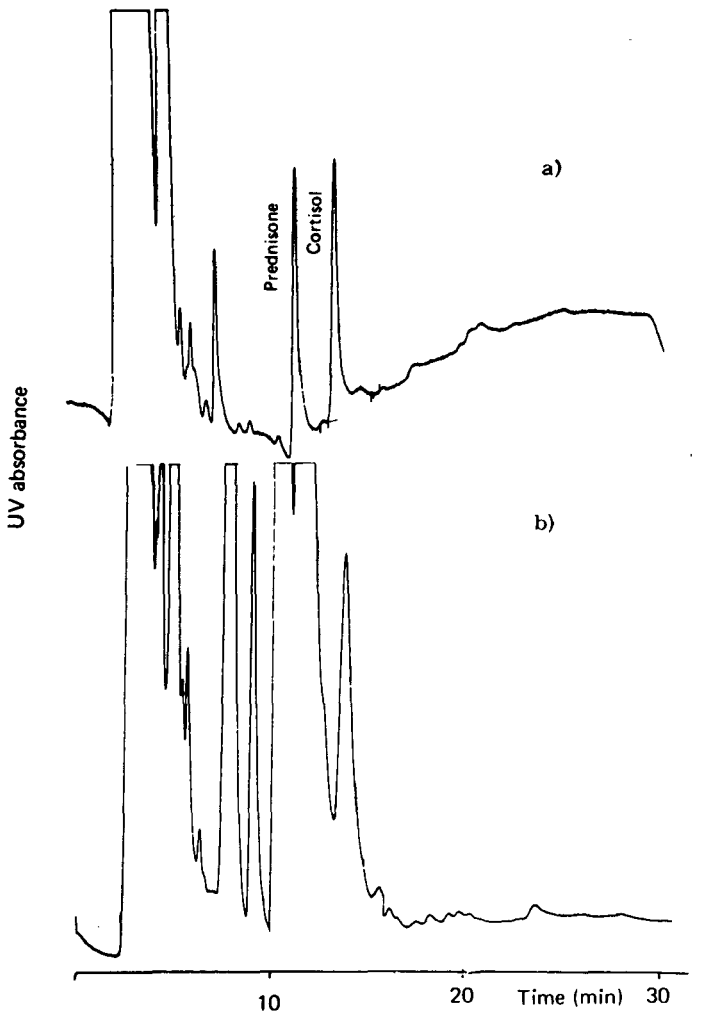

Fig. 2. Representative UV chromatograms from normal serum samples. Ether extracts of 1 ml of serum were applied. Chromatographic conditions as in Fig. 1. The chromatogram in Fig. 2a was well suited for peak height evaluation. In Fig. 2b, the reference peak of prednisone was completely covered by unspecific UV-absorbing compounds of the serum sample.

detection limit, 20 nmol/l; intra- and interassay variability, 7.05% and 10.5% (coefficient of variation), respectively; equation of correlation, $cortisol_{found} = -6.7$ (nmol/l) + 1.01 · $cortisol_{added}$ ($r = 0.99$). These data are comparable to those obtained by the RIA technique [14].

However, 21% of all samples studied, exhibited a UV chromatogram similar to that in Fig. 2b. In these samples, absolute serum cortisol concentrations were not at all, or only speculatively assessable because of considerable absorptions of the serum background, thus overlapping the peaks of prednisone, of cortisol itself, or of both steroids.

Due to the constant absorption background of the serum samples of an individual subject, the problem of unspecific interferences was reduced in samples of function tests. In these cases, the relative changes of the cortisol peak are often sufficient for a chemical diagnosis.

When correlating the results obtained from the 145 samples assessable by UV absorption (method I) with those obtained by HPLC—RIA (method III) as reference method, the following equation was found: $cortisol_{method\ I} = -9.6$ (nmol/l) + 1.25 · $cortisol_{method\ III}$ ($r = 0.965$). The corresponding correlation between method II and III was: $cortisol_{method\ II} = 60.0$ (nmol/l) + 1.41 · $cortisol_{method\ III}$ ($r = 0.971$).

In conclusion, the present data emphasize the findings of other workers [8,9] that HPLC assessment of serum cortisol is feasible in principle and that the analytical quality of HPLC may achieve a level comparable to the current RIA techniques. If, however, UV absorbance is used for quantitation, a reliable estimation of all samples arising in routine laboratory analysis is not guaranteed. The fluorescence method recently published for cortisol quantitation after HPLC [11] may be a potential solution to this problem.

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REFERENCES

1 W. Wortmann, C. Schnabel and J.C. Touchstone, J. Chromatogr., 84 (1973) 396—401.
2 F.K. Trefz, D.J. Byrd and W. Kochen, J. Chromatogr., 107 (1975) 181—189.
3 J.H. van den Berg, C.R. Mol, R.S. Deelder and J.H. Thijssen, Clin. Chim. Acta, 78 (1977) 165—172.
4 D.C. Garg, J.W. Ayres and J.G. Wagner, Res. Commun. Chem. Pathol. Pharmacol., 18 (1977) 137—146.
5 G.E. Reardon, A.M. Caldarella and E. Canalis, Clin. Chem., 25 (1979) 122—126.
6 J.Q. Rose and W.J. Jusko, J. Chromatogr., 162 (1979) 273—280.
7 G. Cavina, G. Moretti, R. Alimenti and B. Gallinella, J. Chromatogr., 175 (1979) 125—140.
8 P.M. Kabra, L.L. Tsai and L.J. Marton, Clin. Chem., 25 (1979) 1293—1296.
9 N.R. Scott and P.F. Dixon, J. Chromatogr., 164 (1979) 29—34.
10 M.D. Smith, J. Chromatogr., 164 (1979) 129—137.
11 T. Kawasaki, M. Maeda and A. Tsuji, J. Chromatogr., 163 (1979) 143—150.
12 M. Schöneshöfer, A. Fenner and H.J. Dulce, Clin. Chim. Acta, 106 (1980) 63—73.
13 R. Wehner and A. Handke, Clin. Chim. Acta, 93 (1979) 429—431.
14 J. Seth and L.M. Brown, Clin. Chim. Acta, 86 (1978) 109—120.

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Note

Mass fragmentographic quantification of naproxen in human plasma

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(First received June 16th, 1980; revised manuscript received October 13th, 1980)

There are publications on various methods for the determination of naproxen (Naprosyn®) (6-methoxy-α-methyl-2-naphthalene-acetic acid) (NPX) in human plasma, e.g. gas chromatography [1], liquid chromatography [2] and spectrofluorometry [3]. The plasma concentrations after ordinary therapeutic treatment do not cause any sensitivity problems in respect of any of the above-mentioned methods. However, naproxen is extensively bound to plasma albumin (>99%) at therapeutic levels [4, 5], and the degree of the protein binding has been demonstrated to be affected by the total plasma concentration [3]. This might influence the therapeutic effect, since it is generally assumed that only the unbound drug will be available to the receptors. Consequently, it is of clinical interest to examine the influence of total naproxen plasma concentration on the magnitude of the free fraction. Thus, a method sufficiently sensitive to quantify approximately 0.1% of the total concentration had to be developed, since neither gas chromatography nor liquid chromatography have the required sensitivity. The spectrofluorometric method [3] demonstrated sufficient sensitivity, but the method does not include a separation procedure. This can influence the specificity, since any chemical substance showing fluorescence will be co-determined. Therefore, a mass fragmentographic method was developed. This method can quantify naproxen far below any plasma concentration of unbound drug, and specificity tests revealed no interference with ordinary, simultaneously administered drugs.

EXPERIMENTAL

Reagents and glassware

Toluene, analytical grade from E. Merck (Darmstadt, G.F.R.) was distilled once before use. N,O-Bis(trimethylsilyl)acetamide (BSA) of a particularly

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purified grade from Pierce (Rockford, IL, U.S.A.) was used for silylation of the compounds. The glassware was cleaned with 0.1 *N* sulphuric acid in an ultrasonic bath for half an hour and rinsed with distilled water twice.

Extraction procedure

To a centrifuge tube containing 1 ml of plasma, 200 μl of 10% hydrochloric acid were added. The internal standard, trideuterium labelled NPX (NPX-D_3) (60 μg) was added, and the compounds were extracted with 6 ml of toluene by mixing for 5 min in a rotary mixer (20 rpm). After centrifugation for 5 min, the organic phase was transferred to a 10-ml glass stoppered tube containing 1 ml of distilled water and 150 μl of 6.6 *N* sodium hydroxide. The compounds were extracted into the aqueous phase by mixing for 5 min. After centrifugation, the organic phase was discarded. By adding 600 μl of 10% hydrochloric acid and 3 ml of toluene to the aqueous phase, the compounds were extracted into the organic phase, by mixing for 5 min in a rotary mixer. The organic phase was, after centrifugation, transferred to an ethanol-moistened tapered tube and evaporated to dryness under a stream of nitrogen. The residue was dissolved in 100 μl of toluene containing 3% (v/v) BSA and was derivatized for 15 min at 40°C. An amount of 2 μl was injected into the combined gas chromatograph—mass spectrometer.

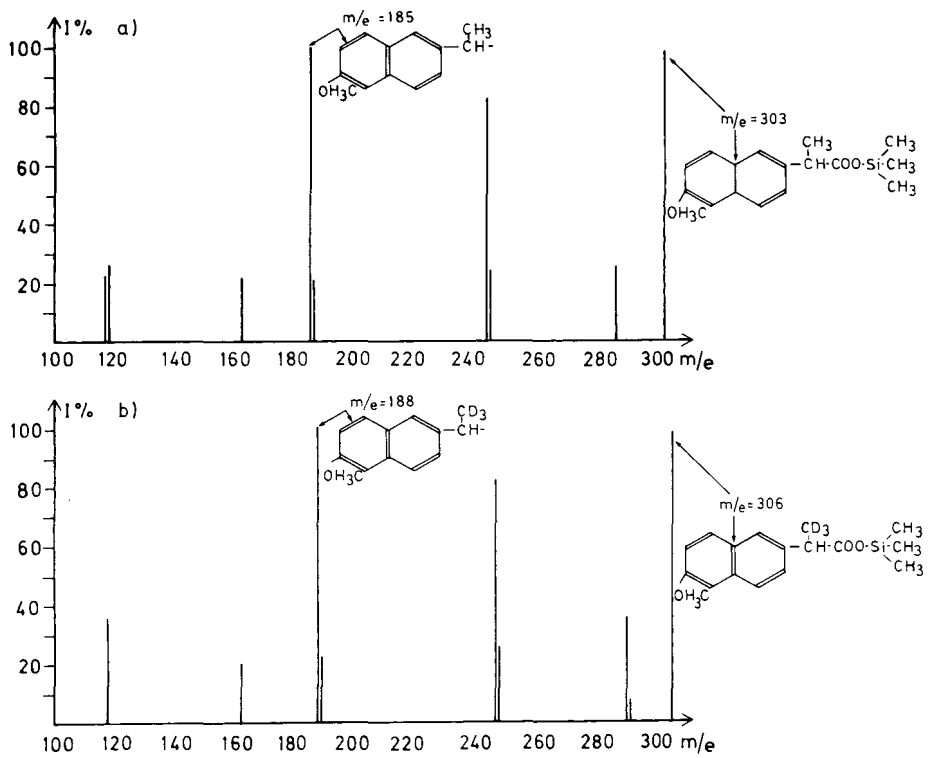

Fig. 1. Mass spectra of (a) silylated naproxen (NPX) and (b) silylated trideuterium labelled naproxen (NPX-D_3). The molecular ions for NPX and NPX-D_3 are *m/e* 303 and *m/e* 306, respectively. Fragments below *m/e* 100 and/or with intensities below 10% have been omitted.

Mass fragmentography

The instrument was a combined gas chromatograph—mass spectrometer (LKB 9000). The mass fragmentographic conditions were as follows: ionization energy, 27 eV; trap current, 60 μA; ion source temperature, 250°C; molecular separator temperature, 235°C; column temperature, 215°C. The column was glass (0.9 m × 2 mm I.D.) packed with 5% FFAP on Diatomite CLQ (100—120 mesh) (J.J.'s Chromatography, Norfolk, Great Britain). NPX and NPX-D_3 are quantified on mass fragments 303 and 306, respectively. Mass spectra of the silylated derivatives of NPX and NPX-D_3 are shown in Fig. 1a and b. Mass fragmentograms of plasma samples containing increasing amounts of NPX are shown in Fig. 2.

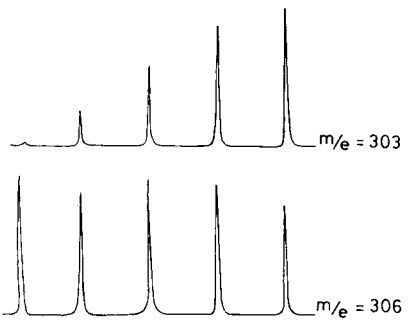

Fig. 2. Mass fragmentograms of plasma samples containing (from left to right), 0, 20, 40, 60, and 80 mg/l of naproxen.

Calculations

The plasma concentrations were read from standard curves drawn on the basis of chromatograms of plasma samples containing varying, but known amounts of naproxen giving concentrations from 20 to 80 mg/l. The peak height ratios between NPX—BSA and NPX-D_3—BSA were plotted against the concentrations. A straight line through the origin was obtained.

RESULTS AND DISCUSSION

The main reason for the development of this mass fragmentographic method was the need for an adequate method for the measurement of the unbound fraction of NPX in human plasma, i.e. concentrations of about 0.1 mg/l. The constitution formula of NPX shows the presence of a carboxylic acid group. In spite of the use of an FFAP stationary phase, which has been developed particularly for the chromatographic separation of compounds containing COOH groups, some tailing on the peaks representing NPX and the internal standard was observed. Attempts to quantify NPX in concentrations of 0.1 mg/l failed, probably owing to absorption phenomena in the glass column. However, in the case of silylation with BSA, concentrations far below 0.1 mg/l are detectable with narrow and symmetric peaks (Fig. 2). The figure illustrates mass fragmentograms of plasma samples containing NPX in varying, but known amounts. The left peak shows a plasma blank with added internal standard. The detector measures NPX on the fragment of *m/e* 303 and the internal standard on the

TABLE I

ACCURACY TEST FOR NAPROXEN AT DIFFERENT CONCENTRATION LEVELS

Concentration added	No. of samples	Observed concentration* (%)
20 μg/l	10	21.6 ± 8.4
40 μg/l	10	39.0 ± 3.8
60 μg/l	10	60.5 ± 2.1
80 μg/l	10	78.5 ± 2.1
20 mg/l	20	21.7 ± 3.6
40 mg/l	20	40.2 ± 1.8
60 mg/l	20	59.5 ± 1.9
80 mg/l	20	77.7 ± 2.1

*Mean ± C.V.

fragment of *m/e* 306. As will be seen from Fig. 2, blank plasma samples produce only a negligible signal on the fragment of *m/e* 303 because the internal standard contains small amounts of unlabelled compound. From a clinical point of view, this small deviation from zero of the peak heights of NPX and NPX-D_3 is of no significance.

Accuracy tests were performed on 120 plasma samples containing NPX in varying concentrations (Table I). The lower limit for quantification (sensitivity) was found to be below 5 μg/l in the case of a plasma volume of 1 ml. The precision of the method gave a coefficient of variation below 10% even in the lowest concentration range (20 μg/l).

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REFERENCES

1 R. Runkel, M. Chaplin, G. Boost, E. Segre and E. Forchielli, J. Pharm. Sci., 61 (1972) 703.
2 D. Westerlund and A. Theodorsen, J. Chromatogr., 144 (1977) 27
3 A. Mortensen, E.B. Jensen, P.B. Petersen, S. Husted and F. Andreasen, Acta Pharmacol. Toxicol., 44 (1979) 277.
4 K.M. Piafsky and O. Borgå, Clin, Pharmacol. Ther., 22 (1977) 545.
5 D.J. Ellis and B. Martin, Fed. Proc., Fed. Amer. Soc. Exp. Biol., 30 (1971) 864.

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Note

Rapid determination of anticonvulsant drugs by isothermal gas—liquid chromatography

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(First received June 24th, 1980; revised manuscript received October 6th, 1980)

Several gas chromatographic methods are available for measuring anticonvulsant drugs in serum. A large number of these techniques use chromatography in the form of their derivatives; methylation is the most commonly used procedure [1—4], another possibility is silylation [5,6]. Recently, two special column packings, GP 2% SP 2510 DA and 2% SP 2110/1% SP 2510 DA on Supelcoport 100—120 mesh have been developed for the separation of anticonvulsant drugs as their metabolites in the underivatized form [7]. Temperature programming was used with the packing 2% SP 2510 DA [7], but working in a routine laboratory it is necessary to use a fast, simple and accurate technique.

In this paper some further experience with the packing 2% SP 2110/1% SP 2510 is reported in the determination of all the more commonly used anticonvulsant drugs without derivatization under isothermal conditions.

Phenobarbital (PB), carbamazepine (CZ), primidone (PD) and phenytoin (DPH) were analysed at 240°C on a column 2 m × 2 mm I.D. and at 225°C on a column 1 m × 2 mm I.D.; ethosuximide (Etx) was analysed at 120°C and at 110°C on the respective columns.

MATERIALS AND METHOD

Reagents

The drugs and internal standards were obtained from Supelco (Bellefonte, PA, U.S.A.). Methylene chloride was analytical grade (E. Merck, Darmstadt, G.F.R.).

The combined internal standard was prepared by convenient dilution in

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methanol of individual stock solutions (1 mg/ml in methanol) to make a solution of 100 μg/ml for 5-methyl-5-phenylhydantoin (MPH) and for 5-(*p*-methylphenyl)-5-phenylhydantoin (MPPH), and 200 μg/ml for α,α-dimethyl-β-methylsuccinimide (MS).

A reference mixture solution of anticonvulsant drugs was formed from individual stock solutions (1 mg/ml in methanol) to contain the following concentrations (μg/ml in methanol): 40.0 Etx, 20.0 PB, 10.0 CZ, 10.0 PD and 20.0 DPH; these solutions were stable at −20°C.

Procedure

Groups of extraction tubes were prepared by adding 100 μl of combined internal standard solution to 10-ml screw-capped glass tubes; the methanol was evaporated with nitrogen at room temperature. These tubes were capped and stored at 4°C until use. A 0.5-ml aliquot of a patient's serum was added to the extraction tubes, followed by one drop of hydrochloric acid. After each addition the mixture was mixed for 30 sec in a rotamixer. Then 5 ml of methylene chloride were added and the tube contents vortex-mixed for 5 min at 25 rpm. After centrifugation at 3400 *g* the aqueous layer was aspirated carefully and the organic layer transferred to 8-ml PTFE-lined screw-capped tubes (Kimax 13 × 100 mm 45066), and evaporated carefully to dryness with nitrogen at room temperature. The dried extract was dissolved in 60 μl of methylene chloride and 1.5 μl were injected into the column.

A reference mixture and a serum blank were treated in the same way as the patient's serum. For the reference mixture, 0.5 ml of reference mixture solution is added to the extraction tube and mixed for 30 sec; the methanol is then evaporated to dryness and 0.5 ml of drug-free serum is added to the tube without internal standard.

Chromatography

Two glass columns (2 m × 2 mm I.D. and 1 m × 2 mm I.D.) were filled with 2% SP 2110/1% SP 2510 DA on Supelcoport 100—120 mesh and were mounted in a Perkin-Elmer gas chromatograph Model 3920B equipped with flame-ionization detectors. Nitrogen (50 ml/min for each column) was the carrier gas. Hydrogen (30 ml/min) and air (400 ml/min) were supplied to the detector.

Injector and detector temperatures were 250°C. The analysis was run isothermally at 240°C and 225°C on the 2 m and 1 m columns, respectively, for MPH, PB, CZ, PD, DPH and MPPH, and at 120°C and 110°C on the two columns, respectively, for Etx and MS; later the column temperature was elevated to elute the other drugs. Amplifier settings were: range 10; attenuation 16.

RESULTS AND DISCUSSION

Fig. 1 shows chromatograms obtained with a drug-free serum supplemented with the reference mixture solution, and a serum blank. Phenylmethyl malonamide (PEMA), a metabolite of Primidone, elutes before MPH. Cholesterol did not interfere with any of the drugs — it has a retention time longer than those of the drugs studied — and under our conditions it is not detected. In the blank no peaks at the retention times of the drugs were observed. The only problem

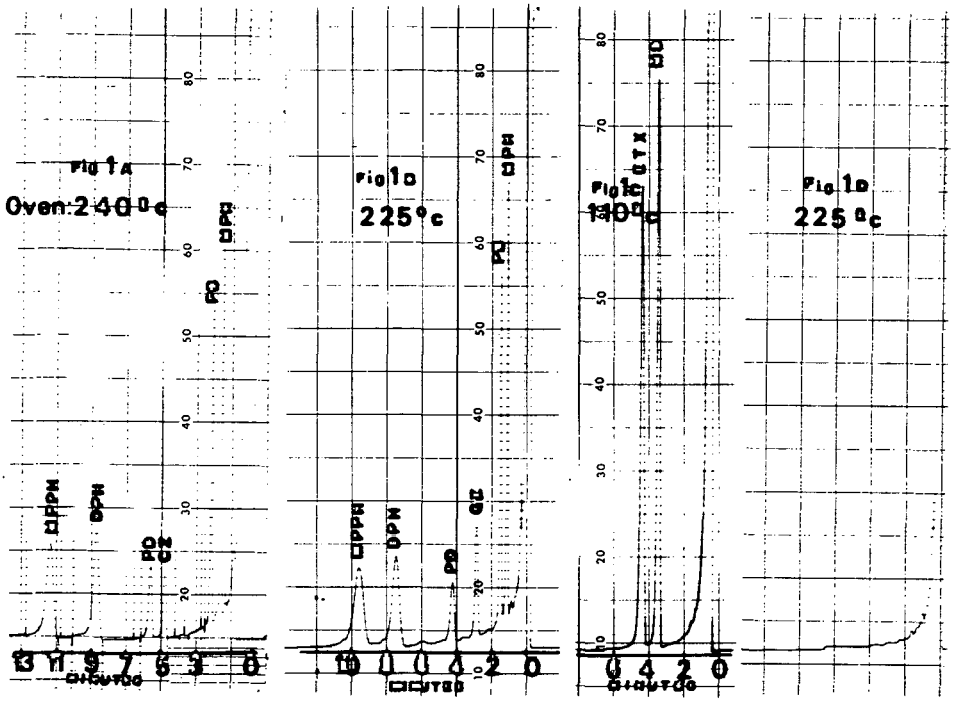

Fig. 1. Chromatograms of a drug-free serum supplemented with the reference mixture solution (A, B, C) and a serum blank (D). Column measurements: 2 m × 2 mm (A), and 1 m × 2 mm (B, C, D).

encountered with the blank was an unidentified interfering substance which co-eluted with CZ and originated from a single lot of methylene chloride.

MPH was used as internal standard for PB, CZ and PD, MPPH for DPH, and MS for Etx. The peak areas were measured with an integrator M2 (Perkin-Elmer). To calculate the concentrations the peak area was compared with that obtained for the reference compound. Linearity of response versus concentration was determined for all drugs studied (Fig. 2). The calibration factors were similar for PB, PD, DPH and Etx on the columns of both lengths, but for CZ it was about 0.94 on the 1-m column.

The analytical recovery was evaluated by spiking drug-free serum with reference mixture solution, and comparing the results with those obtained by direct injection of the same amount of reference mixture solution; the percentages recoveries were about 97 (PB), 104 (CZ), 85 (PD), 98 (DPH) and 104 (Etx).

The precision of the analysis was determined using a pool of drug-free serum spiked with PB, CZ, PD, DPH, and Etx, which was mixed and divided into aliquots and frozen at —20°C; twelve samples were analysed simultaneously and twelve at different times. The results are given in Table I.

The accuracy was established by analysis of serum samples from the Anti-epileptic Drug Level Control Program.

The column of 1 m length was preferred, the analysis time being 11 min and the column temperature was lower than when using the 2-m column. This

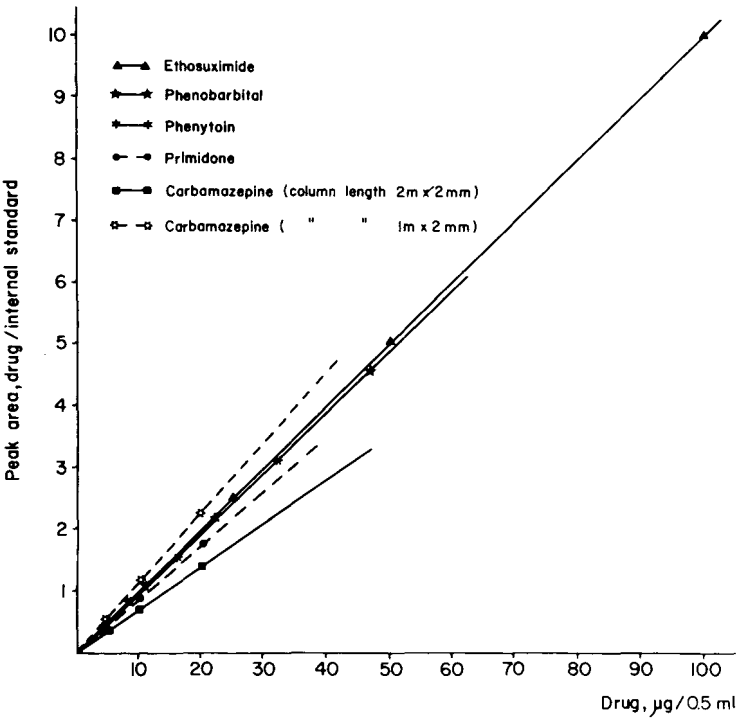

Fig. 2. Calibration curves for each drug studied after extraction and GLC. The area ratio value for ethosuximide is multiplied by 2.

TABLE I

PRECISION OF ANTIEPILEPTIC DRUG ASSAY IN SERUM

Measurements were made after extraction and GLC on a column 2 m × 2 mm I.D. (n = 12).

Drug	Mean concentration (µg/ml)	C.V. (%)	
		Within-day	Between-day
Ethosuximide	49.4	5.2	5.8
Phenobarbital	24.2	2.6	3.4
Carbamazepine	8.4	5.7	6.0
Primidone	9.2	7.0	7.4
Phenytoin	16.0	4.3	6.7

is better for the life of the stationary phase and also the calibration factor for CZ is closer to 1.

Bredensen [8] recently reported the analysis of PB, CZ, PD and DPH without derivatization, with a mixture GP 2% SP 2510 DA and 3% OV-17 under isothermal conditions, but he used only one internal standard for quantitation of the four drugs and the packing is not available commercially. With pure 3% OV-17 the isothermal separation of anticonvulsant drugs is not satisfactory [9].

REFERENCES

1 H. Malkus, P.J. Jatlow and A. Castro, Clin. Chim. Acta, 82 (1978) 113.
2 A. Kumps and Y. Mardens, J. Chromatogr., 182 (1980) 116.
3 E.M. Baylis, D.E. Fry and V. Marks, Clin. Chim. Acta, 30 (1970) 93.
4 J. MacGee, Anal. Chem., 42 (1970) 421.
5 T. Chang and A.J. Glazko, J. Lab. Clin. Med., 75 (1970) 145.
6 H.J. Kupferberg, J. Pharm. Sci., 61 (1972) 284.
7 W. Godolphin and J. Thoma, Clin. Chem., 24 (1978) 483.
8 J.E. Bredensen, Clin. Chem., 25 (1979) 1669.
9 J.E. Bredensen and S.I. Johannessen, Epilepsia, 15 (1974) 611.

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Note

Rapid quantitation of flurazepam and its major metabolite, N-desalkylflurazepam, in human plasma by gas—liquid chromatography with electron-capture detection

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Flurazepam is a 1,4-benzodiazepine widely used as a hypnotic agent [1, 2]. Some investigations have provided evidence that flurazepam is extensively metabolized to derivatives that show a pharmacological activity like the parent drug.

The major metabolite, N-desalkylflurazepam, shows a plasma concentration in man after flurazepam administration that is at least 50 times as high as the flurazepam concentration. Moreover, the elimination half-life of this metabolite is about 47—100 h, while the elimination half-life of flurazepam is very short [3, 4]. Therefore it is possible that in the evaluation of eventual correlations between plasma levels and clinical effects of this drug, the concentrations of both flurazepam and N-desalkylflurazepam must be carefully considered.

For these reasons, we have developed a rapid and sensitive gas chromatographic method for the determination of plasma concentrations of flurazepam and N-desalkylflurazepam in man using electron-capture detection (ECD).

EXPERIMENTAL

Reagents and standards

Flurazepam and N-desalkylflurazepam were obtained from Robin Co. (Milan, Italy), clobazam (the internal standard) was from Hoechst (Milan, Italy), benzene and acetone from Merck (Darmstadt, G.F.R.) and buffer solution (sodium tetraborate, pH 9.00) from Riedel De Haen (Hannover, G.F.R.).

Apparatus

A Carlo Erba Fractovap 2150 gas chromatograph equipped with a ^{63}Ni elec-

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tron-capture detector, an ECD control Model 250 operating at a constant current, and a Hewlett-Packard 3380A recorder—integrator were used. The glass column (1 m × 3 mm I.D.) was pre-treated with dimethyldichlorosilane (Carlo Erba, Milan, Italy) and packed with 3% OV-17 on 100—120 mesh Gas-Chrom Q (Applied Science Labs., State College, PA, U.S.A.). The injector temperature was 300°C, column temperature 250°C, detector temperature 300°C, and the flow-rate of the carrier gas (nitrogen) was 50 ml/min.

Standard solutions

Stock solutions of flurazepam, N-desalkylflurazepam and clobazam were prepared in acetone to give a concentration of 1 mg/ml for each compound. Plasma standards of 5, 10, 25, 50, 100, 200 and 400 ng flurazepam and N-desalkylflurazepam per millileter of plasma (calibration samples) were prepared by adding exact volumes of standard solutions of 50 ng/ml of both compounds to drug-free pooled plasma. Plasma standards were then divided into 1-ml samples, stored at 4°C and analyzed within two weeks.

Extraction procedure

To 1-ml plasma standards, 0.5 ml of buffer solution and 4 ml of benzene were added. The test-tubes were mechanically shaken for 10 min, then centrifuged and the organic phase was evaporated to dryness with a vacuum evaporator at 45°C. The residue was redissolved in variable volumes (100—800 μl) of internal standard solution (clobazam, 0.5 ng/ml in acetone) as specified in the Results and discussion section. One or two microliters of this solution were injected into the gas chromatograph.

RESULTS AND DISCUSSION

The correlation between the peak area ratios (drugs/internal standard) and plasma concentrations when all extracts were redissolved in 100 μl of internal standard solution is shown in Fig. 1. The graphs show a non-linear correlation between detector response and plasma concentration over 50 ng/ml (about

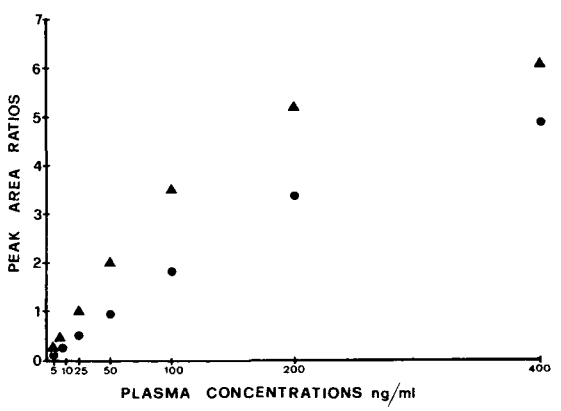

Fig. 1. Correlation between reading and plasma concentrations of flurazepam (•) and N-desalkylflurazepam (▲) in calibration samples obtained by redissolving the residues in 100 μl of internal standard solution.

500 pg injected into the gas chromatograph). In order to obtain a linear correlation between plasma concentration and detector response, new calibration curves with further dilutions of extracts from plasma at initial concentrations of both compounds over 50 ng/ml were made. Extracts of plasma standards at concentrations (for both substances) of 100, 200 and 400 ng/ml were redissolved, respectively, in 200, 400 and 800 μl of internal standard solution, while extracts at concentrations of 5, 10, 25 and 50 ng/ml were redissolved in 100 μl of internal standard solution as described before. The curves so obtained are shown in Fig. 2.

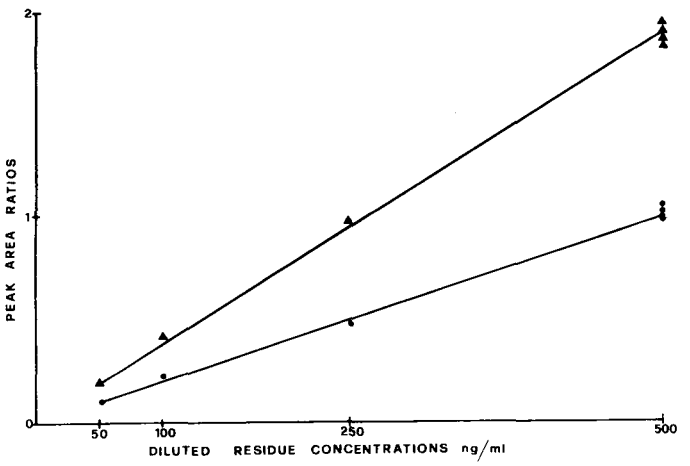

Fig. 2. Calibration curves for flurazepam (•) and N-desalkylflurazepam (▲). The residues obtained from the calibration series were redissolved in different volumes of internal standard solution in order to inject compound quantities within the linear range (see text for explanation).

A previous study [3] utilizing a spectrofluorimetric method has demonstrated that after a single oral dose (30 mg) in man, peak plasma concentrations of N-desalkylflurazepam range from 10 to 20 ng/ml, and that after two weeks of treatment (30 mg daily) plasma concentrations rose to 49—142 ng/ml; flurazepam plasma concentrations were below the sensitivity limit of the assay (3—4 ng/ml) throughout the study.

Thus it is possible that, when using our method, clinical samples containing unknown quantities of the analytes must be injected and read twice: for flurazepam after a first redissolution into 100 μl of internal standard solution and for N-desalkylflurazepam after a further redissolution into a multiple volume of internal standard solution depending on the first reading. This procedure of injecting total concentrations lower than 500 pg and thus in the linear range of the curve, seems to be preferable to the use of two different internal standards at different concentrations or to the other possible procedures. Readings are finally corrected for the dilution.

Fig. 3 shows representative chromatograms of two extracts from calibration curve samples. Plasma concentrations of flurazepam and its metabolite were 10 ng/ml of each (A) and 400 ng/ml of each (B); the relative residues were re-

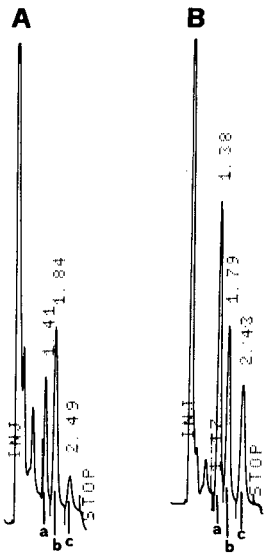

Fig. 3. Gas chromatographic response of two calibration samples. (A) Extract from 1 ml of plasma containing 10 ng of each drug redissolved in 100 μl of internal standard solution. (B) Extract from 1 ml of plasma containing 400 ng of each drug redissolved in 800 μl of internal standard solution. a= N-desalkylflurazepam; b= clobazam; c= flurazepam.

TABLE I

RECOVERY AND REPRODUCIBILITY FROM HUMAN PLASMA SAMPLES

Amount added	Recovery* (% ± S.D.)		Reproducibility** (amount found ± S.D.) (C.V.)	
	Flurazepam	N-Desalkyl-flurazepam	Flurazepam	N-Desalkyl-flurazepam
5	90 ± 3.8	84 ± 4.1	4.9 ± 0.30 (6.0)	5.0 ± 0.31 (6.2)
10	91 ± 3.2	83 ± 3.9	9.9 ± 0.50 (5.0)	10.1 ± 0.54 (5.4)
25	89 ± 3.5	86 ± 4.0	25.1 ± 1.51 (6.0)	24.9 ± 1.60 (6.4)
50	92 ± 4.0	81 ± 3.8	49.0 ± 2.60 (5.2)	50.1 ± 3.25 (6.5)
100	89 ± 3.8	83 ± 3.9	102.0 ± 2.4 (4.8)	100.1 ± 7.11 (7.1)
200	93 ± 3.9	84 ± 4.1	203.2 ± 8.9 (4.4)	199.2 ± 13.11 (6.5)
400	93 ± 4.1	83 ± 4.0	404.1 ± 17.1 (4.3)	400.3 ± 22.31 (5.6)

* Mean of five determinations.

** Mean of ten determinations performed over a two-week period.

dissolved, respectively, in 100 μl and 800 μl of internal standard solution so that the quantities injected were in the linear range.

The minimal detectable concentration of flurazepam and its metabolite was 1 ng/ml of plasma.

The recovery and reproducibility of the method at seven different concentrations are shown in Table I.

To determine the potential usefulness of the procedure, we checked for possible interferences from other benzodiazepines (diazepam, 3-hydroxydiazepam, N-desmethyldiazepam, oxazepam, clonazepam, flunitrazepam, lorazepam, nitrazepam and chlordiazepoxide) by chromatographing solutions of pure standards. We did not observe any interference in the chromatograms that might have altered the flurazepam and N-desalkylflurazepam analyses.

From the reported results we consider that the method is sufficiently rapid, sensitive and specific for utilization in pharmacological studies in man.

REFERENCES

1 A. Kales, E.O. Bixler, M.B. Scharf and J.D. Kales, Clin. Pharmacol. Ther., 19 (1976) 576.
2 M.B. Balter and M.L. Bauer, in A.D. Clift (Editor), Sleep Disturbance and Hypnotic Drug Dependence, Excerpta Medica, Amsterdam, 1975, p. 262.
3 S.A. Kaplan, J.A.F. de Silva, M.L. Jack, K. Alexander, N. Strojny, R.E. Weinfeld, C.V. Puglisi and L. Weissman, J. Pharm. Sci., 62 (1973) 1932.
4 D.D. Breimer, in R.G. Priest, A. Pletsher and J. Ward (Editors), Proceedings of the Northern European Symposium on Sleep Research, Basle, September 26—27, 1978, MTP Press, Basle, 1979, p. 63.

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Note

Simple and rapid gas—liquid chromatographic method for estimating carbamazepine in serum

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A simple and rapid gas—liquid chromatographic (GLC) method for estimating serum carbamazepine is presented. The method has been in continuous use for more than two years in this laboratory. The extraction and chromatography are based on the work of Gardner-Thorpe et al. [1], Toseland et al. [2] and Teasdale [3]. Carbamazepine is chromatographed underivatised and the resultant column effluent has been confirmed to be carbamazepine by infrared spectroscopy.

MATERIALS AND METHCD

The instrument used was a Perkin-Elmer F11 gas chromatograph equipped with dual flame ionisation detectors. A single 1 m × 2 mm I.D. glass column packed with 1% cyclohexane dimethanol succinate in methylene chloride on Diatomite C.Q. 100—120 mesh (Pye Unicam, Cambridge, Great Britain) was used. The column was conditioned for 15 h overnight at 230°C with a carrier flow-rate of 10 ml/min.

The following reagents are required for the extraction procedure: chloroform (A.R.), 5 *M* sodium hydroxide and an internal standard solution containing 0.7 mg dehydroepiandrosterone (BDH, Poole, Great Britain) in 100 ml chloroform. The extraction is performed as follows: 1 ml of internal standard solution is added to a glass-stoppered centrifuge tube (105 × 14 mm) and the contents evaporated to dryness; 1 ml of sample (or standard), 1 ml of 5 *M* sodium hydroxide and 5 ml chloroform were added to the tube. The contents

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were shaken mechanically for 10 min, then centrifuged at 650 *g* for 5 min. The upper aqueous layer including the protein precipitate was removed by suction. The chloroform layer was transferred to a 105 × 15 mm conical centrifuge tube and evaporated to dryness under a stream of nitrogen at 60°C. The side of the tube was washed with 1 ml chloroform and again evaporated to dryness. The residue was dissolved in 20 μl chloroform and a 3-μl aliquot was injected on to the column under the following conditions: flow-rates, carrier gas (helium), 45 ml/min; hydrogen, 18 ml/min; air, 29 ml/min; temperatures, injection port, 275°C; oven, 250°C and amplifier setting 1×10^2.

To facilitate effluent collection a Perkin-Elmer Sigma 2 gas chromatograph equipped with 10 : 1 effluent splitter was used. The column and analytical conditions were identical with the F11 operation and identical peaks were obtained on both gas chromatographs.

The effluent was examined by infrared spectroscopy using a Perkin-Elmer 720 infrared spectrometer. The samples were supported as Nujol mulls between sodium chloride plates.

RESULTS AND DISCUSSION

A typical chromatogram is shown in Fig. 1. The retention times of dehydroepiandrosterone and carbamazepine were 5.5 min and 6.8 min, respectively. Precision was assessed by analysing a pooled serum of unknown value in replicate (n = 29) within one day. The coefficient of variation was 8.66% (mean = 23.31 μmol/l, S.D. = 2.02 μmol/l). A further pooled specimen was analysed on each subsequent day of analysis to assess day-to-day variation. The coeffi-

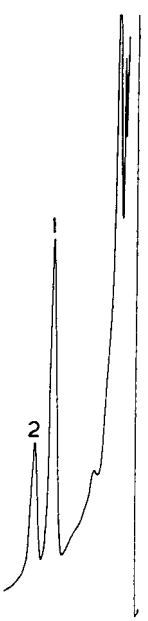

Fig. 1. Chromatogram of serum extract from a patient taking carbamazepine medication showing dehydroepiandrosterone (1) and carbamazepine (2). Chromatography conditions are as given in Materials and Method. The patient's carbamazepine value is 20.2 μmol/l.

cient of variation was 9.18% (mean 23.7 μmol/l; S.D. = 2.17 μmol/l; n = 40).

Participation in the International Quality Control Scheme run by Dr. Alan Richens, provided us with an assessment of accuracy of our results. There is a good correlation (r = 0.96, n = 23) between the authors' laboratory results and the mean of the international results. The equation of the regression line between the two sets of results is $y = 0.986x + 0.96$ (y = authors' result, x = inter-

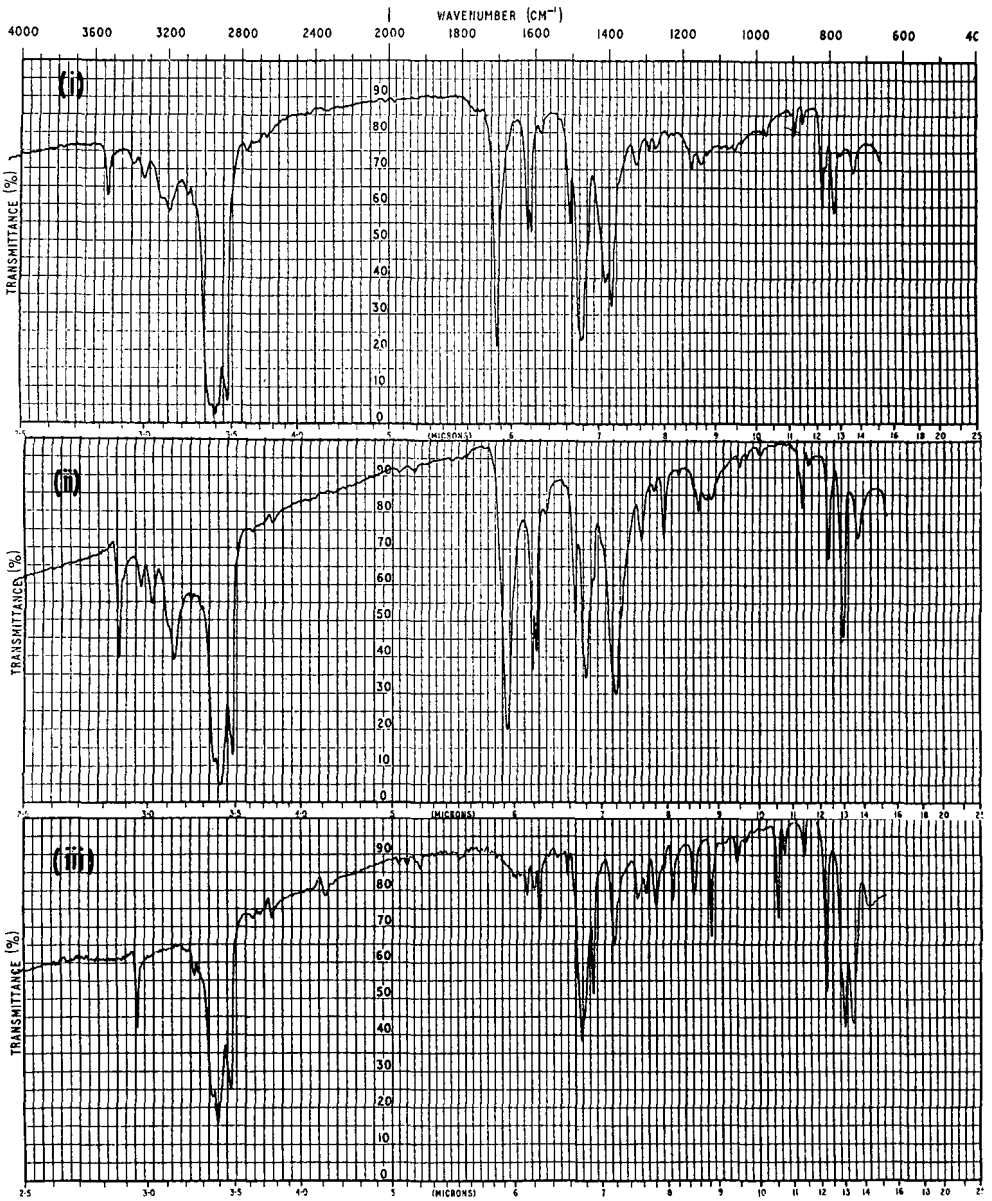

Fig. 2. Infrared spectra of (i) column effluent, (ii) carbamazepine and (iii) iminostilbene measured in Nujol with sodium chloride windows.

national mean). The method is linear up to at least 100 μmol/l, and recoveries for the method are between 97.5% and 108%.

Interference from other commonly prescribed antiepileptic drugs has not been encountered, indicating the method to be highly specific for carbamazepine.

Morselli and Frigerio [4] have shown that the major problem associated with the GLC determination of therapeutic levels of carbamazepine in plasma is the weak thermal stability of the drug and the ease with which it undergoes on-column, acid catalysed degradation and rearrangement to multiple products. In the method described by Chambers and Cooke [5], carbamazepine is reported to undergo complete hydrolysis to iminostilbene. However, our work has shown that the extent of hydrolysis of carbamazepine was insignificant. The infrared spectra of carbamazepine, iminostilbene and column effluent are shown in Fig. 2, from which the column effluent is clearly identified as carbamazepine.

The numerous published methods for estimating carbamazepine in serum testify to problems which arise with various methods. The method of Friel and Green [6] has a rather long extraction step and the chromatogram is also longer than with our method. Derivative formation methods such as the method of Kupferberg [7] although overcoming the problem of degradation to iminostilbene usually have prolonged extraction times. Although Mashford et al. [8] report a rapid extraction procedure for their method, our attention is drawn to the rather low recoveries by this method. Sheehan and Beam [9] give an excellent account of the problems associated with iminostilbene formation, indicating the need to use the relative response of both iminostilbene and carbamazepine in calculating the blood levels of carbamazepine. Even so, their chromatograms are too long and also require temperature programming to a very high temperature. Chambers [10] relies on the complete conversion of carbamazepine to iminostilbene. His method is also used to estimate the metabolite carbamazepine-10,11-epoxide, but seems to have rather low recoveries.

The authors' method is simple, rapid, yields good recoveries and is interference-free from other antiepileptic drugs. However, first indications suggest that it may be necessary to temperature programme in order to accommodate the epoxide on the chromatogram. The authors are currently examining this situation in more detail. A further improvement in the method may result from using cyheptamide or possibly imipramine as the internal standard because the chemical structures of these compounds are very similar to carbamazepine. However, the method itself using dehydroepiandrosterone as the internal standard has proved to be very satisfactory and has enabled the laboratory to offer a very rapid and accurate service for estimating carbamazepine in serum.

REFERENCES

1 C. Gardner-Thorpe, M.J. Parsonage, P.F. Smethurst and C. Toothill, Clin. Chim. Acta, 36 (1972) 223.
2 P.A. Toseland, J. Grove and D.J. Berry, Clin. Chim. Acta, 38 (1972) 321.

3 P.R. Teasdale, personal communication.
4 P.L. Morselli and A. Frigerio, Drug Metab. Rev., 4 (1975) 97.
5 R.E. Chambers and M. Cooke, J. Chromatogr., 144 (1977) 257.
6 P. Friel and J.R. Green, Clin. Chim. Acta, 43 (1973) 69.
7 H.J. Kupferberg, J. Pharm. Sci., 61 (1972) 284.
8 M.L. Mashford, P.L. Ryan and W.A. Thomson, J. Chromatogr., 89 (1974) 11.
9 M. Sheehan and R. Beam, J. Pharm. Sci., 64 (1975) 2004.
10 R.E. Chambers, J. Chromatogr., 154 (1978) 272.

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Note

Determination of bromazepam by gas—liquid chromatography and its application for pharmacokinetic studies in man

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(First received August 18th, 1980; revised manuscript received October 24th, 1980)

Bromazepam is a relatively new drug of the well known 1,4-benzodiazepine class of anxiolytic compounds. It is increasingly used and knowledge of its pharmacokinetic properties may contribute to a safe and rational therapy. This necessitates a specific and sensitive measurement in biological fluids. In the past different methodological approaches have been used, such as direct gas—liquid chromatographic (GLC) measurements of unchanged substance [1] or as hydrolyzed 2-amino-5-bromobenzoylpyridine (ABBP) [2, 3] as well as thin-layer chromatography either directly or after derivatization of bromazepam to an azo-dye on the plate [4]. Apparently, all three methods suffer from different pitfalls and limitations (see Discussion). Because bromazepam is avidly absorbed onto the stationary phases of gas chromatographic column packings, we developed a new assay based on the methylation of bromazepam to its N^1-methyl derivative, resulting in a marked improvement of its GLC performance. The method can easily be applied to monitoring plasma levels in patients treated with bromazepam.

EXPERIMENTAL

Chemicals

Diazepam, bromazepam and N^1-methylbromazepam were kindly donated by Hoffmann-La Roche (Grenzach, G.F.R.). Diethyl ether (nanograde) was purchased from Mallinckrodt (Promochem, Wesel, G.F.R.); iodomethane and tetrabutylammonium iodide were from Fluka (Buchs, Switzerland).

Apparatus

A gas chromatograph Varian 2700 equipped with a ^{3}H-Sc-ECD was used and coupled to a 1-mV data recorder (chart paper speed 0.5 cm/min).

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GLC conditions

Purified nitrogen, at a flow-rate of 40 ml/min, was used as carrier gas. The injection port and the electron-capture detector (ECD) were maintained at 320°C. The temperature of the 1-m coiled glass column filled with 0.5% OV-17 Chromosorb G HP 100—200 mesh (Applied Science Labs., State College, PA, U.S.A.) was 270°C. The new column was conditioned once with Silyl-8 (Pierce, Rockford, IL, U.S.A.) and every working day about three injections with a test solution containing diazepam and methylbromazepam were carried out.

Sample preparation

Stock solutions of diazepam (100 ng/ml) and bromazepam (500 ng/ml) were made with benzene. To 0.5—1 ml plasma 10 ng diazepam (internal standard) and 2 ml 0.1 *N* sodium hydroxide were added. A calibration curve between 10 and 100 ng bromazepam per ml was run with blank plasma. Plasma samples were extracted twice with 6 ml diethyl ether. The combined organic phases were concentrated under nitrogen to about 4 ml. After addition of 100 μl iodomethane and 250 μl tetrabutylammonium iodide (0.025 *M* in 0.1 *N* sodium hydroxide) samples were shaken for 1 h at room temperature. Following this methylation reaction the separated ether phase was extracted with 3 ml 1 *N* hydrochloric acid. After centrifugation the upper organic layer was discarded, the aqueous phase alkalinized with 1.5 ml 2.5 *N* sodium hydroxide and back extracted into diethyl ether (7 ml). This separated and purified ether extract was vaporized in silanized glasses and the residue was redissolved in 20 μl of benzene. A 2-μl aliquot of this solution was injected into the gas chromatograph.

Assignment of the peaks

Qualitative peak assignment was carried out in two ways: first, by comparing the retention times of the peaks to those of known reference compounds and secondly, by comparing the mass spectra of the different peaks. Quantitative assignment was performed by calculating the ratios of the peak heights of the drug to that of the internal standard and relating this to a concomitantly constructed linear calibration curve over the concentration range of 10—100 ng/ml.

RESULTS

Many different liquid phases and various procedures for column conditioning were tried to perform the direct measurement of bromazepam according to De Silva et al. [1]. However, due to the strong absorption of this compound broad and excessively tailing peaks were observed (Fig. 1a). Multiple injections of identical standard solutions as well as of biological samples (after different extraction procedures) resulted in a very wide variation of peak height and areas. Besides the very poor reproducibility, sensivity was also bad (lower limit varying between 50 and 100 ng/ml).

The methylation of bromazepam to its N^1-methyl derivative resulted in a significant improvement of the GLC measurements (Fig. 1c). Multiple direct injections of standard solutions of methylbromazepam and diazepam revealed

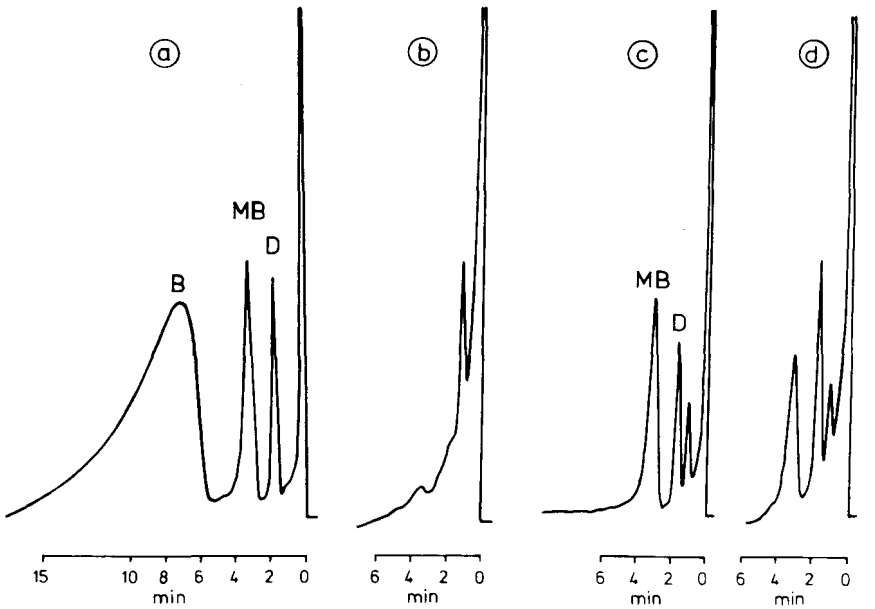

Fig. 1. (a) Chromatogram of standard solutions of the internal standard diazepam (D), N^1-methylbromazepam (MB) and bromazepam (B); concentration of B 25 times higher than that of MB. (b) Chromatogram of an extracted and derivatized control (blank) plasma (two-fold amplified in comparison to a, c and d). (c) Chromatogram of an extracted and derivatized standard plasma spiked with bromazepam and diazepam. (d) Chromatogram of an extracted and derivatized patient's sample.

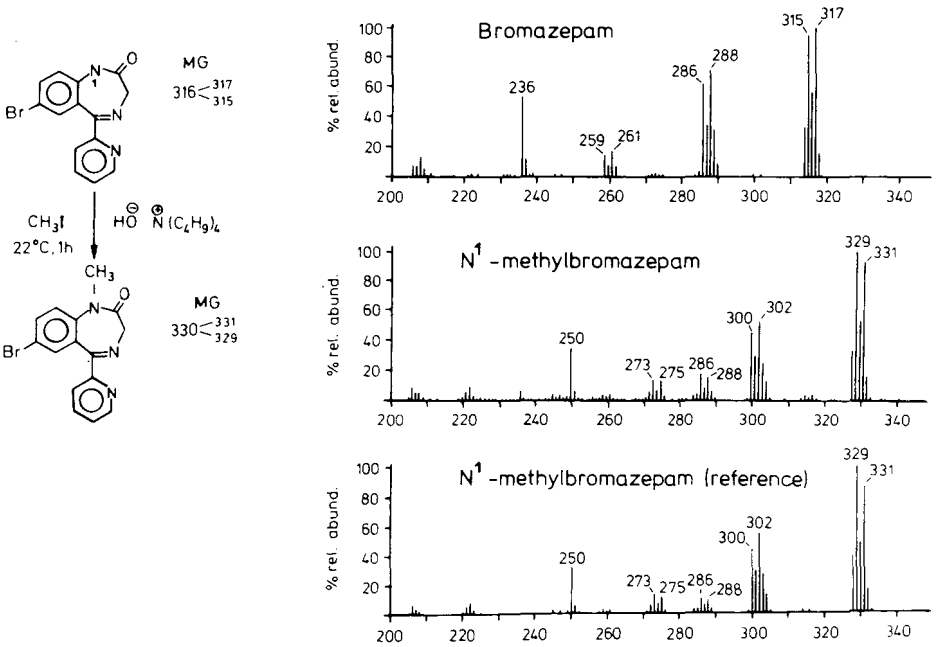

Fig. 2. Mass spectra and formulae of bromazepam (top) and its N^1-methyl derivative (middle), in addition the conditions for the methylation reaction are given (left); for comparison the mass spectrum of an authentic sample of N^1-methylbromazepam (Ro 5-4547) is shown below.

that the variation between different injections was only 3% indicating stabile GLC performance. After the complete extraction and derivatization procedure, recovery averaged 60% and multiple daily analyses (n=10) of a biological quality control sample containing 50 ng/ml revealed a value of 49.8±3.4 (mean ±S.D.).

The GLC peak pattern as well as analysis of the mass spectra proved that the derivatization reaction resulted in a quantitative and specific methylation of the nitrogen in position 1 (Fig. 2). The extensive clean-up procedure was primarily necessary to separate peaks with retention times shorter than that of diazepam. Thereby the samples could be measured at a higher amplifier setting and sensitivity was increased. After the complete sample preparation diazepam and methylbromazepam gave well-shaped, symmetrical peaks with retention times of 1.8 and 3.2 min, respectively. Thus every 5 min a sample could be injected (see Fig. 1). No interfering peaks could be observed in samples of patients not receiving bromazepam (see Fig. 1b). 3-Hydroxybromazepam (major metabolite in plasma) and the benzoylpyridine derivative (major urinary metabolite; 1) have been separated by the multiple steps of derivatization and extraction.

Calibration curves were linear over the concentration range tested (10—100 ng/ml) and the lower limit of sensitivity (5 ng/ml plasma) allows the measure-

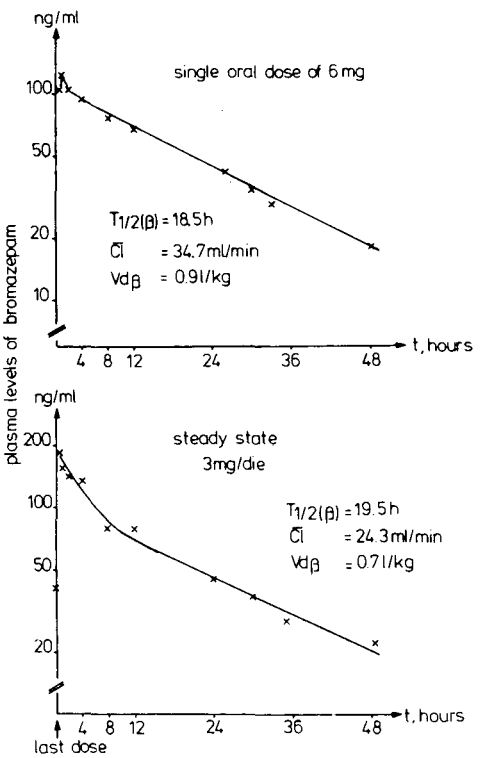

Fig. 3. Plasma concentration—time profiles of bromazepam in the same subject following a single oral dose of 6 mg/day (top) and multiple dosing with 3 mg/day (bottom). The arrow indicates the time of the fifth oral dose of bromazepam. $T_{1/2(\beta)}$ = elimination half-life; $\overline{Cl}$ = total body plasma clearance; Vd_β = apparent volume of distribution.

ment of clinically relevant plasma levels of bromazepam. This can be directly seen from our pharmacokinetic studies. Following a single therapeutic dose of 6 mg bromazepam (Lexotanil®) or the multiple oral dosing with 3 mg per day, plasma concentrations were followed for 48 h in healthy volunteers (Fig. 3). Thus, the new method can be applied for the calculation of all important pharmacokinetic parameters either from single-dose studies or under steady state conditions.

DISCUSSION

Several methods have been published so far for the quantitative analyses of the anxiolytic compound bromazepam [1—4]. However, all assays apparently seem to have their limitations. The direct measurement of bromazepam by GLC—ECD according to De Silva et al. [1] did not give, at least in our hands, satisfactory results. Due to the strong absorption of bromazepam to a variety of stationary phases tested no consistent and reproducible results could be obtained. This problem could not be solved by injecting control blood extracts as suggested by De Silva et al. Their method was also complicated by absorption phenomena since they had to make "10-μl injections of the external standards between every two or three consecutive biological samples". Thus, it appears that the application of this method is not suitable for routine use.

Another approach was the acid hydrolysis of bromazepam to ABBP [2, 3]. This method is not specific for bromazepam since two metabolites of bromazepam yield the same hydrolysis product [1]. This interference could be particularly troublesome after multiple dosing with bromazepam when these metabolites might accumulate in plasma.

More recently Haefelfinger [4] reported a method with thin-layer plates. The direct measurement of the UV reflectance of bromazepam or the colorimetric measurement of ABBP preceded by an extraction procedure does not include an internal standard and the assay shows interferences by unknown plasma constituents and probably by other drugs. Therefore its use in clinical practice might be limited.

Consequently a specific, sensitive and more practicable assay needed to be developed. From our experience with the ECD—GLC measurements of diazepam and its demethylated major metabolite N-desmethyldiazepam [5] we assumed that loss of the nitrogen in position 1 is associated with increasing absorption to the column filling. Therefore derivatization of bromazepam to its N^1-methyl analogue was performed. Such methylation has been successfully applied to the analysis of clonazepam and nitrazepam [6]. As can be seen from Figs. 1 and 2 methylation of bromazepam to N^1-methylbromazepam was specific and quantitative. In contrast to the parent drug the reaction product was not absorbed on the stationary phase and yielded reproducible results with high specificity and sufficient sensitivity. The presented method can be performed by any laboratory equipped with a GLC—ECD system and about 20 samples can be run during one day. From our application to pharmacokinetic questions it can be concluded that the assay can be used also for routine plasma level monitoring. Some important pharmacokinetic parameters have been calculated from the plasma concentration—time profiles (see Fig. 3) and the presented values

are in agreement to the limited literature data [7, 8]. Recently a direct GLC assay for bromazepam was reported [9]; however, the lower limit of sensitivity (600 ng/ml) considerably exceeds the maximal plasma levels (between 50 and 230 ng/ml) observed in man. Thus, this method can not be applied to pharmacokinetic investigations as suggested by the authors.

In summary, this new assay has overcome the difficulties and limitations of published methods and seems to be a method of choice for the determination of bromazepam in human biological fluids.

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REFERENCES

1 J.A.F. de Silva, I. Bekersky, M.A. Brooks, R.E. Weinfield, W. Glover and C.V. Puglisi, J. Pharm. Sci., 63 (1974) 1440.
2 J.A.F. de Silva and J. Kaplan, J. Pharm. Sci., 55 (1966) 1278.
3 J.P. Cano, A.M. Baille and A. Viala, Arzneim.-Forsch., 25 (1975) 1012.
4 P. Haefelfinger, Chromatographia, 11 (1978) 10.
5 U. Klotz, G.R. Avant, A. Hoyumpa, S. Schenker and G.R. Wilkinson, J. Clin. Invest., 55 (1975) 347.
6 J.A.F. de Silva and I. Bekersky, J. Chromatogr., 99 (1974) 447.
7 S.A. Kaplan, M.L. Jack, R.E. Weinfeld, W. Glover, L. Weissmann and S. Cotler, J. Pharmacokin. Biopharm., 4 (1976) 1.
8 J. Raaflaub and J. Speiser-Courvoisier, Arzneim.-Forsch., 24 (1974) 1841.
9 T. Kaniewska and W. Wejman, J. Chromatogr., 182 (1980) 81.

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Note

Simultaneous determination of verapamil and its seven metabolites by high-performance liquid chromatography

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Verapamil is an effective antiarginal and antiarrhythmic agent which exerts its effect through inhibition of membrane transport of calcium [1]. Pharmacokinetic studies have shown that verapamil undergoes extensive biotransformation in men and animals, and several N-dealkylated and O-demethylated metabolites have been identified [2,3]. Thus, it seems to be desirable to measure verapamil and its N-dealkylated and O-demethylated metabolites in biological fluids in order to evaluate the pharmacodynamics and disposition kinetics of verapamil.

Recently, Harapat and Kates [4] reported a simple and sensitive high-performance liquid chromatographic (HPLC) method for simultaneous determination of verapamil and norverapamil (N-demethylated metabolite) in plasma. This method is found to be adequate for the determination of verapamil and norverapamil, but it does not allow measurement of the other N-dealkylated metabolites or, moreover, the O-demethylated metabolites.

This paper describes a simple, rapid and selective HPLC method for the simultaneous determination of verapamil and its seven major metabolites (Fig. 1) in plasma. The procedure involves a simple extraction, paired-ion chromatography with reversed-phase column and fluorescence detection. The method can detect 2.5 ng/ml of each component in plasma, which is sufficiently sensitive for pharmacokinetic studies in human subjects.

EXPERIMENTAL

Materials

Verapamil, its seven metabolites (PR21, PR22, PR23, PR24, PR25, D617, D620) and internal standard (D600) were kindly supplied from Knoll (Ludwigs-

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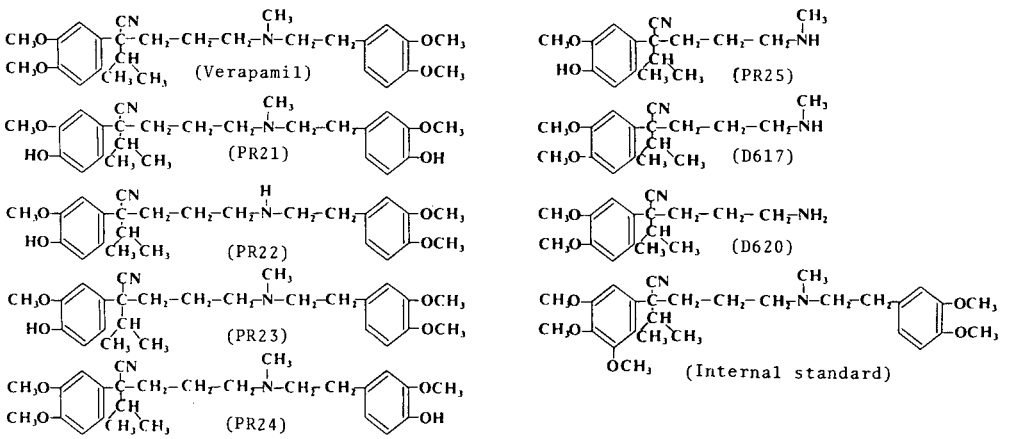

Fig. 1. Chemical structures of verapamil, its seven metabolites and the internal standard.

hafen, G.F.R.). The HPLC-grade acetonitrile and distilled water used for a mobile phase preparation were obtained from Wako Pure Chemical (Tokyo, Japan). The paired-ion reagent, 1-heptanesulfonic acid sodium salt, was obtained from Tokyo Kasei Kogyo (Tokyo, Japan).

Instrumentation

Chromatography was performed on a component system consisting of a Model 100A pump (Altex, Berkeley, CA, U.S.A.), Model 7120 injector (Rheodyne, Berkeley, CA, U.S.A.), Model FS-970 fluorescence detector (Schoeffel, Westwood, NJ, U.S.A.) and Model U-225M dual-pen recorder (Nihon Denshi Kogyo, Tokyo, Japan).

Chromatographic separation was carried out on a 20 cm × 4.6 mm I.D. Nucleosil $5C_{18}$ column (Macherey, Nagel & Co., Düren, G.F.R.).

Extraction procedure

Samples (1 ml) of fresh human plasma spiked with known amounts of verapamil and seven metabolites were transferred to PTFE-lined screw-cap tubes, in which 25 μl (25 ng) of an aqueous solution of internal standard and 1 ml of distilled water were added. The mixture was extracted with 5 ml of diethyl ether for 20 min on a reciprocating shaker. After centrifugation (1000 *g*, 5 min), the aqueous layer was frozen in a dry-ice–acetone bath and the ether layer was decanted into a clean tube. A second extraction was performed in the same way. The combined ether layers were evaporated to dryness under a stream of nitrogen. The residue was reconstituted by vortex mixing in 200 μl of HPLC mobile phase and injected into the column.

Chromatographic conditions

The mobile phase consisted of acetonitrile and aqueous buffer solution. The buffer solution was prepared by dissolving 0.71 g of sodium hydrogen phosphate, 300 μl of phosphoric acid and 2 g of 1-heptanesulfonic acid sodium salt in 1 liter of distilled water. The solution was filtered through a Type HA

membrane filter (Millipore, Bedford, MA, U.S.A.) in vacuo. The mobile phase was then prepared by mixing buffer solution and acetonitrile (1:1, v/v) and the pH adjusted to 4.60 with 0.1 *M* phosphoric acid solution. Chromatographic analysis using the above solvent was carried out at a flow-rate of 1.5 ml/min.

The column temperature was maintained at 30°C by a regulated water-jacket. The excitation monochrometer of a fluorescence detector was fixed at 203 nm and the emission radiation was passed through a 320-nm cut-off filter. A detector range setting of 1.0 μA was used together with a recorder setting of 2 and 10 mV output.

RESULTS

Simultaneous separation of verapamil and its seven metabolites by gas chromatography was difficult because it was not possible to resolve verapamil and its O-demethylated metabolites. Reversed-phase and normal-phase chromatography provided incomplete separations. However, using paired-ion chromatography with a reversed-phase column, separation of verapamil and its seven metabolites in biological samples could be accomplished. Separation of each compound with maximum resolution from each other and from co-extracted contaminants was obtained using a mobile phase of acetonitrile—0.01 *M* phosphoric acid—sodium hydrogen phosphate buffer containing 0.2% 1-heptanesulfonic acid sodium salt (1:1, pH 4.60).

Fig. 2 shows the chromatograms of an extract of 1 ml of blank plasma (A), an extract of 1 ml of plasma sample spiked with verapamil, seven metabolites and internal standard (B), and a mixture of verapamil, seven metabolites and internal standard (C). As can be seen, the nine compounds were separated and eluted within 12 min. The retention times of the nine compounds are as follows: PR25 3.5 min; D620 4.0 min; D617 4.9 min; PR21 5.5 min; PR22 6.7 min; PR23 7.4 min; PR24 7.7 min; verapamil 10.7 min; internal standard

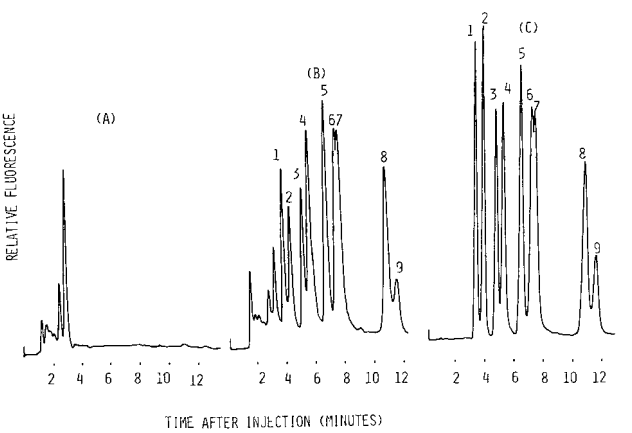

Fig. 2. Chromatograms of (A) an extract of 1 ml of blank plasma, (B) an extract of a 1-ml plasma sample spiked with 25 ng of internal standard and 31.25 ng each of verapamil and seven metabolites, and (C) a mixture of 25 ng of internal standard and 31.25 ng each of verapamil and seven metabolites. Peaks: 1 = PR25, 2 = D620, 3 = D617, 4 = PR21, 5 = PR22, 6 = PR23, 7 = PR24, 8 = verapamil, 9 = internal standard.

11.5 min. The chromatogram of blank plasma demonstrated no interfering peaks under these conditions. PR23 (peak 6) and PR24 (peak 7) were not completely separated from one another (resolution factor 0.60), but quantitation of these compounds was possible by measuring peak heights [5].

Fig. 3 shows the calibration curves which were obtained by analyzing plasma samples spiked with verapamil and seven metabolites in the concentration range 2.5—125 ng/ml. Excellent linearity was achieved for each compound, with the extrapolated plots passing through the origin (correlation coefficient 0.99). The minimum detectable concentration was 2.5 ng/ml of plasma for each compound. The coefficients of variation for the normalized peak height ratios were less than 9.0% for each compound.

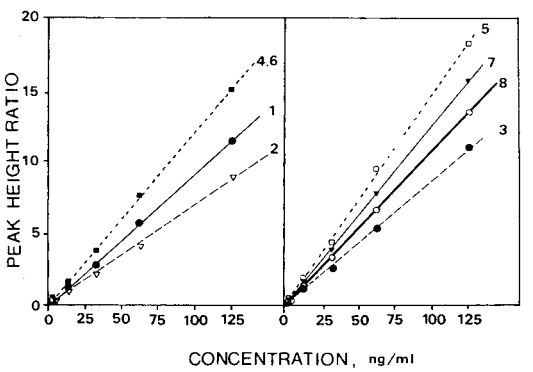

Fig. 3. Calibration curves of verapamil and seven metabolites. 1 = PR25, 2 = D620, 3 = D617, 4 = PR21, 5 = PR22, 6 = PR23, 7 = PR24, 8 = verapamil.

Table I shows the recoveries of each compound over the concentration range stated. The recoveries were estimated by comparing the peak heights of chromatograms obtained from extracted and directly injected samples of each compound. The coefficient of variation for each compound was found to be less than 8.3%. During extraction all compounds were observed to be stable.

TABLE I

RECOVERY OF VERAPAMIL AND ITS SEVEN METABOLITES FROM PLASMA

Values given are percentage recoveries representing the mean ± S.D. of three determinations.

Compound	Concentration in plasma (ng/ml)				
	2.5	12.5	31.25	62.5	125.0
PR25	63 ± 2	63 ± 2	67 ± 4	61 ± 3	58 ± 2
D620	45 ± 3	48 ± 3	55 ± 8	46 ± 5	46 ± 1
D617	55 ± 1	69 ± 8	66 ± 5	71 ± 1	70 ± 2
PR21	80 ± 9	93 ± 8	95 ± 4	97 ± 5	96 ± 2
PR22	80 ± 1	89 ± 5	90 ± 5	94 ± 1	94 ± 3
PR23	87 ± 6	89 ± 4	97 ± 3	99 ± 1	96 ± 4
PR24	93 ± 2	92 ± 4	96 ± 4	99 ± 6	97 ± 4
Verapamil	90 ± 7	90 ± 6	87 ± 4	99 ± 1	98 ± 3

DISCUSSION

An HPLC method for the simultaneous determination of verapamil and norverapamil in plasma has been reported by Harapat and Kates [4]. Their method involves extraction with diethyl ether at basic pH and reversed-phase chromatography using a mobile phase of acetonitrile—0.004 *N* sulfonic acid (pH 2.4). However, this method does not allow one to measure O-demethylated and N-dealkylated metabolites except for norverapamil, since extraction at the basic pH used resulted in a reduction in the extraction efficiency of the O-demethylated metabolites. When a strongly acidic solution is used as mobile phase, the N-dealkylated metabolites are not retained by the reversed-phase column due to the fact that these compounds are ionized in the mobile phase solvent.

In this study, improvement of these disadvantages could be made by changing the extraction procedure and chromatographic conditions. This method has advantages that include simple and specific determination of verapamil metabolites but do not involve special and time-consuming procedures. In addition, the method can detect up to a level of 2.5 ng of verapamil and its seven metabolites in 1 ml of plasma, which is sufficiently sensitive for pharmacokinetic studies.

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REFERENCES

1 B.N. Singh, G. Ellorodt and C.T. Peter, Drugs, 15 (1978) 169.
2 H.M. Mcilhenny, J. Med. Chem., 14 (1971) 1178.
3 M. Eichelbaum, M. Ende, G. Remberg, M. Schomerus and H.J. Dengler, Drug. Metab. Dispos., 7 (1979) 145.
4 S.R. Harapat and R.E. Kates, J. Chromatogr., 181 (1980) 484.
5 L.R. Snyder, J. Chromatogr. Sci., 10 (1972) 200.

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Note

Determination of the melanotropin-inhibiting factor analogue pareptide in urine by high-performance liquid chromatography

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Melanotropin-inhibiting factor (MIF), Pro-Leu-Gly-NH_2, has recently been shown to greatly potentiate the therapeutic efficacy of L-DOPA in the treatment of parkinsonism [1, 2]. However, MIF is hydrolyzed rapidly in tissues [3, 4]. Pareptide*, an analogue of MIF, was synthesized in the Ayerst Research Labs. This compound mimics the physiological function of MIF, and it has been shown to be more potent and long-lasting because of its resistance to degradation, suggesting that it too may have potential use in the treatment of parkinsonism, and of drug-induced extrapyramidal reactions as well.

MIF derivatized with 5-dibutylaminonaphthalene-2-sulphonyl chloride (Bns-Cl) or dansyl chloride (Dns-Cl) was successfully separated from its metabolites by high-performance liquid chromatography (HPLC) [5]. MIF can be quantitated with the aid of pareptide as an internal standard [3]. Pareptide that was added to human blood plasma was studied by HPLC using 7-chloro-4-nitrobenzyl-2-oxa-1,3-oxadiazole (NBD-Cl) as tagging reagent. However, it gave a high blank and it needed a calibration curve for every experiment [6].

We report here a method that was successfully used for quantitative and qualitative determination of pareptide in urine after oral administration of the drug. The sample is first purified by cation-exchange chromatography, then derivatized with Dns-Cl and studied by HPLC and thin-layer chromatography (TLC). MIF can serve as an internal standard, increasing the speed and

*Pareptide is the commercial name for L-prolyl-N-methyl-D-leucyl-glycinamide.

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the specificity of the method. The method in principle is suitable for following the metabolic rate of administered peptides.

EXPERIMENTAL

Chemicals and reagents

Pareptide was obtained from Ayerst Research Labs. (Montreal, Canada). Dns—Cl was obtained from Pierce (Rockford, IL, U.S.A.). MIF was purchased from Sigma (St. Louis, MO, U.S.A.). Reagent-grade solvents obtained commercially were used in TLC without further purification. In HPLC, the HPLC-grade acetonitrile was obtained from Waters Assoc. (Milford, MA, U.S.A.). The solvent systems were prepared immediately before use; for HPLC they were filtered (0.45 μm) and degassed before use. TLC polyamide precoated microplates (5 × 5 cm, 25 μm thick) were obtained from Schleicher & Schuell (Keene, NH, U.S.A.). Analytical-grade cation-exchange resin AG 50W-X4, 200—400 mesh, H^+ form, was obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.). Radial Pak C_{18} column (200 × 8 mm) was obtained from Waters Assoc. The size of the spherical packing particle was 10 μm. Other chemicals were of reagent grade and were obtained from available commercial sources.

Chromatographic equipment

The HPLC system consisted of a Waters Model 6000 pump, a Waters U6K septumless injector with a 2-ml injection loop, and a Waters radial compression module RCM 100; chromatographic elution was monitored by a Schoeffel FS 970 fluorescence detector (Schoeffel, Westwood, NJ, U.S.A.). Detector output was recorded and processed by a Waters data module.

Measurement of pareptide in urine samples

Three normal subjects (DF-1, DF-2, and DF-3) were given 1.5 g of pareptide, the oral dose recently used for the treatment of parkinsonism, in capsules at 6 a.m. Urine samples were collected 0, 2, 4, 6, 8, 12, and 24 h after the administration of pareptide. The samples were kept at −20°C before the assay.

A 50-μl volume of 6 *N* hydrochloric acid was added to a 0.5-ml urine sample. The sample was then loaded on a small AG 50W-X4 cation-exchange column (5.5 × 0.5 cm), the resin of which was prewashed and swollen with 0.1 *N* hydrochloric acid. After loading of the sample, the column was washed with 1 ml of 0.1 *N* hydrochloric acid followed by 5 ml of water; it was then eluted with 1 *N* ammonia. When the eluate became alkaline, 3 ml of it was collected.

MIF (1.08 μmol) was mixed with 100 μl of the eluate. The mixture was dried by a stream of nitrogen at 60°C. The residue was mixed with 40 μl of water and 10 μl of 0.5 *M* sodium bicarbonate. A 20-μl volume of Dns-Cl in acetone (1 mg/ml) was added to the sample, and the dansylation was carried out at 60°C for 30 min. After the dansylation, 200 μl of acetonitrile was added to the sample, which was mixed well and centrifuged at 8000 *g*; portions of the supernatant were used for TLC and HPLC at ambient temperature.

A 1-μl volume of the dansylated mixture was applied to the TLC plate with the aid of a Hamilton microsyringe. The plate was first developed with solvent system 1: formic acid—water (3:97). When the solvent front reached the top of

the plate, it was removed and dried by a stream of warm air. The plate was then turned 90° and developed with solvent system 2: benzene—acetic acid (9:1). After the development in the second direction, the plate was air dried and examined under UV light (365 nm).

Solvent system acetonitrile—0.01 *M* sodium sulfate buffer pH 7.0 (45:55) was pumped isocratically at a flow-rate of 3 ml/min, resulting in a pressure of 215—430 kg/cm^2. Volumes of 10—20 μl of the samples were used for the study. The elution was detected by fluorescence with the excitation set at 350 nm and the emission at 470 nm, which are the maximal wavelengths for excitation and emission of Dns-MIF and its related peptides [5]. The other settings of the fluoromonitor were: sensitivity, 4.0; full-scale expansion fluorometric response range, 0.02 μA; and time constant, 6.0 sec.

Degradation of MIF and pareptide by tissue

A 45-nmol amount of MIF or pareptide was incubated with tissue homogenate in 150 μl of 0.05 *M* Tris buffer, pH 7.5, at 37°C for 1 h. The reaction was stopped with 7.5 μl of 20% trichloroacetic acid. The sample was centrifuged, and the supernatant was dansylated and studied by HPLC as previously described [3].

RESULTS AND DISCUSSION

Specificity of the method

MIF and its possible metabolites were derivatized with Dns, ethansyl, propansyl, Bns, and monoisopropansyl chloride [5]. NBD has been used as a derivatizing reagent for the determination of pareptide in plasma; however, fluorescence with Dns-pareptide is about five times greater than with the NBD-pareptide derivative [7]. Dns-MIF and its Dns metabolites could be detected at the lowest levels. Dansylation was not used before because the relative instability and high background of plasma derivatized with Dns offsets the higher sensitivity of the determination with this reagent [6]. Since pareptide is more hydrophobic than the natural MIF, its retention time in the reversed-phase column was longer. The retention time for MIF was 6.7 min, and for pareptide it was 9 min (Fig. 1B).

Passing the urine sample through the ion-exchange column purified and stabilized it. Pareptide could be fully (100%) recovered from the column. MIF was eluted from the ion-exchange column earlier than pareptide, and thus any MIF (a natural peptide in the body) was separated from the pareptide. Since pareptide could be fully recovered, MIF added after the ion-exchange column could serve as an internal standard.

Dansylated MIF and pareptide were resolved from each other by reversed phase HPLC using solvent systems containing acetonitrile in phosphate buffer. With excitation at 360 nm and emission at 487 nm the fluorescence of the two compounds was equal. However, when we used the optimal excitation and emission wavelengths (350 nm and 470 nm, respectively) [5] the sensitivity of the detection was increased tenfold (minimal detectable amount was 20 pmol) and pareptide fluorescence was two times that of MIF. The amount of pareptide or MIF derivatized with Dns-Cl had a linear relationship to the

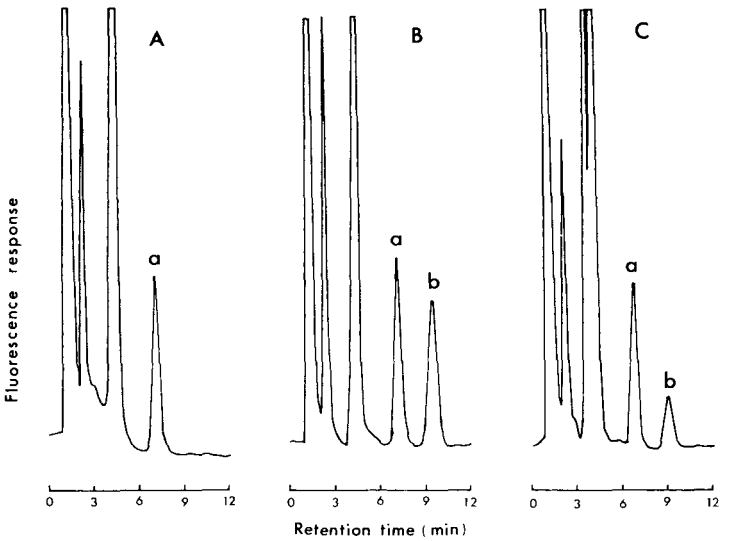

Fig. 1. HPLC chromatograms of urine samples after oral administration of pareptide. A 0.5-ml volume of urine was acidified and purified by cation-exchange chromatography. The isolated pareptide fraction was mixed with internal standard (1.08 μmol MIF), dansylated, and analyzed by HPLC. For details, see Experimental section. (A) 0 time; (B) authentic pareptide (0.54 μmol); (C) 2 h after oral administration of pareptide. Peaks: a = MIF, b = pareptide.

peak heights (or areas) in HPLC [3]. The peak heights or areas of the equivalent amounts of MIF and pareptide were in a straight line, with a ratio of 0.5 within the range of 100 pmol—20 nmol. The experimental error of the method was within 5%. The use of fluorescence had another advantage: there was some absorption at the pareptide region of the HPLC when UV_{254} was used for monitoring. When both MIF and pareptide were derivatized with Dns-Cl, their concentration and the height of their corresponding separated peaks were in linear relationship. MIF was a good internal standard for pareptide, and vice versa, in HPLC.

Two-dimensional TLC on polyamide layers has proved to be a fast and high-resolution qualitative method not only for Dns-amino acids [8] but also for dansylated small peptides. MIF has been separated from its metabolites by such a method [5]. Since pareptide is an analogue and more hydrophobic than MIF, its R_F is greater than that of MIF in each of the two solvent systems we used. With solvent system 2, the R_F values are 0.71 and 0.84, respectively. Dns-MIF and Dns-pareptide are resolved from each other clearly. Even though the compounds are separated at one run, two-dimensional separation isolated them from other dansylated compounds from the urine that interfere in the observation.

At zero time, we did not find any peak in the region of 9 min (pareptide peak) in the chromatogram (Fig. 1A). This proves that after the ion-exchange chromatography and dansylation compounds in the urine did not interfere in our assay by HPLC, which was monitored with fluorescence with excitation at 350 nm and emission at 470 nm. In TLC, there was no fluorescence zone around the Dns-pareptide region.

When authentic pareptide was added to the urine (0 time), we found a symmetrical peak (retention time, 9 min) in the HPLC chromatogram and a confined zone on the TLC plate with an R_F of 0.61 for solvent system 1, and 0.84 for solvent system 2. Pareptide was found by HPLC in the urine collected 2—4 h after oral administration of 1.5 g of pareptide, and the findings were confirmed by TLC.

Peptide absorption and excretion

Pareptide appeared in the urine 2 h after oral administration (Table I) and was present in the urine 12—24 h after its administration. The highest concentration of pareptide in the urine was found 2—4 h after drug administration. In 24 h the total amount of pareptide excreted by urine was 13 mg, which is 0.9% of the amount given orally. In a separate study, using NBD-Cl as a derivatizing agent, the highest peptide concentration (360 ng/ml) was found in blood 2 h after 1.5 g of pareptide was administered orally; this is compatible with our other findings.

The poor absorption of pareptide from the gastrointestinal tract after oral administration was not due to its rapid degradation. When we compared the degradation of MIF with that of pareptide in vitro, we found that virtually no pareptide was split by rat brain, liver, intestinal mucosa, spleen, lung, or plasma (Table II). Pareptide was resistant to hydrolysis even after 19 h of incubation. Under conditions where all of the added MIF was metabolized, pareptide remained intact [4].

Peptides are transported less efficiently than amino acids into the mucosal tissue by carriers different from those that transport amino acids [9]. A number of proline peptides less sensitive to peptidases can pass intact through sacs of everted rat intestine [10]. The absorption of several, perhaps most, of the unmetabolized peptides is very limited. Since the carriers for peptides are specific, and pareptide is an N-methylated D-leucine compound, it is likely that most of the administered drug was not taken up. Pepstatin, a potent cathepsin D inhibitor and a peptide analogue, is not metabolized and is vir-

TABLE I

PAREPTIDE IN URINE AFTER ORAL ADMINISTRATION

Urine samples were collected at the indicated time intervals after oral administration of 1.5 g of pareptide. A, B, and C represent three normal subjects.

Time (h)	Urine collected (ml)			Total amount excreted (mg)		
	A	B	C	A	B	C
0	174	165	146	0	0	0
0— 2	58	69	40	1.3	0.7	0.9
2— 4	150	67	74	5.7	2.8	2.7
4— 6	102	87	93	3.5	1.4	1.8
6— 8	151	83	204	2.9	2.7	1.8
8—12	401	176	374	2.4	3.9	1.7
12—24	266	589	401	0	1.6	1.0
Total	1302	1231	1332	15.8	13.1	9.9

TABLE II

THE DEGRADATION OF MIF AND PAREPTIDE IN RAT TISSUES

An amount of 4.5 nmol of MIF or pareptide was incubated with 0.63 mg tissue in 150 ml of 0.05 *M* Tris buffer, at 37°C for 1 h. The reaction was terminated with 7.5 μl of 20% trichloroacetic acid. The sample was centrifuged, and the supernatant was dansylated and assayed by HPLC. Results for MIF are given; under these experimental conditions there was no detectable metabolism of pareptide by any of the tissue tested. The specific activity for plasma is expressed as nmol MIF split per ml plasma per hour.

Tissue	MIF metabolized (nmol) per mg tissue per hour
Brain	19
Liver	21
Intestinal mucosa	19.5
Spleen	2.8
Lung	1.2
Plasma	53.1

tually unabsorbed on oral administration, but it is excreted in feces [11]. In its absorption in the gastrointestinal tract, pareptide is similar to pepstatin. The fate of pareptide in the body could be studied more quantitatively with the aid of radioactive labelling, which is not yet available. Even though pareptide is poorly absorbed after oral administration, it is still effective as a drug for the treatment of parkinsonism in various laboratory models (Ayerst Research Labs., unpublished observations). Neuropeptides are potent drugs, only a few molecules of which are needed at the target cell to trigger a series of reactions. Furthermore, the affinity between the drug and the receptor plays a critical role in the drug effect. The affinity of MIF and pareptide to a receptor remains to be shown.

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REFERENCES

1 A.J. Kastin and A. Barbeau, Can. Med. Assoc., 107 (1972) 120.
2 F. von Gerstenbrand, H. Binder, C. Kozma, S. Pusch and T. Reismer, Wien. Klin. Wochenschr., 87 (1975) 883.
3 K.-S. Hui, K.-P. Cheng, K.-H. Wong, M. Salshutz and A. Lajtha, J. Neurochem., 35 (1980) 471.
4 K.-S. Hui, A. Lajtha and I. Boksay, Proc. Int. Soc. Neurochem., 1 (1979) 387.
5 K.-S. Hui, M. Salshutz, B.A. Davis and A. Lajtha, J. Chromatogr., 192 (1980) 341.
6 G.T. Krol, J.M. Banovsky, C.A. Mannan, R.E. Pickering and B.T. Kho, J. Chromatogr., 163 (1979) 383.
7 G.T. Krol, C.A. Mannan, R.W. Pickering, D.V. Amato, B.T. Kho and A. Sonnenschein, Anal. Chem., 49 (1977) 1836.
8 N. Seiler and B. Knödgen, J. Chromatogr., 97 (1974) 286.
9 D.M. Matthews, in D.M. Matthews and J.W. Payne (Editors), Peptide Transport in Protein Nutrition, Elsevier, Amsterdam, 1975, p. 61.
10 L.J. Saidel and I. Edelstein, Biochim. Biophys. Acta, 367 (1974) 75.
11 T. Aoyagi and H. Umezawa, in E. Reich, D.B. Rifkin and E. Shaw (Editors), Proteases and Biological Control, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1975, p. 429.

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Note

High-performance liquid chromatographic analysis of pentamethylmelamine and its metabolites in biological fluids

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Pentamethylmelamine (PMM) (Fig. 1), an antitumor agent currently in clinical trial, is an aqueous soluble demethylated metabolite of hexamethylmelamine (HMM). HMM is effective in the treatment of a number of solid tumors, particularly ovarian carcinoma and lung cancer [1]; however, because of its water insolubility HMM is administered orally. PMM has activity similar to that of HMM against a number of animal tumors [2, 3], and both undergo extensive demethylation in vivo [4, 5]. These demethylated metabolites also have antitumor activity [2, 3].

Previous methods for determination of HMM and PMM include gas chromatography [5—7], gas chromatography—mass spectrometry [4], high-performance liquid chromatography (HPLC) [8], and thin-layer chromatography [9]. We now report a rapid, specific HPLC assay that will allow for the determination of the pharmacokinetics of not only PMM but also its demethylated metabolites.

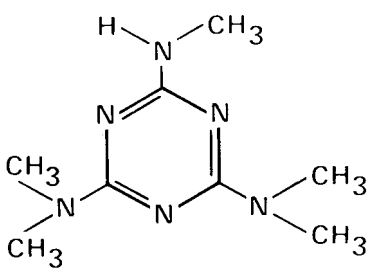


Fig. 1. Structure of pentamethylmelamine.

MATERIALS AND METHODS

Chemicals

Pentamethylmelamine (PMM) and its metabolites N^2,N^2-dimethylmelamine (2,2-DMM); N^2,N^4,N^6-trimethylmelamine (2,4,6-TrMM); N^2,N^2,N^4-trimethylmelamine (2,2,4-TrMM); N^2,N^2,N^4,N^4-tetramethylmelamine (2,2,4,4-TeMM); N^2,N^2,N^4,N^6-tetramethylmelamine (2,2,4,6-TeMM), and monomethylmelamine (MMM) were kindly supplied by Leonard H. Kedda (Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute). Glass-distilled methanol was obtained from Burdick and Jackson Labs. (Saginaw, MI, U.S.A.). All other chemicals were obtained from regular commercial suppliers.

Sample preparation

Biological samples were obtained from patients receiving 80—1500 mg/m^2 of PMM. Blood was drawn into tubes containing heparin and centrifuged to obtain plasma. Urine was collected as voided, and cerebrospinal fluid (CSF) was obtained by lumbar puncture. To 3-ml Clin-Elut tubes (Analytichem International, Lawndale, CA, U.S.A.) containing inert cellulose were added 2 ml of the biological fluid. The tubes were eluted twice with 3 ml of ethyl acetate, with a 5-min delay between elutions. The eluents were collected, combined, and evaporated with a stream of nitrogen. The residues were reconstituted with 100 μl methanol. Aliquots of 10 μl were analyzed by HPLC. Control plasma extracted by the same procedure showed no interfering peaks.

HPLC analysis

Analyses were performed on a Waters Assoc. liquid chromatograph (Milford, MA, U.S.A.) equipped with a M6000 pump, U6K injector, and Model 440 UV detector operating at 254 nm. Peak areas and retention times were determined by a Shimadzu (Kyoto, Japan) Chromatopec-EIA electronic integrator. Separations were achieved on a μ Bondapak C_{18} (10-μm particle size) column using 0.01 *M* ammonium formate (pH 3.5)—methanol (60:40) as eluent at a flow-rate of 1 ml/min.

RESULTS AND DISCUSSION

Fig. 2 shows a chromatogram of an aqueous solution of PMM and its demethylated metabolites. Except for the trimethylmelamines, the PMM metabolites are all well separated, with MMM eluting near the void volume. The capacity factors (k') for the compounds are: PMM, 2.54; 2,2,4,6-TeMM, 1.42; 2,2,4,4-TeMM, 0.91; 2,2,4-TrMM, 0.52; 2,4,6-TrMM, 0.42; DMM, 0.15; MMM, 0.01. The coefficients of variation for three determinations of the extracts of a 1 μg/ml plasma standard were: PMM, 1.9; 2,2,4,6-TeMM, 1.7; 2,2,4,4-TeMM, 1.2; 2,4,6-TrMM, 1.1; 2,2,4-TrMM, 0.64; DMM, 0.98; MMM, 2.4.

Fig. 3 shows the elution profile of plasma from a patient 8 h after the intravenous administration of 640 mg/m^2 of PMM. In this patient, as in most patients studied, no 2,2,4-TrMM was detected; however, all other metabolites were present. The metabolite 2,2,4-TrMM was detected in the plasma of only

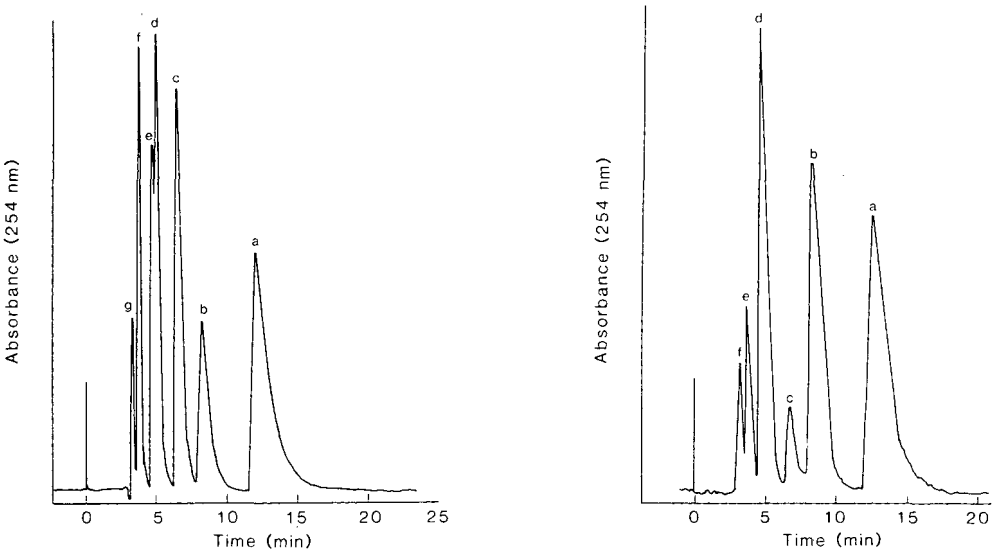


Fig. 2. Elution profile of an aqueous standard of PMM and its demethylated metabolites. Peaks: a = PMM, b = 2,2,4,6-TeMM, c = 2,2,4,4-TeMM, d = 2,2,4-TrMM, e = 2,4,6-TrMM, f = DMM, g = MMM.

Fig. 3. Chromatogram of patient's plasma 8 h after intravenous administration of 640 mg/m² of PMM. Peaks: a = PMM, b = 2,2,4,6-TeMM, c = 2,2,4,4-TeMM, d = 2,4,6-TrMM, e = DMM, f = MMM.

three of 21 patients, whereas 2,2,4,4-TeMM was observed in the plasma of two patients.

The plasma clearance of PMM and metabolites in a patient after 1 g/m² of PMM was administered is shown in Fig. 4. PMM and 2,2,4,6-TeMM are

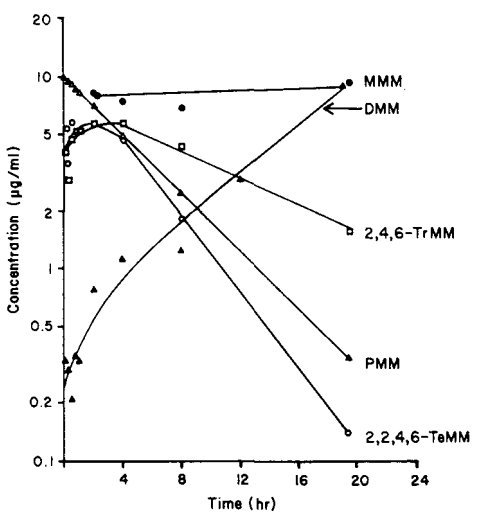
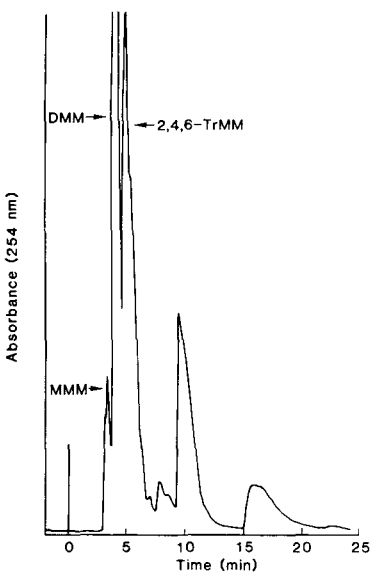


Fig. 4. Plasma clearance of PMM and metabolites after administration of 1 g/m² of PMM.

rapidly cleared from plasma, but 2,4,6-TrMM is cleared more slowly. DMM and MMM concentrations continue to rise during this time.

The urinary excretion of PMM and metabolites was low. Fig. 5 shows the elution profile of a patient's urine during the infusion of 1.5 g/m^2 of PMM. As shown, the major urinary excretion products are 2,4,6-TrMM and DMM. The chromatographic profile of a patient's CSF 24 h after administration of 2.0 g/m^2 of PMM is shown in Fig. 6. Again, the major constituents observed are the TrMM and DMM.

Thus, the HPLC assay described is an efficient and effective method for the determination of PMM and its metabolites in biological fluids. This assay is currently being used to study the clinical pharmacology of PMM.



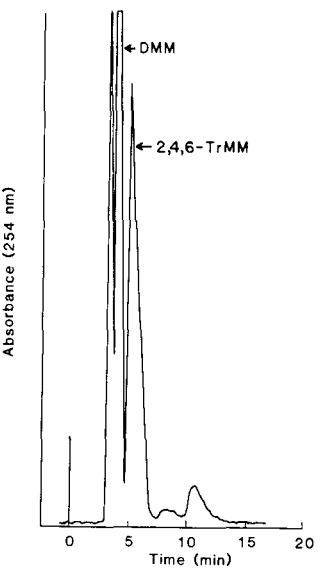


Fig. 5. Chromatogram of patient's urine during infusion of 1.5 g/m^2 of PMM.

Fig. 6. Elution profile of patient's CSF 24 h after administration of 2 g/m^2 of PMM.

ACKNOWLEDGEMENTS

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REFERENCES

1 S.S. Legha, M. Slavid and S.K. Carter, Cancer, 38 (1976) 27.
2 L.M. Lake, E.E. Grunden and B.M. Johnson, Cancer Res., 35 (1975) 2858.
3 C.J. Rutty and T.A. Connors, Biochem. Pharmacol., 26 (1977) 2385.

4 J.F. Wgrzolla, B.M. Johnson, G. Ramirez and G.T. Bryan, Cancer Res., 33 (1973) 2810.
5 M.M. Ames, G. Powis, J.S. Kovach and R.T. Eagan, Cancer Res., 39 (1979) 5016.
6 M. D'Incalci, P. Morazzoni and C. Pantarotto, Anal. Biochem., 99 (1979) 441.
7 A. Hulshoff, J.P. Neijt, C.F.A. Smulders, A.C. van Loenen and H.M. Pinedo, J. Chromatogr., 181 (1980) 363.
8 A. Gescher, M. D'Incalci, R. Fanelli and P. Farina, Life Sci., 26 (1980) 147.
9 C.J. Rutty, T.A. Connors, Nguyen-Hoang-Nam, Do-Cao-Thang and H. Hoellinger, Eur. J. Cancer, 14 (1978) 713.

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Erratum

J. Chromatogr., 221 (1980) 327—335
Page 331, Table IV should read:

TABLE IV

AMOUNTS OF PLASMA AND PLASMA STANDARDS TO BE USED FOR THE DETERMINATION OF BUFURALOL

Expected concentration range of bufuralol (ng/ml)	Amount of plasma to be extracted (ml)	Amount of internal standard in 0.2 ml of 2.5% NaOH (ng)	Amount injected (μl)	Plasma standards to be used (No. from Table III)
$\geqslant 25$	0.2	10	80 40*	2, 3 and 4, if necessary 1 for high concentrations
<25	0.5	4	80	5, 6 and 7, if necessary 4

*Plasma concentrations above 100 ng/ml.

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