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VOL. 230, NO. 2 JOURNAL OF CHROMATOGRAPHY JULY 9, 1982
(*Biomedical Applications, Vol. 19, No. 2*)

CONTENTS

(Abstracts/Contents Lists published in Analytical Abstracts, Biomedical Abstracts, Biological Abstracts, Chemical Abstracts, Chemical Titles, Current Contents/Physical, Chemical & Earth Sciences, Current Contents/Life Sciences, Index Medicus, Mass Spectrometry Bulletin, and Science Citation Index)

Plasma concentrations of *p*- and *m*-hydroxyphenylacetic acid and phenylacetic acid in humans. Gas chromatographic—high-resolution mass spectrometric analysis
by B.A. Davis, D.A. Durden and A.A. Boulton (Saskatoon, Canada) (Received February 17th, 1982). 219

Lipid class and molecular species interrelationships among plasma lipoproteins of Type III and Type IV hyperlipemic subjects
by A. Kuksis, J.J. Myher, K. Geher, W.C. Breckenridge and J.A. Little (Toronto, Canada) (Received February 1st, 1982) 231

A method for high-performance liquid chromatographic screening of UV-positive components in urine eluate from Sephadex G-10 and modifications for determination of urinary salicylic, salicyluric and gentisic acids
by N. Fornstedt (Uppsala, Sweden) (Received February 9th, 1982) 253

Enzymatic detection of urinary conjugated steroids after gel chromatography
by Y. Yamaguchi and C. Hayashi (Osaka, Japan) (Received February 23rd, 1982). . 263

Reversed-phase liquid chromatography with amperometric detection of lipophilic dopamine analogues and determination of brain and serum concentrations after sample clean-up on small Sephadex G-10 columns
by M.G.P. Feenstra, J.W. Homan, D. Dijkstra, T.B.A. Mulder, H. Rollema, B.H.C. Westerink and A.S. Horn (Groningen, The Netherlands) (Received February 9th, 1982) 271

High-performance liquid chromatographic purification of endogenous tumour cell growth inhibitory peptides
by N.M. Barfod (Aarhus, Denmark) (Received February 22nd, 1982) 289

Quantitative enzymatic hydrolysis of tRNAs. Reversed-phase high-performance liquid chromatography of tRNA nucleosides
by C.W. Gehrke, K.C. Kuo, R.A. McCune, K.O. Gerhardt and P.F. Agris (Columbia, MO, U.S.A.) (Received November 12th, 1981) 297

Determination of nicotinamide and metabolic products in urine by high-performance liquid chromatography
by R.W. McKee, Y.A. Kang-Lee, M. Panaqua and M.E. Swendseid (Los Angeles, CA, U.S.A.) (Received February 23rd, 1982) 309

Separation of nanogram quantities of hydroxy metabolites of vitamin D_3 in plasma by thin-layer chromatography on silica gel
by V. Justová, Z. Wildtová and L. Stárka (Prague, Czechoslovakia) (Received February 5th, 1982) 319

The effect of ampholytes on ferritin isoelectric focussing patterns
by B.L.F. Bredenkamp and S.M. Joubert (Congella, Republic of South Africa) (Received February 19th, 1982). 327

(Continued overleaf)

Contents (continued)

Unexpected metabolites produced from clomethiazole
by A. Grupe and G. Spiteller (Bayreuth, G.F.R.) (Received February 8th, 1982). . . 335

Studies of isoniazid metabolism in isolated rat hepatocytes by mass fragmentography
by A. Noda, K.-Y. Hsu, Y. Aso, K. Matsuyama and S. Iguchi (Fukuoka, Japan) and M. Hirata (Osaka, Japan) (Received February 10th, 1982). 345

Micro-determination of clonazepam in plasma or serum by electron-capture gas—liquid chromatography
by N.R. Badcock and A.C. Pollard (North Adelaide, Australia) (Received February 23rd, 1982) 353

Ion-pair high-performance liquid chromatographic assay of 4-aminopyridine in serum
by Y. Shinohara, R.D. Miller and N. Castagnoli, Jr. (San Francisco, CA, U.S.A.) (Received February 15th, 1982). 363

Quantitation of 6-mercaptopurine in biologic fluids using high-performance liquid chromatography: a selective and novel procedure
by P.K. Narang, R.L. Yeager and D.C. Chatterji (Bethesda, MD, U.S.A.) (Received February 17th, 1982). 373

Rapid assay for plasma chlorambucil and phenyl acetic mustard using reversed-phase liquid chromatography
by M. Zakaria and P.R. Brown (Kingston, RI, U.S.A.) (Received February 12th, 1982) 381

Simultaneous determination of amitriptyline, nortriptyline and their respective isomeric 10-hydroxy metabolites in plasma by liquid chromatography
by R.F. Suckow and T.B. Cooper (Orangeburg, NY, U.S.A.) (Received February 18th, 1982) 391

High-performance liquid chromatographic determination of pipotiazine in human plasma and urine
by Y. le Roux, J. Gaillot and A. Bieder (Antony, France) (Received February 16th, 1982) 401

Notes

High-performance gel permeation chromatography of collagens
by Z. Deyl, K. Macek, M. Adam and M. Horáková (Prague, Czechoslovakia) (Received February 4th, 1982). 409

Rapid and sensitive sialidase assay by high-performance liquid chromatography and its application to detection of sialidase in human urine
by K. Omichi and T. Ikenaka (Osaka, Japan) (Received February 15th, 1982) 415

Improved method for the evaluation of 3-hydroxyproline and 4-hydroxyproline in the urine
by G. Bellon, A. Bisker, F.X. Maquart, H. Thoanes and J.P. Borel (Reims, France) (Received February 8th, 1982) 420

Determination of morphine, morphine-3-glucuronide and (tentatively) morphine-6-glucuronide in plasma and urine using ion-pair high-performance liquid chromatography
by J.-O. Svensson, A. Rane, J. Säwe and F. Sjöqvist (Huddinge, Sweden) (Received February 22nd, 1982). 427

Simultaneous high-performance liquid chromatographic assay for quinidine, disopyramide and the mono-N-dealkylated metabolite of disopyramide
by R.L.G. Norris, J.T. Ahokas and P.J. Ravenscroft (Brisbane, Australia) (Received February 17th, 1982) 433

High-performance liquid chromatography assay of acebutolol and two of its metabolites in plasma and urine
by J.N. Buskin, R.A. Upton, R.M. Jones and R.L. Williams (San Francisco, CA, U.S.A.) (Received February 10th, 1982) 438

Improved acetaminophen assay sensitivity by modification of a high-performance liquid chromatography technique
by J.N. Buskin, R.A. Upton and R.L. Williams (San Francisco, CA, U.S.A.) (Received February 10th, 1982) 443

High-performance liquid chromatographic analysis of propafenone in human plasma samples
by S.R. Harapat and R.E. Kates (Stanford, CA, U.S.A.) (Received February 11th, 1982) 448

Specific and sensitive assay of celiprolol in blood, plasma and urine using high-performance liquid chromatography
by J.N. Buskin, R.A. Upton, F. Sörgel and R.L. Williams (San Francisco, CA, U.S.A.), E. Lang (Erlangen, G.F.R.) and L.Z. Benet (San Francisco, CA, U.S.A.) (Received January 29th, 1982) 454

Fluorodensitometric determination of nadolol in plasma and urine
by M. Schäfer-Korting and E. Mutschler (Frankfurt/Main, G.F.R.) (Received February 18th, 1982). 461

Book Review

Immunochemical techniques for the identification and estimation of macromolecules (by J. Clausen), reviewed by Z. Deyl 466

Related articles published in Journal of Chromatography, Vol. 238—241 468

Author Index 474

Subject Index 479

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PLASMA CONCENTRATIONS OF *p*- AND *m*-HYDROXYPHENYLACETIC ACID AND PHENYLACETIC ACID IN HUMANS

GAS CHROMATOGRAPHIC—HIGH-RESOLUTION MASS SPECTROMETRIC ANALYSIS

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(First received October 13th, 1981; revised manuscript received February 17th, 1982)

SUMMARY

Conjugated and unconjugated phenylacetic acid and *m*- and *p*-hydroxyphenylacetic acid have been determined in the plasma of normal, healthy subjects after fasting, consumption of a meal and ingestion of deuterium-labelled amine precursors, by high-resolution gas chromatography—high-resolution mass spectrometry with selected ion monitoring of their trifluoroethyl-pentafluoropropionyl derivatives.

We observed that all three conjugated acids are higher in fasting than in non-fasting subjects, and unconjugated phenylacetic acid was lower. Ingestion of deuterium-labelled amine precursors resulted in the appearance in the blood of the correspondingly labelled acids, a peak in the concentrations being reached about 1 h after consumption. Conjugated and unconjugated acids as expected increased following the consumption of a meal.

Unconjugated phenylacetic acid was significantly higher in females than in males. Most values tended to increase with age, with male unconjugated and conjugated *m*-hydroxyphenylacetic acid and female conjugated phenylacetic and *m*-hydroxyphenylacetic acids increasing significantly.

INTRODUCTION

It has been claimed that phenylacetic acid levels in blood plasma in aggressive psychopaths are elevated [1]. In cerebrospinal fluid, it has similarly been claimed to be elevated in schizophrenics [2] but reduced in depressive patients [3]. Such findings have been interpreted as indicating a role for phenylethylamine in the aetiology of these disease states.

We have previously reported that some of the trace amines, phenylethyl-

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amine and *m*- and *p*-tyramine, are abnormally excreted in Parkinsonism [4] and schizophrenia [4, 5]. In addition it has been shown that the trace amines are found in the brain and in nerve endings [6] and exhibit potent physiological effects when iontophoresed either singly or in combination with noradrenaline and dopamine [7, 8]. Some of them are differentially affected by stimulant and neuroleptic drugs [9] and are taken up and released by unique carrier systems [10—14]. In addition, their role in the brain has been proposed to be that of synaptic activators or modulators [15]. As a preliminary step in a study of the trace amine deamination products (i.e. trace acids) in the blood plasma of aggressive psychopaths, schizophrenics, depressives and agoraphobics, we have first investigated these levels in normal, healthy individuals. In particular, we were interested to investigate the effects of fasting on their plasma concentrations, to assess whether values obtained from the same individual but on different days were reproducible, and to examine the effect of ingestion of some of the trace amines suitably labelled with deuterium on plasma trace acid levels. In addition, the effects of sex and age on the acids levels were also determined.

EXPERIMENTAL

Materials

Phenylacetic acid-α,α-d_2 was synthesized from benzoic acid by reduction with $LiAl^2H_4$ to give benzyl alcohol-α,α-d_2, which was converted to benzyl chloride-α,α-d_2 with thionyl chloride, and finally by treatment of the Grignard complex of benzyl chloride with dry ice to give phenylacetic acid-α,α-d_2. Phenylacetic acid-ring-d_5 was synthesized similarly, starting, however, from toluene-d_8 which was oxidized with permanganate to give benzoic acid-d_5. $LiAlH_4$ was used in the reduction.

m- and *p*-hydroxyphenylacetic acids-α,α-d_2 were prepared starting from the hydroxybenzoic acids. The acids were esterified and the phenolic groups benzylated, reduced with $LiAl^2H_4$ to obtain the deuterated alcohols, which were converted to the chlorides with thionyl chloride and then to the nitriles using potassium cyanide (deuterated solvents required). Hydrolysis of the nitriles in 30% NaO^2H in ^2H_2O gave the O-benzyl acids. Hydrogenolysis over Pd/C yielded *m*- and *p*-hydroxyphenylacetic acids-α,α-d_2. The yield for *m*-hydroxyphenylacetic acid was high, but that for the *p*-hydroxyphenylacetic acid was lower since the nitrile appeared to undergo some kind of dimerization during the basic hydrolysis.

m-Hydroxyphenylacetic acid-d_5 (ring-d_3,α,α-d_2) and *p*-hydroxyphenylacetic acid-d_4 (ring-d_2,α,α-d_2) were synthesized as described previously by exchanges in $^2HCl/^2H_2O$ at elevated temperatures [16]. The syntheses of phenylethylamine-β,β-d_2, *m*- and *p*-tyramine-β,β-d_2 have also been previously described [12].

HPLC grade ethyl acetate, hexane, benzene and triethylamine (Caledon, Georgetown, Canada) were used. All other reagents were obtained at the highest available purity. All glassware was soaked before use in chromic acid, washed in Contrad detergent and finally rinsed several times with distilled water.

Methods

Venous blood samples (20 ml) were drawn into tubes containing sodium citrate in the morning between 8 a.m. and 9 a.m. before breakfast for the fasting group and between 9 a.m. and 10 a.m. after breakfast for the non-fasting group. Blood samples from a few individuals were taken twice, on different days, under otherwise identical conditions to assess the constancy of the concentration in the same person. No control was exercised over the diet of the non-fasting group. The plasma was separated and stored at —70° C until analysis.

For the kinetic study, a fasting blood sample was taken at 11:45 a.m. Lunch was eaten from 12:00 to 12:30 p.m. (beef stroganoff, vegetable soup, salad and tea) and immediately after the meal at 12:30 p.m.) a gelatin capsule containing 25 mg of each of the hydrochloride salts of phenylethylamine-β,β-d_2, *m*- and *p*-tyramine-β,β-d_2 was swallowed. Blood samples were then taken 0.5, 1, 2 and 4 h after swallowing the capsule.

In order to determine unconjugated acid levels, a plasma sample (0.5 ml), to which was added an aqueous solution (100 μl) containing 160 ng of phenylacetic acid-α,α-d_2 (PAA-d_2), 157 ng of phenylacetic acid-ring-d_5 (PAA-d_5), 174 ng of *p*-hydroxyphenylacetic acid (PHPA-d_2), and 49 ng of *m*-hydroxyphenylacetic acid-α,α-d_2 (MHPA-d_2), was diluted to 1 ml with distilled water. For the measurement of labelled acids arising from the ingested labelled trace amines, PAA-d_5 (174 ng), MHPA-d_5 (29 ng) and PHPA-d_4 (48 ng) were used as internal standards. Protein was precipitated from this mixture by adding 0.2 *M* zinc sulfate (1 ml) and 0.2 *M* barium hydroxide (1 ml) followed by centrifugation at 6500 *g* for 20 min. The supernatant was decanted from the precipitate, acidified with 1 *N* hydrochloric acid (200 μl) and the clear solution saturated with about 0.5 g sodium chloride. This mixture was then extracted with ethyl acetate (3 $\times$ 2 ml) using a vortex mixer and a small centrifuge for breaking the emulsions. The extracts were combined in a conical test tube and concentrated to about 200 μl by evaporation in a stream of nitrogen while being warmed (about 60° C) in a water bath. After transfer of the concentrated extract and washings to 0.3-ml Reacti-vials (Pierce, Rockford, IL, U.S.A.), triethylamine (40 μl) was added to prevent loss of PAA and the solution evaporated to dryness in a stream of nitrogen. To ensure that the residue was free of moisture, 100 μl of benzene were added followed by re-evaporation. 2,2,2-Trifluoroethanol (150 μl) and pentafluoropropionic anhydride (40 μl) were then added to the residues and heated at 80° C for 1 h. After concentration of the reagents to 30—40 μl in a stream of nitrogen, hexane (150 μl) and pentafluoropropionic anhydride (40 μl) were then added and the mixture again heated for 1 h at 80° C. After cooling, 100 μl of phosphate buffer (1 *M*, pH 6.0) was added and the mixture shaken for 30 sec. The hexane (upper) layer was withdrawn, concentrated to about 20 μl under a stream of nitrogen, and submitted to high-resolution gas chromatographic—high-resolution mass spectrometric analysis using selected ion monitoring (SIM).

For these analyses, 0.3—0.5 μl of the hexane solution was injected without splitting onto a 50-m OV-101 SCOT capillary column, which was mounted in a Hewlett-Packard 5710A gas chromatograph with a splitless injector, and coupled directly via SGE 0.4 mm I.D. glass-lined tubing to the ion source of a

VG 70 70F high-resolution double-focussing mass spectrometer which was equipped with a digital selected ion monitor and a VG M82 multispec data system. The column was operated isothermally at 170°C with a helium flow-rate of 29 cm/sec. Usually after every fourth injection the temperature was increased to 220°C to remove residual compounds. The mass spectrometer was set up for selected ion monitoring at 5000 resolution such that the following ions could be analyzed: molecular ion of PAA [as trifluoroethyl (TFE) derivative] at *m/z* 218.0555; molecular ion of PAA-d_2-TFE at *m/z* 220.0680; molecular ion of PAA-d_5-TFE at *m/z* 223.0868; fragment ion resulting from loss of $COOCH_2CF_3$ for MHPA and PHPA [as pentafluoropropionyl (TFE) derivative], at *m/z* 253.0288; and MHPA-d_2 and PHPA-d_2 at *m/z* 255.0413. Since all three acids are well separated by the capillary column, only three or four masses needed to be recorded at any one time, the mass reference ion, the proteo ion and the deuterated internal standard ions. The retention times of the acid derivatives of PAA, MHPA and PHPA were 4.1 min, 5.8 min and 6.3 min, respectively. The labelled and unlabelled compounds were almost isographic for each of the three acids with the labelled acids eluting a few seconds before the unlabelled acids [16] (see Fig. 1).

Calibration curves for each acid were prepared by carrying through the entire procedure known concentrations of the labelled and unlabelled acids (see Fig. 2). This permitted the results obtained for the blood samples to be corrected for chemical and isotopic impurities in the internal standards and also for differences in the mass spectrometric sensitivity between the labelled and unlabelled compounds. The absolute amount of any particular acid in plasma is given by the equation:

$$A_{\text{plasma}} = \frac{A_{\text{int. std.}} \times H_{\text{plasma}}}{H_{\text{int. std.}}} \times F \times 2 \text{ ng/ml}$$

where A_{plasma} is the amount in ng of acid in the plasma, $A_{\text{int. std.}}$ is the amount in ng of deuterated internal standard added, H_{plasma} and $H_{\text{int. std.}}$ are the heights of the mass spectrometric SIM signals for the plasma (unlabelled acid) and internal standard (labelled acid) respectively, and F is the correction factor obtained from the calibration curves (always less than 1). The whole is multiplied by 2 to give the answer in ng/ml. The peak heights were used instead of peak areas since the widths were constant due to very reproducible chromatography.

With each batch of eight plasma samples, two blanks and two standards were run simultaneously through the entire procedure. In the case of blanks this meant starting with 0.5 ml distilled water and for the standards 0.5 ml of an aqueous solution containing a known amount of the three unlabelled acids.

Total acids (that is, unconjugated plus conjugated) were determined using 0.25 ml plasma. The internal standards were added and the protein precipitated as described for the unconjugated acids, 2 ml concentrated hydrochloric acid were then added and the solutions heated for 2 h in a heating block at 100°C. After cooling, each solution was partially neutralized with 9 pellets of sodium hydroxide and then extracted and processed as described above for the unconjugated acids. The only difference in procedure was that triethylamine

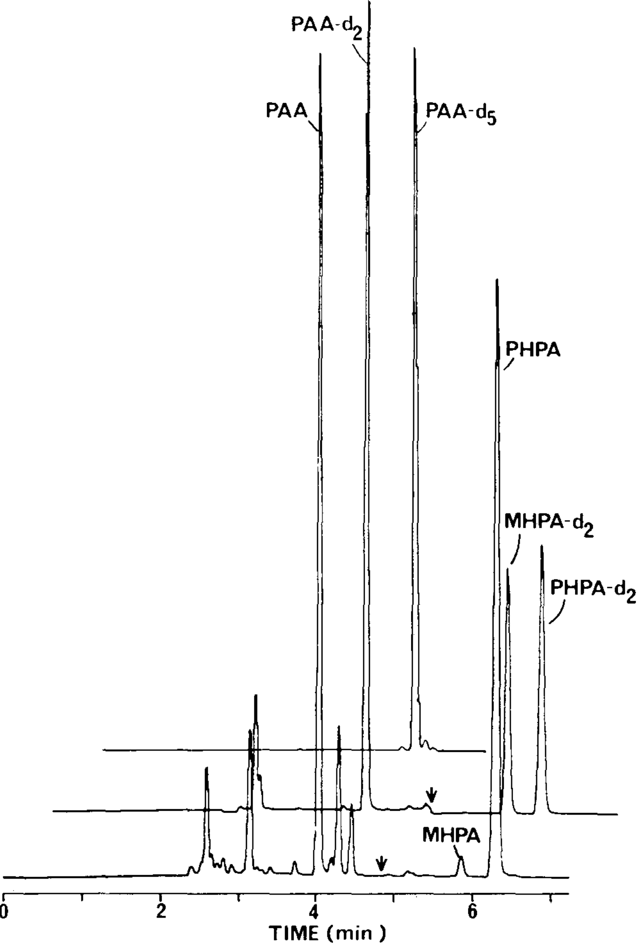

Fig. 1. Mass chromatogram of a human plasma sample showing elution of phenylacetic acid (PAA) at *m/z* 218·0555 and its internal standards PAA-d_2 at *m/z* 220·0680 and PAA-d_5 at *m/z* 223·0868, *m*-hydroxyphenylacetic acid (MHPA) and MHPA-d_2, and *p*-hydroxyphenylacetic acid (PHPA) and MHPA-d_2 at *m/z* 253·0288 and *m/z* 255·0413, respectively. The masses monitored by the SIM unit were changed at the time indicated by the arrows.

was not added after the ethyl acetate had been carefully evaporated just to dryness.

Experiments were undertaken to determine the extent, if any, of exchange of the deuterated standards during the procedure both for the unconjugated and for the total acids. In both cases, known amounts of the labelled acids (PAA-d_5, PAA-d_2, MHPA-d_2, PHPA-d_2) were separately carried through the respective procedures (in duplicate). For these analyses, the mass spectrometer was adjusted so as to measure d_2, d_1 and d_0 for the PAA-d_2, MHPA-d_2, PHPA-d_2, and d_5, d_4 and d_3 for the PAA-d_5.

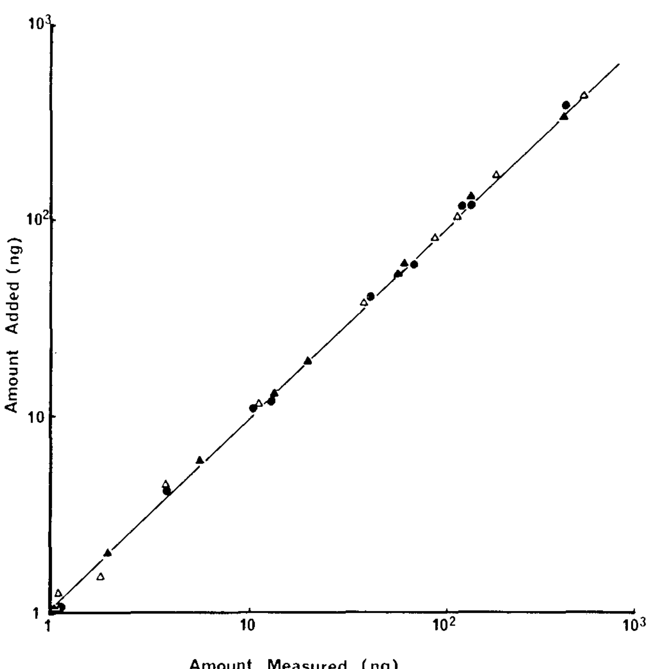

Fig. 2. Calibration curves for PAA (△); MHPA (▲); PHPA (●), using constant amounts of internal standards: 128 ng PAA-d_2, 58 ng MHPA-d_2, 172 ng PHPA-d_2.

RESULTS AND DISCUSSION

The calibration curves for the three trace acids were linear at least from 1—400 ng (Fig. 2), with slopes of 0.94, 1.00 and 0.95 for PAA, MHPA and PHPA, respectively. The three curves were so closely coincident that only one is depicted in the figure. The corresponding correlation coefficients (r) were 0.9998, 0.9985 and 0.9998, respectively. Minimum detectable quantities were 2.0 ng for PAA, 0.8 ng for MHPA and 1.5 ng for PHPA; these are the quantities in 0.5 ml plasma which give rise to signals twice those of the blanks when 40 ng of internal standard are used.

The coefficients of variation for replicate standard samples done on the same day were 4.9% for PAA, 5.7% for MHPA and 1.6% for PHPA, and for replicate samples done on different days were 5.5, 3.5 and 2.3%, respectively. For replicate plasma samples analyzed on different days the coefficients of variation for the unconjugated acids were 4.1% for PAA, 8.9% for MHPA and 4.3% for PHPA, and for total acids they were 7.4, 16.0 and 12.1%, respectively.

In Tables I, II and III are presented the plasma concentrations of total unconjugated and conjugated (calculated by difference) PAA, MHPA and PHPA for 42 different individuals (28 fasting, 14 non-fasting). Previously reported values by Sandler et al. [1] for PAA and Karoum et al. [17] for MHPA and PHPA are also included for comparative purposes for each acid.

It appears that food ingestion affects plasma levels of these acids and that there are, in some cases, significant differences between the levels of the fasting and non-fasting group (see Tables I, II and III). Thus fasting unconjugated PAA

TABLE I

TOTAL, UNCONJUGATED AND CONJUGATED PHENYLACETIC ACID LEVELS IN HUMAN PLASMA (ng/ml)

Number of individuals in each group are indicated in parentheses. Results are expressed as mean ± S.D.

Controls	Total	Unconjugated	Conjugated
Fasting (28)	416 ± 195	107 ± 61*	309 ± 165**
Non-fasting (14)	383 ± 182	155 ± 83.3*	228 ± 132**
Sandler et al. [1] (10)	493 ± 187	107 ± 34	386 ± 160

*These values are significantly different at $p = 0.05$ level.
**These values are significantly different at $p = 0.02$ level.

TABLE II

TOTAL, UNCONJUGATED AND CONJUGATED *m*-HYDROXYPHENYLACETIC ACID LEVELS IN HUMAN PLASMA (ng/ml)

Number of individuals in each group are indicated in parentheses. Results are expressed as mean ± S.D. n.m. = Not measured.

Controls	Total	Unconjugated	Conjugated
Fasting (28)	26.7 ± 23.8	21.5 ± 20.1	5.4 ± 5.6*
Non-fasting (14)	16.9 ± 4.2	17.9 ± 5.2	−1 ± 2.6*,**
Karoum et al. [17] (10)	n.m.	1.0 ± 0.3 (S.E.)	n.m.

*These values are significantly different at $p = 0.001$ level.
**This value is not significantly different from zero.

TABLE III

TOTAL, UNCONJUGATED AND CONJUGATED *p*-HYDROXYPHENYLACETIC ACID LEVELS IN HUMAN PLASMA (ng/ml)

Number of individuals in each group are indicated in parentheses. Results are expressed as mean ± S.D. n.m. = Not measured.

Controls	Total	Unconjugated	Conjugated
Fasting (28)	64.7 ± 40.8	54.1 ± 34.1*	10.6 ± 17.2*
Non-fasting (14)	70.8 ± 28.2	69.0 ± 26.7*	1.8 ± 16.3*
Karoum et al. [17] (10)	n.m.	11.3 ± 0.9 (S.E.)	n.m.

*These results are significantly different, fasting compared to non-fasting, at $p = 0.20$.

was found to be significantly lower than non-fasting unconjugated PAA at $p < 0.05$. Conversely, fasting conjugated PAA appeared to be higher than non-fasting conjugated PAA, but the difference was significant only at $p < 0.20$ with the number of individuals tested. Fasting MHPA levels appear to be higher than non-fasting levels, but only the conjugated acid was significantly higher ($p < 0.001$, Table II). As was the case with PAA, unconjugated PHPA was higher in non-fasting individuals whereas conjugated PHPA levels were higher in the fasting group. Fasting values were obtained on a separate occasion for ten individuals in the non-fasting group. The relationships of the various acid levels were the same as shown by comparison of the two larger groups, but statistical significance could not be obtained due to the small number of samples.

As can be seen in Table I, agreement with the data of Sandler et al. [1] for PAA is good; in the case of MHPA and PHPA (Tables II and III), however, agreement with those of Karoum et al. [17] is poor. In this latter case because Karoum et al. used different preparative procedures (protein was not precipitated for example) we undertook some additional experiments in which the plasma sample was prepared for analysis according to Karoum's method. The results obtained for each of the three acids, by the two different procedures were in agreement and so we conclude that this, at least, was not the cause of the discrepancies.

Because the fasting and non-fasting plasmas were obtained about 60 days apart, we tested for the effect of this by obtaining two fasting blood samples from three different individuals on different days separated by about four weeks. The results for the unconjugated acids with the possible exception of PHPA exhibited relatively little variation (see Table IV).

TABLE IV

COMPARISON OF UNCONJUGATED ACID LEVELS IN TWO PLASMAS TAKEN ONE MONTH APART FROM THREE INDIVIDUALS (ng/ml)

Sample	PAA		MHPA		PHPA	
	Plasma 1	Plasma 2	Plasma 1	Plasma 2	Plasma 1	Plasma 2
1	140.1	119.0	17.0	10.5	28.2	42.1
2	68.6	58.0	0.0	0.8	83.2	44.6
3	317.0	371.2	1.6	2.2	711.8	559.2

Ingestion of the deuterium-labelled amines, phenylethylamine-β,β-d_2, *m*- and *p*-tyramine-β,β-d_2, resulted in the appearance, in the blood, of their deuterated metabolites, PAA-d_2, MHPA-d_2 and PHPA-d_2. In a recent paper, it has been shown that α,α-deuterium-labelled phenylethylamine and *m*- and *p*-tyramine are considerably poorer substrates for monoamine oxidase than the non-deuterated amines, whereas the β,β-dideutero- (and ring-deuterated) amines are either deaminated at the usual rate or at a slightly enhanced rate [18]. As can be seen from Figs. 3, 4 and 5 all three deuterated acids could be detected at all the indicated times of analysis. A peak level was observed in all cases 1 h following the ingestion except in the case of conjugated MHPA and PHPA

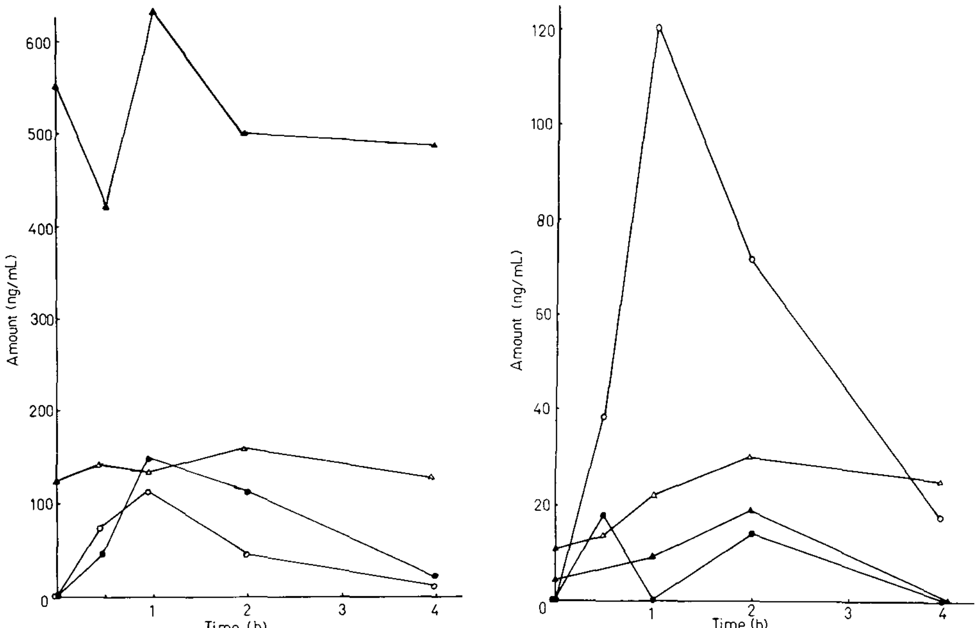

Fig. 3. Plasma levels for phenylacetic acid and phenylacetic acid-α,α-d_2 after consumption of a meal and deuterium-labelled amine precursors. (△) Unconjugated PAA; (▲) conjugated PAA; (○) unconjugated PAA-d_2 ; (●) conjugated PAA-d_2.

Fig. 4. Plasma levels for *m*-hydroxyphenylacetic acid and *m*-hydroxyphenylacetic acid-α,α-d_2 after consumption of a meal and deuterium-labelled precursors. (△) Unconjugated MHPA; (▲) conjugated MHPA; (○) unconjugated MHPA-d_2; (●) conjugated MHPA-d_2.

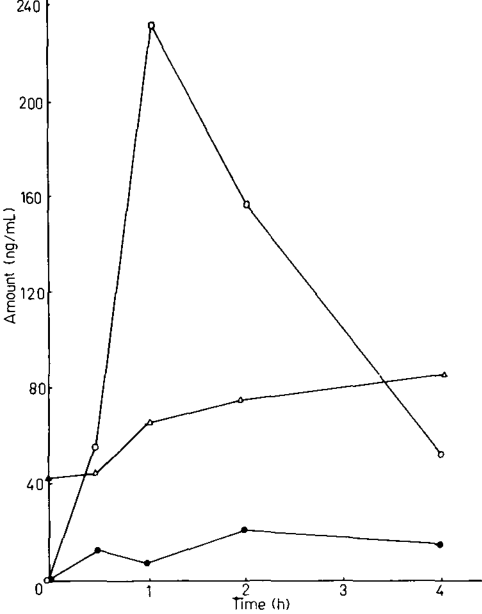

Fig. 5. Plasma levels for *p*-hydroxyphenylacetic acid and *p*-hydroxyphenylacetic acid-α,α-d_2 after consumption of a meal and deuterium-labelled precursors. (△) Unconjugated PHPA; (○) unconjugated PHPA-d_2; (●) conjugated PHPA-d_2. No conjugated PHPA was detected.

which were present only in relatively tiny quantities. A small contribution to MHPA-d_2 and PHPA-d_2 will arise by hydroxylation of the ingested phenylethylamine-d_2 as has recently been shown by Davis and Boulton [19].

The levels of unlabelled acids in the plasma of one individual were measured following the consumption of a meal (see Figs. 3, 4 and 5). Unconjugated PAA rose modestly and MHPA peaked at about 2 h whereas unconjugated PHPA levels continued unexpectedly to rise up to 4 h (samples were not obtained after 4 h). Conjugated PAA increased only slightly and reached a maximum at 1 h, whereas conjugated MHPA reached a maximum at 2 h. Conjugated PHPA could not be detected.

The results obtained in these studies indicate that in order to obtain consistency in trace acid measurements in blood, samples should be taken prior to eating the first meal of the day or at least 3 h after a meal.

The results from the fasting samples were analyzed for differences due to age and sex (Table V). Females were found to have significantly higher unconjugated PAA than males ($p < 0.01$), which is in agreement with the observations of Sandler et al. [1] that unconjugated PAA is higher in cerebrospinal fluid of control (not significant) [2, 3], depressive ($p < 0.005$) [3] and schizophrenic ($p < 0.01$) [2] females than for males.

TABLE V

CORRELATIONS OF TRACE ACID LEVELS WITH SEX AND AGE

	Mean ± S.D. (ng/ml)	Correlation coefficient (r)	p (correlation with age)
Males			
Unconjugated PAA	79.5 ± 47.6*	−0.13	n.s.**
Conjugated PAA	296.4 ± 148.3	−0.06	n.s.
Unconjugated MHPA	16.6 ± 12.9	+0.51	<0.05
Conjugated MHPA	10.0 ± 14.8	+0.49	<0.05
Unconjugated PHPA	66.6 ± 76.1	+0.14	n.s.
Conjugated PHPA	17.7 ± 14.1	−0.30	n.s.
Females			
Unconjugated PAA	119.5 ± 50.5	+0.24	n.s.
Conjugated PAA	365.6 ± 156.9	+0.51	<0.05
Unconjugated MHPA	22.6 ± 21.9	+0.22	n.s.
Conjugated MHPA	5.5 ± 4.7	+0.48	<0.05
Unconjugated PHPA	59.6 ± 39.0	+0.42	<0.07
Conjugated PHPA	13.0 ± 15.7	+0.37	n.s.

*This value is significantly different at $p < 0.01$ from female unconjugated PAA.
**n.s. = not significant.

When combined male and female values were subjected to linear regression analysis no trends according to age were observed. When the values were divided into male and female groups, however, trends were observed (Table V). Most values tended to increase with age, with male unconjugated and conjugated MHPA and female conjugated PAA and conjugated MHPA having

correlation coefficients of about 0.5, all of which proved to be significant at the $p < 0.05$ level. The mean ages were 35.7 years for males (range 24—52) and 30.2 years for females (range 21—50).

In order to determine total acid levels (or conjugated levels which are obtained by subtracting unconjugated values from the total value) plasma must be hydrolyzed by heating with hydrochloric acid. The customary practice of adding the internal standards to the plasma before processing can lead to errors, since back-exchange of some of the label is possible, depending on the location of the deuterium atoms. Deuterium atoms on the side-chain undergo exchange during acid hydrolysis. Under the conditions used here (approximately 5 N hydrochloric acid at 100°C for 2 h) 23% of the PAA-d_2 was converted to PAA-d_1 (about 90%) and PAA (about 10%). Similar amounts of exchange were observed for MHPA-d_2 and PHPA-d_2. This problem can be circumvented in a number of ways. The internal standards can be added before hydrolysis and the results corrected for the exchange; alternatively the internal standards can be added after hydrolysis. We have analysed each of the three acids using both methods and have found that the results compare very well. A third option would be to use standards whose label cannot undergo exchange. This is not feasible in the case of MHPA and PHPA (where most of the ring protons or deuterons are exchangeable), but it is feasible for PAA. Another alternative would be to use ^{13}C- or ^{18}O-labelled acids; however, the high cost of the very highly enriched isotopically-labelled compounds which would be required makes them impractical for routine use in most laboratories. We have opted for, and therefore recommend, the addition of both PAA-d_2 (side-chain) and PAA-d_5 (ring) as well as MHPA-d_2 and PHPA-d_2 (both side-chain) prior to hydrolysis. A comparison of the results obtained using PAA-d_5 as internal standard with those using PAA-d_2 permits the determination of the correction factor for every sample (this turns out to be consistently in the range 0.76—0.78 for PAA-d_2). This correction factor for PAA-d_2 has been found also to apply to MHPA-d_2 and PHPA-d_2. It should be noted that the extent of exchange is highly temperature dependent. At 110°C, nearly 35% of the $^2H_2$-acid is lost, whereas at 95°C, about 13% is lost.

No exchange was found to occur during the determination of unconjugated acids, where no heating under strong acid conditions is required.

The method described here is sensitive and specific as a consequence of both the high resolution of the capillary column in the gas chromatograph and of the high-resolution double-focussing mass spectrometer. The method is presently being applied to an analysis of PAA, MHPA and PHPA in plasma obtained from patients suffering with schizophrenia, manic depression, agoraphobia and violent criminal behavior.

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LIPID CLASS AND MOLECULAR SPECIES INTERRELATIONSHIPS AMONG PLASMA LIPOPROTEINS OF TYPE III AND TYPE IV HYPERLIPEMIC SUBJECTS

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SUMMARY

As a further appraisal of lipoprotein interconversion and equilibration of lipid components a detailed examination was made of the chemical class and molecular species interrelationships among the major fasting plasma lipoprotein fractions within each of six male Type III and Type IV hyperlipemic subjects subsisting on free choice diets. The lipoprotein fractions were prepared by conventional ultracentrifugation and the lipid class and molecular species composition of the corresponding lipoprotein fractions were determined by gas chromatography of the intact glycerol esters and ceramides. In general, each lipoprotein fraction possessed a well defined lipid class composition, which was characterized by a dramatically decreasing triacylglycerol and increasing phospholipid and cholesteryl ester content, when progressing from the very low (VLDL) to the low (LDL) and high (HDL) density lipoproteins, as already established for normolipemic subjects. Likewise, the LDL_1 and LDL_2 of the hyperlipemic subjects contained about two times higher proportion of total phospholipid as sphingomyelin than VLDL and HDL. Furthermore, the sphingomyelins of the HDL fraction contained about 30% more of the higher and 30% less of the lower molecular weight species than the sphingomyelins of the VLDL. Smaller differences were seen in the molecular species composition of the phosphatidylcholines, cholesteryl esters and triacylglycerols among the corresponding lipoproteins. In comparison to normolipemic subjects analyzed previously, the hyperlipemic subjects showed greater individual variability. Despite this variability the lipid class and molecular species composition in the hyperlipemic subjects was again incompatible with the hypothesis which postulates direct VLDL conversion into LDL and HDL under the influence of lipoprotein lipase and lecithin: cholesterol acyltransferase. The main differences between normolipemic and hyperlipemic plasma were

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found to reside in the number of the VLDL and LDL_1 lipoprotein particles and not in their chemical composition or physical structure, or in the apparent mechanism of their metabolic interconversion.

INTRODUCTION

Several laboratories [1—4] have advanced more or less detailed models for a metabolic conversion of very low (VLDL) into low (LDL) and high (HDL) density lipoproteins via lipoprotein lipase and lecithin:cholesterol acyltransferase. The postulated, largely self-contained processes [5, 6] of cascading transformations of the lipoproteins dictate definite interrelationships among the various components of the precursor and product particles [7, 8]. We [9] have recently completed an extensive gas chromatographic examination of the precursor—product mass relationships among the major lipid classes and molecular species of the polar surfaces and neutral lipid cores of the VLDL, LDL_2 and HDL particles in individual samples of plasma from fasting normolipemic subjects on free choice diets. While the data obtained were consistent with the basic idea of VLDL degradation into LDL and HDL, significant differences were seen in their lipid composition, which suggested a much more complex series of transformations than those previously considered [1—4]. The findings were similar to those reported for normolipemic subjects subsisting on controlled experimental diets containing saturated or unsaturated fat [10].

We have sought further confirmation of the latter results in the present study of Type III and Type IV hyperlipoproteinemia subjects. In this we have been mindful of the possibility that the serious abnormalities in the production and/or clearance of the plasma lipids in the hyperlipemic subjects might show as yet unrecognized alterations in the usual relationships among the lipid classes and molecular species of different lipoprotein fractions. The results obtained confirm the complex relationships previously seen in the normolipemic subjects and suggest that the basic differences between normolipemic and hyperlipemic plasma lie in the number of the different lipoprotein particles and not in their structure or in the mechanism of their metabolic interconversion.

MATERIALS AND METHODS

Blood samples were obtained in the fasting state (12—14 h) from twelve hyperlipoproteinemic male subjects: six Type III and six Type IV (24—65 years old) at the St. Michaels Hospital Lipid Clinic, Toronto, Canada. The subjects lived at home and subsisted on their usual diets. The Type III (dysbetalipoproteinemia) and Type IV (hyperprebetalipoproteinemia) hyperlipoproteinemia condition in each subject was established on the basis of clinical history and the biochemical criteria suggested by the Lipid Research Clinics Program [11].

Isolation and characterization of lipoproteins

Lipoproteins were isolated essentially according to Hatch and Lees [12], as described in detail previously [9]. In the present instance, however, an LDL_1

(d = 1.019 g/ml) fraction was also recovered and its lipid classes and molecular species profiles determined. The identity of the different lipoprotein fractions was independently established by double immunodiffusion against rabbit anti-human albumin, anti-human LDL and HDL, as previously described [13]. The protein concentration in each lipoprotein fraction was determined by the method of Lowry et al. [14] using bovine serum albumin as standard. Preparations of VLDL and LDL_1 were extracted with diethyl ether after color development.

Dephosphorylation and isolation of lipids

Portions of the solutions of the various density fractions (equivalent to 0.1—0.2 ml of plasma) were hydrolyzed with phospholipase C in the presence of an excess of calcium chloride as previously described [9, 10]. The released diacylglycerol and ceramide moieties of the diacylglycerolphospholipids and sphingomyelins were then recovered, along with the original neutral lipids of each lipoprotein particle, by extraction with chloroform—methanol (2:1) in the presence of 200 μg of tridecanoylglycerol as internal standard. The extracts were centrifuged to break emulsions and the lipid extracts dried with sodium sulfate, evaporated to dryness and trimethylsilylated as previously described [9, 10].

Gas—liquid chromatography

The quantitative lipid profiles of the various lipoprotein particles were determined by means of an automated gas—liquid chromatography (GLC) system as described [15]. The gas chromatograph was equipped with an automatic liquid sampler, unheated on-column injector, and the system was capable of programmed heating, cooling and equilibration cycles. The separations were performed on a 50 × 0.2 cm I.D. stainless-steel column packed with 3% OV-1 (a methylsilicone polymer) on 100—120 mesh Gas-Chrom Q (Applied Science Labs., State College, PA, U.S.A.) using nitrogen as carrier gas in the temperature range 175—355°C. The retention times and peak areas were recorded by means of an electronic integrator on a punched paper tape. The peak identification and composition of samples was performed in relation to the tridecanoylglycerol internal standard using a modification of a commercially available computer program, and the results were expressed as mg% and as characteristic molar ratios of lipid classes, as previously described [15,16].

The carbon numbers of isolated diacylglycerols [17] and ceramides [18] were determined by GLC of the *tert.*-butyldimethylsilyl ethers. These GLC analyses were performed on a Beckman GC-4 gas chromatograph (Beckman Instruments, Fullerton, CA, U.S.A.) equipped with a 50 cm × 0.2 cm I.D. stainless-steel column packed with 3% OV-1 on Gas-Chrom Q (100—120 mesh). Alternatively the ceramide di-*tert.*-butyldimethylsilyl ethers were resolved by capillary GLC [19]. A 5-m column coated with SP2100 (Supelco, State College, PA, U.S.A.) was installed in a Hewlett-Packard 5880A gas chromatograph equipped with a level IV Terminal. The injector port was maintained at 280°C and the detector at 330°C. After a 1-min isothermal period at 270°C the oven was temperature programmed at 2.5°C/min up to a final temperature of 330°C. The column bleed was automatically subtracted using the single-column compensation mode of the terminal.

The fatty acid methyl esters of the various lipid ester classes were prepared by transmethylation with 6% sulfuric acid in methanol for 2 h (acylglycerols) and 8 h (ceramides and cholesteryl esters), respectively. The methyl esters were isolated, identified and quantitated by GLC on 10% EGSS-X (an ethylene glycol succinate silicone copolymer), as previously described [20].

Calculations

The peak areas were corrected for differences in flame ionization response and recovery of different components by means of appropriate standards. The content of phosphatidylcholines and sphingomyelins of the lipoprotein fractions was calculated on the basis of the areas of peaks C_{36}—C_{38} and peak C_{34}, respectively, using a series of corrections, as previously described [21]. The validity of this calculation was verified by isolating and determining the carbon number distribution of the phosphatidylcholines and sphingomyelins in the various lipoprotein classes in a representative series of Type III and Type IV subjects [19].

The core radii of the lipoprotein particles were calculated on the basis of the surface to volume ratio of a sphere as previously described [18, 22]. The total radii of the particles were obtained by adding the thickness of the surface monolayer, 2.0 nm [23]. The number of neutral lipid molecules in the particle cores were calculated by dividing the appropriate proportion of the core volume by the volume of the average cholesteryl ester and triacylglycerol molecules, respectively. Likewise, the number of the polar molecules in the surface shell were calculated by dividing the appropriate proportion of the total area by the cross-sectional area of the average phospholipid and free cholesterol molecules, respectively.

RESULTS

Total lipid profiles

Fig. 1A and B shows representative total lipid profiles of the VLDL, LDL_1, LDL_2 and HDL fractions of Type III and Type IV hyperlipoproteinemia subjects. In these elution patterns the various lipid subclasses are represented by their total acyl (plus 2), acyl plus sterol, or acyl plus sphingosine carbon numbers. Peak 27 represents free cholesterol and peak 30 the internal standard, tridecanoylglycerol, which has been added in equal amounts to each of the lipoprotein samples. In general the total lipid profiles for the corresponding lipoprotein classes of Type III and Type IV subjects are similar and, except for LDL_1, not unlike those previously described for normolipemic subjects [9, 10]. There are differences, however, in the cholesteryl ester/triacylglycerol ratios, as shown below. In addition, both Type III and Type IV subjects possess LDL_1 as a major plasma lipoprotein of the fasting state. This lipoprotein class possesses a total lipid profile intermediate between those of VLDL and LDL_2 (Table I).

Quantitative composition

The weight percentages of protein and lipid in the VLDL, LDL_1, LDL_2 and HDL fractions isolated from the individual Type III and Type IV subjects are

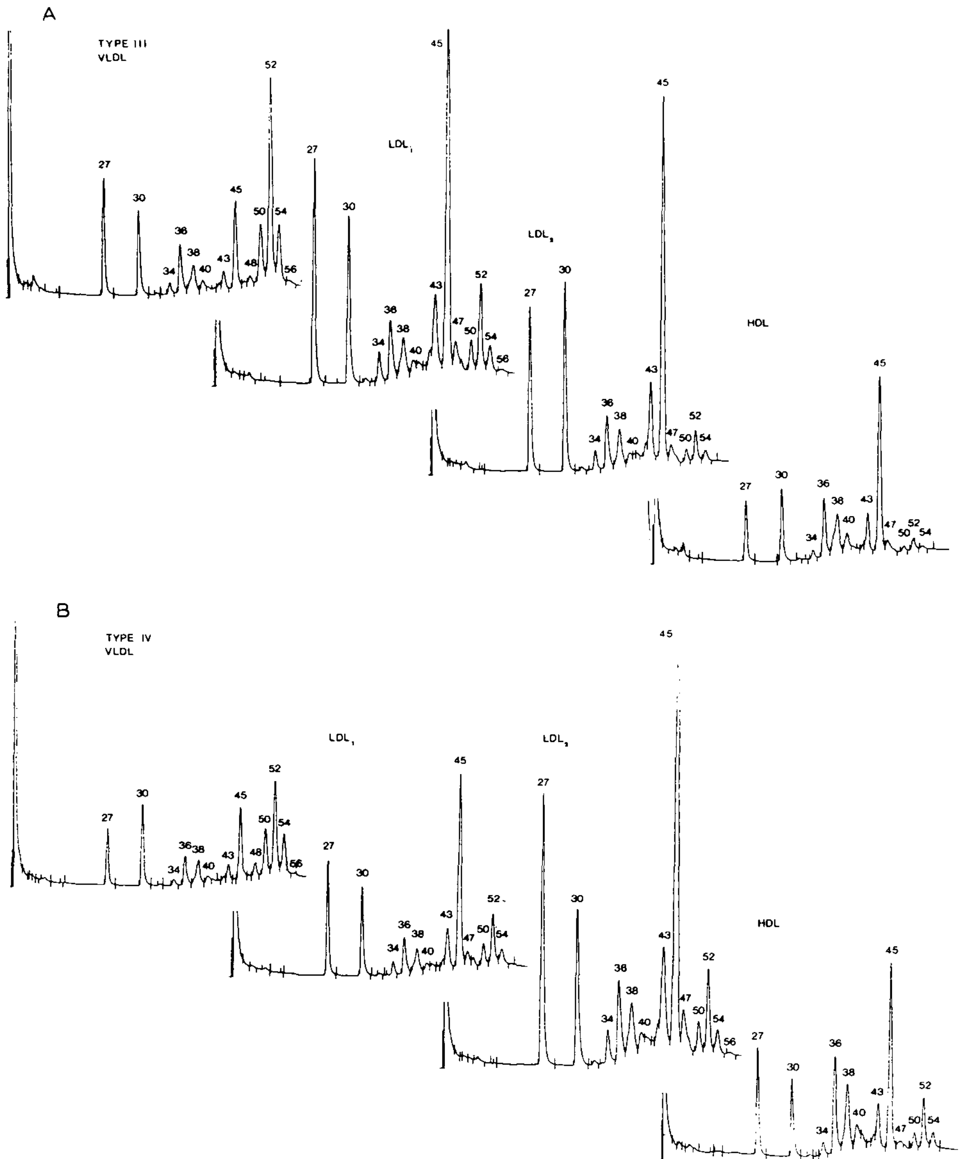

Fig. 1. GLC profiles of total lipids of plasma lipoproteins of representative subjects with Type III (A) and Type IV (B) hyperlipoproteinemia. VLDL, d < 1.006 g/ml; LDL$_1$, d < 1.019 g/ml; LDL$_2$, d < 1.063 g/ml; HDL, d < 1.21 g/ml. Conditions of high-temperature GLC as given in text. Peaks: 27, trimethylsilyl ether of cholesterol; 30, tridecanoylglycerol, internal standard; 34, trimethylsilyl ether of palmitoylsphingosine; 36—42, trimethylsilyl ethers of diacylglycerols of a total number of 34—40 acyl carbons; 43—47, cholesteryl esters of fatty acids with 16—20 acyl carbons; 48—56, triacylglycerols with a total number of 48—56 acyl carbons. Sample size: 1 μl of approximately 1% solution in silylation mixture. Attenuation: 100 times full sensitivity.

TABLE I

OVERALL COMPOSITION OF VLDL, LDL_1, LDL_2 AND HDL PARTICLES OF FASTING PLASMA OF HYPERLIPEMIC SUBJECTS

VLDL, $d < 1.006$ g/ml; LDL_1, $d < 1.019$ g/ml; LDL_2, $d < 1.063$ g/ml; HDL, $d < 1.21$ g/ml.

Chemical component*	Weight (%)													
	Type III							Type IV						
	1125	0996	1224	0973	1015	1222	Average	0745	0718	0738	0720	0011	0012	Average
VLDL														
Total protein	9	8	5	7	6	5	7 ± 2	6	5	5	6	9	9	7 ± 2
Total lipid	91	92	95	93	94	95	93 ± 2	94	95	95	94	91	91	93 ± 2
PC	20	16	13	14	13	14	14 ± 3	15	15	15	15	18	17	16 ± 1
SPH	6	3	3	3	4	2	4 ± 1	4	3	3	3	3	2	3 ± 1
CE	35	33	28	13	17	23	25 ± 9	27	14	13	18	14	11	16 ± 6
TG	29	39	49	64	61	55	50 ± 13	47	63	64	57	60	65	60 ± 7
FC	10	8	7	5	5	6	7 ± 2	7	5	5	6	5	4	5 ± 1
LDL_1														
Total protein	10	17	8	13	13	8	12 ± 2	20	14	16	17			17 ± 2
Total lipid	90	83	92	87	87	92	88 ± 1	80	86	84	83			83 ± 1
PC	17	17	18	19	19	19	18 ± 1	16	15	17	19			17 ± 1
SPH	8	9	8	6	8	7	8 ± 1	7	6	4	6			6 ± 1
CE	44	45	48	31	39	35	40 ± 2	48	37	16	37			34 ± 3
TG	21	18	15	37	25	31	25 ± 2	19	35	58	29			35 ± 7
FC	10	11	11	7	9	9	10 ± 1	10	7	5	9			8 ± 1
LDL_2														
Total protein	20	19	19	20	21	21	20 ± 1	20	26	24	18	20	19	21 ± 3
Total lipid	80	81	81	80	79	79	80 ± 1	80	74	76	82	80	81	79 ± 3
PC	22	21	21	21	23	21	23 ± 2	22	22	20	20	26	23	22 ± 3
SPH	8	7	8	7	4	5	7 ± 2	6	6	6	6	4	7	6 ± 1
CE	45	49	47	39	56	40	46 ± 6	48	50	50	57	48	54	51 ± 4
TG	16	13	14	21	10	26	17 ± 6	13	15	12	9	12	6	11 ± 3
FC	10	11	10	6	7	8	9 ± 1	11	7	10	9	10	10	10 ± 1
HDL														
Total protein	51	51	51	50	56	58	53 ± 3	68	54	40	50	58	51	54 ± 9
Total lipid	49	49	49	50	44	42	47 ± 3	32	46	60	50	42	49	47 ± 12
PC	43	40	46	41	40	43	42 ± 2	40	44	44	40	46	46	43 ± 3
SPH	5	4	5	4	4	5	5 ± 1	4	4	5	5	4	4	4 ± 1
CE	38	34	32	24	35	38	34 ± 5	36	41	35	20	31	38	34 ± 7
TG	8	17	11	27	17	10	15 ± 7	11	6	10	31	17	7	14 ± 9
FC	6	5	6	4	4	4	5 ± 1	6	5	6	4	3	4	5 ± 1

*PC, phosphatidylcholine; SPH, sphingomyelin; CE, cholesterol ester; TG, triacylglycerol; FC, free cholesterol.

given in Table I. The values for total lipids were obtained by summing the GLC estimates for the individual lipid classes, while the estimates for total protein were based on independent determinations of nitrogen on aliquots of the lipoprotein solution. It is seen that the percentage of protein in VLDL from both Type III and Type IV subjects averages 7 ± 2%, which is only slightly lower than the 9 ± 1% found previously in our laboratory for the VLDL of normolipemic subjects on free choice diets [9] but significantly lower than 12—15% noted for the VLDL of normolipemic subjects on high fat diets [19], although all of these values are within the ranges tabulated by Eisenberg and Levy [24] for VLDL of normolipemic subjects. The percentage of protein in the LDL_1 fraction from these subjects was much more variable and averaged 15 ± 4% and both Skipski [25] and Lee [26] have tabulated comparable values for this lipoprotein class from Type III and Type IV hyperlipoproteinemia subjects. The weight percentage of protein in the LDL_2 and HDL fractions averaged 23% and 53%, respectively, and corresponded to the values recorded for these lipoprotein classes in normolipemic subject [9, 10]. Therefore, these results show that the Type III and Type IV subjects in this study possess the normal protein/lipid ratios for their lipoprotein particles.

Table I also gives the weight percent composition of the major lipid classes as measured by GLC. The VLDL is seen to contain an average of 20% total phospholipid and 5—7% free cholesterol, the rest being made up of triacylglycerols and cholesteryl esters. This polar/non-polar lipid ratio is closely similar to that of VLDL of normolipemic subjects [25]. In contrast to the normolipemic subjects (EC/TG = 0.29—0.33), however, the Type III patients contained nearly twice as much cholesteryl ester and correspondingly less triacylglycerol in their VLDL (EC/TG = 0.58), while the cholesteryl ester/triacylglycerol ratio in the Type IV subjects was approximately normal (EC/TG = 0.34). Two of the six Type III patients, however, exhibited nearly normal cholesteryl ester/triacylglycerol ratios, while one of the four Type IV patients exhibited the abnormal cholesteryl ester/triacylglycerol ratio seen in Type III patients. Abnormally high cholesteryl ester/triacylglycerol ratios have previously been reported for some Type III patients by Stromberg et al. [27]. The LDL_1 and LDL_2 contained closely similar relative proportions of the different lipid classes. The total phospholipid content averaged about 30% for both Type III and Type IV subjects, while the free cholesterol ranged from 9—10%, again in close agreement with the composition of LDL_2 from normolipemic subjects. Furthermore, both groups of subjects contained about the same ratios of cholesteryl esters and triacylglycerols in these lipoprotein classes. However, in both groups some subjects possessed triacylglycerol levels which were nearly double that of normolipemic subjects for this lipoprotein class. Likewise, the HDL fraction of both Type III and Type IV patients possessed essentially normal polar/non-polar lipid class ratio, as well as a normal free cholesterol content (5%). The content of triacylglycerols was elevated (14—15%), when compared to that (5—6%) of normolipemic subjects analyzed previously [9]. There was a corresponding decrease in the content of the cholesteryl esters. Elevated triacylglycerol content in the LDL_2 fraction of Type III [28] and in the HDL fraction of Type IV [29] hyperlipoproteinemia subjects has been previously reported, but no exhaustive comparisons of the lipid class proportions have been made.

TABLE II

LIPID CLASS RATIOS IN VLDL, LDL_1, LDL_2 AND HDL PARTICLES OF FASTING PLASMA OF HYPERLIPEMIC SUBJECTS

Chemical components*	Mole/mole											
	Type III							Type IV				
	1125	0996	1224	0973	1015	1222	Average	0745	0718	0738	0720	Average
VLDL												
FC/TC	0.18	0.20	0.24	0.20	0.15	0.26	0.20	0.32	0.35	0.40	0.36	0.36
FC/PL	0.76	0.84	0.87	0.58	0.58	0.75	0.73	0.73	0.55	0.56	0.67	0.62
SPH/PC	0.12	0.11	0.10	0.10	0.10	0.12	0.11	0.27	0.20	0.20	0.20	0.22
EC/TG	1.2	0.8	0.6	0.2	0.3	0.4	0.58	0.6	0.23	0.20	0.32	0.34
FC/SPH	3.2	5.3	4.7	3.3	2.5	6.0	4.2	3.5	3.3	3.3	4.0	3.5
FC/PC	1.0	1.0	1.1	0.7	0.8	0.9	0.91	0.93	0.7	0.7	0.8	0.78
LDL_1												
FC/TC	0.27	0.29	0.28	0.28	0.27	0.29	0.28	0.29	0.24	0.34	0.29	0.29
FC/PL	0.76	0.82	0.85	0.58	0.67	0.69	0.73	0.87	0.67	0.48	0.72	0.68
SPH/PC	0.47	0.53	0.44	0.31	0.42	0.37	0.42	0.43	0.40	0.23	0.31	0.34
EC/TG	2.0	2.5	3.2	0.8	1.6	1.1	1.9	2.5	1.0	0.3	1.3	1.3
FC/SPH	2.5	2.4	2.7	2.3	2.3	2.6	2.5	2.9	2.3	2.5	3.0	2.7
FC/PC	1.2	1.3	1.2	0.7	0.9	0.9	1.0	1.3	0.9	0.6	0.9	0.9
LDL_2												
FC/TC	0.27	0.26	0.26	0.21	0.17	0.23	0.23	0.27	0.19	0.27	0.20	0.23
FC/PL	0.68	0.78	0.68	0.35	0.51	0.61	0.60	0.78	0.50	0.77	0.70	0.69
SPH/PC	0.36	0.32	0.36	0.25	0.17	0.24	0.33	0.27	0.27	0.30	0.30	0.28
EC/TG	2.8	3.8	3.3	1.9	5.6	1.6	3.2	3.7	3.3	4.2	6.3	4.4
FC/SPH	2.6	3.1	2.5	1.8	3.5	3.2	2.8	3.7	2.3	3.3	3.0	2.8
FC/PC	0.90	1.0	0.9	0.44	0.6	0.76	0.76	1.0	0.7	0.1	0.9	0.90
HDL												
FC/TC	0.18	0.20	0.24	0.20	0.15	0.26	0.20	0.25	0.27	0.26	0.20	0.24
FC/PL	0.25	0.23	0.23	0.18	0.18	0.17	0.20	0.27	0.17	0.24	0.18	0.21
SPH/PC	0.12	0.11	0.10	0.10	0.12	0.12	0.11	0.10	0.10	0.12	0.12	0.11
EC/TG	4.7	2.0	2.9	0.9	2.0	3.8	2.7	3.3	6.8	3.5	0.6	3.5
FC/SPH	2.4	2.5	2.4	2.0	2.0	2.0	2.2	3.0	2.0	2.4	1.6	2.2
FC/PC	0.28	0.25	0.26	0.20	0.20	0.19	0.23	0.31	0.19	0.27	0.20	0.24

*TC, total cholesterol; PL, total phospholipid; other abbreviations as in Table I.

Lipid class ratios

The molar ratios of the various lipid classes in the major lipoprotein fractions of each hyperlipoproteinemic individual are given in Table II. These ratios are relatively constant and show significant differences between the Type III and Type IV subjects, and between these hyperlipoproteinemic subjects and normal subjects analyzed previously [9, 10]. Thus, the FC/TC ratio for VLDL averages about 0.20 for Type III and 0.36 for Type IV patients, while the corresponding values for normolipemic subjects average 0.44 (9, 10]. This free cholesterol occurs in a ratio of 1 molecule of sterol per 1.35 molecules of total phospholipid (0.73) for the Type III and of 1 molecule of cholesterol per 1.6 molecules of phospholipid (0.62) for Type IV patients, the normolipemic subjects approaching a ratio of 1:2 for these polar lipid components of VLDL [9, 10]. Furthermore, the Type IV subjects showed elevated SPH/PC ratios (0.22) when compared to Type III (0.11) and normolipemic (0.12—0.13) subjects [9, 10] for the VLDL fraction. The VLDL of Type III subjects possessed an increased esterified cholesterol/triacylglycerol ratio when compared to the normolipemic subjects or to the Type IV subjects, as noted above.

The lipid class ratios of LDL_2 are likewise relatively constant, but with some marked differences between the two groups of patients and between normal and hyperlipemic subjects. The FC/TC ratio in LDL_2 is the same (0.23) in both patient groups and only slightly lower than that (0.27—0.29) of the LDL_2 fraction of normolipemic subjects [9, 10]. The FC/PL ratio is about the same in both Type III and Type IV patients (0.60—0.69) and very close to that of normolipemic subjects for LDL_2 (0.70—0.72). An FC/PL ratio of 0.87—0.93, however, has been recorded for total LDL of normolipemic subjects on high fat diets [10]. The ratio of free cholesterol to sphingomyelin is the same (2.2—2.8) in both LDL_2 and HDL, while that in VLDL is significantly higher (3.5—4.2). The ratios of the other lipid classes in the different lipoproteins are similar to those in the normolipemic subjects, although there are minor differences.

Particle size distribution

The calculated particle size distribution for the various lipoprotein fractions for each subject is given in Table III. The calculated core radii for the VLDL, LDL_1, LDL_2 and HDL averaged 212, 104, 94 and 55 for the Type III and 185, 134, 93 and 53 for the Type IV, respectively. These values are closely similar for both types of subjects and are only slightly higher than the corresponding values for the VLDL, LDL and HDL particles calculated for normolipemic subjects [9]. The particle size for LDL_1 is intermediate between that of VLDL and LDL_2 as would be anticipated on the basis of the centrifugation data. Table III also includes the calculated mass of the various lipoprotein particles and demonstrates a close correspondence between the two types of patients and between these hyperlipoproteinemia subjects and normal subjects [9].

Surface and core composition

The calculated concentrations of the lipids at the surface and in the core of the VLDL, LDL_1, LDL_2 and HDL particles as the number of molecules for each of the Type III and Type IV subjects are given in Table IV. Despite considerable individual variation the average estimates for the phosphatidyl-

TABLE III

AVERAGE SIZE DISTRIBUTION OF VLDL, LDL_1, LDL_2 AND HDL PARTICLES OF FASTING PLASMA OF HYPERLIPEMIC SUBJECTS

Parameter	Type III							Type IV						
	1125	0996	1224	0973	1015	1222	Average	0745	0718	0738	0720	0011	0012	Average
VLDL														
Core radius*	101	152	192	207	208	206	212 ± 81	164	198	198	183	169	198	185 ± 15
Particle weight**	4.3	12.7	23.3	28.8	29.1	29.2	21.2 ± 10	15.2	25.0	25.0	20.4	16.9	26.0	21.4 ± 4.6
LDL_1														
Core radius	104	95	95	123	101	107	104 ± 10	113	147	169	110			134 ± 28
Particle weight	4.7	4.0	3.6	7.5	4.5	5.0	4.9 ± 1	6.6	12.4	18.2	6.0			11 ± 6
LDL_2														
Core radius	85	88	87	86	109	110	94 ± 12	87	105	96	104	84	83	93 ± 10
Particle weight	3.2	3.5	3.4	3.3	6.1	6.2	4.3 ± 1.4	3.3	5.9	4.5	5.3	3.1	2.9	4.2 ± 1.3
HDL														
Core radius	49	60	44	62	63	53	55 ± 8	54	52	47	62	53	48	53 ± 5
Particle weight	1.3	2.2	1.0	2.2	2.7	1.9	1.9 ± 0.6	2.5	1.6	1.0	2.3	1.8	1.2	1.7 ± 0.6

*Particle radius (Å) = core radius (Å) plus thickness of outer shell (20 Å).
**Particle weight in daltons × 10^{-6}

TABLE IV

CALCULATED CONCENTRATION OF LIPIDS AT THE SURFACE AND IN THE CORE OF VLDL, LDL_1, LDL_2 AND HDL PARTICLES OF FASTING PLASMA OF HYPERLIPEMIC SUBJECTS*

Chemical component	Type III							Type IV						
	1125	0996	1224	0973	1015	1222	Average	0745	0718	0738	0720	0011	0012	Average
Surface components														
VLDL														
PC	983	2394	3632	4784	4493	4735	3504 ± 1532	2715	4501	4511	3668	3499	5138	4005 ± 874
SPH	310	471	879	1075	1450	709	816 ± 415	759	944	946	770	612	634	778 ± 144
FC	1005	2446	3997	3492	3532	4148	3103 ± 1188	2590	3067	3074	2999	1987	2471	2698 ± 433
LDL_1														
PC	911	714	753	1571	934	1095	996 ±	1073	2018	3284	1187			1890 ±
SPH	446	393	348	516	409	419	421 ±	488	840	804	390			630 ±
FC	1096	945	940	1183	904	1060	1021 ±	1371	1925	1974	1149			1604 ±
LDL_2														
PC	699	728	713	888	1412	1321	960 ± 323	742	1212	883	1088	802	690	903 ± 206
SPH	267	254	285	241	258	330	273 ± 32	212	347	278	342	129	220	254 ± 84
FC	650	780	695	403	879	1028	739 ± 213	758	788	902	1001	631	613	782 ± 151
HDL														
PC	340	538	275	576	600	424	459 ± 133	419	411	318	569	444	354	419 ± 87
SPH	40	55	30	57	61	50	49 ± 12	43	38	37	73	39	31	44 ± 15
FC	97	137	73	114	123	81	104 ± 25	129	76	89	116	59	63	89 ± 29
Core components														
VLDL														
TG	1310	5360	12577	20093	19370	17092	12634 ± 7772	7815	17370	17683	12805	10715	18048	14073 ± 4284
CE	2117	6073	9624	5465	7228	9570	6680 ± 2828	6011	5168	4810	5415	3348	4090	4807 ± 958
LDL_1														
TG	1034	695	576	2810	1129	1641	1314 ±	1170	4327	10293	1665			4363 ±
CE	2902	2328	2470	3153	2359	2481	2616 ± 334	3960	6125	3802	2844			4183 ± 1385
LDL_2														
TG	467	414	437	634	564	1502	670 ± 416	402	759	487	450	340	165	434 ± 195
CE	1760	2090	1965	1577	4232	3095	2453 ± 1019	1991	3390	2715	3814	1822	1994	2621 ± 831
HDL														
TG	58	210	60	348	234	91	167 ± 117	106	51	66	405	151	50	138 ± 136
CE	370	562	236	415	646	461	448 ± 144	464	471	312	350	369	360	388 ± 65

*Number of molecules per average particle of lipoprotein.

TABLE V

DISTRIBUTION OF MOLECULAR SPECIES OF LIPIDS IN VLDL, LDL_1, LDL_2 AND HDL PARTICLES OF FASTING PLASMA OF HYPERLIPEMIC SUBJECTS

Carbon numbers*	Mole % of lipid class													
	Type III							Type IV						
	1125	0996	1224	0973	1015	1222	Average	0745	0718	0738	0720	L793	L794	Average
VLDL														
36	33	30	35	40	36	36	35 ± 3.3	39	37	35	35	33	40	36 ± 2.7
38	38	39	39	40	39	40	39 ± 0.8	38	41	42	40	39	39	40 ± 1.5
40	29	31	26	20	25	24	26 ± 3.9	23	22	23	25	28	21	24 ± 2.5
43	25	24	25	27	26	21	25 ± 2.1	21	24	24	28	25	24	24 ± 2.2
45, 47	75	76	75	73	74	79	74 ± 2.1	79	76	76	72	75	76	76 ± 2.2
48	6	7	8	11	7	5	8 ± 2.1	8	11	10	12	5	7	9 ± 2.6
50	18	18	21	28	22	14	20 ± 4.8	19	23	24	23	17	25	22 ± 3.1
52	58	48	45	40	53	41	47 ± 7.0	43	41	43	43	57	52	46 ± 6.4
54	18	27	26	21	18	40	25 ± 8.3	30	25	23	22	21	16	23 ± 2.8
LDL_1														
36	35	33	38	40	33	43	37 ± 4.0	39	40	37	39	—	—	39 ± 1.2
38	36	40	39	41	38	44	40 ± 4.0	36	36	46	44			40 ± 5.2
40	29	27	23	19	29	13	23 ± 6.4	25	24	17	17			21 ± 4.3
43	23	20	21	30	23	20	23 ± 3.8	21	24	26	20	—	—	20 ± 3.3
45, 47	77	80	79	70	77	80	77 ± 3.8	79	76	74	80			77 ± 2.7
48	1	5	3	12	8	6	6 ± 4	5	6	8	12			8 ± 3.1
50	24	21	24	28	21	18	23 ± 3.4	20	21	24	23	—	—	22 ± 1.8
52	49	44	46	40	42	44	44 ± 3.1	47	43	43	41			44 ± 2.5
54	26	30	27	20	29	32	27 ± 4.1	28	30	25	24			27 ± 2.8

LDL_2														
36	34	36	39	40	36	39	37.3 ± 2.3	38	39	40	37	35	37	37.7 ± 1.8
38	37	45	41	44	40	34	40.2 ± 4.2	37	41	40	42	36	34	38.3 ± 3.1
40	39	22	20	16	24	27	23.0 ± 4.7	25	20	20	12	29	29	22.5 ± 6.5
43	21	22	21	30	20	19	22.2 ± 4.0	21	32	28	20	24	24	24.8 ± 4.5
45	67	70	71	59	74	72	68.8 ± 5.3	68	58	60	74	62	67	64.8 ± 5.9
47	12	8	8	11	6	8	8.8 ± 2.2	11	10	12	16	14	9	12.0 ± 2.6
48	9	6	7	11	6	7	7.7 ± 2.0							
50	23	21	21	24	26	16	21.8 ± 3.4	28	34	32	29	29	30	30.3 ± 2.2
52	41	41	44	38	45	43	42.0 ± 2.5	58	49	53	46	48	48	50.3 ± 4.4
54	20	29	26	24	19	28	24.3 ± 4.1	14	17	15	21	23	22	18.7 ± 3.8
56	7	3	2	3	3	6	4.0 ± 2.0							
HDL														
36	35	29	35	35	34	36	34.0 ± 2.5	39	38	34	35	32	38	36.0 ± 2.8
38	43	42	42	41	40	45	42.2 ± 1.7	42	45	45	44	38	40	42.3 ± 2.9
40	22	29	23	24	25	19	23.7 ± 3.3	19	17	21	21	30	22	21.7 ± 4.5
43	20	20	16	26	20	13	19.2 ± 4.4	24	17	21	21	24	23	21.7 ± 2.7
45	65	65	80	62	70	82	70.7 ± 8.4	67	78	70	66	62	66	68.2 ± 5.4
47	15	15	4	12	10	5	10.2 ± 4.8	9	5	9	13	14	11	10.2 ± 3.2
48	6	8	10	14	15	6	9.8 ± 3.9	5	8	9	7	6	5	6.7 ± 1.6
50	26	22	16	26	21	13	20.7 ± 5.3	16	20	23	18	23	29	21.5 ± 4.6
52	42	43	47	38	40	46	42.7 ± 3.4	59	44	43	51	60	46	50.5 ± 7.5
54	25	27	27	22	24	35	26.7 ± 4.5	20	28	25	24	11	20	21.3 ± 5.9

*Carbon numbers 36—40, phosphatidylcholines with 34—38 acyl carbons; carbon numbers 43—47, cholesteryl esters with 16—20 acyl carbons; carbon numbers 48—56, triacylglycerols with 48—56 total acyl carbons.

TABLE VI

CARBON NUMBER DISTRIBUTION OF PHSOPHATIDYLCHOLINES OF VLDL, LDL_1, LDL_2 AND HDL PARTICLES OF FASTING PLASMA OF HYPERLIPEMIC SUBJECTS

Carbon number	Mole %									
	Type III					Type IV				
	1125	1224	0973	1015	Average ± S.D.	0745	0718	0738	0720	Average ± S.D.
VLDL										
32	2.0	2.9	2.5	1.2	2.1 ± 0.7	1.8	2.2	2.0	1.5	1.9 ± 0.3
33	1.3	2.0	0.5	0.7	1.1 ± 0.7	0.2	1.0	1.1	0.5	0.7 ± 0.4
34	40.4	33.6	40.7	37.6	38.1 ± 3.3	44.4	44.8	36.0	39.4	41.3 ± 4.2
36	40.0	37.7	38.8	40.4	39.2 ± 1.2	37.4	40.4	40.5	40.5	39.8 ± 1.5
38	14.9	19.2	14.5	15.6	16.1 ± 2.1	13.9	9.9	15.8	16.3	14.0 ± 2.9
40	1.4	4.6	3.0	4.5	3.4 ± 1.5	2.2	1.6	3.3	1.7	2.2 ± 0.8
LDL_1										
32	2.6	3.5	3.6	5.2	3.7 ± 0.9	2.1	2.8	1.5	2.3	2.2 ± 0.5
33	1.2	1.6	0.9	1.3	1.3 ± 0.3	0.7	1.1	0.8	1.1	0.9 ± 0.2
34	40.1	41.4	41.5	40.3	40.8 ± 0.7	42.5	44.8	34.8	40.4	40.6 ± 4.3
36	37.2	37.3	37.9	36.2	37.3 ± 0.8	37.2	40.4	41.9	39.4	39.7 ± 2.0
38	16.4	13.1	13.5	13.5	14.1 ± 1.5	14.8	9.9	16.0	14.5	13.8 ± 2.7
40	2.5	2.8	2.5	3.6	2.9 ± 0.5	2.7	1.6	3.5	2.2	2.5 ± 0.8
LDL_2										
32	2.1	3.1	2.2	1.8	2.3 ± 0.6	1.7	1.8	2.2	1.8	1.9 ± 0.2
33	1.1	1.6	0.3	1.0	1.0 ± 0.5	0.8	1.1	1.3	0.6	1.0 ± 0.3
34	41.7	38.9	38.7	39.8	39.7 ± 1.4	40.9	36.3	35.0	39.4	37.9 ± 2.7
36	38.2	37.7	40.4	37.8	38.5 ± 1.3	38.4	43.6	38.3	39.5	40.0 ± 2.8
38	13.8	15.2	15.7	15.9	15.2 ± 0.9	15.4	14.8	14.1	16.1	15.1 ± 0.9
40	3.1	3.4	2.7	4.2	3.4 ± 0.6	2.8	2.4	5.0	2.5	3.2 ± 1.2
HDL										
32	1.5	3.2	2.0	1.1	2.0 ± 0.9	1.9	1.7	1.0	1.6	1.6 ± 0.4
33	1.3	2.1	0.3	0.5	1.1 ± 0.8	0.7	1.0	0.6	0.5	0.7 ± 0.2
34	32.8	36.9	35.1	35.1	35.0 ± 1.7	43.9	38.4	31.3	41.5	38.8 ± 5.5
36	40.1	36.9	40.6	39.6	39.3 ± 1.7	38.8	43.4	41.5	38.6	40.6 ± 2.3
38	19.7	16.7	18.4	18.9	18.4 ± 1.3	12.3	14.7	20.7	14.6	15.6 ± 3.6
40	4.6	4.1	3.5	4.8	4.3 ± 0.6	2.5	0.7	4.9	3.1	2.8 ± 1.7

choline, sphingomyelin and free cholesterol molecules compared closely among the corresponding lipoprotein classes in the two types of subjects. Likewise, closely comparable are the average estimates for the sum of the cholesteryl ester and triacylglycerol molecules in the corresponding lipoprotein classes of the Type III and Type IV subjects, although there is much variation among the individual subjects in the cholesteryl ester/triacylglycerol ratios. These estimates are of the order of those obtained for the VLDL, LDL_2 and HDL particles of normolipemic subjects [9] and therefore probably represent the same complex lipoprotein interrelationships. These include the presence of less than one-half of the number of cholesteryl ester molecules in the LDL_2 compared to the VLDL particles. In many instances, however, the number of cholesteryl ester molecules in the VLDL, LDL_1 and LDL_2 particles is the same. On the basis of the data presented in Tables III and IV it is obvious that the corresponding lipoprotein particles in the Type III and Type IV subjects possess closely similar structures, although the proportions of the neutral lipid classes in the particle cores and of the polar lipid classes in the particle surface may possess significant differences.

Carbon number distribution

The distribution of the carbon numbers of the cholesteryl esters, triacylglycerols and phosphatidylcholines in the various lipoprotein fractions from the individual hyperlipoproteinemic subjects is given in Table V. It is seen that the variations among the individual subjects are of about the same order as those among the average values of different lipoprotein fractions. Furthermore, the carbon number distribution of the cholesteryl esters is about the same in all four lipoprotein classes for any one subject, although the contribution of the cholesteryl arachidonate to VLDL and LDL_1 is not readily apparent because of an overlap with the excess shorter chain triacylglycerols. The triacylglycerols of the VLDL were present in the highest amounts and allowed the most detailed assessment of their profiles. However, LDL_1 and LDL_2 fractions also contained sufficient amounts of triacylglycerols for the recognition of characteristic carbon number distributions. Clearly all of these lipoprotein fractions contained closely similar carbon number distributions, as did the HDL fraction, which contained only small amounts of triacylglycerols. We have shown elsewhere [30] that the similarities in the carbon number distribution of these triacylglycerols extend also to the fatty acid composition, but the molecular association and stereospecific distribution of the fatty acids has been determined only in the VLDL. The similarities in the carbon number distribution seen for the cholesteryl esters and triacylglycerols appear to extend to the phosphatidylcholines and sphingomyelins, which are estimated from their diacylglycerol and ceramide profiles. Since the ceramides and diacylglycerols partially overlap, the resulting carbon number profiles tend to obscure any differences among the different lipoprotein classes. Detailed analysis of the carbon numbers of the diacylglycerols and the ceramides requires a prior resolution of the two lipid classes by thin-layer chromatography in the form of the parent phospholipids or as the derived neutral lipid moieties.

Distribution of molecular species

Table VI gives the complete carbon number distribution of the diacylglycerol moieties of the phosphatidylcholines from the individual lipoprotein classes of four subjects each of the Type III and Type IV hyperlipoproteinemia. These analyses were made on capillary columns containing a non-polar liquid phase, which allowed the resolution of both carbon number and certain types of unsaturation. It is seen that the molecular species of phosphatidylcholines are closely similar in all classes of lipoproteins when isolated from the same or from different subjects. Furthermore, there are no significant differences in the distribution of the carbon number of the species between the Type III and Type IV hyperlipoproteinemia. These data are consistent with the hypothesis of an essentially complete equilibration of the molecular species of the phosphatidylcholines among the different plasma lipoprotein classes. The results of the distribution of the sphingomyelins among the VLDL, LDL_2 and HDL fractions of the Type III and Type IV subjects have already been reported [19]. The present analyses extend these findings to the LDL_1 fraction and show that its composition is clearly similar to that of the LDL_2 fraction in each of the subjects examined in both Type III and Type IV hyperlipoproteinemia (data not shown). For the present purpose it is pertinent to note that in these hyperlipoproteinemia subjects as in the normolipemic subjects analyzed previously [9] the sphingomyelin species did not fully equilibrate among the different lipoprotein classes. Specifically, both Type III and Type IV subjects possessed about 30% more of the longer chain species (C_{20}—$C_{24:1}$ acid amides) in the HDL fraction than in the VLDL fraction, with the LDL_1 and LDL_2 fractions containing intermediate proportions of the long-chain and short-chain species.

Table VII gives the fatty acid composition of the cholesteryl esters of the individual lipoprotein classes for these patients of each of the two hyperlipoproteinemia types. It is seen that the average composition of the fatty acids and therefore the molecular species of the cholesteryl esters is closely similar among the four lipoprotein classes of the Type III subjects, which compare closely to the observations made for the different lipoprotein classes of normolipemic subjects [9, 10]. In contrast to the normolipemic subjects, however, there is no evidence of an increased content of the characteristic dietary acids (oleic and palmitic) in the VLDL fraction. The cholesteryl ester composition of the different lipoprotein classes of the Type IV subjects was markedly different, with the VLDL and LDL_1 containing significantly less oleic and more linoleic acid than the HDL fraction. This suggests that these cholesteryl esters originate from different pools of fatty acids and do not equilibrate. The ratio of C_{16}/C_{18} fatty acids in the HDL fraction of the Type IV samples, however, was significantly lower than the corresponding ratio of the intact cholesteryl esters and raised the possibility that some of the linoleic acid could have been lost on storage of the samples, as the fatty acids were analyzed subsequent to the determination of the total lipid profiles. The relative content of the arachidonic acid was about the same in the cholesteryl esters of all lipoprotein classes, although a significant individual variation was also noted. In general, the molecular weight distribution of the fatty acids of the cholesteryl esters corresponded rather closely to the carbon number distribution of the cholesteryl esters given in Table V, except for VLDL and LDL_1, where the

TABLE VII

FATTY ACID COMPOSITION OF CHOLESTERYL ESTERS OF VLDL, LDL_1, LDL_2 AND HDL PARTICLES OF FASTING PLASMA OF HYPERLIPEMIC SUBJECTS

Fatty acids	Mole %							
	Type III				Type IV			
	1125	0793	1015	Average	0745	0718	0720	Average
VLDL								
14:0	1.68	2.17	1.48	1.77	1.15	1.73	1.27	1.3
16:0	16.4	19.9	14.4	16.9	16.8	15.9	16.9	16.5
16:1	3.9	3.4	3.6	6.7	4.3	5.4	4.5	4.7
18:0	1.8	1.5	1.9	1.7	2.1	1.8	1.8	1.9
18:1	25.4	26.2	19.8	23.8	22.2	27.9	26.3	27.1
18:2	46.2	33.8	5.8	43.9	44.9	41.1	45.5	43.8
18:3	0.5	1.1	0.9	0.8	0.5	2.3	0.8	1.2
20:3	0.4	0.3	0.7	0.5	0.7	1.3	0.3	0.7
20:4	3.4	2.6	4.3	3.4	2.3	2.4	2.4	2.3
LDL_1								
14:0	0.5	3.1	2.5	2.0	1.2	3.9	1.1	2.0
16:0	13.8	16.5	15.0	15.1	17.5	15.7	14.8	16.3
16:1	2.7	9.5	2.0	4.7	3.5	4.0	2.2	3.2
18:0	1.7	1.8	2.4	1.9	2.5	2.9	2.1	2.5
18:1	23.8	24.5	20.1	22.8	28.5	22.5	22.0	24.3
18:2	51.1	38.3	52.6	47.3	43.6	45.7	50.4	46.5
18:3	0.4	0.8	1.2	0.8	0.7	0.7	1.4	0.9
20:4	6.0	5.5	4.2	5.2	2.5	4.6	6.0	4.3
LDL_2								
14:0	1.2	2.5	1.8	1.8	1.8	2.5	2.5	2.2
16:0	16.0	22.8	14.9	17.8	20.0	16.0	20.4	18.8
16:1	3.8	12.6	4.3	6.9	5.0	6.1	4.5	5.2
18:0	1.5	2.0	1.4	1.6	2.1	1.6	1.5	1.7
18:1	22.6	29.3	16.2	22.7	26.1	23.9	22.4	24.1
18:2	49.2	29.9	54.4	44.1	42.3	44.0	44.7	43.6
18:3	0.6	0.4	0.9	0.6	0.4	2.3	0.8	1.2
20:3	0.5	0.3	1.0	0.6	0.6	0.8	0.4	0.6
20:4	4.3	0.9	4.9	3.4	1.5	2.6	2.5	2.2
HDL								
14:0	1.6	1.8	1.8	1.7	1.7	4.7	3.6	3.3
16:0	20.0	19.5	14.6	18.0	24.9	28.8	27.1	26.9
16:1	5.0	12.6	5.0	22.6	9.3	7.5	8.4	8.4
18:0	1.7	1.6	1.1	1.4	2.6	3.7	2.9	3.0
18:1	26.8	26.4	16.8	23.3	35.4	34.5	30.7	33.5
18:2	43.4	32.8	53.3	43.1	24.1	14.6	25.7	21.4
18:3	0.6	1.0	1.2	0.9	0.8	1.4	0.7	0.3
20:4	2.2	2.9	4.8	3.3	0.5	3.7	0.5	1.6

cholesteryl arachidonate contribution was masked by the excess triacylglycerol.

The carbon number distribution of the molecular species of the triacylglycerols of the various lipoprotein classes for each subject with Type III and Type IV hyperlipoproteinemia is given in Table V. Despite considerable individual variation, there is rather close correspondence among the average values. The best estimates are obtained for the VLDL and LDL_1 fractions, which account for a high proportion of the total lipoprotein in both Type III and Type IV hyperlipoproteinemia and which are also richest in the triacylglycerols. It is not known whether or not the similarities in the carbon number distribution extend to the composition and positional distribution of the fatty acids. We have performed [30] detailed stereospecific analyses of the triacylglycerols of VLDL on a small number of normolipemic and Type III and Type IV hyperlipemic subjects and have found certain similarities in the triacylglycerol structure. However, no comparisons have yet been made among the triacylglycerol structures of different lipoprotein classes from any normolipemic or hyperlipemic subjects.

DISCUSSION

On the basis of the detailed analysis of the lipid class and molecular species composition it is obvious that the various plasma lipoprotein fractions examined in the present study possess essentially normal composition. The accumulation of the LDL_1 fraction in both Type III and Type IV subjects appears to represent an excess of VLDL remnants (LDL_2), which possess a reasonable similarity to LDL_1 according to a variety of criteria, including the increased content of sphingomyelin. The similarities in the chemical composition of the VLDL, LDL_1, LDL_2 and HDL particles between the normolipemic and hyperlipemic subjects include the calculated particle diameters. The above findings are in accordance with certain previous studies. Stromberg et al. [27] have claimed that in Type III hyperlipoproteinemia all VLDL subfractions contain increased concentrations of total cholesterol and triacylglycerols and are relatively enriched in total cholesterol, when compared to VLDL from normolipemic subjects. The ratios of total cholesterol to triacylglycerols in the LDL subfractions of Type III hyperlipoproteinemia, however, were not significantly different from those in the LDL subfractions of the normolipemic subjects and the protein composition was also similar. Packard et al. [28] have shown that the enrichment in total cholesterol of the VLDL subfractions in Type III patients is due largely to cholesteryl esters, which are accumulated at the expense of some of the triacylglycerol. In the Type IV group, all VLDL subfractions were normal in composition. The lipid composition of the LDL subfractions of Type III and Type IV groups showed no significant deviation from that of normolipemic subjects. There were no significant differences in particle size among the Type III and Type IV patients and normal subjects of the corresponding higher density VLDL and LDL subfractions, although the lower density VLDL subfractions of the hyperlipemic subjects showed somewhat greater particle size than those of the corresponding normolipemic subjects.

On the basis of chemical analyses and computation of number of particles

Redgrave and Carlson [31] have concluded that the hyperlipidemia of Type IV subjects is accounted for by an increase in total number of VLDL and a shift towards higher particle size. Although our data cannot exclude possible changes in particle size of a minor subclass of any one lipoprotein class, the possibility of a significant deviation of the average particle size from that of normal particles is unlikely unless there has been a significant structural change in these particles, for which no evidence has yet been advanced. As a result, the well defined hyperlipoproteinemias of the Type III and Type IV, therefore, must be attributed largely to the accumulation of abnormal numbers of essentially normal lipoprotein particles, especially those of the VLDL, LDL_1 and LDL_2 density range.

Havel [32] and Hazzard and Bierman [33] have suggested that in Type III hyperlipoproteinemia there is defective conversion of VLDL into LDL with accumulation of LDL_1, while Stromberg et al. [27] have claimed that the defect may lie not only at the stage of conversion of VLDL into LDL, but also earlier in the metabolic cascade. The reason for the proposed defect in catabolism of VLDL remains uncertain. Stromberg et al. [27] have suggested that it may be related to the relative proportions of the various tetramethylurea soluble apolipoproteins, while Packard et al. [28] have considered the possibility of decreased fluidity resulting from a higher concentration of cholesteryl esters in the smaller VLDL particles as a contributing factor. In fact, the validity of the direct lipoprotein interconversion hypothesis itself must be questioned, but a satisfactory explanation has not been forthcoming.

We have recently examined the precursor—product relationship of the lipid components required for a direct conversion of VLDL into LDL and HDL postulated by Eisenberg et al. [1] and have shown that such a simple relationship does not exist in normolipemic subjects and that much more complex series of events must be involved. A similar examination of the precursor—product relationship in the present study appears to support these conclusions. The present study shows that the LDL_1 and LDL_2 fractions are closely related in their qualitative and quantitative lipid class and molecular species composition. The major differences between VLDL and LDL_1, and LDL_1 and LDL_2 are confined to the relative proportions of the triacylglycerols and cholesteryl esters. A gradual increase in the cholesteryl ester content would be anticipated if the particles were the products of VLDL degradation by lipoprotein lipase, which hydrolyses triacylglycerols but not cholesteryl esters. A simple conversion of a VLDL particle into a LDL_1 or LDL_2 particle, however, would appear to be excluded as the resulting particles contain only about one half the number of cholesteryl ester molecules present in the original VLDL. Clearly, the VLDL particle must have lost a considerable amount of cholesteryl ester along with the bulk of the triacylglycerol. Alternatively, the residual VLDL particle could have been cleaved into the LDL_1 and LDL_2 particles. Neither of these possibilities have been considered by the lipoprotein interconversion hypothesis. Neither a simple cleavage of the VLDL particle nor an indiscriminate loss of cholesteryl ester can account for the differences in the fatty acid composition of the VLDL and the LDL fractions. Since neither LDL_1 nor LDL_2 are known to support cholesteryl ester synthesis via lecithin: cholesterol acyltransferase, the differential composition of the cholesteryl

esters must have resulted from some other mechanism, such as cholesteryl ester exchange [34] or particle fusion [35], both processes having been demonstrated to occur among the plasma lipoproteins, but not included as prominent transformations in the lipoprotein interconversion hypothesis of Eisenberg et al. [1].

Just like the LDL_1 and LDL_2 of the normolipemic subjects, those of the hyperlipemic subjects also showed a marked relative increase in the sphingomyelin/phosphatidylcholine ratio, although the molecular species of neither phospholipid had undergone significant change. This change in the phospholipid class ratio indicates that the degradation of the VLDL involves in addition to the hydrolysis of the triacylglycerols also a preferential loss of phosphatidylcholine and retention of sphingomyelin in the residual particle. Since the sphingomyelin/cholesteryl ester ratio remains about the same as in VLDL, the loss of any cholesteryl ester must be accompanied by a loss of sphingomyelin, which would favour a cleavage of the VLDL particle at some stage of the degradation. It must therefore be concluded that a direct precursor—product relationship is not realized for the VLDL and LDL_1 or LDL_2 particles in the hyperlipoproteinemic patients. This also was the case in the normolipemic subjects [9, 10]. The hope that the accumulation of the presumed products would help to demonstrate the postulated precursor—product relationship was not realized, although the possibility was not entirely excluded in some modified form.

According to the lipoprotein interconversion hypothesis [1, 36, 37], plasma HDL arises largely or exclusively from the excess surface material of VLDL, which is released as an LPX-like interphase following the triacylglycerol hydrolysis. It would be anticipated that in such an instance the HDL would posses the relative proportions and composition of the sphingomyelins and phosphatidylcholines which are identical to those of VLDL. Even if allowance is made for some distortion of the molecular species of the phosphatidylcholines due to lecithin:cholesterol acyltransferase activity on HDL, which is known to favour the more unsaturated species [4], the molecular species of the sphingomyelins should have retained the composition of the precursor VLDL. The present work with the hyperlipemic plasma lipoproteins shows that the expected relationship again is not realized. Although the total sphingomyelin/phosphatidylcholine ratio in the HDL is rather close to that in the VLDL, and both lipoprotein classes possess about the same species of phosphatidylcholine, there is a marked and irreconcilable difference in the composition of the molecular species of the sphingomyelins between the VLDL and HDL fractions. As noted for normolipemic subjects the HDL contains as much as 30% more of the longer chain species of sphingomyelin than does the VLDL, even when isolated from the same subject. Other transformations than simple salvage of excess phospholipids must therefore be involved even in the VLDL—HDL conversion. We have suggested that perhaps the accumulation of the long-chain sphingomyelins in the HDL could be explained on the basis of a lateral phase separation taking place at the time of the LPX formation. Alternatively, preferential interaction of apo A_1 or apo A_2 with the long-chain sphingomyelins must be considered.

Finally, the HDL from both Type III and Type IV subjects possesses a

relatively high proportion of triacylglycerols. The lipoprotein interconversion hypothesis does not allow for the presence of triacylglycerols in the HDL fraction. Possibly significant amounts of the cholesteryl esters of HDL in the hyperlipemic subjects have been exchanged for triacylglycerols in the LDL_1, LDL_2 or VLDL, with which HDL is believed to undergo catalytic exchange via special carrier proteins [38]. Alternatively, the HDL could have been contaminated with some VLDL or LDL_1 remnants of high density, which are rich in triacylglycerols and are cleared very slowly [39]. We have discussed the above possibilities in the context of the results obtained with the lipoproteins of normolipemic subjects [9], and have proposed that particle fusion and cleavage in plasma in the native form or during ultracentrifugal isolation in the presence of the chelating agents must be advanced as a plausible mechanism for the rationalization of the differential distribution of the various lipid classes among the different lipoprotein fractions.

In view of the absence of any obvious characteristic abnormalities in the major lipid composition and structure, the accumulation of the VLDL and LDL_1 particles in Type III and Type IV hyperlipoproteinemia subjects must be sought elsewhere, e.g., in the apoprotein composition [40, 41] of these lipoproteins or in the composition and structure of their membrane receptors, which may recognize the minor differences noted.

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A METHOD FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SCREENING OF UV-POSITIVE COMPONENTS IN URINE ELUATE FROM SEPHADEX G-10 AND MODIFICATIONS FOR DETERMINATION OF URINARY SALICYLIC, SALICYLURIC AND GENTISIC ACIDS

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SUMMARY

Acidified urine is chromatographed on a small Sephadex G-10 column, and UV-absorbing urine constituents in an appropriate eluate are screened by reversed-phase high-performance liquid chromatography. Chromatograms of morning-urine specimens from healthy adults show 14—19 peaks. The elution positions of eighteen common aromatic urine compounds have been established. Modifications for the determination of urinary salicylic, salicyluric and gentisic acids are also presented.

INTRODUCTION

The combination of aromatic adsorption chromatography on Sephadex gel and reversed-phase high-performance liquid chromatography (HPLC) with UV absorbance detection provides a simple tool for the determination of some aromatic urine metabolites [1—3]. In order to explore the possibilities of its application to determine some other aromatic urine components, the combination using Sephadex G-10 [2,3] is modified for screening some low-molecular-weight aromatic urine constituents and drugs and drug metabolites. For identification purposes, as well as for developing separate determination methods, the retention properties of 25 aromatic reference substances on G-10 and on a reversed-phase column were established. Methods for the measurement of urinary salicylic acid (SA), salicyluric acid (SUA) and gentisic acid (GA) are presented in this report. A preliminary mass spectrometric investigation of an unknown urine component (see x_4 in ref. 3) is also included in the paper.

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EXPERIMENTAL

Apparatus

The equipment and the operation conditions for the Sephadex G-10 chromatographic isolation and the HPLC—UV measurements previously described [3] were utilized, except that the isolation columns had the dimensions of 6 cm × 4 mm I.D. and 4 cm × 4 mm I.D., respectively, for SA and GA and the detection wavelengths were 300, 300 and 320 nm, respectively, for SA, SUA and GA.

Chemicals and reagents

(1) 0.1 *M* ammonium formate buffer, pH 3. (2) Mobile phases: (A) (for monitoring reference substances and for screening) 200 ml acetonitrile—800 ml glass-distilled and degassed water—0.4 ml concentrated sulphuric acid—100 mg sodium lauryl sulphate (SLS), pH 2; (B) (for SA assay) 200 ml aceto nitrile—200 ml glass-distilled and degassed water—0.4 ml concentrated formic acid, pH 3; and (C) (for a purification approach and for SUA and GA assays) 120 ml acetonitrile—280 ml glass-distilled and degassed water—0.4 ml concentrated formic acid, pH 3. (3) Reference substances (Sigma, St. Louis, MO, U.S.A.) were as listed in Table I. Check [1—3] the accuracy of the respective stock solution, 100 µg/ml distilled water, of SA, SUA and GA. Store the stock solutions at 5°C (stable for one month). Prepare the working standards as in ref. 3 and make also a 43.75 µg/ml standard from the 50 and 37.5 µg/ml GA standards. (4) Pack the isolation columns with Sephadex G-10 (Pharmacia, Uppsala, Sweden), equilibrate and store as in ref. 3.

Procedure

Sample preparation. Use freshly voided morning urine [2,3] for the screening and purification purposes and untimed urine (acidify and centrifuge as for the morning urine) for the determination of aspirin metabolites.

Screening of UV-positive components in protein-free urine. The procedure for the determination of HVA in urine, described in ref. 3, was used with some modifications. Place a set of one to ten equilibrated G-10 columns (12 cm × 4 mm I.D.) over one to ten sets of three 15-ml test tubes, graduated for 10 ml. Run 200 µl of the urine specimen as described. Discard the first fraction (3 ml). Pool the second and the third fractions (10 ml each) and freeze-dry. Dissolve the residue in 0.5 ml of the buffer, centrifuge and inject 20 µl of the supernatant onto the reversed-phase column (for checking of the HPLC—UV system, see ref. 3) and screen for 30 min.

Purification of the component corresponding to peak No. 16 in Fig. 1 (x_4 in ref. 3) from urine. Chromatograph (see calibration process in ref. 3) 200 µl of the specimen on the equilibrated G-10 column (12 cm × 4 mm I.D.) and establish the elution range of component 16, recognizable by its retention characteristics (V_e/V_t = 4.40—4.66 on G-10; t_R on ODS-2 is 20 min in mobile phase A and 7.1 min in C). For isolation and enrichment on an analytical scale, use the following technique. Place two sets of ten columns over 20 sets of two 15-ml

test tubes, graduated for 10 ml, and run a 200-μl specimen on each column as described [3]. Discard the first 5-ml fractions. Keep the second 3.5-ml fractions (a typical chromatogram for the analysis of No. 16 is illustrated in Fig. 2A), pool and freeze-dry. Dissolve the residue in 200 μl of the mobile phase, centrifuge and inject ten times 20 μl of the supernatant onto the ODS-2 column and collect the eluate containing component 16 (see the markings in Fig. 2B), pool and freeze-dry. Dissolve the residue in about 50 μl of the mobile phase, centrifuge and check the spectrometric purity of the component on HPLC—UV (Fig. 2C).

For a mass spectrometric measurement, repeat the described procedure (avoid use of plastic materials all through) at least four times after regenerating the columns overnight.

Determination of SA, SUA and GA in urine. Run 200 μl of the specimen on the appropriate isolation column as described under Determination of HVA in urine in ref. 3. Discard the first fraction of 2.5, 5.0 and 2.5 ml and keep the second fraction of 4.5, 4.0 and 4.0 ml for the SA, SUA and GA assay, respectively, for further separation and quantitative determination on the HPLC—UV system (for checking of HPLC—UV, see ref. 3). Obtain the concentration by means of the respective calibration curve (peak height in mm versus concentration in μg/ml) constructed using working standards run in the same way as the samples.

RESULTS AND DISCUSSION

As can be seen in G-10 chromatograms of 24-h urine specimens [2], many UV-positive urine components are strongly adsorbed by elution with 0.1 *M* ammonium formate buffer, pH 3. Hence, the eluate from G-10 containing low-molecular-weight components in urine was subjected to screening by HPLC—UV. Freshly voided morning urine was utilized throughout the study in order to avoid interference from dietary metabolites and the risk of inadequate collection and of decomposition during storing associated with 24-h urine. The compounds eluting from G-10 at between 2.1 and 15.3 bed volumes (V_t = 1.5 ml) could be resolved into 14—19 peaks on ODS-2 with the chosen conditions. Specimens from healthy adults (n = 15) screened by the present technique, showed chromatographic patterns similar to those illustrated in Fig. 1.

For tentative identification of the peaks, 25 reference substances, mostly aromatic acids and their hydroxyl derivatives frequently found in urine, were checked on G-10 and on ODS-2 columns separately, and their chromatographic properties compared with those of the above-mentioned peaks. A list of the retention data is given in Table I. As can be seen in the table of the retentions on G-10, half of the tested substances showed a V_e/V_t value greater than 4. The affinity of phenolic compounds to Sephadex may be due to interactions between phenolic hydroxyls and the ether oxygens of the crosslinks [4]. Accordingly, the masking of a phenolic hydroxyl by an alkyl group results in a considerably lower retention (Table I). On the other hand, the higher adsorp-

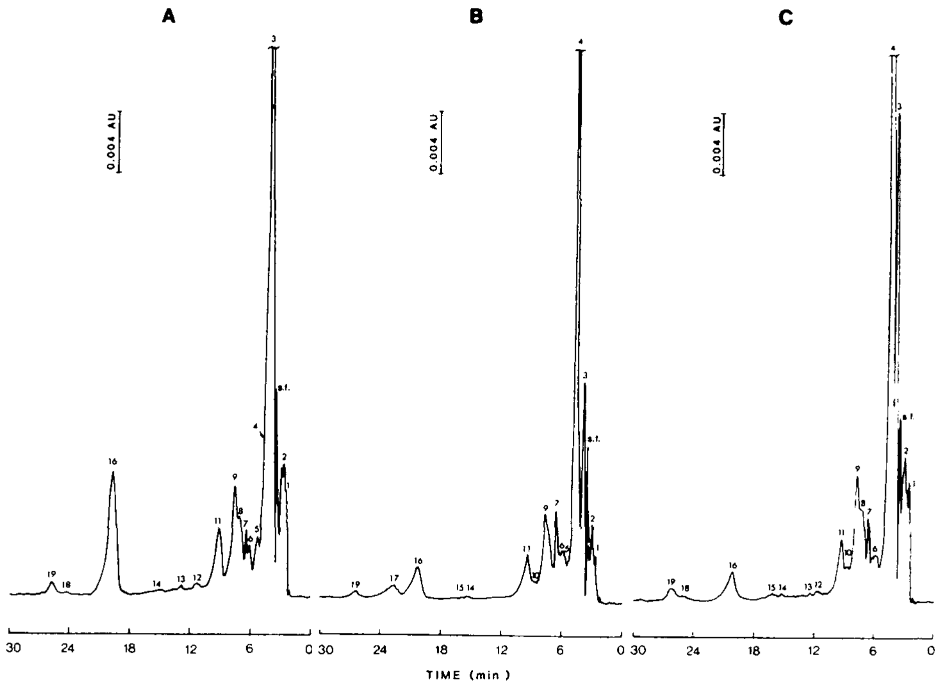

Fig. 1. Typical chromatographic patterns of the HPLC—UV screening of UV-positive components in protein-free urine. Morning urine specimens were obtained from the healthy subjects: (A) female, age 52, weight 55 kg, urine volume 320 ml; (B) male, age 64, weight 79 kg, urine volume 325 ml; (C) female, age 20, weight 58 kg, urine volume 400 ml.

tion of IAA and 5-HIAA, already reported [2], may be caused by a high π-electron-donating ability of such indolic compounds; the presence of uncharacterized π-electron-acceptor groups in Sephadex was suggested earlier [5]. Furthermore, the aromatic acids with unsaturated α—β linkages also adsorb strongly on G-10, as can be seen in the table of the retention values of cinnamic acid and its hydroxyl derivatives. On the other hand, the retentions on ODS-2 seem to be mainly due to reversed-phase interactions. (None of the reference compounds showed any ion pairing with SLS.) Thus, the 2-hydroxy-substituted aromatic acids were retained much more than the 4-hydroxy-substituted ones. By means of the retention values for the reference compounds which elute from the G-10 column within 15 bed volumes and from the ODS-2 column within 30 min, the elution position of several common urine compounds could be determined in the screening diagram. Thus, the position of UA coincides with peak 1, of 3,4-DHMA with peak 2, of 3-HMA, 4-HMA, 3,4-DHPAA, 4-HLA and HGA with peak 3, of 4-HPAA with peak 7, of 3-HPAA, HVA, HA, 4-HBA and GA with peak 8 and of IAA with peak 17. Additionally, SUA and caffeine appear close to peak 12 and ASA close to peak 16. The position of CA is at t_R = 27 min. Although most of the peaks correspond to more than one component, some of them may correspond to a single urine component only. Regarding peak 16, the purification process on an

TABLE I

RETENTION BEHAVIOUR OF REFERENCE COMPOUNDS, RUN ON SEPHADEX G-10 AND ON ODS-2 SEPARATELY

A 200-μl volume of freshly prepared reference solution (100—200 μg/ml) was chromatographed [3] on a G-10 column (12 cm × 4 mm I.D.)* and 10—50 fractions of 0.5 ml monitored** by HPLC—UV (conditions as described in Experimental). t_R values were obtained by direct injections from the respective reference solution.

Compound	V_e/V_t on G-10	t_R (min) on ODS-2
Creatinine	0.73— 1.00	6.0
1,3,7-Trimethylxantine (caffeine)	0.73— 1.00	10.8
2,6,8-Trioxypurine (uric acid, UA)	1.40— 1.66	2.3
Benzoylaminoacetic acid (hippuric acid, HA)	1.40— 1.66	6.9
2-Hydroxyhippuric acid (salicyluric acid, SUA)	4.40— 4.66	11.1
4-Hydroxyphenyllactic acid (4-HLA)	2.06— 2.33	4.3
2-Hydroxybenzoic acid (salicylic acid, SA)	7.06— 7.33	34.0
4-Hydroxybenzoic acid (4-HBA)	10.40—10.66	6.8
2-Acetoxybenzoic acid (acetylsalicylic acid, ASA)	2.06— 2.33	19.3
2,5-Dihydroxybenzoic acid (gentisic acid, GA)	10.73—11.00	6.9
3,4-Dihydroxybenzoic acid (3,4-DHBA)	10.73—11.00	4.3
3-Hydroxymandelic acid (3-HMA)	2.06— 2.33	3.7
4-Hydroxymandelic acid (4-HMA)	2.40— 2.66	3.5
3-Methoxy-4-hydroxymandelic acid (VMA)	1.73— 2.00	3.3
3,4-Dihydroxymandelic acid (3,4-DHMA)	2.73— 3.00	2.6
3-Hydroxyphenylacetic acid (3-HPAA)	3.73— 4.00	7.1
4-Hydroxyphenylacetic acid (4-HPAA)	3.73— 4.00	6.3
3-Methoxy-4-hydroxyphenylacetic acid (HVA)	3.40— 3.66	6.8
2,5-Dihydroxyphenylacetic acid (2,5-DHPAA, HGA)	4.73— 5.00	3.5
3,4-Dihydroxyphenylacetic acid (3,4-DHPAA, DOPAC)	4.73— 5.00	4.0
Indole-3-acetic acid (IAA)	15.77—16.33	22.3
5-Hydroxyindole-3-acetic acid (5-HIAA)	19.20—20.00	5.6
Cinnamic acid (CA)	10.06—10.33	27.0
4-Hydroxycinnamic acid (4-HCA)	23.20—24.00	12.9
3,4-Dihydroxycinnamic acid (3,4-DHCA, caffeic acid)	34.20—36.00	6.8

*G-10 chromatography of 5-HIAA, 4-HCA and 3,4-DHCA carried out on 4 cm × 4 mm I.D. columns.
**Creatinine monitored at 240 nm (not detectable at 280 nm).

analytical scale resulted in a spectrometrically pure isolated substance (Fig. 2C). Furthermore, the compound in question eluted as a single peak in a number of mobile phases with different acetonitrile/water ratios (unpublished results). A preliminary mass spectrometric investigation resulted in the fragmentation patterns illustrated in Fig. 3. Base ions appear at m/z 161 (Fig. 3A) and 44 (Fig. 3B). Prominent ions were the fragments at m/z 132, 147 and 186 (Fig. 3A) and at m/z 28, 29 and 46 (Fig. 3B).

Certainly, gas chromatography (GC) combined with mass spectrometry (MS) is recognized as the main technique for screening and identifying constituents present in human urine (publications until 1977 are reviewed in ref. 6). However, GC is restricted to volatile substances and compounds con-

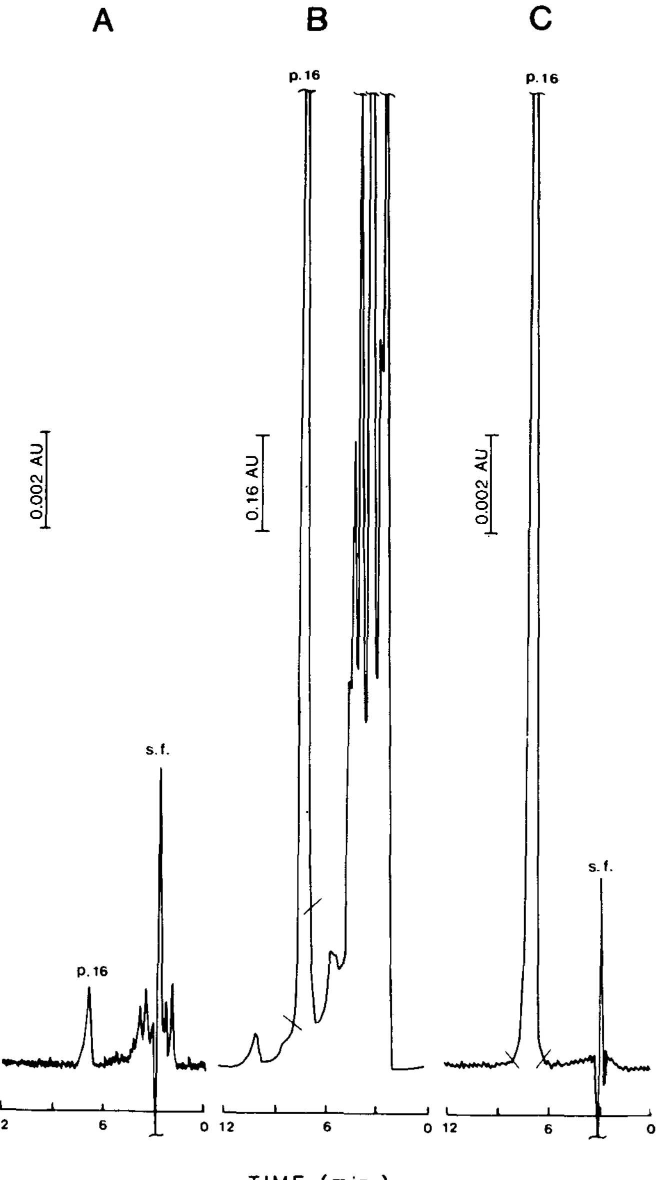

Fig. 2. HPLC diagrams for checking the isolation and enrichment stages for component 16 in Fig. 1 from urine (conditions as in Experimental).

vertible into volatile derivatives and is not suitable for screening high-molecular-weight and/or temperature-labile constituents. Consequently, the HPLC technique constitutes a powerful complement to GC [7,8] by its rapidity, simplicity, reproducibility and efficiency. The simple HPLC—UV technique worked out in this study may be used for preliminary checking of alterations in urinary phenolic acid status, since the formation of methyl esters of such aromatic

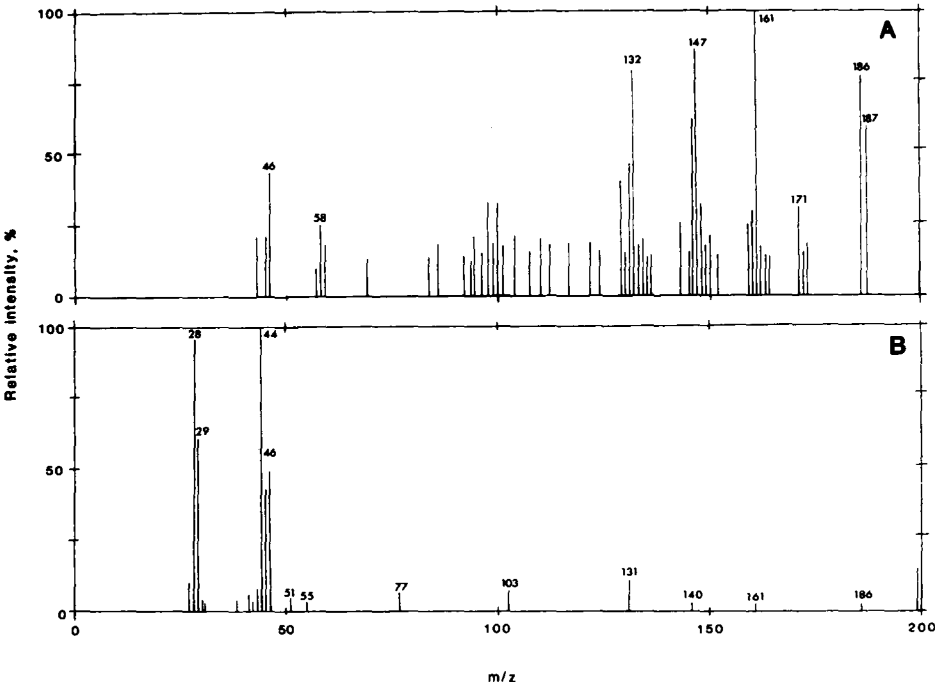

Fig. 3. The 12-eV electron-impact (A) and the 70-eV electron-impact (B) spectra of urine compound 16, obtained on an LKB-9000 mass spectrometer by scanning the region m/z 20—400. Operating conditions were: direct inlet temperature, 90°C; ion source temperature, 290°C; trap current, 60 μA.

acids for multiple-component analysis by GC also results in the formation of methyl ethers leading to multiple derivatives [9].

Retention data in Table I were also of use in selecting and developing some new two-step chromatographic determination methods. Typical chromatograms for the determination of some aspirin metabolites are given in Fig. 4. From a dose of one aspirin (about 10 mg of aspirin per kg body weight) 1.1% was excreted after 8 h as SA, 39.2% as SUA and 0.9% as GA. The calibration curves of SA, SUA and GA, which pass through the origin on extrapolation, are linear from 3.125 to, respectively, 50, 37.5 and 43.75 μg/ml. Higher concentrations require dilution of the urine samples. For quantities less than about 2 μg/ml the second fractions will be concentrated [2]. The lowest detectable amounts of SA, SUA and GA in urine are, respectively, 0.5, 0.5 and 0.25 μg/ml, which correspond to 10, 10 and 5 ng injected. The recoveries of added (25 μg/ml urine) SA, SUA and GA were found to be, respectively, 98.7% (0.4% R.S.D., n = 5), 96.1% (0.5% R.S.D., n = 5) and 99.1% (0.5% R.S.D., n = 5).

Furthermore, methods for the measurement of urinary cinnamic acid and its hydroxyl derivatives are possible by small modifications of the described determination methods.

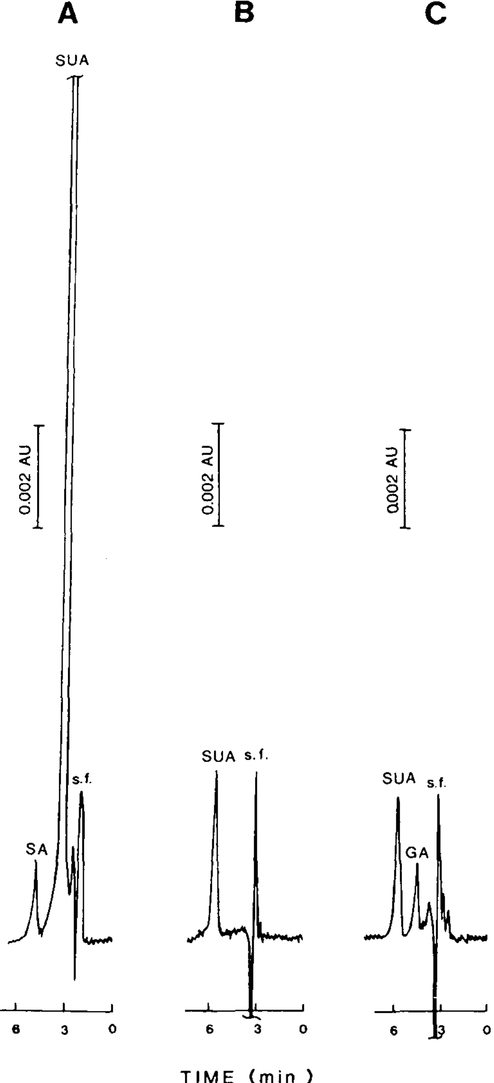

Fig. 4. Typical HPLC diagrams of (A) SA, (B) SUA and (C) GA in urine obtained by the described methods. Concentrations in urine, 8 h after an oral dose of one aspirin tablet (500 mg ASA), were 20.7, 702.0 and 16.4 μg/ml, respectively (voided urine volume = 280 ml; SUA analysis on 20-fold diluted urine).

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ENZYMATIC DETECTION OF URINARY CONJUGATED STEROIDS AFTER GEL CHROMATOGRAPHY

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SUMMARY

An enzymatic detection method is described for urinary conjugated steroids after chromatographic fractionation with Sephadex G-25. The principle of the method is as follows. Part of a 24-h urine sample, (1—2 ml of urine) is applied directly, to a short column of Sephadex G-25 and eluted with acetate buffer solution. Steroid conjugates in each fraction are hydrolyzed with steroid sulfatase—β-glucuronidase. After enzymatic hydrolysis, an enzymatic color development reagent for steroids, either 3α-hydroxysteroid dehydrogenase or 3β-hydroxysteroid oxidase, are added and the dye formed is measured spectrophotometrically. Excretion patterns of steroid-3β-sulfates, and steroid-3α-glucuronides and steroid-3α-sulfates are shown with some patients' samples. A precision of the assay values for steroid-3α-glucuronide, steroid-3α-sulfate and steroid-3β-sulfates in urine samples and assay values for normal subjects are also studied.

This simple enzymatic method for detecting the excretion patterns of urinary conjugated steroids may have a diagnostic value for clinical tests.

INTRODUCTION

Urinary steroids have been determined by colorimetric methods such as the Porter—Silber reaction and the Zimmerman reaction after hydrolysis of steroid conjugates and extraction with organic solvent [1,2].

Enzymatic detection of steroids such as neutral 3α-hydroxysteroids [3,4], 3β-hydroxysteroids [5,6], acidic 3α-hydroxysteroids [7], and 17β-hydroxysteroids [8,9] has been reported previously.

Amberlite XAD-2 and Sephadex G-25 resins have been used for extraction of conjugated steroids instead of organic solvent [10,11], and Sephadex LH-20 and DEAE-Sephadex have been used for fractionation of free steroids and conjugated steroids [12,13].

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In this paper, direct enzymatic detection of urinary conjugated steroids such as steroid-3β-sulfate, steroid-3α-glucuronide and steroid-3α-sulfate is described. The principle of the reactions is as follows:

$$\left.\begin{array}{l}\text{Steroid sulfate}\\ \text{Steroid glucuronide}\end{array}\right. \xrightarrow[\beta\text{-glucuronidase}]{\text{steroid sulfatase}} \text{free steroids} \qquad (1)$$

$$3\alpha\text{-Hydroxysteroids} + NAD^+ \xrightarrow{3\alpha\text{-hydroxysteroid dehydrogenase}} 3\text{-oxosteroids} + NADH \qquad (2)$$

$$NADH + INT \xrightarrow{\text{diaphorase}} \text{formazan}$$

$$3\beta\text{-Hydroxysteroids} \xrightarrow{3\beta\text{-hydroxysteroid oxidase}} 3\text{-oxosteroids} + H_2O_2 \qquad (3)$$

$$H_2O_2 + 4\text{-Aminoantipyrine} + EMAE \xrightarrow{\text{peroxidase}} \text{quinoneimine dye}$$

MATERIALS AND METHODS

All the reagents used were of analytical grade, and together with β-glucuronidase from *Escherichia coli* (EC 3.2.1.31) and bovine liver, and sulfatase—β-glucuronidase (EC 3.1.6.1 and EC 3.2.1.31) from *Helix pomatia*, were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.

All standards of steroids and conjugated steroids, 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride (INT) and diaphorase (EC 1.6.99.2, from *Clostridium kluyveri*) were also purchased from Sigma. 3α-Hydroxysteroid dehydrogenase (3α-HSD) from *Pseudomonas testosteroni* (EC 1.1.1. 50) and β-NAD^+ were purchased from Nyegaard (Oslo, Norway). 3β-Hydroxysteroid oxidase from *Brevibacterium sterolicum* (EC 1.1.3.6), peroxidase from horseradish (EC 1.11.1.7), 4-aminoantipyrine (4-AA) and N-ethyl-N-(3-methylphenyl)-N′-acetylethylenediamine (EMAE) were purchased from Kyowa Medics Co., Tokyo, Japan.

Sephadex G-25 fine and the column were purchased from Pharmacia (Uppsala, Sweden).

Preparation of reagents

Sulfatase—β-glucuronidase (200 units of sulfatase and 3000 Fishman units of β-glucuronidase per 1 ml of 0.05 *M* acetate buffer, pH 5.0) from *H. pomatia* (type H-1), β-glucuronidase (500 Fishman units per 1 ml of 0.1 *M* phosphate buffer, pH 6.8) from *E. coli*, and β-glucuronidase (1500 Fishman units per 1 ml of 0.05 *M* acetate buffer, pH 5.0) from bovine liver (type B-3) were prepared. All these enzymes were used without addition of activator for a step in the hydrolysis of steroid conjugates.

For the preparation of enzyme reagent for color development of 3α-hydroxysteroids [3,4] dissolve 60 mg of INT in 100 ml of 0.2 *M* K_2HPO_4 (pH 9.0) containing per 100 ml: 10 U of 3α-HSD, 500 U of diaphorase and 50 μmol of

β-NAD^+. The enzyme reagent for color development of 3β-hydroxysteroids was as described previously [5,6]. Determiner FC '555' was purchased and used for color development of 3β-hydroxysteroids.

Preparation of urine sample

A 24-h urine specimen is collected and an aliquot is centrifuged for 3 min at 2500 *g*. A volume of 1—2 ml of the supernatant is directly applied to the Sephadex G-25 column (1 × 24 to 29 cm).

Sephadex gel chromatography

Sephadex G-25 is swollen by heating a suspension of the particles in 0.05 *M* acetate buffer (pH 5.0) for 4 h at 90°C under constant stirring. The fines are removed by several decantations and the slurry is poured directly into the column which is then washed for 3 h with acetate buffer solution.

After application of the urine sample, chromatographic separation is performed with 0.05 *M* acetate buffer (pH 5.0). One fraction of effluent contains 1.3 ml; ten fractions are run within 30 min and 35 fractions are collected.

Procedure for detection of steroid-3α-glucuronide and steroid-3α-sulfate

To 0.4 ml of each chromatographic effluent is added 0.1 ml of sulfatase—β-glucuronidase solution and incubated for 20 h at 37°C. After hydrolysis of steroid conjugates, 1 ml of color development reagent for 3α-hydroxysteroids is added and incubated for 30 min at 37°C. Absorbance at 500 nm is read against the first effluent fraction.

Procedure for determination of steroid-3β-sulfate

To 0.6 ml of each effluent fraction is added 0.1 ml of sulfatase-β-glucuronidase solution and incubated for 20 h at 37°C. After hydrolysis, 1 ml of color development reagent for 3β-hydroxysteroids is added and incubated for 20 min at 37°C. Absorbance at 550 nm is read against the first effluent fraction.

RESULTS

Gel chromatography of conjugated steroids

One millilitre of dehydroepiandrosterone-sulfate (DHEA-S), androsterone-glucuronide (A-G), estrone-sulfate (E_1-S) and estriol-16α-glucuronide (E_3-G) solution (100—500 μg/ml) was applied to the column. DHEA-S and A-G were detected by the present methods, and E_1-S and E_3-G were detected spectrophotometrically at 260 nm. Their chromatogram is shown in Fig. 1.

Sulfuric ion in samples on chromatogram

Sulfuric ion in urine, which is an inhibitor of sulfatase, was detected with barium chloride solution; the elution position of sulfuric ion is shown in Fig. 2. The sample used is from a patient with adrenal virilizing tumor.

Effect of incubation time with sulfatase on hydrolysis rate

After the addition of sulfatase solution, the hydrolysis rates at 2 h and 20 h were determined and found to be 59% and 86%, respectively.

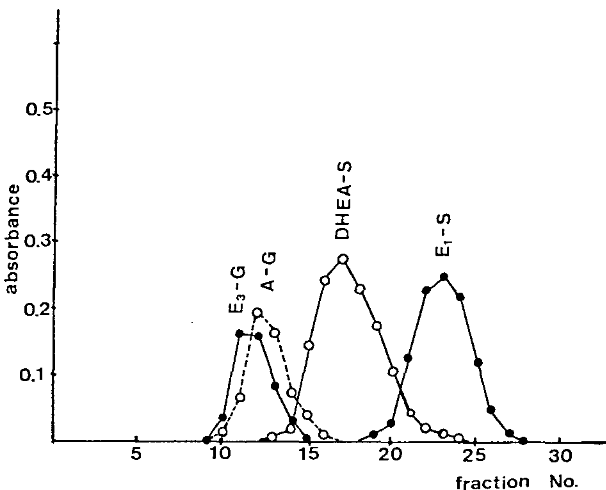

Fig. 1. Gel chromatography of steroid conjugates of standard. DHEA-S = dehydroepiandrosterone-sulfate; A-G = androsterone-3-glucuronide; E_1-S = estrone-3-sulfate; E_3-G = estriol-16-glucuronide. Color development of DHEA-S and A-G was performed as described in Methods; E_1-S and E_3-G were detected spectrophotometrically at 260 nm.

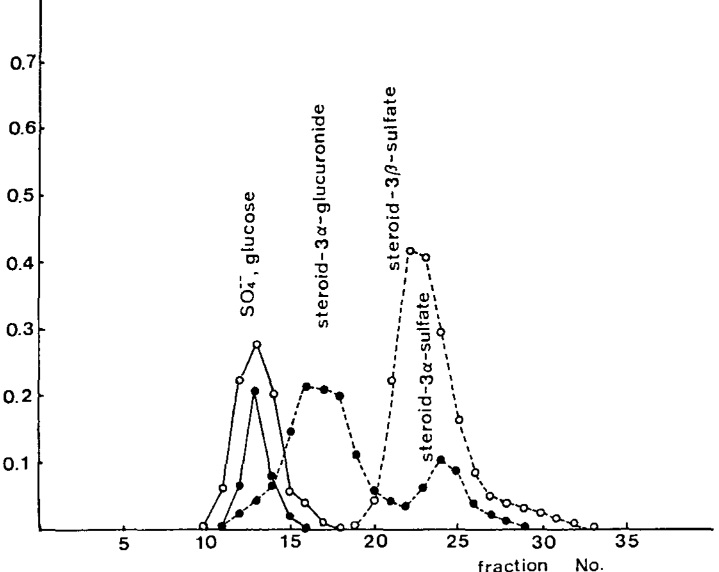

Fig. 2. Elution position of sulfuric ion in urine sample. (○) sulfuric ion; (●) glucose (detected by the glucose oxidase method). Column length was 29 cm. The sample used to detect the elution position on the chromatogram is from a patient with adrenal virilizing tumor.

Gel chromatography of urine samples for detection of steroid-3α-glucuronide and steroid-3α-sulfate

β-Glucuronidase from *E. coli*, bovine liver and *H. pomatia* were used for comparing hydrolysis of urinary steroid conjugates; the results are shown in Fig. 3.

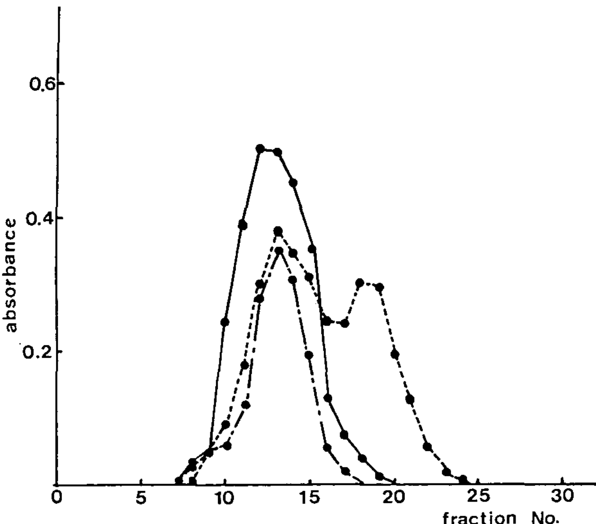

Fig. 3. Color development of 3α-hydroxysteroids after hydrolysis with β-glucuronidase from *E. coli* (• —•), bovine liver (•— · —•), and *H. pomatia* (• — — — •). Sample used for analysis is from a patient with adrenogenital syndrome. After hydrolysis with various β-glucuronidases, steroids were color-developed with 3α-hydroxysteroid dehydrogenase as described in Methods.

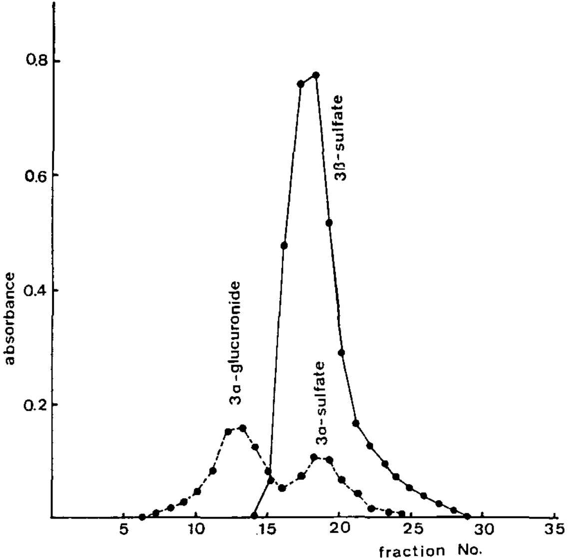

Fig. 4. Excretion patterns of steroid-3α-glucuronide, steroid-3α-sulfate and steroid-3β-sulfate in a urine sample from a patient with adrenal tumor. (• — — — •) 3α-hydroxysteroids; (•———•) 3β-hydroxysteroids. Hydrolysis of steroid conjugates was performed with sulfatase—β-glucuronidase from *H. pomatia*; enzymic color development was performed by the method described in Methods.

Precision of the method

The coefficient of variation (C.V.) of day-to-day assay using a sample of a patient with Cushing's disease was 6.9% (mean 25.5 ± 1.8 mg/day, duplicate assay for three days) for steroid-3α-glucuronides, 7.2% (mean 23.7 ± 1.5 mg/day) for steroid-3α-sulfates, and 20.1% (mean 4.2 ± 0.8 mg/day) for steroid-3β-sulfates. Values of the individual steroid groups are calculated from standard curves of androsterone-glucuronide for steroid-3α-glucuronides, androsterone-sulfate for steroid-3α-sulfate, and dehydroepiandrosterone-sulfate for steroid-3β-sulfates. All values were obtained by submitting these standard compounds to the whole procedure and the sum of the measured absorbance of each fraction was used for calculation of the sample values.

Normal values of urinary steroid conjugates

Values determined for five normal subjects were 13.8 ± 6.2 mg/day for steroid-3α-glucuronides, and 5.7 ± 2.1 mg/day for steroid-3α-sulfates; steroid-3β-sulfates were not detectable.

Excretion pattern of steroid conjugates in some patients

The excretion patterns of urinary steroid conjugates in patients with adrenal tumor and Cushing's disease are shown in Figs. 4 and 5, respectively.

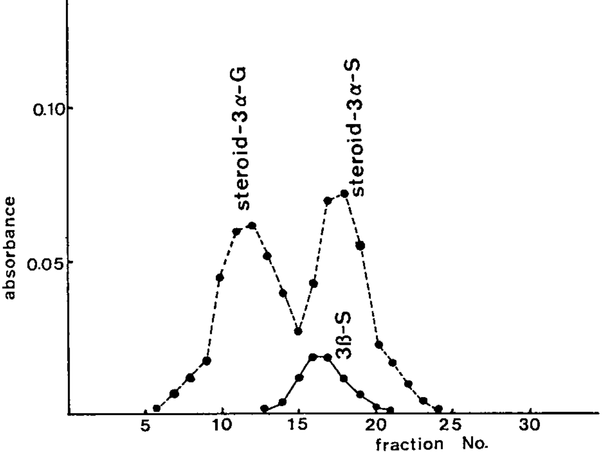

Fig. 5. Excretion patterns of steroid-3α-glucuronide, steroid-3α-sulfate and steroid-3β-sulfate in a urine sample from a patient with Cushing's disease. (• — — — •) 3α-hydroxysteroids; (•——•) 3β-hydroxysteroids. Hydrolysis of steroid conjugates was performed with sulfatase—β-glucuronidase from *H. pomatia*; enzymic color development was performed by the method described in Methods.

DISCUSSION

The hydrolysis enzyme β-glucuronidase from various sources has a well-known specifity for substrate and some inhibitors or activators [14,15], but in the present method chromatography on Sephadex G-25 was used to eliminate the influence of inhibitor. A selection of suitable enzymes for hydrolysis is still an important factor (Fig. 3).

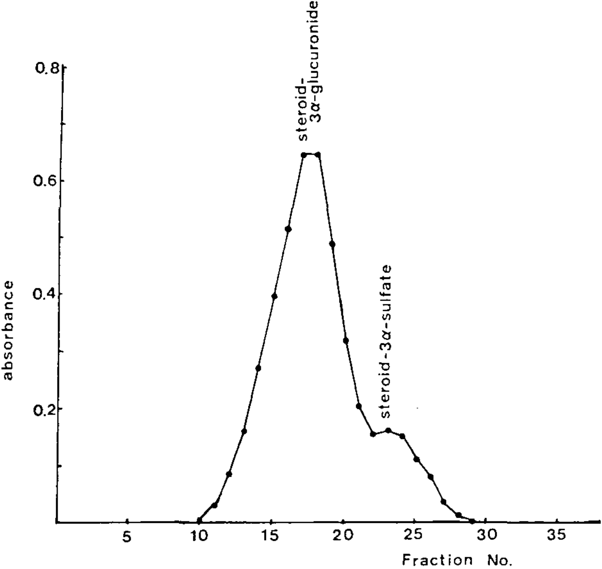

Fig. 6. Excretion pattern of steroid-3α-glucuronide and steroid-3α-sulfate in a urine sample from a patient with adrenogenital syndrome caused by enzyme deficiency.

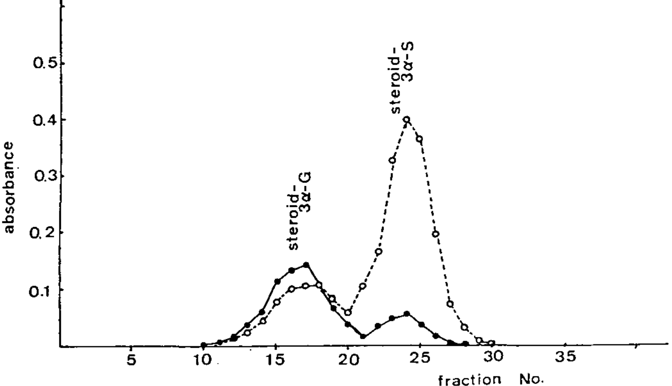

Fig. 7. Excretion patterns of steroid-3α-glucuronide and steroid-3α-sulfate in a urine sample from a patient with acute hepatitis (○) and from a normal subject (●).

The sample volume which can be applied to the column is small, so a high excretion of steroid conjugates is required as in the case of adrenogenital syndrome, Cushing's disease and some other forms of abnormal steroid metabolism.

In application to clinical diagnosis, significant differences between normal subjects and pathological urine were observed; for example, elevated steroid-3β-sulfate in patients with adrenal virilizing tumor (Fig. 4), elevated excretion of steroid-3α-glucuronide in patients with adrenogenital syndrome caused by enzyme deficiency (Figs. 3 and 6), and elevated steroid-3α-sulfate in patients with acute hepatitis (Fig. 7). From these observations, we conclude that this simple enzymatic method for detecting the excretion patterns of steroid conjugates may have a significance for diagnosis of steroid abnormal metabolism.

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REVERSED-PHASE LIQUID CHROMATOGRAPHY WITH AMPEROMETRIC DETECTION OF LIPOPHILIC DOPAMINE ANALOGUES AND DETERMINATION OF BRAIN AND SERUM CONCENTRATIONS AFTER SAMPLE CLEAN-UP ON SMALL SEPHADEX G-10 COLUMNS

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SUMMARY

The liquid chromatographic determination of N-alkylated analogues of dopamine is described. The retention and separation of these compounds, ranging from dopamine to N,N-dibutyl-dopamine, was studied on four bonded-phase columns, of which Nucleosil 5 C_{18} was chosen for routine use. The compounds were detected by a rotating disc amperometric detector. Samples of rat brain and serum were taken through a clean-up step on small Sephadex G-10 columns from which the dopamine analogues eluted in the same fraction as dopamine. The overall recovery was 70—90% from brain tissue and 60—70% from serum or plasma. The limit of detection for the catechol-containing compounds in tissue was 40—100 pg, for O-methylated ones 100—200 pg. The method is applied to the determination of dopamine analogues in rat brain after peripheral administration.

INTRODUCTION

High-performance liquid chromatography (HPLC) in combination with amperometric detection is now widely used for the determination of catecholamines and their metabolites in various tissues and body fluids [1—4]. Much less attention has been paid to the application of this technique to the analysis of compounds that are structurally related to catecholamines and that have been designed to exert a similar pharmacological action. Recent interest in dopaminergic drugs has led to the preparation of a series of dopamine (DA) receptor agonists which contain many of the structural features of DA itself [5—8]. Although the pharmacological properties of these compounds have

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TABLE I

CHEMICAL FORMULAE OF THE COMPOUNDS USED

Compound	Abbreviation	Formula	R_1	R_2	R_3
Dopamine	DA		H	H	H
Epinine	Epi		CH_3	H	H
N,N-Dimethyl-dopamine	DiMe-DA		CH_3	CH_3	H
N,N-Diethyl-dopamine	DiEt-DA		C_2H_5	C_2H_5	H
N,N-Dipropyl-dopamine	DiPr-DA		C_3H_7	C_3H_7	H
N,N-Dibutyl-dopamine	DiBu-DA		C_4H_9	C_4H_9	H
3-Methoxy-tyramine	3-MT		H	H	CH_3
3-O-Methyl-N,N-dipropyl-dopamine	3-O-Me-DiPr-DA		C_3H_7	C_3H_7	CH_3
2-Amino-6,7-dihydroxy-tetralin	6,7-ADTN		H	H	H
2-N,N-Dimethylamino-6,7-dihydroxy-tetralin	DiMe-6,7-ADTN		CH_3	CH_3	H
2-N,N-Dipropylamino-6,7-dihydroxy-tetralin	DiPr-6,7-ADTN		C_3H_7	C_3H_7	H
2-Amino-6-hydroxy-7-methoxy-tetralin	7-O-Me-6,7-ADTN		H	H	CH_3
2-N,N-Dipropylamino-5,6-dihydroxy-tetralin	DiPr-5,6-ADTN		C_3H_7	C_3H_7	H
Apomorphine	APO		—	—	—

been studied extensively, in most cases the important information about distribution (for example, in the brain), metabolism and excretion is still lacking. This knowledge would be of great help in designing new centrally acting DA agonists that may be of value in the study and treatment of diseases such as Parkinsonism and schizophrenia [9].

The prototype of these compounds is apomorphine (APO), which is more lipophilic than DA itself and is able to penetrate the brain and activate central DA receptors [10,11]. APO has been assayed by various fluorimetric [12,13], gas chromatographic [14] and radioenzymatic [15] methods. Recently, HPLC methods have been described using spectrophotometric [16,17] or amperometric [18] detection. This last method can be applied for the determination of many DA analogues that possess a catechol group, and thus are easily oxidized. However, the potential of this method is only realized when used in combination with a reliable and fast sample clean-up procedure.

Isolation on small Sephadex G-10 columns was shown to be an excellent method for the isolation of catecholamines and their metabolites from brain tissue [4,19], cerebrospinal fluid [20] and urine [21]. In our laboratory we have been using this method for over two years in determining brain and serum concentrations of various DA agonists [18,22] and their metabolites [23] in experimental animals. The described methods were applied, except for APO, to rather hydrophilic compounds, that could be expected to show a chromatographic and electrochemical behaviour similar to DA.

In this article we report on the chromatographic behaviour, detection characteristics and sample clean-up of more lipophilic DA analogues of the DA and 2-aminotetralin series (Table I). We also describe the application of these methods for the determination of the DA analogues in rat brain and serum after peripheral administration.

METHODS

Materials

2-Aminotetralin and DA derivatives were synthesized according to known methods [24,25]. Dopamine · HCl (Serva, Heidelberg, G.F.R.) and apomorphine · ½H_2O · HCl (Brocades, Maarssen, The Netherlands) were commercially obtained. All liquids and reagents used were analytical grade and obtained from E. Merck (Darmstadt, G.F.R.). Water was deionized and glass-distilled.

Apparatus

Use was made of a Spectra-Physics 740 pump with a 740C pump control and 714 pressure monitor, and occasionally a Waters 6000A solvent delivery unit. Samples were injected by a Rheodyne 70-10 injection valve normally equipped with a sample loop of 200 μl.

Analytical columns (15 cm × 0.46 cm I.D.; Chrompack, Middelburg, The Netherlands) were packed with Nucleosil 5 C_{18} (5 μm; Macherey-Nagel & Co., Düren, G.F.R.), LiChrosorb RP-2 (5 μm) and RP-8 (5 μm) (Merck) and Spherisorb S 5 Phenyl (5 μm; Phase Separations, Queensferry, Great Britain). Columns were packed using a Phase Sep packing bomb, a Waters 6000A pump

and suspensions of the materials in carbon tetrachloride—methanol (20:80). After packing the columns were purged with methanol and then water.

The detector was a rotating disc electrode [4, 26], packed with carbon paste [made by carefully mixing 3.25 g of Spektralkohle RW-A (Ringsdorff) and 1.75 g of high vacuum silicon grease (DOW Corning)]. The surface of the electrode was normally polished on smooth paper for about 1 min. This was repeated when a high offset current or a decreased sensitivity appeared. The electrode was usually rotated with a speed of about 30 rps. The detector cell was further equipped with a platinum auxiliary electrode and a saturated calomel reference electrode. The detector was controlled by a potentiostat LC-2A (Bioanalytical Systems). The applied oxidation potential was 500 mV, except where otherwise indicated. Recordings of the chromatograms were made on a Kipp BD 41 recorder with a 0.5–5 V full-scale deflection. Sephadex G-10 columns (7 cm × 0.5 cm I.D.) were prepared in small pasteur pipettes as previously described [27].

Chromatography

Mobile phases consisted of McIllvaine buffer (0.1 *M* citric acid and 0.2 *M* Na_2HPO_4), with methanol or acetonitrile as organic modifiers. The buffer was filtered by suction through a 0.45-μm polycarbonate membrane filter (Schleicher and Schüll), after which methanol was added in a similar manner. This procedure provided sufficient degassing. EDTA (final concentration 10^{-4} *M*) was routinely added as its disodium salt. The flow-rate was usually 1.0 ml/min.

Chromatographic retention of the compounds was studied by varying the methanol and acetonitrile concentrations and the pH of the eluent. Retention is expressed as the capacity ratio $k' = (t_R - t_0)/t_0$, where t_R is the time from injection to the peak maximum and t_0 the time from injection to the first deflection in the detector response.

Separation factors for two compounds were calculated by dividing the capacity ratio of the last eluting compound by that of the first eluting compound. The number of theoretical plates was calculated as $n = 5.54(t_R/w_{0.5})^2$, where $w_{0.5}$ is the peak width at half-height.

Detection

The characteristics of the detector cell were studied by varying the distance between the surface of the rotating disc and the cell floor and by varying the rotation speed of the detector. Response was measured as peak height in nA. Noise in the detector response was measured as the spread in the baseline in nA. The susceptibility to oxidation of some compounds was studied by measuring the response in nA with varying oxidation cell potentials in mV, resulting in voltammograms.

Extraction procedures

Serum and brain tissues were taken from rats of a Wistar-derived strain (CDL, Groningen, The Netherlands). The animals were stunned and decapitated, and the brain tissues were rapidly removed, frozen on dry ice and kept at −80°C.

Blood, obtained by direct decapitation, was allowed to clot. Serum was prepared by centrifuging for 15 min at 1000 *g* and 4°C and stored at −80°C. Plasma was collected in heparinized tubes, centrifuged and stored as serum.

Tissue homogenates were prepared with a Potter-Elvejem homogenizer with a Teflon pestle in 5-ml glass tubes filled with 0.5 ml of 0.1 *M* perchloric acid (PCA). Homogenates were poured into polypropylene tubes. Pestle and glass tube were rinsed with another 0.5 ml of 0.1 *M* PCA, which was added to the homogenate. The homogenates were centrifuged at 3000 *g* and 4°C for 15 min. The supernatants were completely decanted into glass tubes, in which it was found they could be stored overnight at 4°C. Serum or plasma was deproteinized by mixing with 0.5 ml of 0.1 *M* PCA. After centrifugation the supernatants were completely decanted. The supernatants were applied to small Sephadex G-10 columns, which were prewashed with 2 × 1.5 ml of 0.02 *M* ammonia and 2 × 1.5 ml of 0.01 *M* formic acid (see Fig. 1) and were allowed to

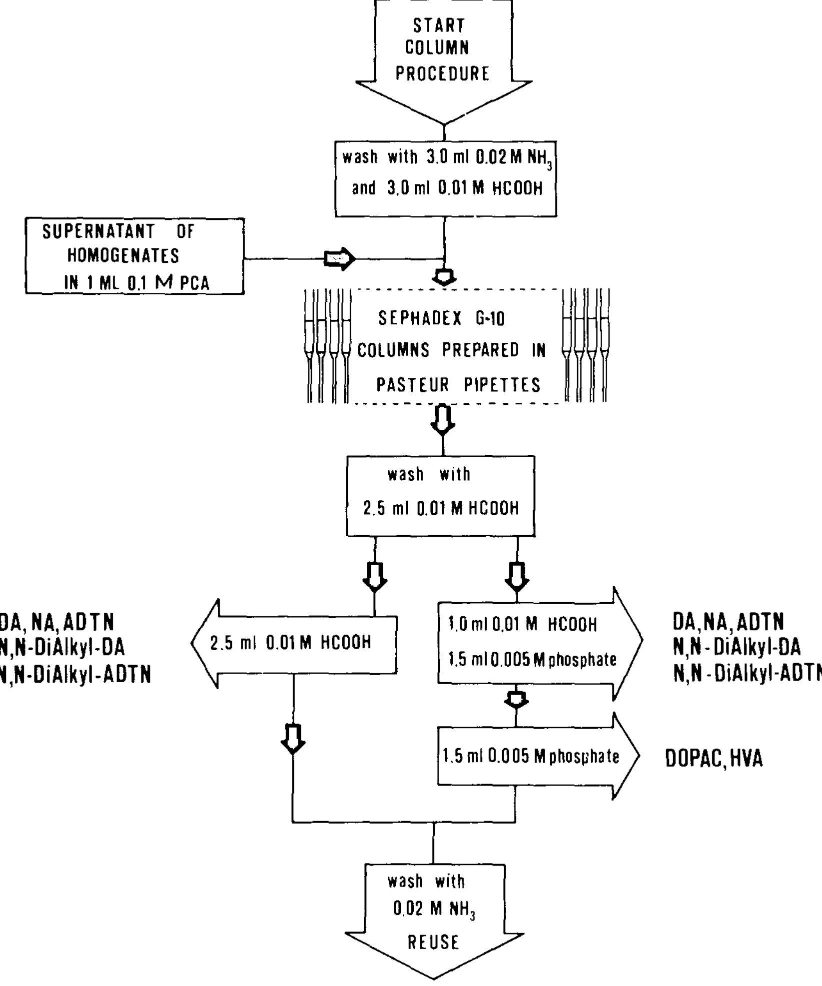


Fig. 1. Flow chart for the isolation procedure.

run dry. After application of the samples the columns were washed with 2.5 ml of 0.01 *M* formic acid and the amines were eluted with 2.0 or 2.5 ml of 0.01 *M* formic acid. When this elution is performed with 1.0 ml of formic acid and 1.5 ml of 0.005 *M* Na_2HPO_4, a second fraction may be collected which contains the DA metabolites homovanillic acid (HVA) and dihydroxyphenylacetic acid (DOPAC) [4,19]. Finally, columns were washed with 2×1.5 ml of 0.02 *M* ammonia and allowed to run dry. A 200-µl volume of the amine fraction was usually taken for the determination of the DA analogues by HPLC with amperometric detection. The remainder was used for the determination of DA, L-DOPA or noradrenaline (NA) [4,19].

Calibration curves

Stock solutions containing 100 µg/ml (referring to the free base) were prepared by weighing the appropriate amounts of the salts, which were dissolved in 0.01 *M* formic acid. These solutions, kept at 4°C, were stable in concentration for at least six months. Solutions of 1 µg/ml were made every month, while the lower concentrations were freshly prepared every day in water and the solution slightly acidified with formic acid.

Calibration curves were made by injecting the various standard solutions, measuring the peak heights in the chromatograms and plotting peak height (in cm or nA) against concentration or amount injected.

Precision was measured by repeatedly injecting the same solution and calculating the coefficient of variation [C.V.% = $(S.D./\bar{x}) \times 100$] of the response.

Recovery

The recovery of the total assay was assessed by adding 25—100 µl of an appropriate standard solution to the glass tube with 0.5 ml of 0.1 *M* PCA and a piece of cerebellar or cortical tissue, and processing as described under Extraction procedure; 200 µl of the amine eluate were injected onto the column. The peak height was measured and compared to that of a standard solution. The recovery was calculated as follows:

$$\text{recovery (\%)} = (h_{rec}/h_{st}) \times (C_{st} \times 2.5/X) \times 100$$

where X = ng added, h_{rec} = peak height of recovery, h_{st} = peak height of standard with concentration C_{st}, 2.5 = volume in ml of the collected fraction.

Recovery from serum or plasma was assessed identically: to 100 or 200 µl of serum or plasma the appropriate amounts were added and the above procedure was followed. The influence of the tissue weight was studied by varying this weight from 20 to 120 mg.

The precision of the recovery was determined by repeating the procedure 4—10 times and calculating the standard deviation (S.D.).

RESULTS AND DISCUSSION

Chromatography

Fig. 2 shows the retention behaviour of a range of DA analogues (Table I) on Nucleosil 5 C_{18} with methanol as organic modifier in pH 4.0 McIllvaine

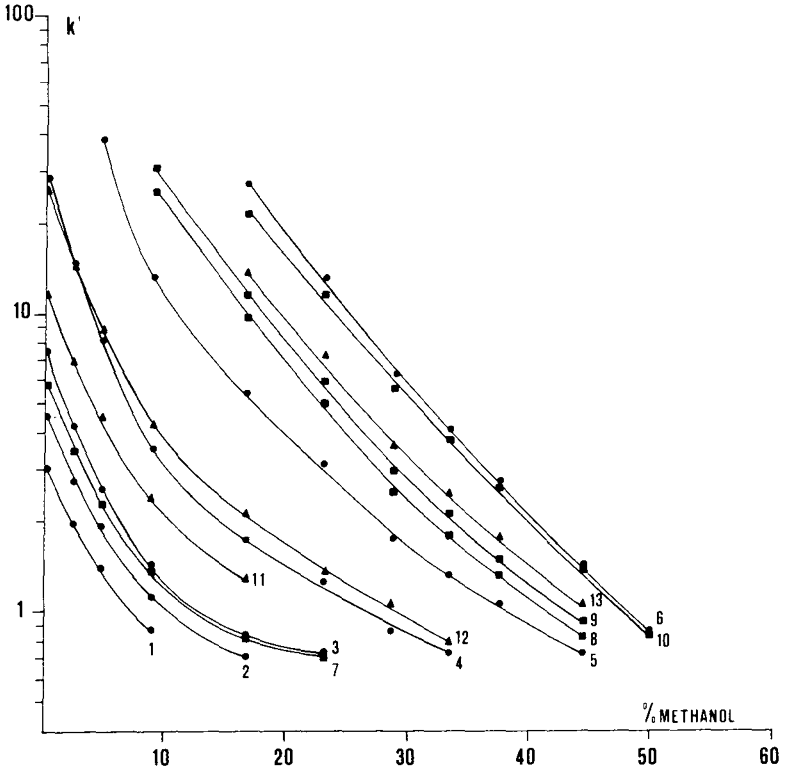


Fig. 2. Relationship between the percentage organic modifier methanol and the capacity factor (log k'). Experimental conditions: column, Nucleosil 5 C_{18}; eluent, pH 4 McIllvaine buffer; flow-rate, 1.0 ml/min; detector, rotating disc amperometric detector; oxidation potential, 700 mV; injected volume, 200 μl; concentration of the injected solutions, 200—1000 ng/ml. (●) Dopamine-derived catechols: 1 = DA, 2 = Epi, 3 = DiMe-DA, 4 = DiEt-DA, 5 = DiPr-DA, 6 = DiBu-DA. (■) 2-Aminotetralin-derived catechols: 7 = 6,7-ADTN, 8 = DiPr-6,7-ADTN, 9 = DiPr-5,6-ADTN, 10 = APO. (▲) O-Methyl derivatives: 11 = 3-MT, 12 = 7-O-Me-ADTN, 13 = 3-O-Me-DiPr-DA.

buffer. Similar curves were obtained using acetonitrile as organic modifier (results not shown). The relation between the percentage organic modifier and log k' is not linear, as was expected from the literature data [28, 29]. The strongest curvature appeared at $k' < 2$ and about 10% organic modifier. For the remaining part of the curves a linear approximation may be adequate. Schoenmakers et al. [29] state that the curves are parallel with acetonitrile but divergent with methanol and tetrahydrofuran. However, the separation factors we obtained for several pairs of compounds were found to increase steadily with decreasing concentrations of methanol or acetonitrile, thus indicating divergent curves regardless of the organic modifier. No specific effects of acetonitrile were noted compared to methanol, except that APO is eluted faster with acetonitrile. The only difference between methanol and acetonitrile seems, therefore, to be that the same percentage acetonitrile reduced the retention by about 50%. As acetonitrile presented no advantages, further experiments were performed exclusively with methanol as organic modifier.

Essentially the same results were obtained with the Phenyl, RP-8 and RP-2 columns. The relation between percentage methanol and log k' is shown in Fig. 3 for two compounds, DA and DiPr-DA, on the four columns used. For the lipophilic compounds the Phenyl column causes the strongest retention and the RP-2 the weakest, while C_{18} and RP-8 do not differ very much from each other. However, for the hydrophilic compounds only the latter two columns gave any retention. The order of elution is generally the same, only APO is relatively more retained on the RP-8 column and less on the Phenyl column, compared to the C_{18} column. Thus, the separation factors for chemical substituents (3-OMe-DiPr-DA against DiPr-DA; DiBu-DA against DiPr-DA; DiPr-5,6-ADTN against DiPr-6,7-ADTN; etc.) are mostly comparable for all columns, with the above-mentioned two exceptions: (1) further "cyclization" of aminotetralins to APO results in a deviating chromatographic behaviour;

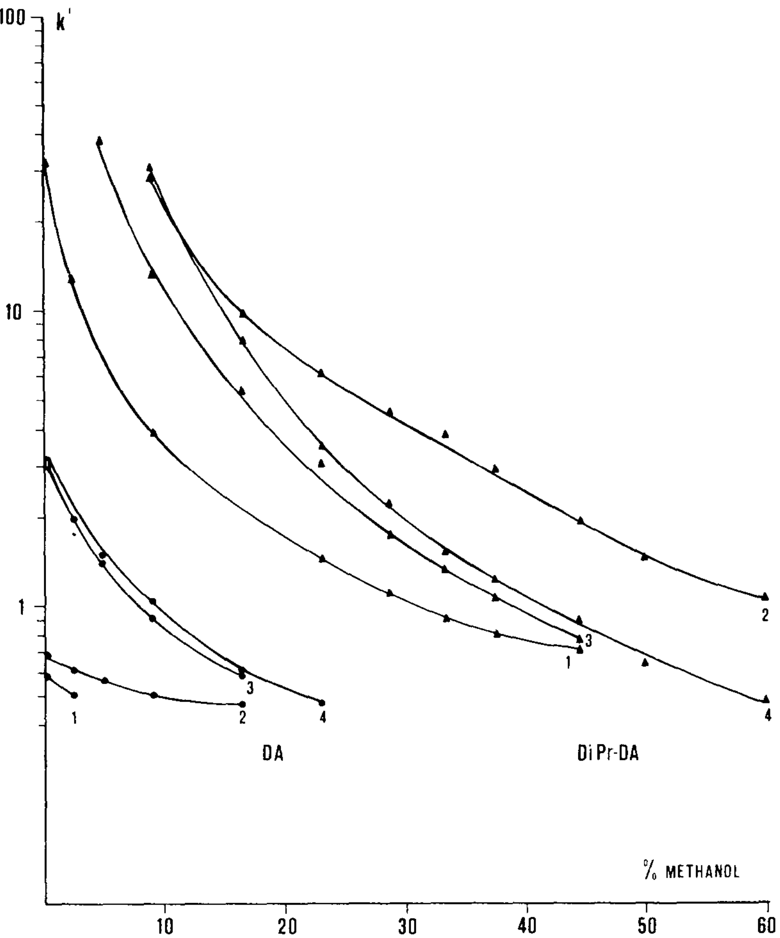


Fig. 3. Relationship between the percentage organic modifier (methanol) and the capacity factor (log k') on four different bonded-phase columns. 1 = LiChrosorb RP-2; 2 = Spherisorb S5 Phenyl; 3 = Nucleosil 5 C_{18}; 4 = LiChrosorb RP-8. (●)DA; (▲) DiPr-DA. Further experimental conditions as in Fig. 2.

(2) the RP-2 but especially the Phenyl column gives large separation factors between hydrophilic compounds like DA and weak lipophilic ones like DiMe-DA.

A McIllvaine buffer was chosen for the aqueous mobile phase as it is easily prepared and has a wide pH range (2.5—8.0). It has been shown that using this buffer with a properly chosen pH even strongly hydrophilic compounds such as NA and L-DOPA are sufficiently retained on Nucleosil 5 C_{18} without the need for a strong ion-pairing agent like octylsulphate in the mobile phase [4]. The addition of citrate to a phosphate buffer apparently increases the retention compared to a simple phosphate buffer as used by Molnar and Horváth [30]. Freed and Asmus [31] reported that citric acid, like trichloroacetic acid (TCA), increases retention of amines by ion-pair formation. The McIllvaine buffers seem to have an intermediate position between simple acids and ion-pairing agents like TCA and octylsulphate. No rapid column deterioration by citric acid [31] was observed. When used daily for biological samples, columns seemed to have a median lifetime of six months.

The chromatographic efficiency of the columns is strongly dependent on the structure of the test compounds. The efficiency of the column is substantially lower for amines than for acids like the natural metabolites of DA (DOPAC, HVA) and serotonin (5-hydroxyindoleacetic acid, 5-HIAA).

Typical plate numbers on the Nucleosil 5 C_{18} column are, for amines like DA and DiBu-DA with a k' of 4—6, n = 2500 (17,000 per m), and for acids like HVA and 5-HIAA with a k' of 4—6, n = 5000 (33,000 per m). The efficiencies obtained with the other packings were generally somewhat lower, while the tailing was more pronounced on the RP-2 column for all compounds and on the RP-8 column for APO and the O-methylated compounds.

The influence of the pH on the retention of acids is well known [30]. For amines an increase in retention with higher pH [30], accompanied by an increase in peak asymmetry [32], has been reported. We found by varying the pH from 4.0 to 6.5 that the more lipophilic amines are retained more at higher pH. The effect on the peak shape is detrimental, however.

Buffers more acidic than pH 4 may be used but they sometimes cause unstable baselines, especially when the Spectra-Physics pump was used.

From the above summarized results it was concluded that a system consisting of a column packed with Nucleosil 5 C_{18}, a pH 4 citrate—phosphate buffer as eluent and methanol as organic modifier represents a universal system for DA analogues. The separation of DiEt-DA, DiPr-DA, DiPr-6,7-ADTN, DiPr-5,6-ADTN and DiBu-DA on Nucleosil 5 C_{18} with 23.1% methanol in the eluent is shown in Fig. 4.

Detection

In Fig. 5 the sensitivity in nA/pmol for DA and some of its N,N-dialkylated analogues is shown as a function of the oxidation potential. The percentage methanol in the eluent was varied to keep k' at about 3 for all compounds. The sensitivity for DA is about twice that of the N,N-dialkylated analogues, while the O-methylated compound is only oxidized at potentials higher than 600 mV. Potentials higher than 700 mV were usually not applied for three reasons. Firstly, the offset current increases to sometimes unacceptable values;

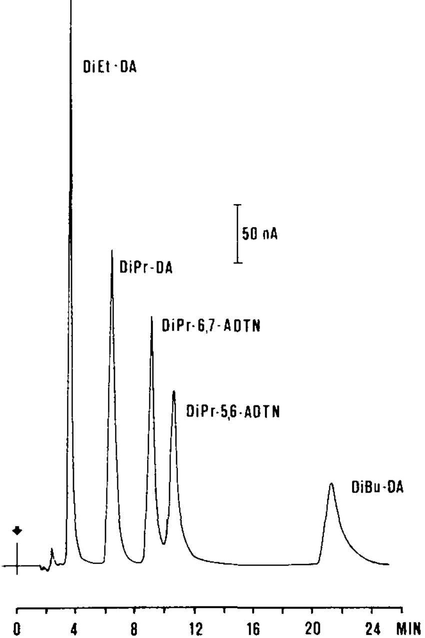


Fig. 4. Separation of five lipophilic DA analogues. Experimental conditions: column, Nucleosil 5 C_{18}; eluent, pH 4 McIllvaine buffer with 23.1% methanol; flow-rate, 1.0 ml/min; detector, rotating disc amperometric detector; oxidation potential, 700 mV; injected volume, 200 μl; potentiostat setting, 500 nA/V; concentrations of the injected solutions, 1000 ng/ml.

secondly, the baseline is not as stable as with lower potentials; and thirdly, the chance that endogenous compounds will interfere is increased.

The influence of the percentage methanol in the eluent was assessed by coupling the detector inlet directly to the injector outlet. The same difference between DA and its alkylated analogues exists, regardless of the methanol concentration. Also the sensitivities in this experiment were the same with 0 and 23% methanol. It may thus be concluded that the sensitivity is not affected by a relatively large concentration of methanol.

The influence of the pH on the sensitivity is hard to assess because of the serious disturbance of peak shapes at higher pH. However, at pH 5.5 there is no indication of an increased sensitivity for the N,N-dialkylated DA analogues, while at pH 6.5 the response is strongly decreased.

The distance of the electrode surface to the cell floor (where the inlet is located) is, depending on the cell geometry, an important parameter in the performance of the rotating disc electrode [4,33]. A very small distance (less than 0.2 mm) gives a high but fluctuating response, while the electrode surface is very easily damaged. Rotation causes a small increase in sensitivity. Increasing the distance results in a steadily decreasing sensitivity when the electrode is not rotating. For the rotating electrode an optimum can be found, where the sensitivity is at least as high as at a very small distance. At this optimum the difference in sensitivity between a rotating and stationary electrode is a factor of 5. The response at this height shows a better repro-

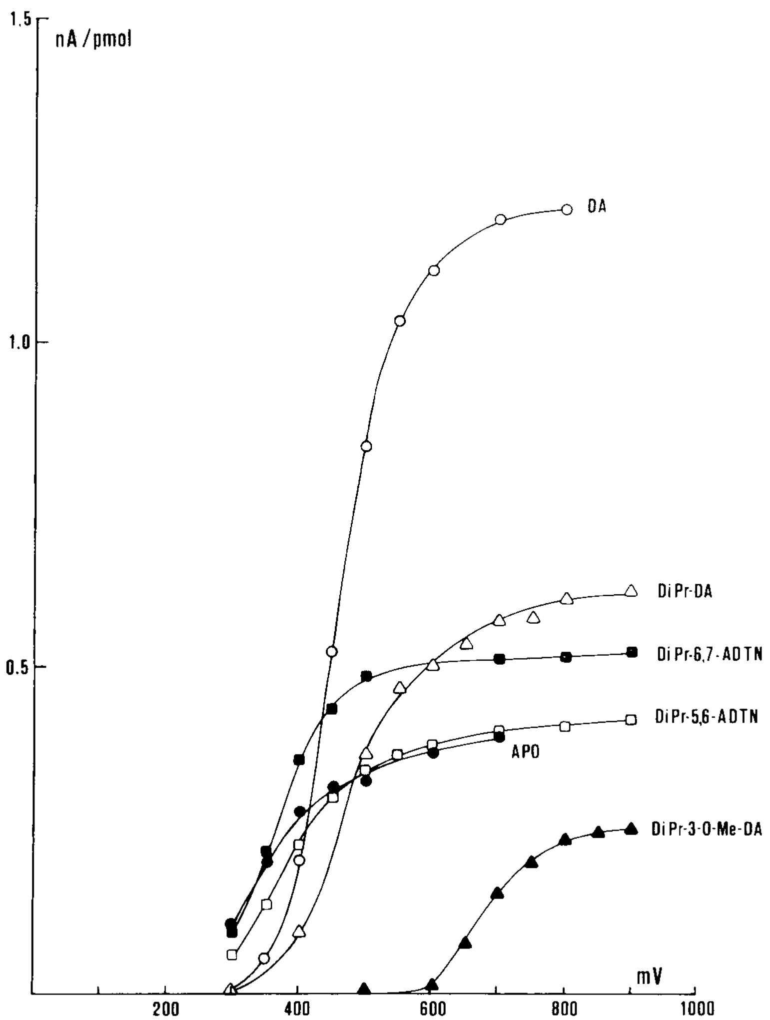


Fig. 5. Relationship between the response in nA/pmol and the oxidation potential in mV. Experimental conditions: column, Nucleosil 5 C_{18}; eluent, pH 4 McIllvaine buffer with varying percentages of methanol; flow-rate, 1.0 ml/min; detector, rotating disc amperometric detector; injected volume, 200 μl; concentrations of the injected solutions, 100—400 ng/ml. The percentage methanol was chosen so that the capacity factor was about 3 for all compounds.

ducibility, while the chance of damaging the electrode surface is minimal. Rotation provides a very thin diffusion layer at the electrode surface. Thus, the effective cell volume is very small compared to the total cell volume and peak broadening is minimal. All work reported here was carried out with the electrode at the optimal height (about 2.8 mm). Rotating the electrode only slightly increases the detector noise, and the signal-to-noise ratio is, like the sensitivity, increased by a factor of 5. This ratio is constant for a rotation speed up to 40 rps. Faster rotation results in a lower ratio. At a 500-mV oxidation potential the noise was usually 5—10 pA. The noise is mainly dependent

on the earthing of the rotating electrode. Further influences are rotation speed, cell geometry, condition of the carbon paste surface and the pH of the eluent. When a high sensitivity was used (0.5 or 1 nA/V) a Faraday cage around the detector was sometimes needed to prevent baseline instability.

The sensitivities reported by us (see also ref. 4) compare well to those reported in the literature (for example, ref. 34) for the more commonly used thin-layer detector cells. The rotating disc electrode is easy to work with and has a short stabilisation time; typically 15—30 min after applying the oxidation potential, a stable baseline was obtained at a setting of 1 nA full-scale. The carbon paste may be easily made in any laboratory and the electrode is readily repacked. The surface area of the electrode was about 40 mm^2.

Sample clean-up and recovery

N,N-Dialkylated DA and DA analogues of the DA and 2-aminotetralin series behave in much the same way on the small Sephadex G-10 columns as was reported for DA, NA, L-DOPA [4], 6,7- and 5,6-ADTN [22] and the O-methylated derivatives of DA (3-methoxytyramine, 3-MT [35]) and 6,7-ADTN (7-O-methyl-ADTN [23]). Thus the 2.0- or 2.5-ml fraction that is eluted with formic acid contains amines from DA to DiBu-DA with recoveries from 70 to 90%. As O-methylation tends to result in an earlier elution (for 3-MT see ref. 19), 3-O-Me-DiPr-DA has a lower recovery of about 60%. The preceding fraction, which indeed contains these compounds, is, however, less suited to sensitive determination as it gives a broad negative front.

Retention on Sephadex G-10 for amines is dependent on the formation of ion pairs between the amine and the perchlorate ion from the homogenization mixture [4]. This occurs at a low pH when the amine is protonated. The ion pair slowly dissociates and the amine is eluted. At a low pH the acidic (DA) metabolites are also retained. These acids, however, elute when the columns are washed with a phosphate solution of pH 7—8. Homogenisation in sulfuric acid, which is not an ion-pairing agent, or precipitation of the excess PCA in the supernatant, leaves the elution of the acids unaltered. The amines like DA [4,19] and DiPr-DA (unpublished results) are in this case much less retained and do not elute in the DA fraction.

These columns are easy to prepare and may be used for more than 6 months without any deterioration in the recovery of the amines. The recoveries are reproducible over a long period of time, representing 4—5 generations of columns.

Thus, these small Sephadex G-10 columns represent an easy and reliable way to prepare brain samples for the determination (with semi-automated fluorimetry or HPLC with amperometric detection) of biogenic amines and their acid metabolites [4,19,36], for the HPLC determination of their N-alkylated analogues and even for the gas chromatographic determination of the monohydroxy derivatives (Feenstra et al., unpublished results). It must be stressed, however, that introduction of a second phenyl group makes a compound unsuitable for this isolation procedure: APO is not eluted from Sephadex G-10, while 4-phenyl-6,7-ADTN is slowly eluted in all fractions. The increased lipophilicity may be responsible.

The recovery from serum or plasma is less, 55—70%, but with the same

reproducibility as from brain samples. It may be that more is coprecipitated with the proteins. From experiments in which the recovery of 50 ng of DiPr-DA was determined with and without added tissue it was found that about 10% DiPr-DA is lost with the various transfers before and during the column clean-up procedure and that another 10% is lost with the tissue homogenization. Increasing the tissue weight to over 100 mg did not increase this loss.

Fig. 6 shows chromatograms of serum (a, b) and cerebellar tissue (c, d) obtained from rats injected with 100 μmol/kg DiBu-DA and from control rats. No endogenous substances were found to interfere. This also applies to DiPr-DA, DiEt-DA, DiPr-5,6-ADTN, DiPr-6,7-ADTN and 3-O-Me-DiPr-DA. Determination of the more hydrophilic amines requires another pH (DA and NA [4]) or another eluent system (3-MT [35], and DiMe-6,7-ADTN in very low concentrations [37]) as endogenous compounds do interfere.

Quantitation

The present method can be used to determine concentrations of DA analogues in serum and brain tissue. The detection limit (the injected amount that gives a peak height which is four times the noise) for the N,N-dialkylated catecholamines was found to be 25—50 pg per injection from a standard solution and 40—100 pg from a biological sample. Methoxylated compounds have higher detection limits (50—100 pg in a standard solution and 100—200 pg in a biological sample for 3-O-Me-DiPr-DA), because the sensitivity is less (Fig. 5) and the baseline is less stable with the higher potential setting.

The calibration curves for all the compounds are linear from the detection limit at a setting of 1 nA/V to about 500 ng at the maximal attenuation of 500 nA/V. Table II lists results from a calibration and recovery experiment for DiPr-DA and 3-O-Me-DiPr-DA, in which 1, 10 and 100 ng were added to pieces of cerebellar tissue; a 2.0-ml amine fraction was collected, of which 200 μl were injected. Standards were diluted to the same extent with 0.01 *M* formic acid. Linearity, recovery and precision were excellent for DiPr-DA and acceptable for 3-O-Me-DiPr-DA. It might be argued that an even better precision would be obtained if an internal standard was used. To test this we determined the reproducibility of the total procedure with and without tissue. DiPr-6,7-ADTN (50 ng) was added as internal standard to DiPr-DA (50 ng). The concentration of DiPr-DA was calculated with and without the use of the internal standard. The results showed that the reproducibility is not affected by the use of an internal standard; the coefficients of variation were in both cases 4.4% (with added tissue) and 4.0% (without tissue), respectively. Therefore we feel that the use of an internal standard is not indicated.

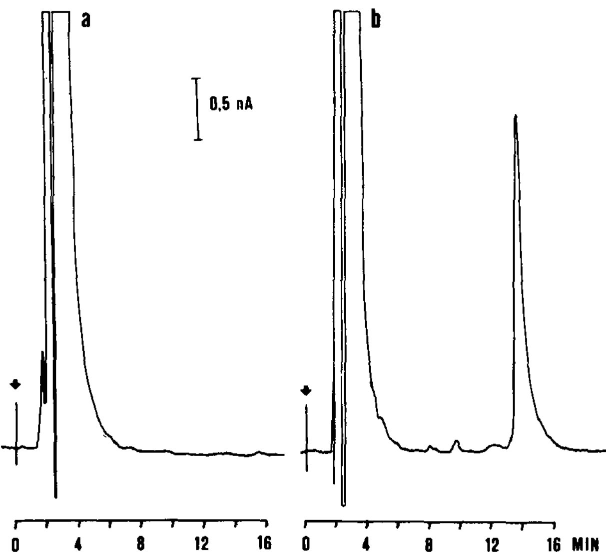
Fig. 7 shows the time—course of serum and brain concentrations of DiPr-DA after intraperitoneal injections of 100 μmol/kg to female Wistar rats of about 200 g. It is clear that the compound rapidly achieved its maximum brain concentration, but only remained a short time in the brain. This short duration is to a large extent explained by the metabolic deactivation by the enzyme catechol-O-methyltransferase. A more detailed account of the brain concentrations of DA analogues will be presented elsewhere.

TABLE II

CALIBRATION AND RECOVERY OF DiPr-DA AND 3-OMe-DiPr-DA

Calibration: both compounds were added in the stated amounts to 2.0 ml of 0.01 *M* formic acid. Recovery: both compounds were added in the stated amounts to a piece of cerebellar tissue, which was treated as described under Extraction procedure. From the 2-ml fractions obtained, 200 μl were injected onto the Nucleosil 5 C_{18} column. Eluent: pH 4 McIlvaine buffer—20 and 28% methanol. Detector potential: 500 and 700 mV (for DiPr-DA and 3-O-Me-DiPr-DA, respectively). Potentiostat: 10 or 1 nA/V. Response was calculated from the peak height. Recovery was calculated as the response of the extracted sample divided by the response of the standard sample. For all groups: $n = 5$. Correlation and slope were calculated after log—log conversion of all individual data.

Compound	Added (ng)	Injected (ng)	Standard		Recovery from brain tissue		
			Response (nA)	C.V. (%)	Response (nA)	C.V. (%)	Recovery (%)
N,N-Dipropyl-dopamine	100	10	12.0	2.4	9.6	4.7	80.0
	10	1	1.2	3.5	0.97	4.7	80.8
	1	0.1	0.12	3.1	0.095	3.6	79.2
Correlation coefficient (r^2)			0.999		0.999		
Log—log slope			1.004		1.005		
3-O-Methyl-N,N-dipropyl-dopamine	100	10	4.95	1.0	3.0	12.7	60.6
	10	1	0.46	8.2	0.3	5.8	65.2
	1	0.1	0.055	24.9	Not detectable		
Correlation coefficient (r^2)			0.994		0.994		
Log—log slope			0.982		0.986		



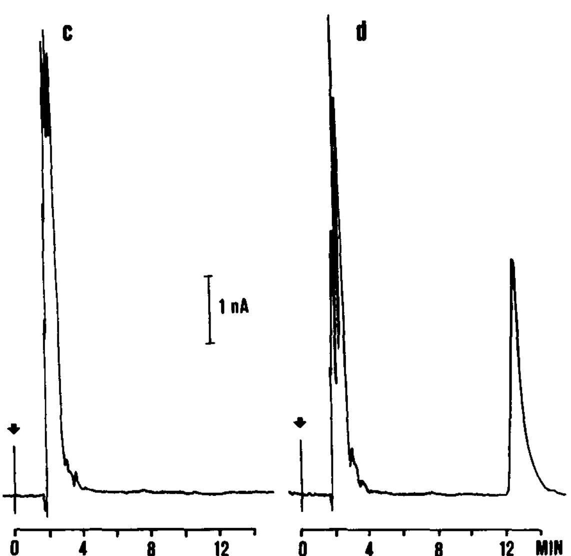


Fig. 6. Detection of DiBu-DA in rat brain and serum. (a) 500 μl of serum from a control rat. (b) 500 μl of serum from a rat that was 10 min previously injected with 100 μmol/kg DiBu-DA. Calculated concentration = 120 ng/ml (injected 4.8 ng). (c) 40.0 mg of cerebellar tissue of a control rat. (d) 40.7 mg of cerebellar tissue of a rat that had been injected 20 min previously with 100 μmol/kg DiBu-DA. Calculated concentration = 1.92 μg/g (injected 6.3 ng). Experimental conditions: column, Nucleosil 5 C_{18}; eluent, McIllvaine buffer pH 4 with 28.6% methanol; flow-rate, 1.0 ml/min; detector, rotating disc amperometric detector; oxidation potential, 500 mV; injected volume, 200 μl.

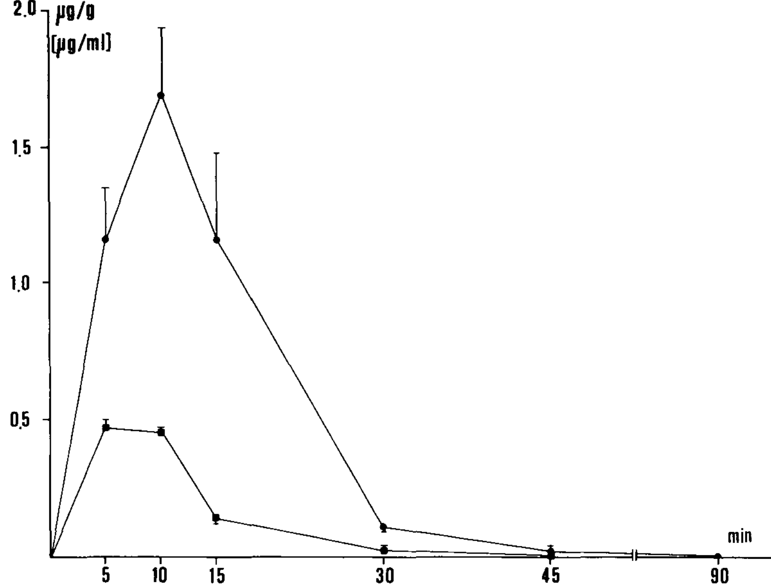


Fig. 7. Concentrations of DiPr-DA in striatum (o) and serum (□) of rats injected intraperitoneally with 100 μmol/kg. Experimental conditions: as in Fig. 6 but 20.0% methanol. The values presented are the means (± S.E.M.) of four rats. The concentrations were corrected for recoveries which were 83.4% (± 7.2% S.D.) for striatum (n = 4) and 67.5% (± 3.4% S.D.) for serum (n = 4).

CONCLUSION

Up until now the only reported method of determination of N-alkylated DA analogues other than APO was a radioenzymatic assay for DiPr-DA [8], which was, however, only used for the measurement of blood levels of this compound. The present method, with its use of a short and easy sample cleanup procedure on Sephadex G-10 columns, and a selective detection with a relatively low oxidation potential of 500 mV after separation on a reversed-phase HPLC column, provides a simple, sensitive and reliable way to detect and quantify DA analogues in serum and small pieces of brain tissue of experimental animals. Furthermore, the method is very versatile as only slight variations in the experimental conditions, such as the pH and the percentage methanol in the eluent or the oxidation potential, also allow the determination of the methoxylated derivatives of the DA analogues as well as a wide range of endogenous catecholamines and metabolites. The effects of the DA analogues on the brain concentrations of DA and its metabolites can be used as a method to assess the dopaminergic potency of the analogues [38].

Thus, the present method makes it possible to determine in one and the same piece of brain tissue the concentration of the pharmacological agent and the effect it exerts on the dopaminergic system.

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CHROMBIO. 1273

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC PURIFICATION OF ENDOGENOUS TUMOUR CELL GROWTH INHIBITORY PEPTIDES

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SUMMARY

Five endogenous growth inhibitors of JB-1 ascites tumour cells have been further purified and characterized. Probably because of the high biological activity of most of the inhibitors which are low molecular weight peptides, these have been extremely difficult to handle using conventional purification methods. However, high-performance liquid chromatography (HPLC) turned out to be a very powerful and useful technique in the last purification steps. Although many types of HPLC packings were tried, only a few of them (Nucleosil 5C-18 and Nucleosil 5CN) behaved satisfactorily for the present purpose.

INTRODUCTION

In a series of studies it has been shown that ascites tumours in their plateau phase of growth contain substances or activity which inhibit the progression of the ascites cells through the cell cycle [1—4]. The biological activity has been tested on various tumour cells in culture using flow cytometry [5] for screening the fraction during the purification procedures. Most of the inhibitory activity turned out to be of peptidic nature [6,7], however, nearly all standard peptide purification methods had their drawbacks as described in the Discussion.

The problems with purification of the active factors were minimized by taking advantage of their good solubility in polar organic solvents [7,8]. The active factors have now been purified by means of Sephadex LH-20 and improved high-performance liquid chromatographic (HPLC) techniques.

MATERIALS AND METHODS

Preparation of tumour cell inhibitory extract

The inhibitory activity was obtained from out-grown JB-1 ascites tumours in

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their plateau phase of growth and was purified by (1) DE-52 cellulose chromatography and (2) Sephadex G-15 chromatography of the methanol-soluble material as recently described [7,8]. The active fractions were further purified by Sephadex LH-20 chromatography and HPLC as described below.

Chemicals and reagents

The water used in all operations was deionized laboratory water purified by the Milli-Q reagent-grade water system (Millipore, Bedford, MA, U.S.A.). Methanol and acetonitrile were of HPLC grade (Rathburn Chemicals, Peeblesshire, Great Britain), and acetic acid was of sequencer grade (Rathburn). Trifluoroacetic acid, 99% pure, was obtained from Pierce Chemical Company, Rockford, IL, U.S.A. Ethanol, 96% or 99% (De Danske Spritfabrikker, Copenhagen, Denmark) was distilled from a mixture with charcoal before use.

Sephadex LH-20 chromatography

Twenty to fifty milligrams of freeze-dried active fraction from Sephadex G-15 were dissolved in 1.5 ml of the mixture ethanol—0.1% trifluoroacetic acid in water (4:1) and applied to a Sephadex LH-20 (Pharmacia, Uppsala, Sweden) column and eluted with the above-mentioned solvent mixture. Five milligrams of glycerol were added to pooled fractions which were evaporated in vacuo at 40°C in a Rotavapor R-110 (Büchi Laboratoriums-Technik, Flawil, Switzerland) and lyophilized to dryness. Aliquots were taken for biological testing.

High-performance liquid chromatography

Two types of columns packed with Nucleosil® 5 μm C_{18} and Nucleosil® 5 μm CN (Macherey-Nagel & Co., Düren, G.F.R.) were used. The columns were packed as described in detail elsewhere [9]. All chromatograms run in the isocratic mode and at room temperature were done using a Waters HPLC apparatus (Waters, Milford, MA, U.S.A.) consisting of two Model 6000A pumps, a Model 660 solvent flow programmer, a Model U6K injector, a Model 440 fixed-wavelength UV detector, having an 8-μl flow-through cell, and an Omniscribe® dual-channel chart recorder (Houston Instruments, Austin, TX, U.S.A.). Samples were injected via the U6K injector with a 25-μl syringe (Hamilton, Reno, NV, U.S.A.) into the column.

For the chromatograms performed with acetonitrile gradients, a Hewlett-Packard 1084B liquid chromatograph (Hewlett-Packard, Geneva, Switzerland) equipped with a processor-controlled sampling and gradient elution system was used. The column was operated in an accessible temperature-controlled compartment. The absorbance of the column eluate was measured with a Cecil CE 588 UV Scanning Spectrophotometer (Cecil Instruments, Cambridge, Great Britain). Samples were injected into the column with the automatic sampling system.

Prior to use, all solvents used for HPLC were filtered under suction through 0.5-μm FH Millipore filters to remove solid particles and to degas solutions.

Fractions were taken from the chromatographic runs by hand as judged from the absorbance profiles of the eluates. After completion of a chromatogram, aliquots were taken for biological testing, the organic modifier was removed by

flushing the fractions at 40°C with a nitrogen stream and, finally, they were freeze-dried.

RESULTS

Peak 1 from the LH-20 column (Fig. 1) had no affinity for the C_{18} columns when using methanol—acetic acid—water (50:8:42), being eluted in the front volume. However, the activity could be purified by eluting the C_{18} column with acetic acid—water (4:96). The activity splits into two activity peaks (I_7 and I_6 in Fig. 2) showing weak retardation in the columns (Fig. 3, left part). These peaks could be further purified with 0.1% trifluoroacetic acid in water developed with an acetonitrile gradient (Fig. 4). The first eluting activity peak (I_7) in Fig. 3, left part, was eluted shortly after the front (after 3.8 min) and before the start of the gradient (Fig. 4, left part). The second activity peak (I_6) in Fig. 3, left part, was slightly more retarded (eluting after 4.4 min) but also appeared before the start of the gradient (Fig. 4, right part). Both activity peaks (I_7 and I_6) appeared chemically homogeneous as evidenced by the accordance of absorbance at 218 nm and the biological activity. After hydrolysis, the following ratios of moles of amino acids were found: I_7: 1 Asx : 1 Ser : 1 Glx : 2

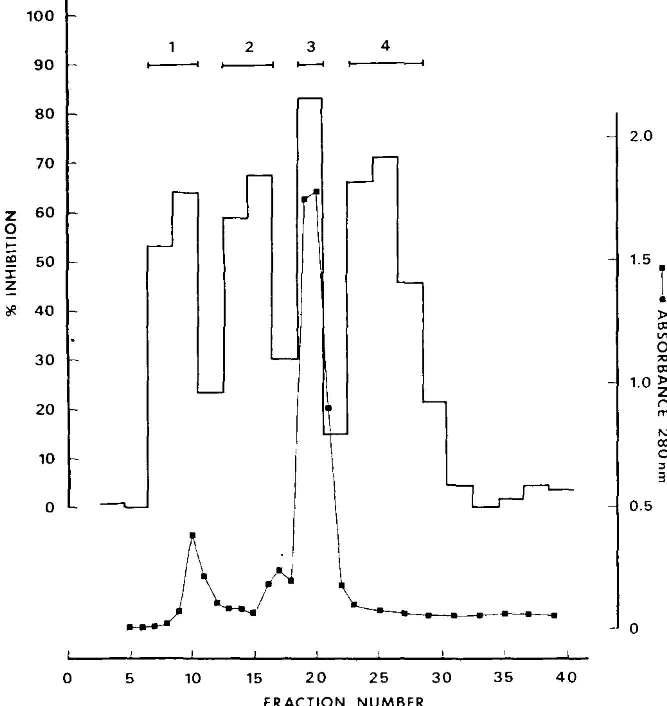

Fig. 1. Purification of tumour cell growth inhibitors by Sephadex LH-20 column (95 × 2.0 cm) eluted with 80% ethanol + 20% water containing 0.1% trifluoroacetic acid using a flow-rate of 36 ml/h and collecting 15 ml per fraction. The active peaks were pooled into four fractions as indicated in the figure by the horizontal bars.

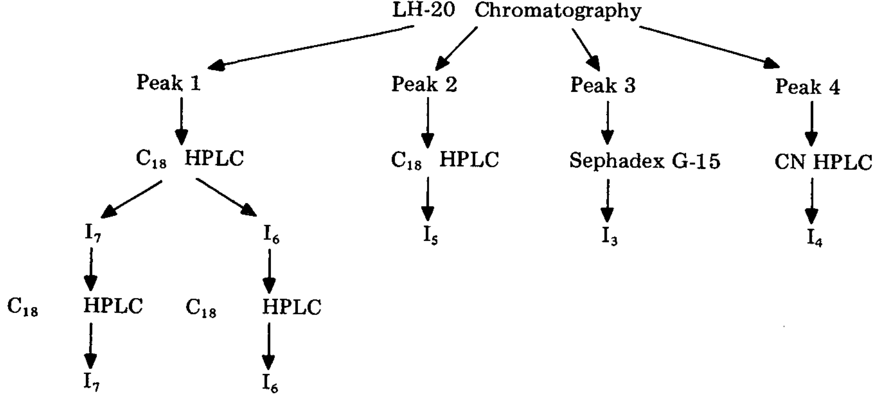

Fig. 2. Scheme of purification.

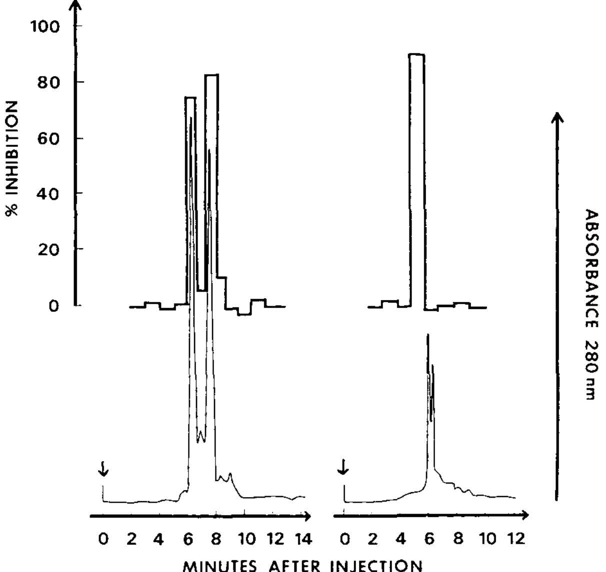

Fig. 3. Reversed-phase HPLC of pool 1 (left part) and pool 2 (right part) from LH-20 column. Column: Nucleosil 5 μm C_{18}, 250 × 4.6 mm I.D. Column temperature: room temperature. Mobile phase: 96% water + 4% acetic acid; flow-rate: 0.5 ml/min. Detection: 1.0 a.u.f.s. at 280 nm. Chart speed: 0.5 cm/min. Sample volume: 25 μl. Upper part of the curves: biological activity in individual fractions.

Gly, and I_6 : 1 Asx : 1 Thr : 1 Ser : 1 Glx : 2 Gly : 1 Ala, as described recently [7].

The activity peak 2 from the LH-20 column was even more hydrophilic, being eluted very near the front of the C_{18} column (Fig. 3, right part). Despite this non-optimal elution behaviour, the compound (I_5) seemed chemically pure, consisting of 1 mole of Asx and at least 4 moles of glucosamine, thus suggesting a very small glycopeptide with an extensive carbohydrate structure [7].

Peak 3 (I_3) could be further purified by Sephadex G-15 chromatography

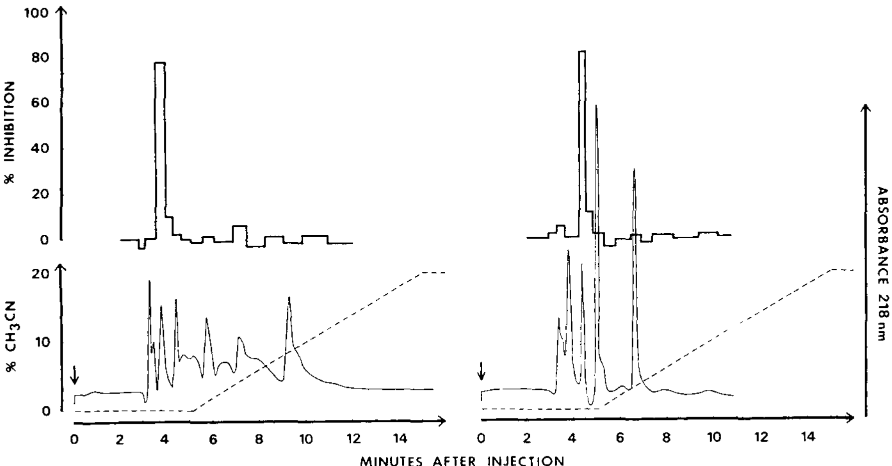

Fig. 4. Reversed-phase HPLC of inhibitors purified as described in Fig. 3, left part. Column: as in Fig. 3: Nucleosil 5C-18. Column temperature: 40°C. Elution conditions: 0.1% trifluoroacetic acid in water isocratic for 2 min followed by a gradient of 2% acetonitrile per min at 1.0 ml/min up to 20% acetonitrile. Detection: 0.2 a.u.f.s. at 218 nm. Chart speed: 1.0 cm/min. Sample volume: 20 μl. Left part: HPLC of first activity peak in Fig. 3, left part (I_7). Right part: HPLC of second activity peak in Fig. 3, left part (I_6). Upper part: As in Fig. 3.

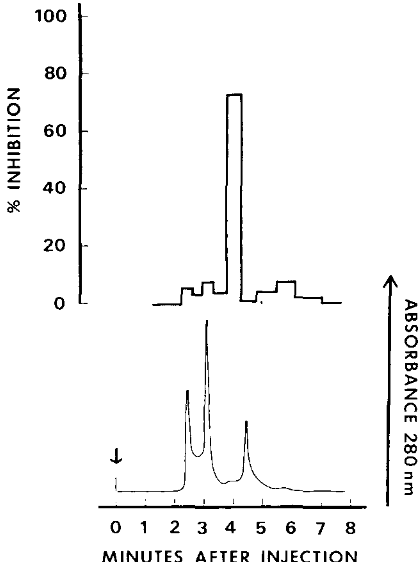

Fig. 5. HPLC of pool 4 from LH-20 column. Column: Nucleosil 5 μm CN, 250 × 4.6 mm I.D. Column temperature: room temperature. Mobile phase: acetonitrile—0.1% trifluoroacetic acid in water (1:1); flow-rate 1.0 ml/min. Detection: 0.5 a.u.f.s. at 280 nm. Chart speed: 0.5 cm/min. Sample volume: 25 μl. Upper part: as in Fig. 3.

eluted with 1.0 *M* acetic acid. As judged from its UV spectrum, showing a maximum absorption at 260 nm, it could be a nucleotide derivative.

The last eluting activity peak from the LH-20 column (peak 4) could be purified on a CN-cyanopropyl column (CN Nucleosil) (Fig. 5). The inhibitory peak

(I_4) appeared homogeneous after analysis, which showed 1 Asx : 1 Ser : 1 Glx : 2 Gly [7].

For more biochemical and cell biological characterization of the purified peptides see ref. 7.

DISCUSSION

The results obtained earlier with purification of endogenous growth inhibitors from the JB-1 ascites tumour indicated problems due to aggregation with other components in the extract, poor recovery and accumulation of toxic substances during the various purification steps [8]. The aggregation problems have been solved by extracting the active substances into methanol and primarily partitioning the activity in organic solvents in the subsequent purification steps, as described in the present paper. The poor recoveries were mainly associated with ion-exchange chromatography and high-voltage paper electrophoresis [6,8]. The bad recovery obtained by high-voltage paper electrophoresis may have been caused by ineffective elution of the factors from the electrophoresis paper. An important drawback of high-voltage paper electrophoresis was the coelution of toxic substance from the paper, possibly generated during the electrophoresis or picked up from the cooling tanks. All these problems have been greatly diminished by using the above-mentioned strategy.

The active extract has been purified recently [7] by HPLC without the first LH-20 step using a Nucleosil 5-C_{18} column eluted with methanol—acetic acid—water (50:8:42). This yielded inhibitors I_5 + I_6 + I_7 eluting together in the front fractions while I_3 and I_4 were more retarded. This procedure could not be used on a preparative scale due to the dominant occurrence of inhibitor I_3, which required a preparative purification step (LH-20) to separate I_3 from the other inhibitors. Besides, the most hydrophobic inhibitor I_4 appeared as a smeared peak in some HPLC experiments.

Other types of column packing material were tried for HPLC purifications. μBondapak C_{18} (Waters Assoc.) gave results similar to Nucleosil C_{18}, but LiChrosorb RP-18 (Altex Scientific, Berkeley, CA, U.S.A.) gave satisfactory separation of only the hydrophilic inhibitors (I_5, I_6 and I_7) while the more hydrophobic inhibitor I_3 produced a smeared peak; in addition, I_4 was totally lost and could not be eluted, even when the concentration of the organic modifier was increased. Spherisorb 5S ODS (Phase Separations, Queensferry, Great Britain) was totally unusable for the purification of any of the inhibitory peptides, all being irreversibly bound to the column. Although it produced satisfactory separation during LH-20 chromatography, Sephasorb™ HP Ultrafine (Pharmacia), which is the HPLC version of Sephadex LH-20, gave no separation of the various peptides: they were all eluted in the front volume although the same solvent was used as in LH-20 chromatography. Lowering the concentration of organic modifier did not improve the resolution since the various inhibitory peaks broadened and smeared into each other.

After LH-20 chromatography inhibitor I_4 could be purified by CN-Nucleosil HPLC, eluting isocratically with acetonitrile—0.1% trifluoroacetic acid in water (1:1) (Fig. 5). Lowering the acetonitrile concentration to 40% gave a better

resolution of the chromatogram. However, the recovery of activity decreased from 95% in the first system to approximately 50%, probably caused by poorer solubility or irreversible binding to the column of some of the active material.

Although very soluble in methanol—water (9:1), inhibitors I_5, I_6 and I_7 seemed rather hydrophilic, showing low affinity for the C_{18} column used. After HPLC purification these inhibitors seemed relatively pure as judged from amino acid analysis. However, a more powerful purification step could perhaps have been obtained by ion-exchange HPLC with Aminex A-28 or similar materials.

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CHROMBIO. 1262

QUANTITATIVE ENZYMATIC HYDROLYSIS OF tRNAs

REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF tRNA NUCLEOSIDES*

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SUMMARY

A rapid quantitative method for enzymatic hydrolysis of microgram amounts of tRNA has been developed, specifically to take full advantage of our precise, accurate, and selective reversed-phase high-performance liquid chromatographic (HPLC) system for separation and measurement of the major and modified nucleosides in tRNA. After study of several enzyme systems, nuclease P1 and bacterial alkaline phosphatase were selected and the hydrolysis parameters were systematically studied. Optimized hydrolysis conditions give quantitative hydrolysis in 2 h and this short incubation time prevents loss of unstable nucleosides. The chromatographic system can tolerate relatively high levels of protein in the sample allowing high enzyme—substrate ratios and direct injection of hydrolysates. This enzymatic hydrolysis—HPLC method is the best described to date for quantitative determination of the nucleoside composition of tRNAs and has already provided important information for investigation of the role of modification in the function of RNAs.

INTRODUCTION

Since the discovery of the wide variety of modified nucleosides present in tRNAs, there has been a great deal of interest in the role of these modified

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nucleosides in the function of tRNA. Current areas of interest include the effect of modifications in, or adjacent to, the anticodon on the fidelity of translation, the importance of modifications in the recognition of tRNA by aminoacyl-tRNA synthetases and the role of modified nucleosides in tRNA–ribosome interactions.

Determination of the modifications present in a tRNA, their position in the sequence, and the extent to which they are modified is essential to the study of modification in tRNA. Sequencing techniques available today are very powerful but often they do not allow identification of the modified nucleoside or exact quantitation of the level of a modification per unit of tRNA. Methodology for accurate identification and quantitation of the complete nucleoside composition of tRNAs is therefore required as a complementary technique to obtain information on the modifications present. Conditions that must be placed on this method are that it be applicable to today's use of microgram amounts of tRNA for sequence determination and allow preparative isolation of unknown modified nucleosides for identification and structure elucidation.

Analysis of the composition of tRNAs at the nucleoside level offers several advantages over analysis at the base or nucleotide level. Nucleosides can be obtained by enzymatic hydrolysis under mild conditions while chemical hydrolysis requiring harsh conditions is needed to obtain the bases and this may alter or destroy structure in sensitive modified nucleosides. Also, chromatographic separation of nucleosides is more efficient than separation of the highly charged nucleotides, and analysis at the base level does not allow the determination of the ribose-methylated nucleosides.

In earlier studies we developed a reversed-phase high-performance liquid chromatographic (HPLC) system for determination of urinary nucleosides [1–3] to use for investigation of tRNA breakdown products as cancer markers [3–5]. Reversed-phase HPLC was found to be ideally suited for separation of ribonucleosides because of their intermediate polarity. We then developed a reversed-phase HPLC method for measurement of the major and modified nucleosides in tRNAs by direct injection of nucleoside mixtures for enzymatic hydrolysates of RNAs [6]. This chromatography has been improved [7] and now allows separation and measurement of more than thirty nucleosides.

Despite a number of investigators reporting the use of various enzymes for hydrolysis of tRNA [8–16], the studies presented in this paper are, to our knowledge, the first systematic investigations on the conditions for enzymatic hydrolysis of tRNA reported and are directed towards developing a hydrolysis procedure that allows us to take full advantage of the chromatographic method. For accurate determination of the nucleoside composition of RNA the hydrolysis procedure must be quantitative, reproducible and capable of hydrolyzing microgram amounts of RNA in microliter volumes without large mechanical loss or chemical breakdown of the resultant nucleosides. Randerath et al. [12] reported some recovery studies with free nucleosides subjected to an enzymatic hydrolysis procedure, which showed conversion of m^1Ado to m^6Ado, and some loss of hUrd, m^3Cyd, and m^7Guo during the 6-h hydrolysis of tRNA with ribonuclease A, snake venom phosphodiesterase, and bacterial alkaline phosphatase. This degradation of nucleosides

was due to the alkaline lability of these particular molecules. We have shown that minimizing the incubation time of the nucleosides at pH 8 eliminated this problem.

In addition to the enzymes used previously for the hydrolysis of tRNA for nucleoside analysis we report the use of nuclease P1, an endonuclease that quantitatively hydrolyzes both DNA and RNA to 5′-mononucleotides with little specificity requirements for the bases.

The 2-h nuclease P1—bacterial alkaline phosphatase (BAP) hydrolysis procedure presented in this paper, together with our HPLC methods developed earlier, provide the tools for the determination of the complete nucleoside composition of tRNAs needed for research on the modified nucleosides in RNAs.

MATERIALS AND METHODS

The HPLC instrumentation, columns, buffer preparation, chromatographic conditions, standard solutions, and reagents used in these studies were described in detail in earlier publications [2, 3, 6]. Buffers for the step gradient separation of ribonucleoside mixtures were slightly different from those in our earlier tRNA analysis studies [6], but were the same as those used more recently for the separation of deoxyribonucleoside mixtures [17] in research on methylated nucleosides in DNA. Buffer A was composed of 2.5% methanol (v/v), 0.01 *M* $NH_4H_2PO_4$, pH 5.3 and buffer B was 8% methanol (v/v), 0.01 *M* $NH_4H_2PO_4$, pH 5.1.

All enzymes were obtained from commercial sources. Nuclease P1 (EC 3.1.4.-), ribonuclease A (EC 3.1.27.5), snake venom phosphodiesterase (EC 3.1.4.1), and calf intestinal alkaline phosphatase (EC 3.1.3.1) were obtained from Boehringer Biochemicals (Mannheim, G.F.R.); bacterial alkaline phosphatase (EC 3.1.3.1) and ribonuclease T_2 (EC 3.1.4.23) were obtained from Sigma (St. Louis, MO, U.S.A.). Brewer's yeast phenylalanine tRNA accepting 966 pmole phenylalanine per A_{260} unit, was also obtained from Boehringer Biochemicals.

The enzymatic hydrolysis of tRNA with ribonuclease A and T_2 was accomplished according to the following procedure. A tRNA solution (5 μl) containing 25 μg of tRNA was heated at 100°C for 2 min, then quenched in an ice-water bath. Ribonuclease A, 15 μl of a 1 mg/ml solution, and ribonuclease T_2, 25 μl of 1000 unit/ml solution, were added and the mixture was incubated for the desired time (0.25—12 h) at 37°C. The pH of the mixture was adjusted by addition of 6 μl of 0.5 *M* Tris, pH 7.9. BAP, 10 μl containing 2 units, was then added and the incubation was continued for an additional 12 h.

The method developed for the hydrolysis of tRNA with nuclease P1 and BAP is described in the following procedure. A tRNA solution of 5—125 μg of tRNA in 25 μl of water was heated for 2 min at 100°C. The sample was rapidly cooled in an ice-water bath. Then the following reagents were added: 2 μl of 20 m*M* $ZnSO_4$, 10 μl of nuclease P1 (1 mg/ml, 200 units/mg, in 30 m*M* sodium acetate, pH 5.3), and 10 μl of BAP (a commercial suspension containing about 190 units/ml, 30 units/mg, diluted 1:100 with water). This

mixture was incubated at 37°C for 1 h. The pH was adjusted by addition of 15 μl of 0.5 *M* Tris, pH 7.9, and the incubation continued at 37°C for another hour. For hydrolysis of different initial volumes of tRNA solution, the amounts of enzymes and buffers were adjusted to maintain the concentrations at about the same level as those used in the above procedure. The ratio of enzymes to RNA was also kept within or above the indicated range when hydrolyzing greater quantities of RNA. Although no systematic studies were made of the stability of the nucleosides in the hydrolysates, we found that the samples were stable for at least two months when frozen at −20°C.

RESULTS AND DISCUSSION

Monitoring enzymatic hydrolysis

Determination of the nucleoside composition of tRNA requires both quantitative hydrolysis of the macromolecule and precise and accurate analysis of the resulting ribonucleoside mixture. For the following investigations, the yield of the hydrolysis under each set of the conditions was determined by reversed-phase HPLC [6, 7] separation and quantitation of the nucleosides. This HPLC method allowed direct injection of the enzymatic hydrolysates onto the column without removal of protein or any other sample preparation. The analyses were performed at the rate of one every 2 h and automated instrumentation allowed unattended overnight operation.

A readily available commercial preparation of phenylalanine tRNA from yeast was chosen as the model substrate for these studies. This tRNA has been widely studied and the complete sequence and three-dimensional X-ray crystallographic structure are known.

Selection of enzymes for RNA hydrolysis

The enzymatic hydrolysis of RNA is a two-step process requiring a nuclease to release the mononucleotides and a phosphatase to remove the phosphate groups yielding nucleosides. Three nuclease systems and three phosphatases were investigated prior to selecting the best combination of enzymes for simple, rapid and complete hydrolysis.

The phosphatases considered were acid phosphatase, calf intestinal alkaline phosphatase, and BAP. The use of an acid phosphatase would allow the entire hydrolysis to be carried out at the pH optimum for the nucleases without incubation at high pH. However, the very low activity of these enzymes would require the addition of an excessive amount of protein to the mixture to ensure complete hydrolysis within a reasonable period of time.

The commercial preparation of calf intestinal alkaline phosphatase was contaminated with very high adenosine deaminase activity and gave almost quantitative conversion of Ado to Ino. This prevented determination of Ino residues present in tRNA and the accurate quantitation of Ado and modified Ado. For this reason we chose the BAP with only a trace of adenosine deaminase activity.

Each lot of BAP was checked for adenosine deaminase activity. If the activity was high enough to convert more than 1% of the Ado to Ino under the conditions used for hydrolysis of RNA, the BAP suspension was heated at

100°C for 5 min to denature the adenosine deaminase [18]. The activity of the BAP was checked before and after this heat treatment to assess how much BAP activity was lost. If 50% or more of the activity remained after heating the BAP could still be used effectively.

Three nuclease systems were investigated for hydrolysis of tRNA to nucleotides: nuclease P1; ribonucleases A and T_2; and ribonucleases A and T_2 with snake venom phosphodiesterase. The use of snake venom phosphodiesterase without other nucleases was not studied. This enzyme is an exonuclease and consequently its activity is decreased in the hydrolysis of oligonucleotides having appreciable secondary or tertiary structure.

Hydrolysis of yeast $tRNA^{phe}$ with nuclease P1 and BAP yielded all of the expected chromatographic peaks for major and minor ribonucleosides (Fig. 1), whereas hydrolysis with ribonucleases A and T_2 failed to release 2′-O-methylcytidine (Cm) and 2′-O-methylguanosine (Gm) as seen by the absence of these two peaks from the chromatogram (Fig. 2). This result was expected since hydrolysis with ribonucleases A and T_2 requires a free 2′-hydroxyl group for formation of a 2′—3′-cyclic nucleotide as an intermediate.

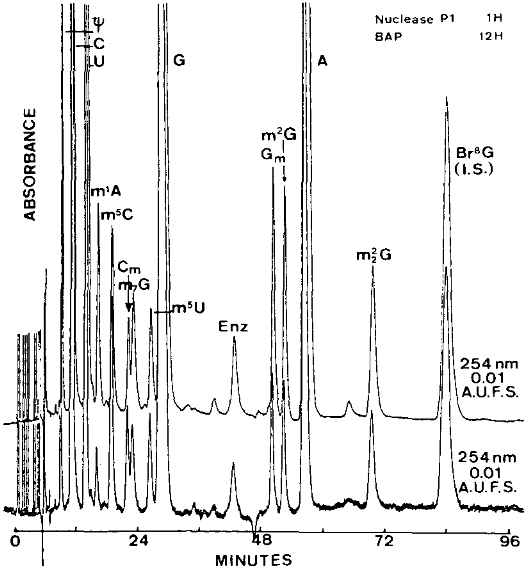

Fig. 1. A representative chromatogram for analysis of yeast $tRNA^{phe}$ hydrolyzed with nuclease P1 and BAP; 5 μg of tRNA were injected.

A quantitative comparison of these two enzyme systems is given in Table I. In addition to the failure of ribonucleases A and T_2 to release the 2′-O-methylated nucleosides, hydrolysis with these enzymes yielded only 80% as much m^7Guo and 50% as much m^2_2Guo as hydrolysis with nuclease P1. These observations reflect a difference in the substrate specificity of the two enzyme systems and not differences in the specific activities for they were compared over a wide range of enzyme incubation times (0.25—12 h).

The addition of snake venom phosphodiesterase as well as ribonucleases

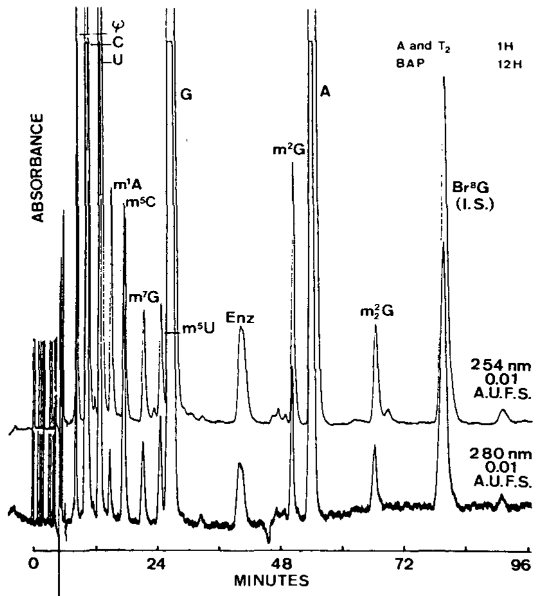

Fig. 2. A representative chromatogram for analysis of yeast $tRNA^{phe}$ hydrolyzed with ribonuclease A, ribonuclease T_2 and BAP; 5 μg of tRNA were injected.

TABLE I

HYDROLYSIS OF $tRNA^{phe}_{yeast}$

Nucleoside	Residues per 73 residues (hydrolyzed with nuclease P1)	Residues per 71 residues (hydrolyzed with nucleases A and T_2)
ψ	2.1	2.1
Cyd	16.2	16.4
Urd	11.6	11.0
m^1Ado	0.88	0.88
m^5Cyd	1.68	1.70
Cm	0.74	—
m^7Guo	0.79	0.64
m^5Urd	0.93	0.92
Guo	18.8	19.0
Gm	0.97	—
m^2Guo	0.87	0.88
Ado	16.5	17.1
m^2_2Guo	0.90	0.49

A and T_2 to the sample gave results essentially identical to those for nuclease P1. Since hydrolysis with a single enzyme is much more desirable than use of a three-enzyme system we chose nuclease P1 for optimization of hydrolysis conditions.

Effect of incubation time on hydrolysis of tRNA^{phe} with nuclease P1 and BAP

A very rapid hydrolysis procedure is desirable to prevent the breakdown of sensitive nucleosides during hydrolysis, and to allow the preparation of hydrolysates within one 8-h working day. Therefore, the time required for complete hydrolysis of tRNA was investigated using high concentrations of nuclease P1 and BAP. HPLC allows injection of high concentrations of protein without interfering with the analysis. This means high enzyme to RNA ratios may be used but column life will be shortened somewhat.

In this and subsequent studies, the tRNA solutions were heat denatured for 5 min at 100°C and immediately quenched in an ice-bath prior to addition of the enzymes. There was no clear evidence that this heat denaturation was necessary; however, denatured tRNA is more easily attacked by nucleases than native molecules and heat treatment does not damage the nucleosides. Thus, a heat treatment prior to hydrolysis is recommended.

Mixtures of denatured tRNA^{phe} (25 μg), nuclease P1 (2 units, 10 μg) and $ZnSO_4$ (10 nmole) in 50 μl of 30 m*M* sodium acetate buffer, pH 5.3, were incubated for 0.25, 0.5, 1, 2, 3, 8 and 12 h. At the end of the incubation period the sample was heated at 100°C for 5 min to stop the nuclease P1 activity. The pH of each sample was then adjusted with 6 μl of 0.5 *M* Tris, pH 7.9, and incubation was continued for an additional 12 h with 3.8 units (63 μg) of BAP in a final total volume of 66 μl.

Less than 15 min of incubation time were required for complete hydrolysis of tRNA^{phe} by nuclease P1 at the concentration used in this study. The qualitative and quantitative results for analysis of the samples from each incubation time were identical indicating the nucleotides released by nuclease P1 were stable under these conditions for at least 12 h.

The effect of incubation time on the BAP hydrolysis of nucleotides to nucleosides was studied in an analogous manner, except the samples were all incubated with 2 units (10 μg) of nuclease P1 for 1 h prior to incubation with BAP for 0.25, 0.5, 1, 2, 3, 4 and 24 h. A BAP concentration of 3.8 units in 66 μl of sample solution hydrolyzed the nucleotides completely in less than 15 min. The levels of all nucleosides except m^{7}Guo remained constant regardless of the BAP incubation time. The m^{7}Guo level in the hydrolysates decreased from 0.84 residues per 73 residues when BAP incubation time was 15 min to 0.61 residues per 73 residues when incubated with BAP for 24 h. This is consistent with the known base lability of m^{7}Guo [12].

Thus, quantitative hydrolysis of tRNA^{phe} (25 μg) to nucleosides was completed in less than 30 min incubation, 15 min with each enzyme, when the enzyme concentrations were high: 40 units nuclease P1 per ml and 570 units of BAP per ml.

Optimization of nuclease P1 concentration for hydrolysis of tRNA^{phe}

To determine the concentration of nuclease P1 required for complete hydrolysis of 25 μg of tRNA^{phe} in 1 h, a series of samples were prepared that were identical except for the nuclease P1 concentration. The use of a very large excess of enzyme is undesirable because injection of too much protein onto the HPLC columns would shorten column life. Obviously, the

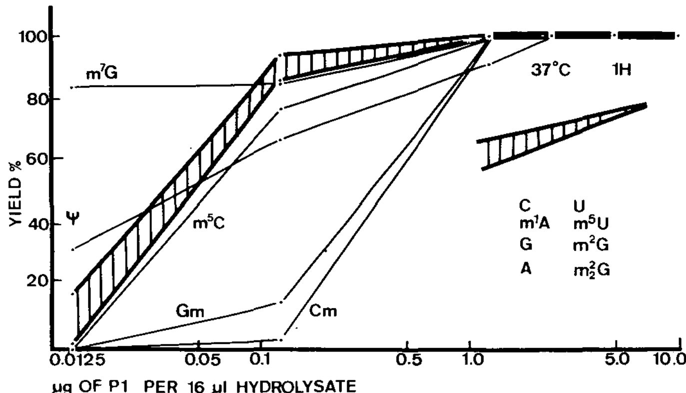

Fig. 3. Effect of nuclease P1 concentration on hydrolysis of yeast $tRNA^{phe}$. Reaction mixtures were prepared containing $tRNA^{phe}$ (25 μg), $ZnSO_4$ (10 nmole) and 0.0125, 0.125, 1.25, 2.5, 5 and 10 μg of nuclease P1 in a final volume of 16 μl (30 m*M* sodium acetate buffer, pH 5.3) and incubated for 1 h at 37°C. Nuclease P1 activity was stopped by heating at 100°C for 5 min. Hydrolysis to nucleosides was then completed by addition of 6 μl of 0.5 *M* Tris, pH 7.9, 10 μl of BAP (3.8 units, 63 μg) and incubation at 37°C for 1 h. The nucleoside levels in each sample were determined by HPLC and expressed as nanomoles per hydrolysate relative to the internal standard added after hydrolysis. The nanomoles per hydrolysate for each nucleoside were converted to per cent yield based on a 100% yield for the hydrolysates having a nuclease P1 concentration of 2.5 μg per 16 μl or greater.

use of too little enzyme would result in incomplete hydrolysis.

The yield of each nucleoside as a function of nuclease concentration is presented in Fig. 3. All nucleosides except ψ were obtained in 100% yields when the nuclease P1 concentration was 1.25 μg per 16 μl or greater. Cm, Gm and ψ exhibited a marked dependence on enzyme concentration while m^7Guo was much less dependent on enzyme concentration.

We suggest that the inhibited release of Cm and Gm was due solely to the nature of the modification, 2′-oxygen-methylation, and its hinderance of phosphate ester cleavage. The yield of these two nucleosides was less than 10% at a nuclease concentration of 0.125 μg per 16 μl and essentially zero below this concentration.

In contrast to the 2′-O-methyl nucleosides the yield of ψ did not drop below 30% for the lowest nuclease concentration, but did not reach 100% until the nuclease concentration was above 1.25 μg per 16 μl. This would suggest that the release of ψ from $tRNA^{phe}$ depends both on the nature of the nucleoside, ψ is the only one with a carbon—carbon ribofuranosyl bond, and on its two different locations within the tRNA structure. One ψ residue is located in the TψCG loop while the other is in the hydrogen-bonded anticodon stem. The high yield of m^7Guo (> 80%) even at the lowest concentration of nuclease P1 could be due to its exposed position in the extra loop.

Based on these studies a nuclease P1 concentration of 2.5 μg per 16 μl is sufficient for complete hydrolysis of $tRNA^{phe}$ in 1 h. Routinely, we use a concentration 2—4-fold higher which gives a margin of safety ensuring complete hydrolysis.

Optimization of BAP concentration for conversion of nucleotides to nucleosides

The study of the concentration of BAP required for complete release of nucleosides from tRNA was analogous to the study of nuclease P1 concentration on extent of enzymatic hydrolysis. Percent yields of the nucleosides from yeast $tRNA^{phe}$ versus phosphatase concentration is presented in Fig. 4.

Only three nucleosides, m^1Ado (45%), m^7Guo (32%) and ψ (not detected) were obtained in yields of less than 70% at the lowest level of BAP studied, 0.0125 μg in 32 μl. All nucleosides were quantitatively released at a BAP concentration of 0.05 μg per 32 μl except m^1Ado and m^7Guo which were obtained in 85% yield. This indicates that phosphatase action on the corresponding nucleotides is influenced by the nature of the modification.

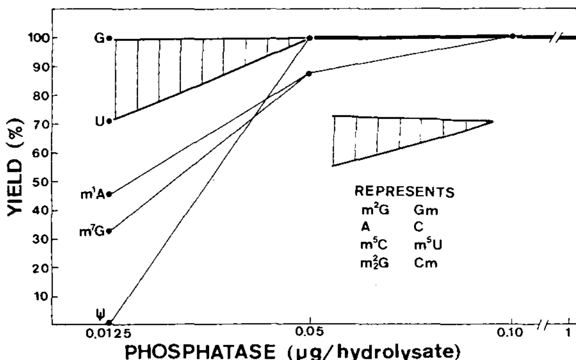

Fig. 4. Effect of phosphatase concentration on hydrolysis of yeast $tRNA^{phe}$. Samples containing 25 μg of $tRNA^{phe}$ were subjected to nuclease P1 (2 units, 10 μg) hydrolysis for 1 h. The resulting nucleotides were then incubated for 1 h with BAP (30 units/mg) at concentrations ranging from 0.0125 μg per 32 μl to 10 μg per 32 μl. Calculations of yields were made in the same manner as those in Fig. 3.

We have concluded that under these conditions, 0.1 μg per 32 μl is the minimum concentration of BAP required for quantitative dephosphorylation of the nucleotides resulting from nuclease P1 hydrolysis of 25 μg of tRNA. In subsequent experiments the concentration of phosphatase was increased 5-fold to ensure complete dephosphorylation.

Since BAP is appreciably active at a pH of 5.3 and was not irreversibly inactivated at this pH there was a decided advantage in adding the phosphatase enzyme at the same time as the nuclease P1. Dephosphorylation of nucleotides would then start at the pH optimum for nuclease P1 and would continue at a higher rate after adjustment of the pH to the optimum (pH 7.9) for BAP. When BAP was added to the samples with nuclease P1, the analytical results were identical to those obtained when BAP was added after completion of hydrolysis with nuclease P1. The combined addition of the enzymes has the added advantage of simplifying sample handling.

Maximum capacity of the enzymatic hydrolysis method

With establishment of the optimum enzyme concentrations and incubation times to assure quantitative hydrolysis of 25 μg of tRNA, the capacity

of the system for hydrolysis of larger quantities of tRNA was studied. Samples were prepared containing 25, 50, 125 and 200 μg of tRNA^{phe} with 10 μg of nuclease P1 and 0.63 μg of BAP. The 200-μg tRNA sample was in an initial volume of 50 μl and the others were in an initial volume of 25 μl. The samples were hydrolyzed for 1 h and then 15 μl of 0.5 *M* Tris, pH 7.9, were added prior to incubation for another hour. Complete hydrolysis of the tRNA was achieved under the above conditions. Normally only a limited amount (1—50 μg) of pure tRNA is available for nucleoside composition analysis so the enzymatic capacity demonstrated here is more than adequate. We have used the HPLC method for the determination of the nucleoside composition of 1 μg of tRNA, so the high capacity is required only for preparative scale isolation of modified nucleosides.

Application of the enzymatic hydrolysis to nucleoside analysis of various tRNAs

The enzymatic procedures reported here (see Materials and methods) have been used to hydrolyze a wide variety of tRNA samples for nucleoside analysis by HPLC. Excellent precision was obtained for replicate analyses of 5 μg of tRNAs with relative standard deviations (R.S.D.) ranging from 0.5 to 1.0% for the major nucleosides and from 2—4% for the modified nucleosides. A representative chromatogram from the analysis of a yeast tRNA^{ser} isoacceptor (Fig. 5) illustrates the HPLC separation of the nucleosides from a tRNA with a greater variety of modification than is found in yeast tRNA^{phe}. This sample is a single pure species; however, quantitative comparison of these results with other serine tRNAs cannot be made since the sequence for this species

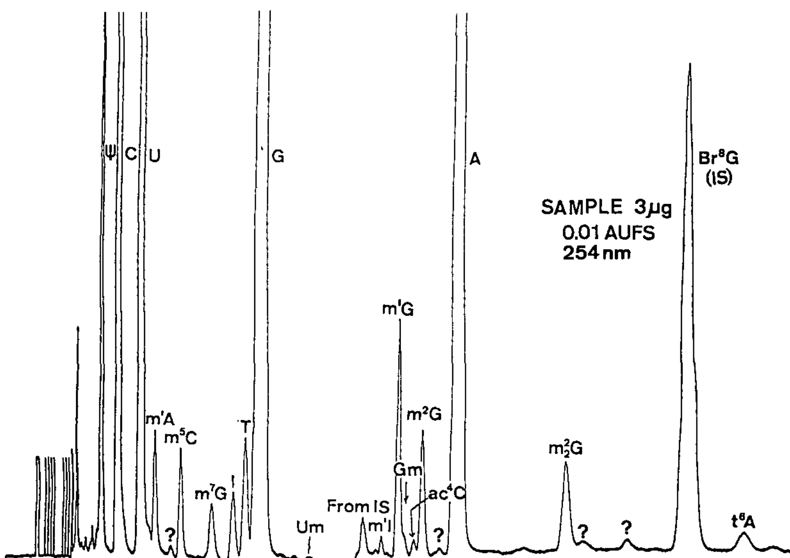

Fig. 5. Reversed-phase HPLC of a 3-μg hydrolysate of a purified species of *S. pombe* tRNA^{ser}. The tRNA was hydrolyzed with nuclease P1 and BAP according to the procedure in the Materials and Methods section.

TABLE II

NUCLEOSIDE COMPOSITION ANALYSIS OF tRNAs BY HPLC

The amount of each nucleoside found is expressed as the number of residues per molecule. The calculation was made using the equation:

$$R_N/\text{molecule} = \left[\frac{\text{nmols } N}{\text{total nmols}}\right] \times [\text{total number of residues } (R) \text{ in the tRNA}]$$

where R_N/molecule is the number of residues of the same nucleoside N per tRNA molecule; nmols N is the measured nanomoles of the nucleoside in the total sample; total nmols is the total nanomoles of all nucleosides in the sample (determined from the analysis); and the total number of residues per molecule is based on the sequence. Dihydrouridine and i^6Ado residues in the sequence were not counted. Dihydrouridine has a very low absorptivity at 254 and 280 nm preventing its detection by UV absorption at these wavelengths and i^6Ado was not quantitated because different chromatographic conditions are required for elution of this strongly retained nucleoside.

Nucleoside	Residues per molecule			
	$tRNA_f^{met}$ (*E. coli*)		$tRNA^{trp}$ (yeast)	
	Analysis	Sequence***	Analysis	Sequence***
ψ	1.01	1	5.8	6
Cyd	25.6	25	15.9	16
Urd	8.2	8	9.6	10
m^1Ado	—	—	0.81	1
Cm	0.91	1	1.81	2
m^7Guo	0.51	1/0*	0.91	1
m^5Urd	1.02	1	1.05	1
Guo	23.9	24	16.1	16
S^4Urd	0.8	1	—	—
m^1Guo	—	—	0.88	1
Gm	—	—	1.09	1
m^2Guo	—	—	0.96	1
Ado	14.1	14/15*	16.4	16
hUrd	NA**	1	NA	3

*The numbers on the right represent the sequence for a minor subspecies.
**This nucleoside was not analyzed.
***Ref. 19.

has not been determined. The presence of all of the expected modified nucleosides, by comparison to all published yeast $tRNA^{ser}$ sequences, indicates nuclease P1 was capable of releasing all of the wide variety of modified nucleosides present in this tRNA. Enzymatic hydrolysis for analysis of many different tRNA samples has shown that nuclease P1 is capable of releasing all of the expected nucleosides regardless of the extent of modification.

The nucleoside compositions determined for $tRNA^{met}$ (from *E. coli*) and $tRNA^{trp}$ (from *S. cerevisiae*) are presented in Table II along with the predicted results based on the published sequences from these tRNAs [19]. The agreement of the values from HPLC analysis with those predicted from the sequence is excellent.

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DETERMINATION OF NICOTINAMIDE AND METABOLIC PRODUCTS IN URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic procedure has been developed for the quantitation of nicotinamide, nicotinic acid, nicotinuric acid, 1-methylnicotinamide and 1-methyl-2-pyridone-5-carboxamide in rat and human urines. The procedure utilizes a Varian Model 5020 liquid chromatograph with a UV detector, and an Altex 15 cm × 4.6 mm Ultrasphere-ODS column, employing a linear ion-pair mobile phase gradient. Solvent A contains 10 m*M* concentrations of pentanesulfonic acid (PSA), tetramethylammonium chloride (TMA) and KH_2PO_4, and solvent B contains PSA, TMA and acetonitrile. Different pH values for solvent A vary the retention times and thus the separation of the five compounds. Temperature of the system is critical. The conditions found most satisfactory were pH 3.30 and 24.5°C.

INTRODUCTION

Rapid, precise analyses of blood and urine for vitamins and their metabolites are of paramount importance in the evaluation of nutritional status. Progress in this field has been greatly accelerated by the use of high-performance liquid chromatography (HPLC). In 1973, Williams and co-workers [1] employed HPLC for separating and quantifying nicotinic acid and riboflavin in aqueous solutions. These investigators [2] modified and extended their reversed-phase HPLC procedure to separate mixtures of vitamins B_2, B_{12}, C, and the four fat-soluble vitamins. Since that time these and other vitamins and derivatives have been separated and measured employing various modifications of HPLC [3—10]. In 1980, De Vries et al. [11] determined nicotinamide in human plasma and urine by ion-pair reversed-phase HPLC.

We present a method for determining nicotinamide and several of its metabolites in rat and human urines with HPLC using a linear ion-pair mobile

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phase gradient. The compounds reported are nicotinic acid (niacin), nicotinamide, 1-methylnicotinamide, nicotinuric acid and 1-methyl-2-pyridone-5-carboxamide. A preliminary report of a method for separating some of these compounds has already appeared [12].

MATERIALS AND METHODS

Apparatus

The apparatus employed was a Varian Model 5020 high-performance liquid chromatograph, incorporating a Varian Vari-Chrom UV detector, a Rheodyne 7126 injector with a 20-μl loop, and an Altex 15 cm × 4.6 mm Ultrasphere-ODS column, particle size 5 μm. The HPLC system was interfaced to a Varian CDS 401 system for gradient programming and data processing.

Chemicals and solvents

The 1-pentanesulfonic acid sodium salt (PSA) and tetramethylammonium chloride (TMA) were obtained from Eastman-Kodak (Rochester, NY, U.S.A.). The water, methanol and acetonitrile were Baker Analyzed HPLC grade reagents (J.T. Baker, Phillipsburg, NJ, U.S.A.). All other chemicals used in preparing the two solvent systems were of analytical reagent grade. The nicotinamide (NAM) was a Calbiochem (Los Angeles, CA, U.S.A.) product; the nicotinic acid (NA), 1-methylnicotinamide chloride (NMN) and nicotinuric acid (NUA) were from Sigma (St. Louis, MO, U.S.A.); and the 1-methyl-2-pyridone-5-carboxamide (2-PYR) was kindly prepared by Dr. Michael Jung of the UCLA Department of Chemistry. All five compounds were kept desiccated.

Several A solvents with different amounts of mobile phase ion-pairing agents, PSA and TMA, and different pH values were prepared and evaluated. The one selected for the work reported here contained 10 m*M* concentrations of each of the two reagents, and 10 m*M* KH_2PO_4 in HPLC grade water. Solvent B consisted of 100 ml HPLC grade water containing 10 m*M* PSA and 10 m*M* TMA, plus 900 ml acetonitrile. The solvents were degassed by sonication for 2—3 min.

Standard solutions

Solutions of different known strengths were prepared in HPLC grade water for each of the five compounds (NAM, NA, NMN, NUA and 2-PYR). They were employed as standards either singly or in combinations and for adding to urine samples to confirm the position and quantification of the peaks. Use of an internal standard has been considered but a suitable one has not been determined. Therefore standard solutions were determined at least twice each day, runs were made and the values determined to be constant as described below under Sensitivity and accuracy.

Urine specimens

Urines (48 h) were collected from three-month-old Sprague-Dawley rats into 1 *N* hydrochloric acid and diluted to 0.1 *N* and a known volume. The animals were maintained on a diet containing 12% casein and 90 mg niacin per kg diet. The human urines were either 24-h collections made into toluene, or spot

samples. There was no diet supplementation with nicotinamide or nicotinic acid. Urine samples not analyzed immediately upon collection were kept frozen. Urine aliquots were filtered through 0.2-μm Metricel Gelman membrane filters and analyzed without further treatment, except that dilution was necessary for some urine samples. Trial treatment with perchloric acid to remove any protein did not alter concentrations of the compounds. No other fractionation was attempted. Repeated freezing, storing at −20°C, and thawing over a period of at least five weeks did not alter the concentrations in either the urines or standard solutions.

Operation of apparatus

The analyses were performed with the UV detector set for 254 nm, a band width of 8 nm, and an absorbance range of 0.05. The CDS 401 was programmed to record the concentration of each compound in μg/ml, with an attenuation of 512 and a plotting rate of 1 cm/min. Aliquots (20 μl) of standards or urines were injected for analysis with a flow-rate of 2 ml/min and a linear ion-pair mobile phase gradient programmed from 0% to 6% solvent B in 6 min. The five compounds were eluted within 4 min. The column was regenerated for 2 min with a solvent A/B ratio of 94:6, and then equilibrated for 5 min with an A/B ratio of 100:0.

RESULTS

Calibration of HPLC apparatus

Each of the five standard solutions was chromatographed separately at several pH values. The average of three determinations was used for the final calibration factor for each of the five standards. Mixtures of the five standards were then chromatographed (Fig. 1A and Table I).

Retention times of the standards were rechecked each time a new batch of solvent was prepared and at least twice during a day of analyzing.

TABLE I

RETENTION TIMES (min) AT 24.5°C FOR THE FIVE COMPOUNDS AT DIFFERENT pH VALUES

Solvents A and B as described in text, were used. Each value is an average of 2—4 assays, with no attempt made to show statistically significant differences. Between-assay retention time variation was ± 0.05 min. Therefore it is important to select conditions for which the retention times from one compound to another differ by at least 0.20 min.

Compound	pH value for solvent A				
	2.20	3.30	4.40	5.50	6.80
NA	1.70	1.35	1.20	1.00	0.85
NMN	1.80	1.90	1.95	2.05	2.30
NAM	2.00	2.85	3.40	3.50	3.50
NUA	3.60	3.10	2.40	1.30	1.20
2-PYR	3.30	3.40	3.35	3.35	3.30

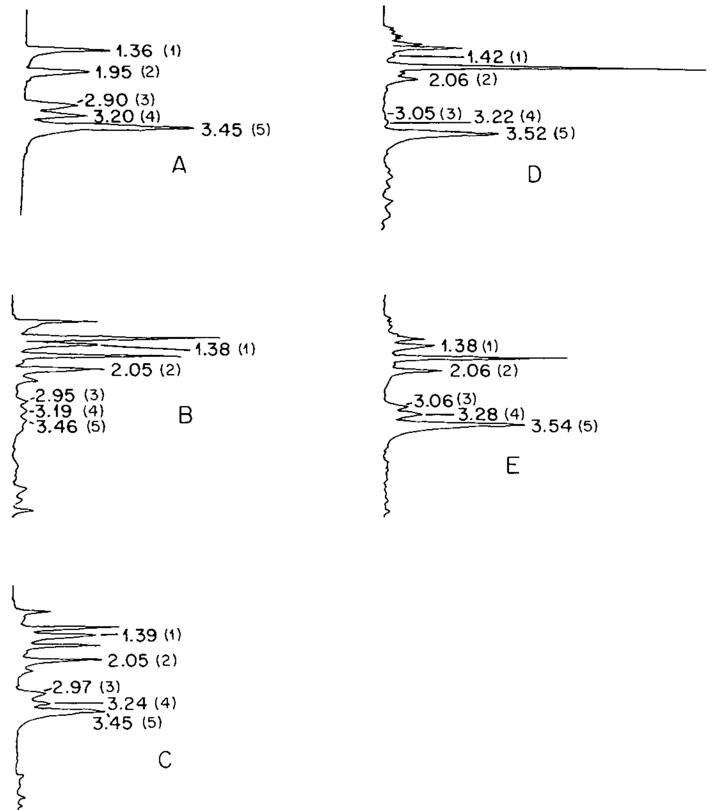

Fig. 1. Tracing of elution pattern of the five compounds: (1) NA, (2) NMN, (3) NAM, (4) NUA, and (5) 2-PYR. Analyses were made with solvent A, pH 3.30, and temperature of 24.5°C. Retention times in minutes are noted in the five tracings: (A) standard curves, (B) rat urine (see also Table III), (C) rat urine plus standards, (D) human urine (see also Table IV), (E) human urine plus standards.

Sensitivity and accuracy

Standards (1 μg) in 20-μl samples (50 μg/ml) could be determined with an accuracy of about 3% and 0.1 μg with an accuracy of about 6%. The following are examples of two 2-PYR standards analyzed over a period of two weeks. Six determinations of one standard gave a mean value of 48.0 μg/ml (46.4–48.8), S.E.M. 0.35. Twelve determinations of a second standard gave a mean value of 4.24 μg/ml (4.03–4.45), S.E.M. 0.034. Amounts as small as 0.01 μg were readily detectable but the determined values varied as much as 50%.

Composition and pH of solvent A

Eight different concentration combinations of PSA and TMA, with a constant amount of KH_2PO_4, were tested at different pH values. The retention time and calibration factors changed with each solvent. The concentrations found to be most satisfactory were 10 m*M* for each of the three components. Concentrations of PSA, TMA and acetonitrile in solvent B were held constant (see Chemicals and solvents section).

Changes in the pH of solvent A greatly altered the retention times and the calibration factors (Table I) so that in going from solvents of different pH the system had to be reprogrammed.

Effects of temperature

Maintenance of temperature of the solvents and column was found to be important for consistent retention times for the five compounds. With variations in temperature, retention times for components having a stronger affinity for the column packing were altered to a greater degree than those components eluted earlier in the run (Table II). At the lower temperature there was a somewhat greater spread of the retention times. For this study solvent A, pH 3.30 and a temperature of 24.5°C were employed.

TABLE II

RETENTION TIMES (min) AT DIFFERENT TEMPERATURES FOR THE FIVE COMPOUNDS

Solvent A, pH 3.30 was used. Each value is an average of 2—4 assays, with no attempt made to show statistical differences. Between-assay retention time variation is ± 0.05 min.

Compound	Temperature of column		
	22.5°C	24.5°C	26.0°C
NA	1.40	1.35	1.35
NMN	2.00	1.90	1.90
NAM	3.00	2.85	2.80
NUA	3.25	3.10	3.00
2-PYR	3.55	3.40	3.30

Chromatography of urine samples

Duplicate analyses were made on the urines from six rats for the five components, and values for one are shown in Table III. Fig. 1B is a tracing of the chromatogram for this urine. The compounds were identified by retention times and by addition of an equal volume of a mixture of the five standards (Fig. 1C). Percent recoveries of the amounts added are shown in Table III.

One rat urine which contained 4.65 μg 2-PYR/ml (three determinations, 4.61—4.71), when diluted with an equal volume of a 2-PYR standard containing 4.24 μg/ml, was determined to have 4.15 μg/ml (five determinations, 3.98—4.36). Theoretically the value should be $4.65/2 + 4.24/2 = 4.45$ μg/ml, a difference of 7%. Another rat urine with only traces of 2-PYR gave values of 0.17—0.49 μg/ml (four determinations) and recoveries of added 2-PYR that were in error by more than 50%.

Also, duplicate analyses were made on the urines from six human subjects for the five components and the compounds identified, as with the rat urines, by retention times and by the addition of an equal volume of a mixture of the five standards (Fig. 1D and E). Data are given in Table IV. These same urines were also analyzed for NMN by the chemical method of Pelletier and Brassard [13]. There was good agreement with values averaging 7% higher for the chemical procedure.

TABLE III

AMOUNTS OF NA, NMN, NAM, NUA AND 2-PYR IN ONE RAT URINE, AND RECOVERIES OF THE FIVE COMPOUNDS ADDED TO THE ONE, AND TO SIX RAT URINES

Aliquots of 20 μl were analyzed by HPLC [(1) and (4)]; for recoveries the urines were diluted with an equal volume of an aqueous mixture of the five compounds.

Component	Rat urine analyses and recoveries					Recovered (%) from six rat urines	
	Amount present (μg/ml) (1)	Amount added (μg/ml) (2)	Sum (1) + (2) (μg/ml) (3)	Total amount measured (μg/ml) (4)	Recovered (%) (4)/(3)	Range	Average
NA	23.8	25.0	48.8	48.4	99	92—99	95
NMN	29.1	25.0	54.1	58.2	108	93—110	102
NAM	16.2	25.0	41.2	37.9	92	85—104	93
NUA	7.4	25.0	32.4	30.0	93	89—111	100
2-PYR	1.8	25.0*	26.8	30.1	112	98—133	117

*Recoveries of 2.12 μg/ml from an additional rat urine gave an error of 7%, see section Chromatography of urine samples.

TABLE IV

AMOUNTS OF NA, NMN, NAM, NUA AND 2-PYR IN ONE HUMAN URINE, AND RECOVERIES OF THE FIVE COMPOUNDS ADDED TO THE ONE URINE, AND TO SIX HUMAN URINES

Aliquots of 20 μl were analyzed by HPLC [(1) and (4)]; for recoveries the urines were diluted with an equal volume of an aqueous mixture of the five compounds.

Component	Human urine analyses and recoveries					Recovered (%) from six human urines	
	Amount present (μg/ml) (1)	Amount added (μg/ml) (2)	Sum (1) + (2) (μg/ml) (3)	Total amount measured (μg/ml) (4)	Recovered (%) (4)/(3)	Range	Average
NA	0	25.0	25.0	25.7	103	92–116	105
NMN	7.1	25.0	32.1	33.4	104	89–105	100
NAM	0	25.0	25.0	23.6	94	75–112	95
NUA	5.0	25.0	30.0	33.6	112	99–112	107
2-PYR	8.7	25.0	33.7	35.8	106	95–108	102

DISCUSSION

The presence of large numbers of components in rat and human urines made separation and quantification of nicotinamide and metabolic products very difficult. Often there was overlapping of peaks of unknown components with one or more of the five compounds studied and often this was the reason for excessively high values for recoveries of the added compounds. This was especially the situation for 2-PYR in rat urines. To obtain better separations, varying concentrations of PSA and TMA in the solvent were found to be beneficial. Varying the pH has been found to be the most beneficial (Table I). With higher pH values the retention times of 2-PYR remained fairly constant while those of the acids shifted to shorter times and the amides to longer times. It also was important to maintain the temperature of the system within 1°C (Table II).

De Vries et al. [11] have determined NMN in human plasma and urine, precleaning the samples with a small column of Sep-Pak C_{18} followed by HPLC separation and the use of an ion-pair in combination with a reversed-phase system. The sensitivity of their method is 0.1 mg/l for plasma and 1 mg/l for urine. At a plasma level of 0.5 mg/l the coefficient of variation (n = 10) was 3.4% and at a urine level of 5 mg/l the coefficient of variation was 6.8%. The variations we obtained for NMN are comparable (Tables III and IV).

It is of interest to note that the rats excreted large quantities of NA, NMN and NAM and relatively small amounts of NUA and 2-PYR. Chang and Johnson [14] reported results that were somewhat different. They found excretion of high levels of NMN, NUA and 4-PYR, and low levels of 2-PYR and NAM. In their procedure they injected 5.9 mg/kg body weight of ^{14}C-labeled NA, collected 24-h urine samples and separated the components using paper chromatography. Petrack et al. [15], employing paper chromatography, analyzed rat urines for NA, NMN, NAM, NUA, 2-PYR, N-methyl-4-pyridone-3-carboxamide and nicotinamide-N-oxide, following injection of the rats with 7-^{14}C-labeled nicotinamide and nicotinic acid. They found only very small amounts of the last three compounds in rat urine. We have not been able to obtain samples of N′-methyl-4-pyridone-3-carboxamide, or of nicotinamide-N-oxide, a metabolic product present in relatively large amounts in the urine of mice [16].

By contrast to rats, our human studies showed large excretions of NMN, NUA and 2-PYR and little or no NA and NAM. The amount of 2-PYR was usually greater than NMN. Mainardi and Tenconi [17] in their studies on humans also found basal values of 2-PYR to be higher than NMN. They utilized ultraviolet measurements before and after passing urine through multilayers of Dowex 1 and 50.

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CHROMBIO. 1255

SEPARATION OF NANOGRAM QUANTITIES OF HYDROXY METABOLITES OF VITAMIN D_3 IN PLASMA BY THIN-LAYER CHROMATOGRAPHY ON SILICA GEL

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SUMMARY

Thin-layer chromatography (TLC) on silica gel coated HPTLC plates, using chloroform—ethanol—water as mobile phase, is highly effective in the quantitative separation of biologically active metabolites of vitamin D. The combination of TLC and competitive protein-binding assay results in a rapid, sensitive and reproducible method for the analysis of nanogram quantities of metabolites of vitamin D_3 (25-hydroxycholecalciferol, 24,25-dihydroxycholecalciferol and 25,26-dihydroxycholecalciferol) in plasma samples.

INTRODUCTION

It is now well established that the actions of the D vitamins on intestine and bone are mediated through their hydroxylated metabolites [1]. At the present time there are many modifications of the assays for hydroxy metabolites of vitamin D [2—5]. All of them involve, prior to the application of a proper quantitative method, preparative chromatography using Sephadex LH-20 [6], Lipidex 5000 [7] and/or, recently, silica Sep-Pak [8,9] and high-performance liquid chromatography for the finest separation of the metabolites of vitamin D in serum (reviewed in refs. 2, 10 and 11). The use of these chromatographic steps results in very good separation of vitamin D metabolites but, on the other hand, it requires apparatus with specialized accessories for separation of larger series for routine analysis which may not be available in laboratories specializing in the methods of saturation analysis.

Extending our previous work on the description of thin-layer chromatographic (TLC) separation of metabolites of vitamin D [12], we present a detailed study of the determination of nanogram quantities of hydroxy

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metabolites of vitamin D_3 [25-OH-D_3, 24,25-$(OH)_2$-D_3 and 25,26-$(OH)_2$-D_3]* in plasma after their chromatographic separation on thin layers of silica gel. We have tried to simplify this separation step. The elution of the separated metabolite from silica gel [12] was omitted, and for final determination diluted osteomalacic plasma as binding protein [13] was applied directly to scraped silica-gel areas.

EXPERIMENTAL

Materials

All solvents (Lachema, Brno, Czechoslovakia) were of analytical grade and were used without further purification.

Crystallized 25-OH-D_3 (a gift from Philips Duphar, Weesp, The Netherlands), 24,25-$(OH)_2$-D_3, 25,26-$(OH)_2$-D_3 and 1,25-$(OH)_2$-D_3 (a gift from Dr. M. Uskokovič, Hoffmann-La Roche, Nutley, NJ, U.S.A.) and D_3 (purchased from E. Merck, Darmstadt, G.F.R.) were dissolved in 96% distilled ethanol to a concentration of 0.24 μM and stored at −20°C.

25-Hydroxy[26(27)-methyl-^{3}H]cholecalciferol (0.43 T Bq/mmol) was purchased from the Radiochemical Centre, Amersham, Great Britain. Ethanolic solution of 8.6 μM was stored at −20°C. Prior to use in the assay, ^{3}H-labelled 25-OH-D_3 was purified by TLC on silica gel in ethyl acetate—*n*-hexane (1:6, v/v).

The following pre-coated plates (for nano-TLC) were tested: silica gel 60 without fluorescent indicator, silica gel 60 F_{254} with fluorescent indicator (both manufactured by E. Merck), and silica gel for HPTLC without fluorescent indicator from Whatman (Maidstone, Great Britain).

Chloroform—ethanol—water (183:16:1, v/v) was used as the mobile phase for the development. The plates were stored at a relative humidity of about 45%.

The charcoal—dextran suspension was prepared by mixing equal volumes of 0.05% (w) dextran (Koch-Light Labs., Colnbrook, Great Britain) and 0.5% (w) suspension of charcoal Norit A (Serva, Heidelberg, G.F.R.) in 0.05 M phosphate buffer, pH 7.2.

Heparinized plasma from a patient suffering from osteomalacia who had not been treated with vitamin D previously, was diluted 1:1500 (v/v) with 0.05 M phosphate buffer, pH 7.2, and used as binding protein for competitive protein-binding assay (CPBA).

Plasma samples for analysis were collected from healthy men aged 40—50 years.

Radioactivity in 0.5-ml aqueous aliquots was counted in 10 ml of Bray's scintillation fluid on a Model 2425 Packard Tricarb liquid scintillation spectrometer with counting efficiency of about 50%.

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*Abbreviations: D_3 = cholecalciferol, vitamin D_3; 25-OH-D_3 = 25-hydroxycholecalciferol; 1,25-$(OH)_2$-D_3 = 1α,25-dihydroxycholecalciferol; 24,25-$(OH)_2$-D_3 = 24,25-dihydroxycholecalciferol; 25,26-$(OH)_2$-D_3 = 25,26-dihydroxycholecalciferol.

Sample preparation

The plasma samples were analysed by a modification of our method previously described [13]. Three volumes of ammonium sulphate (66% saturation) were used to precipitate carrier proteins for metabolites of vitamin D in 1-ml plasma samples. Following centrifugation the precipitate was dissolved in 1 ml of water. Precipitation was repeated three times. After the addition of 1 ml of methanol the metabolites were extracted with 3 ml of toluene. An aliquot of 1 ml of toluene was evaporated and redissolved in 20 μl of ethanol and applied to the silica-gel thin layer.

Thin-layer chromatography

Prior to use the plate was developed in the mobile phase used for a development and dried gently. The sample (2–20 μl) was then applied to the plate. After developing in the solvent mixture microgram quantities of the separated compounds were visualized either as yellow–red spots by spraying the plate with concentrated sulphuric acid [14] or as violet spots on a fluorescent background under a UV source at 254 nm. For smaller quantities, 0.2–4.0 ng, the sample must be run alongside a larger standard sample and the spot position determined with the aid of a template. The spot is then marked out, scraped off and the compound is, after wetting with 50 μl of water, diluted with 20 μl of 96% ethanol [for analysis of 24,25-$(OH)_2$-D_3 and 25,26-$(OH)_2$-D_3], or with 20 μl of the developing solvent (for analysis of 25-OH-D_3). After thoroughly mixing for 20 min the mixture is prepared for CPBA.

Competitive protein-binding assay

The CPBA was performed essentially as we have described elsewhere [13,15]. Ethanol (20 μl) was added to all assay tubes. Incubation was started by the addition of 1 ml of a cold (−4°C) incubation solution. This solution contained, per 1 ml of a solution of binding protein, 20 μl of ethanolic solution of ^{3}H-labelled 25-OH-D_3 of about 22,000 cpm. After the addition of 0.25 ml of dextran-coated charcoal, phase separation was achieved by centrifugation at 1000·*g*. Aliquots of the supernatant (1 ml) were solubilized in Bray's scintillation solution and monitored for radioactivity.

RESULTS AND DISCUSSION

Separation efficiencies of silica-gel-coated HPTLC plates of different origin for the separation of vitamin D metabolites are listed in Table I. Using high-performance silica-gel plates a much higher separation efficiency was achieved than with the other types of TLC on silica gel [12]. Naturally occurring vitamin D metabolites of biological importance, listed in Table I, are distinctly separated by TLC in nanogram quantities.

From studying the binding properties of the metabolites of vitamin D we have found that 25-OH-D_3, 24,25-$(OH)_2$-D_3 and 25,26-$(OH)_2$-D_3 are equipotent in displacing ^{3}H-labelled 25-OH-D_3 from the plasma binding protein (diluted plasma from a patient with non-cured osteomalacia). Neither D_3 (within the concentration range 0–100 ng) nor 1,25-$(OH)_2$-D_3 (within the concentration range 0–100 pg) affects the competitive protein-binding reaction.

TABLE I

R_F VALUES OF METABOLITES OF VITAMIN D AFTER TLC ON SILICA-GEL LAYERS IN CHLOROFORM—ETHANOL—WATER (183:16:1)

HPTLC silica-gel layers	R_F values				
	D_3	25-OH-D_3	24,25-$(OH)_2$-D_3	25,26-$(OH)_2$-D_3	1,25-$(OH)_2$-D_3
Kieselgel with indicator* (Merck)	0.69	0.56	0.41	0.30	0.20
Kieselgel without indicator* (Merck)	0.65	0.51	0.38	0.28	0.22
Silica gel without indicator* (Whatman)	0.70	0.56	0.48	0.39	0.32

*Fluorescent indicator at 254 nm.

Using the above CPBA method for final quantitative determination of metabolites of vitamin D, we have checked for evidence of separation of nanogram quantities of 25-OH-D_3, 24,25-$(OH)_2$-D_3 and 25,26-$(OH)_2$-D_3 by TLC. The characteristics of the CPBA evaluated by establishing standard curves of these metabolites were used in this assay [15]. Determinations of 1,25-$(OH)_2$-D_3 and D_3 — after their separation on TLC — by CPBA using a specific binding protein from the intestine of chicken are under investigation. Our attempts to simplify the procedure of determination of metabolites after their separation on thin layers of silica gel [12] by omitting the elution step are illustrated in Fig. 1 and Table II.

TABLE II

PERCENTAGE RECOVERY AND ACCURACY OF ADDED AMOUNTS OF 25-OH-D_3, 24,25-$(OH)_2$-D_3 AND 25,26-$(OH)_2$-D_3 THROUGH THE PROCEDURE OF TLC + CPBA

Compound	Amount applied to TLC* (ng)	Amount found after TLC + CPBA** (ng)	No. of replicates (n)	Recovery (%)	C.V.*** (%)
25-OH-D_3 24,25-$(OH)_2$-D_3 25,26-$(OH)_2$-D_3	0	0.00 ± 0.01	10	100.01	2.57
25-OH-D_3	0.40	0.38 ± 0.03	5	95.00	7.50
24,25-$(OH)_2$-D_3	0.40	0.39 ± 0.01	6	98.59	1.68
25,26-$(OH)_2$-D_3	0.40	0.39 ± 0.01	5	96.50	3.59
25-OH-D_3	1.00	0.99 ± 0.01	6	99.30	1.72
24,25-$(OH)_2$-D_3	1.00	1.00 ± 0.07	4	100.50	6.57
25,26-$(OH)_2$-D_3	1.00	1.03 ± 0.05	4	103.10	4.67

*On silica gel (Merck, without indicator).
**Mean ± S.D.
***Coefficient of variation.

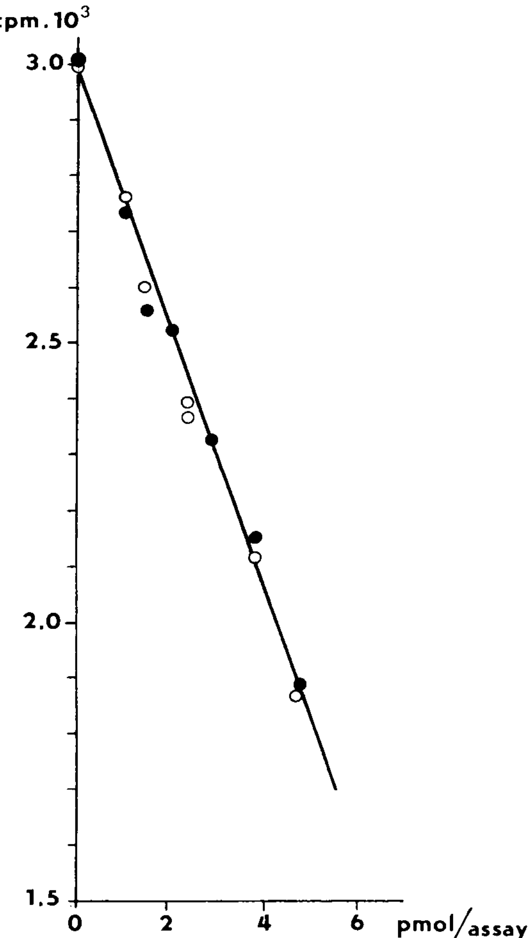

Fig. 1. Competitive protein-binding displacement of 25,26-$(OH)_2$-D_3, within the concentration range 0.0—3.0 ng, without or after TLC on silica gel (Whatman). (●) CPBA, (○) TLC + CPBA in the presence of silica gel.

Standard curves of 25,26-$(OH)_2$-D_3 constructed from the results of CPBA of 25,26-$(OH)_2$-D_3 (without TLC) and the same CPBA (after TLC) in the presence of silica gel resulted in practically the same values. This means that the precision of the above procedure does not decrease the usefulness of TLC; it is the same as for CPBA itself.

Recoveries of added standards of 25-OH-D_3, 24,25-$(OH)_2$-D_3 and 25,26-$(OH)_2$-D_3 to thin layers of silica gel (Merck) after TLC and following CPBA, summarized in Table II, confirm that the presence of silica gel did not interfere with the CPBA of the above metabolites.

Results of the analysis of the metabolites of vitamin D in 1 ml of plasma after TLC and following CPBA are listed in Table III. The accuracy of the procedure was calculated from the values of recoveries of the added amounts of 25-(OH)-D_3, 24,25-$(OH)_2$-D_3 and 25,26-$(OH)_2$-D_3 to the extract of the

TABLE III

ACCURACY OF TLC (SILICA GEL, MERCK) OF PLASMA SAMPLES EVALUATED BY RECOVERY OF ADDED 25-OH-D_3, 24,25-$(OH)_2$-D_3 AND 25,26-$(OH)_2$-D_3 TO PLASMA SAMPLES

Sample	Amount added (ng)			Amount recovered* (ng)					
	25-OH-D_3	24,25-$(OH)_2$-D_3	25,26-$(OH)_2$-D_3	25-OH-D_3	C.V.** (%)	24,25-$(OH)_2$-D_3	C.V. (%)	25,26-$(OH)_2$-D_3	C.V. (%)
Plasma A	0.00	0.00	0.00	3.28 ± 0.18	5.49	0.65 ± 0.04	6.91	0.28 ± 0.01	5.71
Plasma A′	0.00	0.40	0.40	3.28 ± 0.18	5.49	1.03 ± 0.07	7.20	0.66 ± 0.03	5.62
Plasma B	0.00	0.00	0.00	3.82 ± 0.29	7.61	1.02 ± 0.06	6.13	0.21 ± 0.01	6.66
Plasma B′	0.00	1.00	0.30	3.82 ± 0.29	7.61	2.00 ± 0.11	5.62	0.52 ± 0.03	6.42
Plasma C***	0.00	0.00	0.00	2.41 ± 0.09	3.72	0.44 ± 0.04	9.10	0.02 ± 0.00	4.00
Plasma C′	0.60	0.60	0.60	2.95 ± 0.18	6.09	1.13 ± 0.09	7.98	0.70 ± 0.04	5.78

*Mean ±S.D. ($n = 5$).
**Coefficient of variation.
***Plasma C was analysed using TLC on silica gel from Whatman.

plasma sample prior to TLC. Added amounts correspond to those of naturally occurring endogenous vitamin D metabolites in plasma. The criteria (accuracy and precision) showed that TLC can be used for reliable quantitative separation and further determination of metabolites of vitamin D occurring in plasma in nanogram quantities. There are no differences in quantitative parameters using either silica-gel plates from Merck or from Whatman.

TLC on silica gel provides a convenient and fast procedure for the quantitative separation of metabolites of vitamin D in plasma available for routine analysis. The major advantage of using silica-gel-coated HPTLC plates in comparison to the currently used column chromatography on Sephadex LH-20, even when silica Sep-Pak cartridges are used for prepurification [8, 9], is not only saving of time in column packing but complete quantitative separation of all biologically active metabolites of vitamin D including 1,25-$(OH)_2$-D_3 and 25,26-$(OH)_2$-D_3. Both techniques are comparable in cost, convenience, reproducibility and sample recovery. As can be seen from our results, TLC is highly effective for the quantitative separation of nanogram quantities of biologically active metabolites of vitamin D in plasma capable of replacing column chromatography on Sephadex LH-20 with subsequent high-performance liquid chromatography.

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CHROMBIO. 1263

THE EFFECT OF AMPHOLYTES ON FERRITIN ISOELECTRIC FOCUSSING PATTERNS

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(First received October 5th, 1981; revised manuscript received February 19th, 1982)

SUMMARY

Ferritin was subjected to isoelectric focussing (IEF) on agarose gels containing different commercial carrier ampholytes. In two gels protein staining revealed banded patterns which differed from one another, while a third gel yielded zones rather than discrete bands, indicating that the bands may be artefacts.

The differences between banded patterns were studied by isolating bands from an IEF gel and refocussing these on gels containing either the original ampholyte or a different ampholyte preparation. Striking differences were noted.

Chromatofocussing of ferritin resulted in the elution of broad peaks between the same pH limits as indicated by IEF patterns.

INTRODUCTION

The microheterogeneity of ferritin was first demonstrated [1] by isoelectric focussing (IEF) more than a decade ago and this technique has subsequently been widely used for the characterisation of ferritin from various tissues. The multiple bands (isoferritins) in these IEF patterns differ according to the tissue from which the ferritin was isolated [2]. These differences may be explained by a model [3] in which the 24-subunit protein shell of ferritin contains different proportions of two distinct types of subunit, designated H and L, following the general formula L_nH_{24-n}, where n may assume values between 0 and 24.

While the validity of the IEF patterns of ferritin has been challenged [4] experimental evidence that the multiple bands may be artefacts has only recently been published [5]. This evidence is based on the appearance of a striated pattern which appeared with a modified iron stain on an acrylamide gel in which IEF had been carried out. The striations were independent of the presence of protein. When ferritin was present a high degree of correspondence

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was noted between protein bands staining for iron and dark striations in the gel.

In this paper we present evidence which shows that the IEF pattern of ferritin varies with the ampholyte used and thus represents a technique artefact. This is supported by the chromatographic characterisation of ferritin using chromatofocussing, a recently introduced chromatographic technique.

MATERIALS AND METHODS

Ferritin was isolated from human heart and liver, obtained post mortem, as previously described [6]. Samples of ferritin (10—15 μg) were subjected to isoelectric focussing in thin layers (80 mm × 115 mm × 1 mm) of 1% agarose gel (Pharmacia, Uppsala, Sweden, Agarose IEF) containing 12% sorbitol (Merck, Darmstadt, G.F.R., extra pure for microbiology) and 2.5% ampholytes of one of the following types: Ampholine pH 4—6 (LKB, Stockholm, Sweden, Batch No 44); Pharmalyte pH 4—6.5 (Pharmacia, Lot EL 13994); Bio-Lyte pH 4—6 (Bio-Rad Labs., Richmond, CA, U.S.A., Lot No. 21211). Calibration proteins (Pharmacia, low p*I* calibration kit) were run on each gel in addition to the ferritin samples.

Focussing was carried out on an LKB Multiphor 2113 apparatus and constant power (6 W per gel) was supplied by an LKB 2103 power supply. Coolant (10°C) was circulated through the Multiphor apparatus by a Lauda refrigerator/pump. The minimum duration of each run was 2 h during which time the voltage had risen to an essentially constant value. In one trial samples were applied at both the anodal and cathodal ends of a gel containing Pharmalyte carrier ampholytes, and in a further trial the concentration of these ampholytes was increased to a level of 5%. At the end of each run the gels were fixed (10% trichloroacetic acid, 5% sulphosalicylic acid), washed and stained with Coomassie blue R250.

Refocussing of heart ferritin

Semi-preparative scale IEF of heart ferritin was carried out on a slab of agarose containing Biolyte as described above. At the end of the focussing run, portions of the gel containing prominent bands were excised and the ferritin contained in those gel slices was concentrated and freed of ampholyte in an electrophoretic concentrating device [7]. The ferritin fractions thus isolated were refocussed on gels containing Ampholine and Bio-Lyte, respectively. The gels were fixed and stained as before and scanned at 570 nm in a Varian 635 spectrophotometer.

Chromatofocussing

Liver and heart ferritin were chromatofocussed on PBE 94 exchanger with Polybuffer 74 (Pharmacia) according to the methods prescribed by the manufacturers. A glass chromatography column (20 × 0.9 cm) was packed with PBE 94 exchanger and equilibrated with 0.025 *M* histidine·HCl buffer, pH 6.2. Liver or heart ferritin which had been dialysed against this buffer was applied to the column and eluted with Polybuffer 74, diluted with boiled distilled water, and adjusted to pH 4 with hydrochloric acid. The final dilu-

tion was 1:8. All buffers were degassed before use. The column effluent was monitored at 280 nm and the pH of each 3-ml fraction was measured.

RESULTS

Analytical IEF

In each of the three gels, as shown in Fig. 1, the calibration proteins (in order from the cathode: bovine carbonic anhydrase A, p*I* 5.85; β-lactoglobulin A, p*I* 5.20; soybean trypsin inhibitor, p*I* 4.55) focussed into sharp bands. In the gels containing LKB Ampholine (Fig. 1a) and Bio-Lyte (Fig. 1c), respectively, heart ferritin focussed into discrete bands between pH 4.6 and 5.6, while liver ferritin focussed in the range pH 5.0—5.6. The focussing patterns were however different. By contrast, no discrete bands could be seen for either heart or liver ferritin in the gel containing Pharmalyte although the marker proteins focussed at the correct p*I* values (Fig. 1b). However, the pH limits of the zones formed by heart and liver ferritins were similar to the pH limits found for these ferritins on gels with either LKB Ampholine or Bio-Lyte.

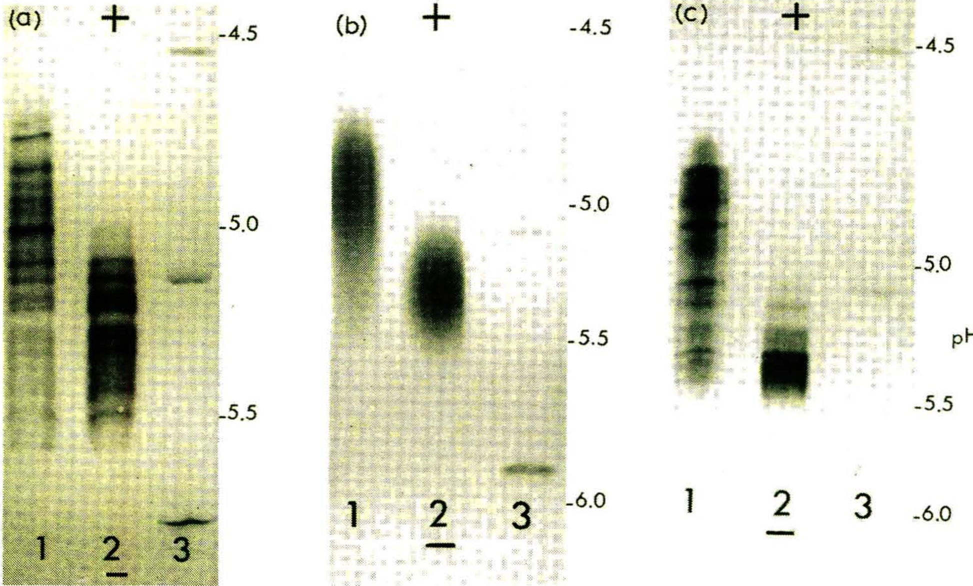

Fig. 1. IEF patterns in gels containing (a) LKB Ampholine ampholyte; (b) Pharmalyte ampholyte; (c) Bio-Lyte ampholyte. Channels 1 were loaded with heart ferritin, channels 2 with liver ferritin and channels 3 with calibration proteins (see text).

Fig. 2 demonstrates that when ferritin samples are applied at both anodal and cathodal ends of a Pharmalyte-containing gel and focussed as described above, the equilibrium position of either liver or heart ferritin is independent of point of application, indicating that equilibrium is attained under the focussing conditions described and that ferritin is not restricted by the gel matrix.

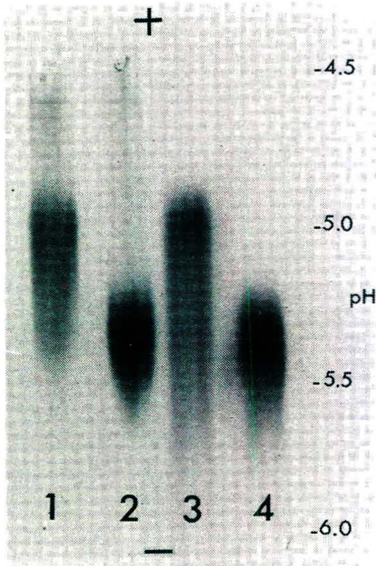

Fig. 2. Demonstration of focussing equilibrium. Samples of heart and liver ferritin were applied to both anodal and cathodal ends of a gel containing Pharmalyte and focussed as described in the text.

A trial in which the concentration of ampholyte was increased to 5% did not influence the ferritin pattern and the differences in focussing behaviour of ferritin with the different ampholyte preparations were reproducible when an alternative agarose of electrofocussing grade was used (Isogel, Marine Colloids Division of F.M.C., Marcus Hooke, PA, U.S.A.) and when running conditions were varied with regard to duration of run, and power applied. In these runs the calibration proteins invariably focussed as sharp bands at the correct p*I* values. Similar behaviour of ferritin isolated from spleen and kidney was observed when focussed with the various ampholyte preparations.

Refocussing of heart ferritin

When fractions of heart ferritin, obtained by semi-preparative scale IEF on agarose gels containing Bio-Lyte, were refocussed on gels containing either Bio-Lyte or Ampholine striking differences could be seen. While it was not possible to isolate a single band in the initial focussing step, it will be seen that in general a single major band with minor contaminants was isolated as revealed by refocussing on Bio-Lyte agarose gels. However, the same fraction on Ampholine-containing gels could show either greater complexity (Fig. 3a and b) or a reduced number of bands (Fig. 3c, d).

Chromatofocussing

Liver ferritin was resolved into two poorly differentiated peaks by this technique (Fig. 4a) while no distinct peaks are to be seen in the elution pattern of heart ferritin (Fig. 4b). However, it will be seen that both proteins were eluted over a wide pH range which corresponds to the characteristic range of p*I* values of the two ferritin types.

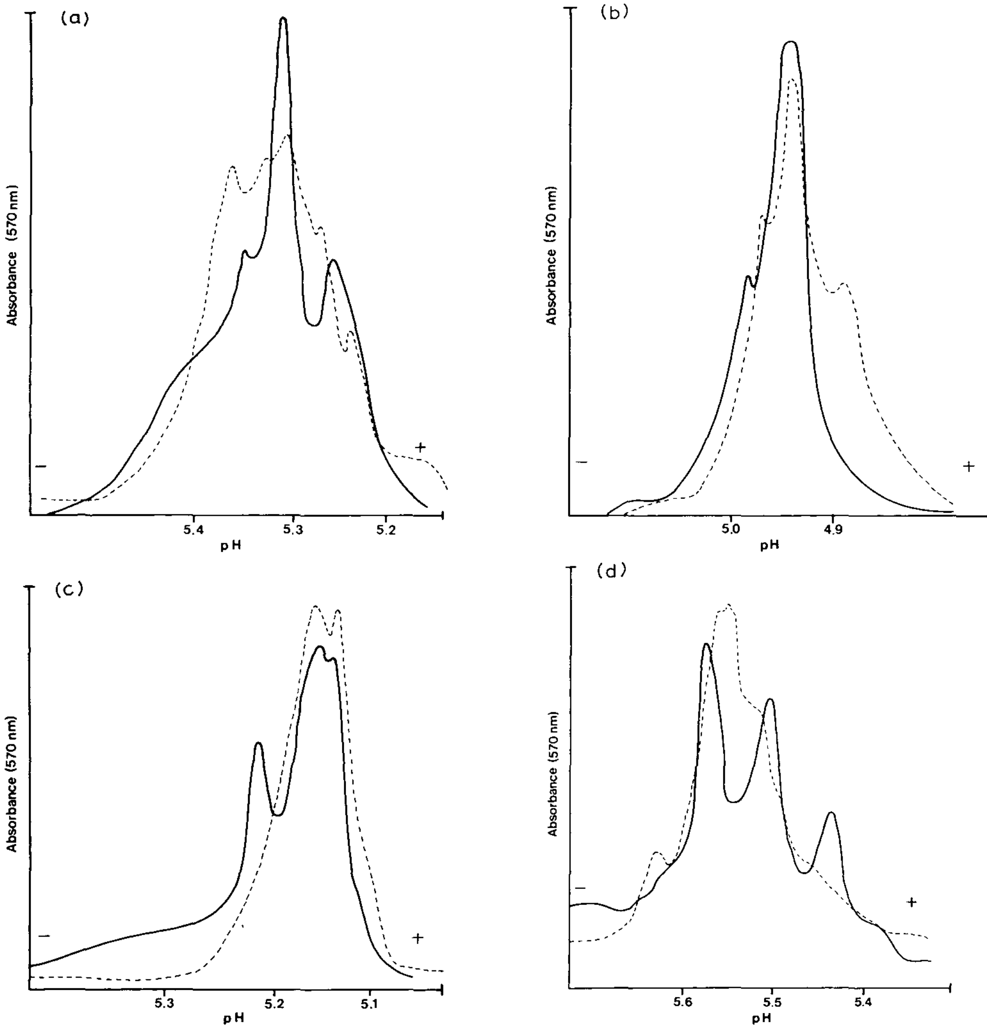

Fig. 3. Densitometric scans of IEF patterns of heart ferritin fractions obtained from a Bio-Lyte IEF separation refocussed on gels containing either Bio-Lyte (———) or Ampholine (- - - -). Scans are superimposed for comparison. (a) and (b) represent scans in which Ampholine gave a greater number of bands than Bio-Lyte; (c) and (d) represent less complex patterns than Bio-Lyte.

DISCUSSION

The evidence presented here clearly demonstrates that the IEF profile of ferritin is a product of the ampholyte preparation. Whereas the patterns seen with LKB Ampholine resemble those previously reported [2], the Pharmalyte ampholytes gave a picture which shows no discrete banding at all, merely zones or areas of localisation between pH limits which are common to all three ampholytes. The fact that marker proteins focussed as discrete sharp bands, and the demonstration that focussing equilibrium was achieved and that the movement of ferritin was not impeded by the agarose gel used, counter

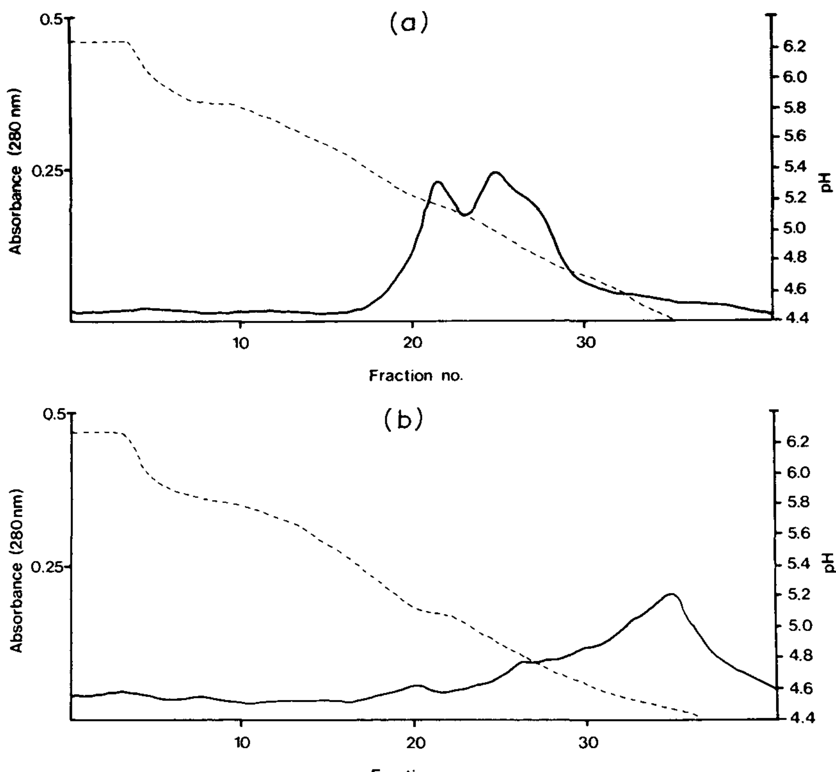

Fig. 4. Elution patterns obtained on chromatofocussing (a) liver ferritin and (b) heart ferritin. ——, Absorbance at 280 nm; — — —, pH.

the interpretation of these zones as technique failure. Ampholytes also apparently differ by batch preparation: with the batch preparation of Pharmalyte used in this study no discrete bands were formed, while in the Pharmalyte preparation used by other workers [5] focussing into discrete bands was observed.

The fact that distinctly different patterns resulted from refocussing ferritin fractions on an alternative ampholyte is strong support for the view that the discrete bands are artefacts rather than the characterisations of distinct biological entities.

The reason for the variation in ferritin IEF profiles with different ampholyte preparations is unknown. Although it has been suggested that micro-steps in the pH gradient are responsible [5], the present study goes no further than demonstrating that marker proteins of known p*I* value invariably focussed appropriately, irrespective of the ampholyte used. It follows from these observations that the IEF profile of ferritins has only relative relevance inasmuch as it should be related to a specific preparation of ampholytes.

While IEF depends on the establishment of a pH gradient by migration of amphoteric substances in an electric field, the recently introduced technique of chromatofocussing achieves the same effect independently of an electrical field. The elution pattern of liver and heart ferritin with this technique con-

firms that ferritin exhibits heterogeneity with regard to isoelectric point, but the lack of distinct peaks again suggests that the discrete bands seen in some IEF patterns are artefacts.

That ferritin exhibits heterogeneity with regard to p*I* is not in dispute. On IEF the ferritins localised between the pH limits characteristic for their tissues of origin, and eluted on chromatofocussing between approximately the same pH limits. For a given ampholyte the IEF patterns were consistently reproducible. Thus the heterogeneity of ferritin is confirmed, and may, perhaps, be greater than formerly thought. We therefore believe that IEF remains a useful technique for the characterisation of ferritin, provided it is used on a comparative basis and the interpretation of patterns is strictly qualified in terms of ampholyte used. Where possible, supporting physical characterisation by other methods is clearly indicated.

ACKNOWLEDGEMENT

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CHROMBIO. 1253

UNEXPECTED METABOLITES PRODUCED FROM CLOMETHIAZOLE*

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SUMMARY

The heterocyclic drug clomethiazole is metabolized by body passage partly to small aliphatic molecules. The occurrence of such unexpected metabolites is usually overlooked. An appropriate method for their detection is the comparison of urine profiles during drug intake and after withdrawal of the drug.

INTRODUCTION

We have compared urine profiles of healthy individuals and patients suffering from chronic diseases [1—3] to establish whether or not there are any metabolic changes. In the course of these studies we obtained a urinary acid profile of a patient, hospitalized for misuse of alcohol (Fig. 1). This profile showed some peaks (Nos. 2, 6, 8, 9, 12, 23), not present in the urine of healthy individuals. A gas chromatographic—mass spectrometric (GC—MS) run revealed one of the unknown compounds (No. 8) to be 4-methylthiazole-5-acetic acid methyl ester (1a). The corresponding acid (1b) is a known metabolite of clomethiazole (2) [5-(2-chloroethyl)-4-methylthiazole; trademarks are Distraneurin® and Hemineurin®] [4].

1a: R = CH_3
1b: R = H

2

Inquiry in the clinical department where the patient was hospitalized assured us that the individual was treated with clomethiazole to repress withdrawal symptoms. So we suspected that the other unknown peaks in the GC run might

*Dedicated to Professor H. Schildknecht on the occasion of his 60th birthday.

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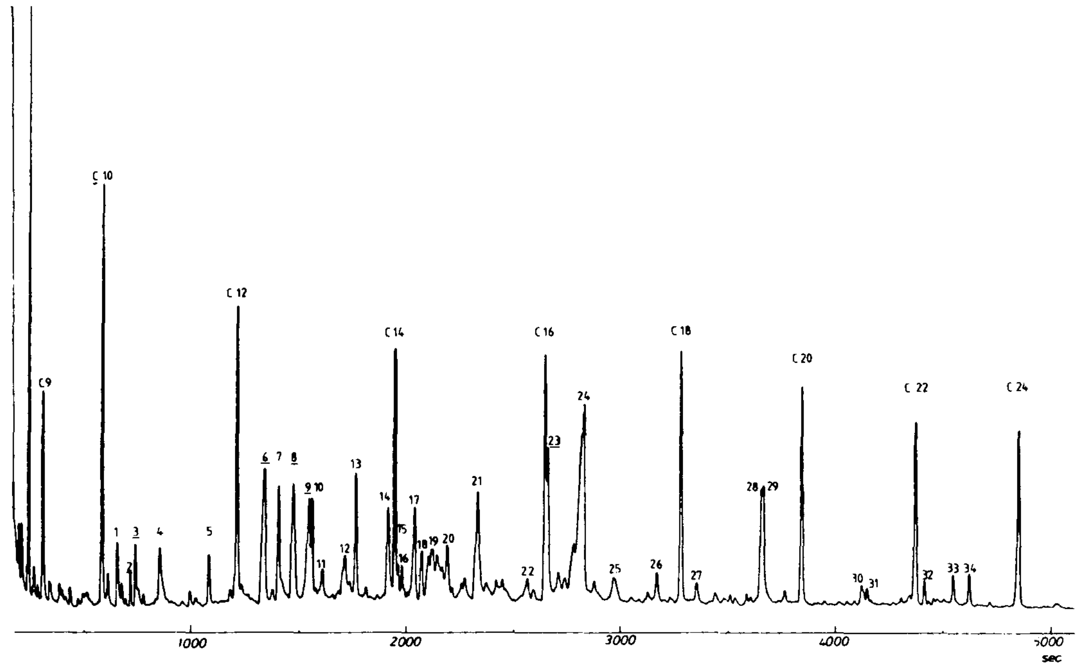

Fig. 1. Profile of the acid fraction obtained from the urine of a patient during treatment with clomethiazole. Peaks: 1 = succinic acid dimethyl ester; 2 = unknown metabolite of clomethiazole (mol.wt. 130); 3 = 3-methyl-mercapto-2,4-pentadione; 4 = unknown; 5 = methylglutaconic acid methyl ester; 6 = thiodiacetic acid dimethyl ester; 7 = 3-methyladipic acid dimethyl ester; 8 = 4-methylthiazole-5-acetic acid methyl ester; 9 = unknown metabolite of clomethiazole; 10 = unknown; 11 = pimelic acid dimethyl ester; 12 = 4-carboxymethoxy-5-vinylthiazole [6]; 13 = phenylbutyric acid methyl ester (internal standard); 14 = 4-methoxyphenylacetic acid methyl ester; 15 = unknown; 16 = unknown dicarboxylic acid dimethyl ester; 17 = decanoic acid dimethyl ester (branched); 18 = decanoic acid dimethyl ester (branched); 19 = unknown; 20 = decanoic acid dimethyl ester (branched); 21 = homovanillic acid methyl ester; 22 = 3,4-dimethoxyphenylacetic acid methyl ester; 23 = unknown metabolite of clomethiazole (mol. wt. 213); 24 = hippuric acid methyl ester; 25 = unknown (mixture); 26 = indoleacetic acid methyl ester; 27 = unknown (mol.wt. 240); 28 = pentylurofuranic acid dimethyl ester; 29 = 4-methoxyhippuric acid methyl ester; 30 = stearic acid methyl ester; 31 = unknown urofuranic acid dimethyl ester; 32 = pimaric acid methyl ester; 33 = isopimaric acid methyl ester; 34 = dehydroabietic acid methyl ester.

also be metabolites of clomethiazole. To verify this hypothesis, another urine sample of the same patient was investigated several days after the withdrawal of the drug. The GC run (Fig. 2) did not show peaks 2, 6, 8, 9, 12 and 23. Some other peaks which had disappeared (32, 33, 34) originated from an impurity present in the Extrelut® columns used [5]; some other peaks (e.g. 16, 21) which are rather small in the GC run reproduced in Fig. 2 compared to Fig. 1, were identified by MS to be common metabolites produced in very different proportions depending on nutritional conditons. Similar investigations carried out with other patients during and after intake of clomethiazole confirmed the differences in the GC runs.

Clomethiazole is used in high doses (2—6 g/day) for the treatment of

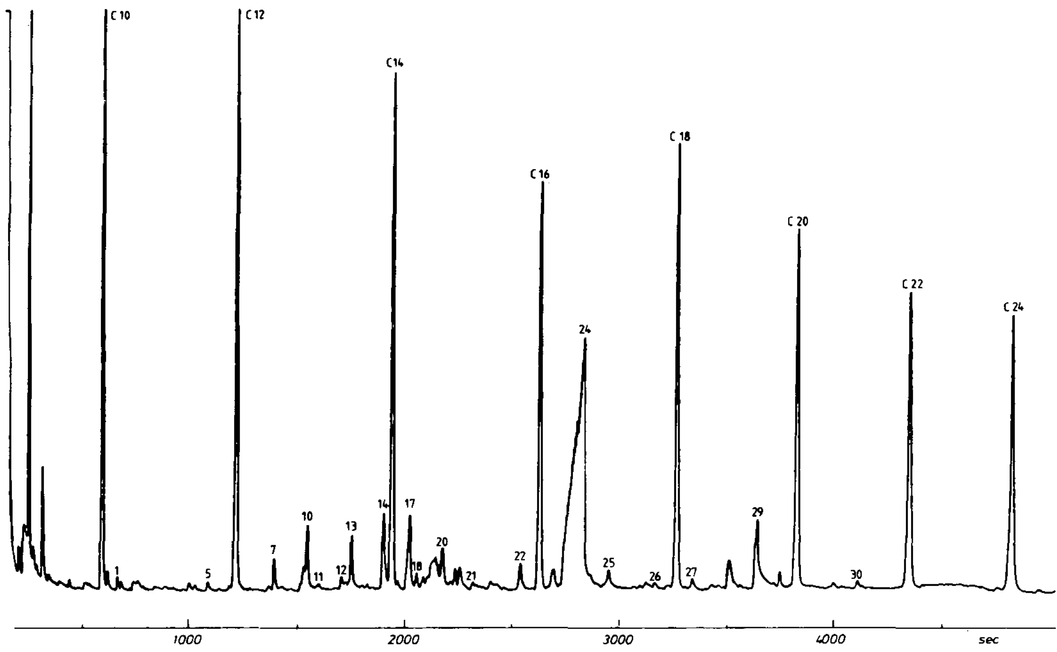

Fig. 2. Profile of the same urine fraction of the same individual as in Fig. 1, three days after treatment with clomethiazole was stopped.

delirium tremens and general withdrawal symptoms [7]. A great number of its metabolites have been detected. They all are oxidation products of the side-chain carbon atoms in position 3 and 4 [8—11]. Nevertheless, and in spite of radioactive labelling at C-2, only a small percentage of (2) was recovered in the form of metabolites. The fate of most of the drug after body passage remains unknown.

Thus the unexpected peaks offered the possibility of obtaining an insight into the still obscure pathway of metabolism.

This paper deals with the structure elucidation of some of the unknown metabolites indicated in the GC runs. It deals further with some conclusions that must be drawn from this investigation.

EXPERIMENTAL

Instruments

The GC apparatus and conditions were as follows: Carlo-Erba gas chromatograph 2900; hydrogen flow-rate: 2 ml/min; 25-m thin-film glass capillary coated with OV-101; injector, 275°C; oven, 80—300°C; temperature program, 2°C/min; flame ionization detector.

For MS measurements an LKB 2091 mass spectrometer was used with electron-impact (EI) ion source, an electron energy of 70 eV, and registration of the total ion current signal at 20 eV. The mass spectrometer was combined with a Pye-Unicam gas chromatograph, a 25-m thin-film glass capillary coated

with OV-101; temperature program as above; helium flow-rate, 2 ml/min. The instrument was combined with an LKB 2130 data system, using a PDP 11 computer.

High-resolution data were obtained with a Varian 312 mass spectrometer with a chemical ionization (CI)/EI ion source, combined with a Varian Model 3700 gas chromatograph with open split; helium flow-rate, 2 ml/min, 25-m thin-film glass capillary column coated with OV-101, conditions as above. Data system: MAT 200, PDP 11/34.

^{1}H-NMR measurements were carried out with a Bruker WM 250 instrument.

Work-up procedure for profiling

To 20 ml of urine 1 ml of standard solution containing 0.2 mg/ml phenylbutyric acid in water were added and then 2 N H_2SO_4 was added drop by drop until pH 1 was reached. This solution was made up with water to 22 ml. This sample was poured on an Extrelut® column (E. Merck, Darmstadt, G.F.R.) and eluted with 60 ml of ethyl acetate.

The extract was evaporated nearly to dryness in vacuo, 1 ml of methanol was added followed by ethereal diazomethane solution until the yellow colour remained. After 5 min the excess diazomethane was evaporated by a nitrogen stream. The solution was evaporated to 0.5 ml; 0.2—1.0 μl of this solution was injected into the gas chromatograph.

Work-up procedure for the separation of clomethiazole metabolites

A 150-ml urine sample from a patient treated with 2 g clomethiazole per day (after the second day of intake) was acidified to pH 1 by the addition of concentrated sulphuric acid (diluted 1:1). The acidified urine was extracted three times with 100 ml of ethyl acetate. The organic extract was dried over sodium sulphate and evaporated to dryness in vacuo. The residue (1.2 g) was dissolved in 1 ml of methanol. Ethereal diazomethane solution was added until the solution remained yellow. The excess diazomethane was evaporated by a nitrogen stream.

The residue was separated on home-made thin-layer chromatography (TLC) plates 20 × 20 cm (1 mm silica-gel layer) using a mobile phase of diethyl ether—cyclohexane (5:3). The zone with an R_F value of 0.42—0.48 was scratched off and eluted with ether. After evaporation to dryness, 14.5 mg of a viscous fluid remained. A glass capillary gas chromatogram revealed that this fraction consisted of 56% of this thiodiacetic acid dimethyl ester (3a).

Semiquantitative measurements

Urine specimens (24 h) of patients treated with known amounts of clomethiazole were collected. After the addition of 2 ml of the standard solution to 20 ml of the urine the sample was worked-up as described above.

Peak areas were measured with an integrator (Spectra-Physics Autolab System I). The amounts of the acetic acid dimethyl ester were put in relation to the standard compound. Recovery of the standard was estimated to be about 100%.

In Table I data of thiodiacetic acid dimethyl ester obtained from two

TABLE I

EXCRETION OF THIODIACETIC ACID DIMETHYL ESTER

	Interval (h)	Amount of drug consumption (g)	Amount of thiodiacetic dimethyl ester (mg)
Patient 1	48	11	462
	24	4	142
	24	2	80
	24	2	68
	24	0	16
Patient 2	48	8	44
	24	4	49
	24	2	29
	24	1.5	34

patients are recorded to show typical examples and the differences in excretion rates.

Sources of reference compounds

(1) Thiodiacetic acid (3b) and mercaptoacetic acid (4b) were obtained from EGA-Chemie, Steinheim, G.F.R.

(2) S-(2-Oxopropyl)-mercaptoacetic acid (5a). One gram (0.01 mol) of mercaptoacetic acid (4b) was dissolved in a saturated $NaHCO_3$ solution; under vigorous stirring 2 g of chloroacetone were added. After 15 min the mixture was filtered and the filtrate washed three times with diethyl ether. The aqueous solution was brought to pH 1 by adding concentrated hydrochloric acid and extracted three times with ether. The ethereal solution was dried with sodium sulphate and evaporated. A viscous resin remained (0.69 g). Distillation (0.2 Torr/150°C) gave a colourless waxy liquid. ^{1}H-NMR (C^2HCl_3): δ = 2.31 ppm (s, 3H, $-C\underline{H}_3$); 3.31 (s, 2H, $-S-C\underline{H}_2-COOH$); 3.5 (s, 2H, $-CO-C\underline{H}_2-S-$); 10.25 (s, 1H, $-O\underline{H}$).

(3) 3-Methylmercapto-2,4-pentadione (6a). (A) 1-Bromo-2,4-pentadione (7). Ten grams (0.1 mole) of freshly distilled acetylacetone were dissolved in dry carbon tetrachloride (dried over P_4O_{10}); 17.8 g (0.01 mole) of N-bromosuccinimide and 200 mg of azobisisobutyronitrile were added and heated under reflux for 2 h. After cooling the precipitated succinimide was filtered off and the remaining solution was concentrated in vacuo. GC—MS analysis showed that 1-bromo-2,4-pentadione (7) was the main product. (B) 3-Methylmercapto-2,4-pentadione (6a). The rough carbon tetrachloride solution of the above reaction was cooled to −10°C. Gaseous mercaptomethane (from S-methylthiourea and potassium hydroxide) was introduced and condensed by cooling. After 10 min at room temperature the solution was washed three times with $NaHCO_3$ solution, dried and evaporated. The mixture was purified by preparative gas chromatography which rendered enough sample to determine an NMR spectrum. ^{1}H-NMR (C^2HCl_3): δ = 2.13 ppm (s, 3H, $-SC\underline{H}_3$); 2.44 (s, 6H, $-COC\underline{H}_3$); 16.97 (s, 1H, $-O\underline{H}$). ^{13}C-NMR (C^2HCl_3): δ = 20.0 ppm (q, $-S-\underline{C}H_3$); 24.3 (q, $-CO-\underline{C}H_3$); 116.4 (s, $-\underline{C}=$); 197.2 (s, $-\underline{C}O$) ppm.

RESULTS

The structures of the unknown compounds represented by peaks 3 and 6 in the glass capillary chromatogram represented in Fig. 1 were clarified by interpretation of the spectra, synthesis of comparison compounds and determination of their identity with the compounds found in the GC run 1.

The mass spectrum of peak 6 (Fig. 3) showed a molecular ion of mass 178. Peaks at M−32, M−59, M−60 and at mass 59 indicated the presence of at least one carbomethoxy group. The isotope ratio of these peaks suggested the presence of sulphur. This assumption was confirmed by a high-resolution measurement which showed the molecular formula to be $C_6H_{10}O_4S$.

Since it was not possible to deduce further structural information from the spectrum, we tried to accumulate sufficient pure material for an NMR investigation. For this purpose the whole acid fraction was methylated and each zone subjected to GC analysis. The zone containing the compound of mol. wt. 178 was eluted. The GC run of this zone showed an enrichment of 56%, sufficient for NMR analysis. The NMR spectrum was very simple. It showed — besides the small peaks of impurities — only two signals in the ratio 2:3 at δ = 3.40 and δ = 3.78 ppm.

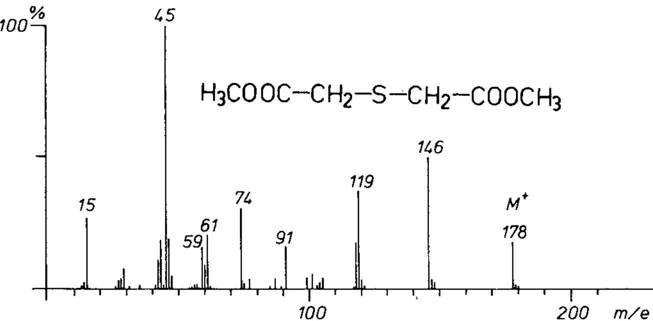

Fig. 3. Mass spectrum of thiodiacetic acid dimethyl ester (3a) (synthetic sample).

Since the compound contained ten hydrogen atoms, the presence of two CH_3– and two CH_2— groups each in a similar surrounding was established. The two methyl groups could be only carbomethoxy groups according to the MS fragmentation pattern. The remaining two CH_2— groups and the sulphur atom consequently must have been present as a CH_2—S—CH_2 group. Therefore the structure of the molecule was derived to be 3a.

ROOC–CH_2–S–CH_2–COOR

3a: R = CH_3

3b: R = H

The mass spectrum (Fig. 4) of peak 3 in Fig. 1 corresponded to a compound of mol. wt. 146. By high-resolution measurement the molecular formula was

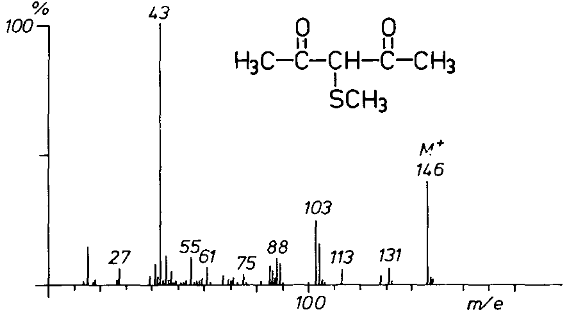

Fig. 4. Mass spectrum of 3-methylmercapto-2,4-pentadione (6a) (synthetic sample).

determined to be $C_6H_{10}O_2S$. The ion of mass 103 (C_4H_7OS) corresponded to the loss of CH_3CO. The presence of such a group was further indicated by the base peak of mass 43. Since the molecule contained only one more double bond equivalent and the spectrum showed no indication of the presence of an OH— or an SH— group (peaks at M−18 and M−34 were missing), the presence of a further CO- -CH_3 was assumed, rendering compound 6a as the most probable structure for the molecule.

$$H_3C-\underset{\underset{O}{\|}}{C}-\overset{\overset{SR}{|}}{CH}-\underset{\underset{O}{\|}}{C}-CH_3$$

6a: $R = CH_3$
6b: $R = H$

Compound 6a was synthesized by bromation of acetylacetone to compound 7 and reaction with $HSCH_3$ to compound 6a.

$$CH_3-\underset{\underset{O}{\|}}{C}-CH_2-\underset{\underset{O}{\|}}{C}-CH_3 \xrightarrow{NBS} \underset{7}{CH_3-\underset{\underset{O}{\|}}{C}-CH=\underset{\underset{OH}{|}}{C}-CH_2-Br} \xrightarrow{HSCH_3} CH_3-\underset{\underset{O}{\|}}{C}-\overset{\overset{SCH_3}{|}}{C}=\underset{\underset{OH}{|}}{C}-CH_3 \rightleftharpoons \underset{6a}{CH_3-\underset{\underset{O}{\|}}{C}-\overset{\overset{SCH_3}{|}}{CH}-\underset{\underset{O}{\|}}{C}-CH_3}$$

Thus in the sample compound 6b was originally present and is methylated by treatment with diazomethane to compound 6a. It proved to be identical in all respects to the metabolite.

It should be mentioned that trace amounts of two other sulphur-containing compounds were detected by a careful inspection of all mass spectra of the dif-

ferent TLC fractions. One metabolite was identified, using the eight-peak index [12], as methylmercaptoacetic acid methyl ester (4a); the other was found to be S-(2-oxopropyl)-mercaptoacetic acid methyl ester (5a) with the aid of reference spectra indicating that the metabolites originally present must have structures 4b and 5b.

$RS-CH_2-COOR$ $CH_3-C(=O)-CH_2-S-CH_2-COOR$

4a: $R = CH_3$ 5a: $R = CH_3$

4b: $R = H$ 5b: $R = H$

DISCUSSION

Thiodiacetic acid (3b) was found to be a metabolite of vinyl chloride [13]. In addition, some drugs containing a $N-CH_2-CH_2-Cl$ chain (e.g. ifosfamide, manomustine) were found to be also metabolized to thiodiacetic acid [14]. It is assumed that the chlorine atom is substituted in a nucleophilic reaction by the sulphur atom of cysteine, and then further degraded by desamination, decarboxylation and oxidation to the final metabolite, thiodiacetic acid (3b).

Since clomethiazole (2) contains a CH_2-CH_2-Cl side-chain, degradation in a similar way by loss of the heterocyclic ring can be envisaged. This in turn leads to the assumption of a primary or secondary attack on the heterocyclic ring. This hypothesis is strengthened by the occurrence of metabolite 6b which is probably produced from the already known metabolite 5-acetyl-4-methyl-thiazole (8). We suppose that metabolite 8 is alkylated at the nitrogen atom to quaternary compound 9.

$CH_3-C(=O)-CH_2-C(=O)-CH_2-Br$ 7

[Structures 8, 9, 10, 11]

Such compounds suffer cleavage — a well-known reaction of vitamin B_1 [15]. In our case we assume formyl derivative 10 to be formed, which could be degraded further by hydrolysis to the imine 11 and to 3-mercapto-2,4-pentadione (6b).

The SH— group is methylated by treatment with diazomethane to form compound 6a. Thus the presence of compound 6b is a strong hint for the already mentioned postulate that clomethiazole is metabolized not only by side-chain oxidation but also by cleavage of the heterocyclic ring.

In addition, Dr. Pal in our laboratory detected recently several metabolites of clomethiazole oxidized at C-2 [6], obviously missing links in the formation of aliphatic degradation products.

The occurrence of compounds 4b and 5b not yet found in the urine of healthy people gives further hints for the total cleavage of the heterocyclic ring of clomethiazole in the body.

While the amount of compound 6a is rather low, thiodiacetic acid (3b) must be one of the major metabolites. A precise quantitative determination of compound 3b was not possible due to lack of an appropriate standard (HOOC—C^2H_2—S—C^2H_2—COOH easily exchanges the deuterium atoms against hydrogens and can not therefore be used as standard). Thus we were only able to estimate the daily excretion by use of an internal standard (phenylbutyric acid). It turned out that the amount of compound 3b produced from compound 2 is very much dependent on individual parameters (see Table I), but at least in one case intake of 11 g of clomethiazole within two days caused a production of about 460 mg of compound 3b. Assuming that the production of 1 mole of compound 3b requires 1 mole of cysteine (if the side-chain is attacked as in other compounds with a CH_2—CH_2—Cl side-chain), the metabolism of compound 2 to compound 3b needs at least the presence of 370 mg of cysteine. This amount of cysteine is lost with the urine. Since the daily excretion of cysteine in healthy adult individuals is about 6—66 mg/day [16], treatment with clomethiazole must cause a decrease of cysteine in the body. This phenomenon must be investigated in the future. It might well be connected with the side-effects observed by continuous treatment with clomethiazole.

The finding of aliphatic metabolites of clomethiazole seems to us important also in an other respect. Usually the metabolic pathway of a drug is evaluated by labelling experiments. The label is introduced into the drug molecule at a position where one can expect that it will not be lost by body passage, e.g. in an aromatic or a heterocyclic ring. The detection of aliphatic major products in the metabolism of clomethiazole demonstrates the danger of such labelling

experiments. For reasons of synthesis C-2 was labelled and this atom is lost — as we now know — preferentially by biochemical degradation. Thus the label is lost and consequently the degradation products escaped detection.

Profiling of all fractions (basic, neutral, acid) obtained by work-up of biological fluids after drug intake followed by MS identification of the same fractions after withdrawal of the drug should therefore be applied as an appropriate and supplementary method to radioactive labelling experiments.

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CHROMBIO. 1256

STUDIES OF ISONIAZID METABOLISM IN ISOLATED RAT HEPATOCYTES BY MASS FRAGMENTOGRAPHY

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SUMMARY

Isoniazid metabolism in isolated rat hepatocytes was studied by mass fragmentography using single ion monitoring. Isoniazid and its metabolites were determined as the trimethylsilylated derivatives of acetylisoniazid and diacetylhydrazine and of the benzaldehyde hydrazones of isoniazid and acetylhydrazine. Deuterated analogues served as internal standards. Hydrazine was quantitated as benzalazine using ^{15}N-labeled hydrazine as an internal standard. The method is well suited for the microanalysis of isoniazid metabolites in specificity and reliability to demonstrate the overall pathway of isoniazid metabolism, from which it was clarified that the greater part of hydrazine, a hazardous metabolite of isoniazid, was formed through the direct hydrolysis of isoniazid itself as expected.

INTRODUCTION

The metabolism of isoniazid (INH), a drug widely used in tuberculosis chemotherapy, has been extensively studied in human and experimental animals, as described in the previous paper [1]. Enzymatic acetylation, hydrolysis and conjugation result in the formation of such diverse metabolites as acetylisoniazid (AcINH), acetylhydrazines, pyruvic hydrazone, isonicotinic acid and isonicotinuric acid. A large portion of INH ingested is excreted into urine as metabolites. Assay techniques established by us, part of which has already been published [1—5], have facilitated the detection and accurate

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determination of such metabolites in biological fluids. Using the method, we detected constant urinary excretion of free hydrazine (Hz) by patients receiving INH, which drew much attention because of its possible hepatotoxicity and mutagenicity [1]. Although the concentration of the compound found in urine reflects the balance of production and elimination in tissues, the amount of Hz at the site of formation could be higher than that found in the urine. In fact, active formation of Hz, as well as AcINH, from INH in isolated rat hepatocytes has been noted [4]. In relation to this finding, we describe here our assay techniques in detail and the time—course of Hz formation from INH using the rapid and sensitive method.

EXPERIMENTAL

Chemicals

Collagenase (*Clostridium histolyticum*) was purchased from Boehringer Mannheim (Mannheim, G.F.R.). Bovine serum albumin (demineralized) was the product of Povite Producten, Amsterdam, The Netherlands. Amino acid mixture (without L-glutamine) was obtained from Grand Island Biological Co., Grand Island, NY, U.S.A. d_3-AcINH and d_3-diacetylhydrazine (d_3-DAcHz) were obtained by the acetylation of INH and acetylhydrazine (AcHz) with deuteroacetic anhydride-d_6 in deuteroacetic acid-d_4. d_3-AcHz hydrochloride was synthesized from *tert*.-butylcarbazate (Sigma, St. Louis, MO, U.S.A.) using a modified method of Nelson et al. [6]. All other chemicals were of reagent grade.

Gas chromatography—mass spectrometry

Gas chromatography—mass spectrometry (GC—MS) was carried out on a system comprising a Shimadzu GC-MS 7000, MID-PM. GC separation was performed by using a glass column (1 m × 3 mm I.D.) packed with 1.5% OV-17 on Shimalite W (80—100 mesh). Helium was used as a carrier gas (flow-rate, 30 ml/min). The analytical conditions are listed in Fig. 1.

Preparation and incubation of isolated rat hepatocytes

Isolated hepatocytes were prepared from male Wistar rats, 280—320 g, by the collagenase perfusion method as described by Moldéus et al. [7]. The viability of the cells was 98—99% according to the lactic dehydrogenase latency test and trypan blue exclusion [8]. The hepatocytes were suspended in Krebs-Hensleit buffer, pH 7.4, containing 1% bovine serum albumin, 10 m*M* glucose, amino acid mixture (Gibco), 13 m*M* Hepes (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid) and penicillin (400 IU/ml), and were incubated with a substrate in a rotating round-bottom flask at 37°C under a stream of oxygen—carbon dioxide (95:5). In order to obtain the apparent K_M and V_{max} values, $5 \cdot 10^{-5}$ to $1 \cdot 10^{-3}$ *M* substrates and $4 \cdot 10^6$ cells/ml of hepatocytes were employed. For constructing a time—course of INH metabolism, 0.5 m*M* INH and $7 \cdot 10^6$ to $8 \cdot 10^6$ hepatocytes/ml were used.

Sample preparation and extraction

After incubation for a certain time period, 4-ml aliquots of the mixture were transferred to a test tube with 4 ml of phosphate buffer solution, pH 6.0,

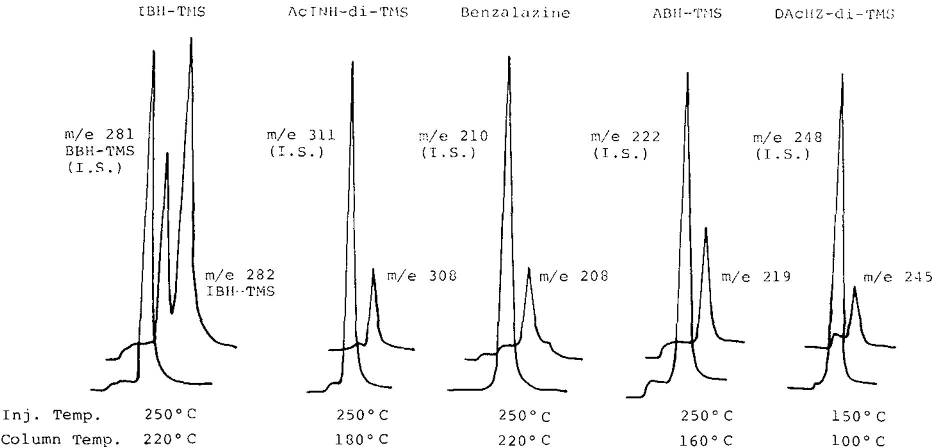

Fig. 1. Mass fragmentograms of derivatives of INH and its metabolites. Column: 1.5% OV-17 on Shimalite W (80—100 mesh), 1 m × 3 mm glass column. MS conditions: accelerating voltage, 3 kV; ionizing current, 60 μA; ionizing energy, initial 20 eV, jump 70 eV; separator temperature, 250°C.

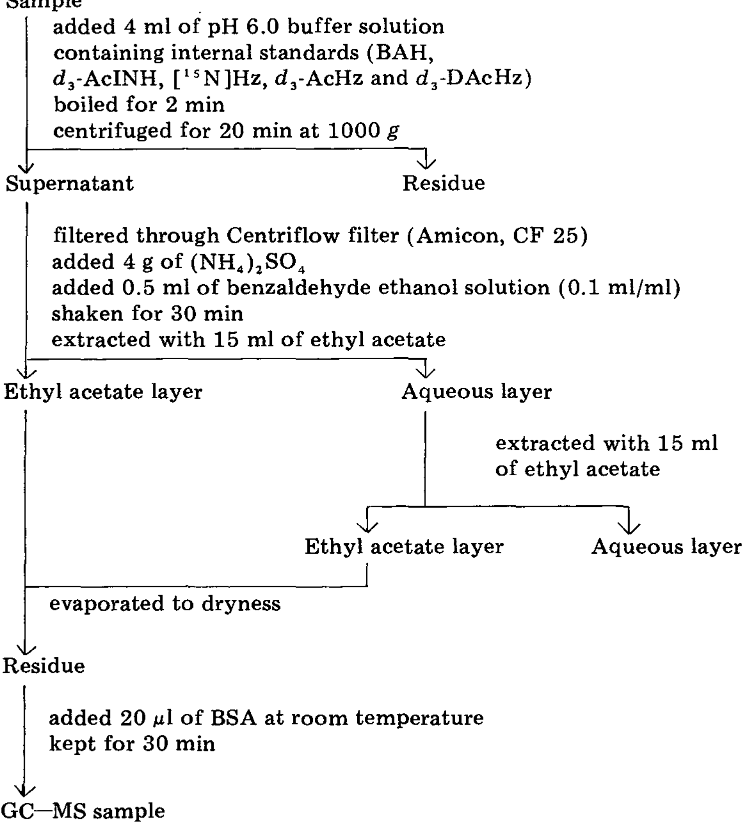

Fig. 2. Sample preparation for mass fragmentography of INH and its metabolites.

containing [^{15}N]Hz (2 μg/ml), d_3-AcINH (10 μg/ml), d_3-DAcHz (2 μg/ml), d_3-AcHz (1 μg/ml) and benzoic acid hydrazide (BAH, 50 μg/ml) as internal standards. The mixed solution was boiled for 2 min to terminate the reaction. After cooling on ice, the tubes were centrifuged for 20 min at 1000 *g* and the supernatants were filtered through Centriflow filters (Amicon, CF 25). The filtrates thus obtained were extracted with ethyl acetate as shown in Fig. 2.

Derivatization for GC—MS

As shown in Fig. 3, INH, BAH, AcHz, d_3-AcHz, Hz and [^{15}N]Hz were derivatized with benzaldehyde to give 1-isonicotinoyl-2-benzylidene-hydrazine (IBH), 1-benzoyl-2-benzylidene-hydrazine (BBH), 1-acetyl-2-benzylidene-hydrazine (ABH), d_3-ABH, benzalazine and [^{15}N]benzalazine, respectively. Further derivatization with N,O-bistrimethylsilylacetamide (BSA) was necessary for IBH, BBH, AcINH, d_3-AcINH, ABH, d_3-ABH, DAcHz and d_3-DAcHz prior to the GC injection to give the corresponding trimethylsilylates as shown in Fig. 4.

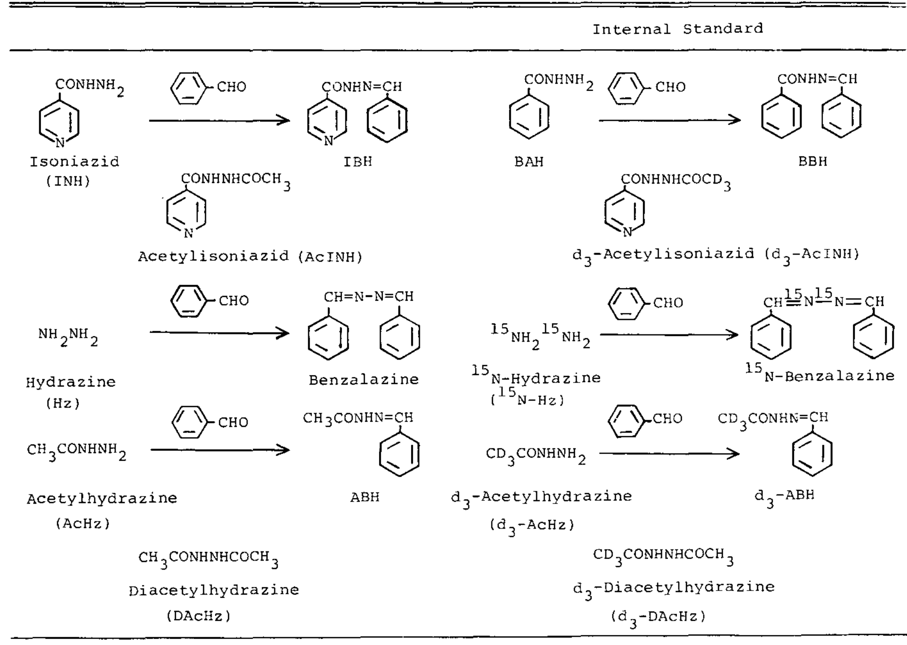

Fig. 3. Derivatization employed for GC—MS.

RESULTS AND DISCUSSION

Determination of INH and its metabolites by mass fragmentography

INH and its metabolites were determined by GC—MS. For mass fragmentography using single ion monitoring, the ions at *m/e* 282 (281), 308 (311), 208 (210), 219 (222) and 245 (248) were selected for INH, AcINH, Hz, AcHz and DAcHz, respectively, with the internal standard in parentheses.

Each metabolite was determined successfully as follows.

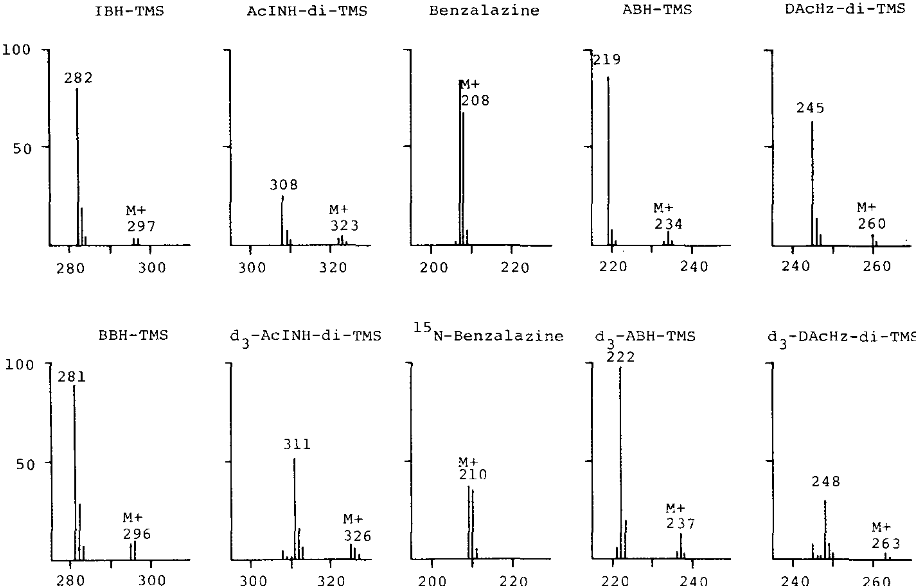

Fig. 4. Mass spectra of derivatives of INH and its metabolites.

(1) Intact INH. The present assay improved the detection limit by a factor of 10 compared to that in the previous method using GC [1].

(2) Hz. The same method that has already been reported was employed [1].

(3) AcINH and DAcHz. In comparison with the GC method used [1] the present assay in which acid hydrolysis was unnecessary prior to the extraction is very simple. Reliable data were obtained by using d_3-AcINH and d_3-DAcHz as internal standards.

(4) AcHz. In the previous work [1] the ion peaks at *m/e* 162 for ABH derived from AcHz and at *m/e* 133 for [^{15}N]benzalazine derived from [^{15}N]Hz (internal standard) were employed for monitoring. However, the method is not suitable for the accurate determination of AcHz, because the difference in mass range of the two peaks is more than 15%. The problem was solved by using d_3-AcHz as an internal standard.

Table I indicates the accuracy of determination of INH and its metabolites. The assay method is very reliable for the microdetermination, since the values of the regression coefficient of all compounds were distributed around 1.00. It is very important for the assay that calibration curves are made every time the experiment is performed and that the standard samples are treated by the same procedure as shown in Fig. 2.

K_M and V_{max} values of INH metabolism in isolated rat hepatocytes

Since the isolated hepatocyte system catalyses sequential drug metabolizing reactions including phase I and II under conditions similar to those in vivo and different from those in rat liver homogenate (S-9 mixture), the system could serve as a suitable model for investigating INH metabolism.

Isolated rat hepatocytes were incubated with each substrate (INH, AcINH, AcHz or Hz) and the product formed was determined by mass fragmentography

TABLE I

ACCURACY OF DETERMINATION OF INH AND ITS METABOLITES BY MASS FRAGMENTOGRAPHY

Each value was obtained from the results of the experiments performed three times.

Compound	Concentration range of calibration curve (μg/ml)	Regression coefficient	Standard deviation (μg/ml)
Isoniazid	2.0—10.0	0.99	± 1.550
Acetylisoniazid	0.8—4.0	1.00	± 0.391
Hydrazine	0.08—0.40	0.99	± 0.062
Acetylhydrazine	0.4—2.0	0.99	± 0.310
Diacetylhydrazine	0.4—2.0	0.98	± 0.438

TABLE II

APPARENT K_M AND V_{max} VALUES OF EACH METABOLIC PATHWAY OF INH IN ISOLATED RAT HEPATOCYTES AT 37°C

Substrate concentration: $5 \cdot 10^{-5}$ to $1 \cdot 10^{-3}$ *M*.

Metabolic pathways	K_M (m*M*)	V_{max} (nmole per min per $4 \cdot 10^6$ cells)
Hydrolysis		
INH → Hz	0.19	1.8
AcINH → AcHz	0.38	1.1
AcHz → Hz	0.88	11.8
Acetylation		
INH → AcINH	0.03	1.5
Hz → AcHz	0.16	2.4
AcHz → DAcHz	0.28	6.0

as mentioned above. Table II indicates the apparent K_M and V_{max} values of INH metabolic routes which were calculated using Lineweaver—Burk plots.

In the case of hydrolysis, the K_M values indicate that the reaction from INH to Hz (K_M = 0.19 m*M*) takes place easier than that from AcINH to AcHz (K_M = 0.38 m*M*) and that from AcHz to Hz (K_M = 0.88 m*M*). Therefore, Hz might be directly formed by a simple hydrolysis of INH itself, and Hz formation from AcHz seems to be negligible.

As for acetyl conjugation, it is concluded that AcINH formation from INH (K_M = 0.03 m*M*) takes place predominantly as expected, in comparison with the formation of AcHz from Hz (K_M = 0.16 m*M*) and with that of DAcHz from AcHz (K_M = 0.28 m*M*).

Time—course of INH and its metabolites in isolated rat hepatocytes

In order to study a definite pathway of INH metabolism, especially for Hz formation, an additional experiment was performed. Isolated rat hepatocytes were incubated with 0.5 m*M* INH, and the amounts of AcINH, Hz, AcHz and DAcHz formed were determined. INH remaining unchanged in the system was

also quantitated simultaneously at 0, 10, 20 and 30 min. Representative data of the percentage INH eliminated and the metabolites formed are listed in Table III. Fig. 5 indicates the average values of three experiments. AcINH was formed in a linear fashion with time at the rate of about 0.3 nmol per 10^6 cells per min. AcHz and DAcHz were also formed, though the amounts were much smaller than that of AcINH. For Hz, 1.92 and 3.95 nmol per 10^6 cells were detected at 5 and 10 min. Particularly interesting is the fact that as much Hz was produced as AcINH after 10 min of incubation, and the Hz produced began to decrease from 10 min and disappeared at 15 min after incubation.

TABLE III

PERCENTAGE INH ELIMINATION AND METABOLITE FORMATION IN ISOLATED RAT HEPATOCYTES

Initial concentration of INH = 0.5 m*M*. Number of liver cells = $8 \cdot 10^6$ cells/ml.

Products	Incubation time (min at 37°C)			
	0	10	20	30
INH	100.0 %	79.2 %	77.8 %	71.4 %
AcINH	0.0	4.1	6.8	10.3
DAcHz	0.0	0.6	0.9	1.6
AcHz	0.0	0.2	0.5	0.8
Hz	0.0	8.4	2.7	0.8

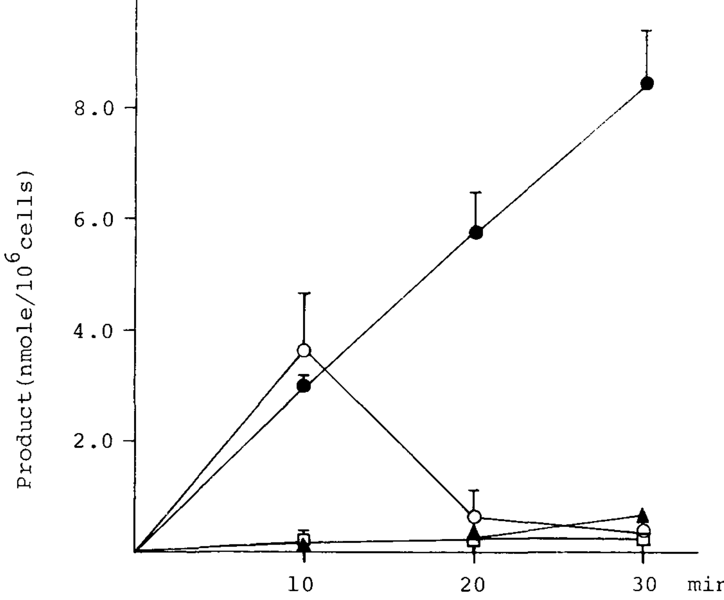

Fig. 5. Time—course of INH metabolism in isolated rat hepatocytes. Isolated hepatocytes were incubated with 0.5 m*M* INH and the metabolites formed were determined as described in the text. Vertical bars represent standard errors of the means. (●—●) AcINH, (○—○) Hz, (▲—▲) AcHz, (□—□) DAcHz.

It is already known that the hydrolytic process of INH is inhibited by AcINH [5]. Therefore, if the rate of Hz degradation remains unaltered, the detectable Hz could decrease as AcINH accumulates. We examined the influence of AcINH on Hz formation from INH in a rat liver homogenate system; the time-course indicated inhibition of Hz formation from INH ($5 \cdot 10^{-4}$ M) by AcINH ($2 \cdot 10^{-4}$ M) from 5 min after incubation. Further experiments are in progress and the details will be reported soon.

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CHROMBIO. 1277

MICRO-DETERMINATION OF CLONAZEPAM IN PLASMA OR SERUM BY ELECTRON-CAPTURE GAS—LIQUID CHROMATOGRAPHY

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(First received September 22nd, 1981; revised manuscript received February 23rd, 1982)

SUMMARY

A rapid method is described for the electron-capture gas chromatographic determination of clonazepam in plasma or serum using methyl-clonazepam as an internal standard. The analysis is performed isothermally on the silicone stationary phase SP-2510DA (Supelco). With this liquid phase, gas chromatographic properties are comparable to methods involving acid hydrolysis or derivatisation. A short pre-column containing another phase is added to enhance resolution. The method involves a single extraction, requires 100 μl of sample and has a detection limit of 3 nmol/l. Response is linear at concentrations from 5—900 nmol/l and thus clonazepam analysis both during therapy and after overdosage is possible. Plasma and serum clonazepam levels are interchangeable.

INTRODUCTION

Clonazepam is an established anticonvulsant agent widely used in the treatment of epilepsy [1—6]. Because of the narrow therapeutic range [7—11] and the risk of increased seizure frequency in overdosage [10], regular monitoring of plasma clonazepam levels during management with the drug is not only useful but necessary [10]. Whilst thin-layer [12] and high-performance liquid chromatographic methods [13,14] have been described, the low therapeutic concentration generally limits routine analysis of clonazepam on capillary samples to gas chromatography (GC) with electron-capture detection [8,9, 15—31].

GC of most benzodiazepines can be satisfactorily performed on phenyl silicone phases such as OV-1, OV-17 and OV-225 [32,33]. Because of difficulty in completely deactivating these liquid phases and the support material, clonazepam usually elutes with prolonged tailing of the peak on these stationary liquid phases, making quantitation difficult [9,15,19,21,26,28].

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Considerably better chromatographic peaks are obtained following modification of the drug, and despite many disadvantages including multiple extractions, long reaction times and loss of specificity, the most widely accepted GC assays for clonazepam are based on indirect determination of clonazepam following either acid hydrolysis to its benzophenone [9,15--19,34] or derivatisation [20—23].

Assays that measure the unchanged drug are the most likely candidates for a reference method in that they are simple and unambiguous [28]. Apart from methods for quantitating clonazepam on a standard OV-17 column [26—31], which, with the poor chromatographic properties of clonazepam, invariably require large sample volumes, column loading and peak quantitation by digital integration, three other GC methods remain for determining clonazepam as the intact moiety. The mass fragmentographic techniques of Min and co-workers [25,35] even when available, are rather expensive for routine analysis. The method of De Boer et al. [24], which uses a support coated open tubular column, suffers the disadvantage of requiring 1 ml of plasma for a single analysis, has nitrazepam as an internal standard and requires a two-step extraction. An expensive argon—methane mixture is required as carrier gas together with a solid injection system. The authors also examined a limited number of drugs for possible interference. Despite the injection and capillary column system used in the micro method described by Edelbroek and De Wolff [8], repeated injections of blank plasma extracts are still required before analysis to obtain peaks that are more gaussian. The organic infranatant is injected without aspiration of the aqueous phase resulting in some carryover of aqueous material in the syringe. Moreover, in our hands, the extraction mixture often formed an emulsion, making the method inconvenient. A four-fold attenuation change is required between the internal standard peak and clonazepam. A typical response curve with linearity to 475 nmol/l is reported.

By using two recently described GC column packings [36,37], 100 μl plasma and only one extraction step, we have developed a rapid, sensitive and selective assay for clonazepam, equally amenable to either routine clinical use, or emergency screening applications. With these phases, chemical modification of clonazepam is not required to obtain the sensitivity and peak symmetry characteristic of methods involving derivatisation or degradation.

EXPERIMENTAL

Reagents and glassware

All reagents are analytical grade. Clonazepam, 5-(*o*-chlorophenyl)-1,3-dihydro-7-nitro-2H-1,4-benzodiazepin-2-one was obtained from Roche Products (Sydney, Australia). The stock clonazepam solution, 250 μmol/l (78.9 mg/l) in absolute ethanol, is stable for at least six months at 4°C. The working clonazepam solution, 250 nmol/l in absolute ethanol, is prepared fresh on the day of analysis.

To prepare the quality control (QC) serum (drug-free pooled human serum containing added clonazepam), rapidly stir 100 ml of serum and slowly add about 3.2 mg of clonazepam, continue stirring the mixture for 1 h. Dilute 100 μl to 100 ml with serum and store in 0.2-ml aliquots at −15°C; this is stable for ten weeks.

Methyl-clonazepam was also obtained from Roche Products. The stock methyl-clonazepam internal standard solution, 100 μmol/l (33.0 mg/l) in ethyl acetate, is stable for at least six months at 4°C. The working methyl-clonazepam internal standard solution, 20 nmol/l in ethyl acetate—cyclohexane (4 : 1, v/v) is prepared fresh on each day of analysis.

Glass vials, 1 ml (Pierce Reactivials, Pierce, Rockford, IL, U.S.A.) were obtained complete with screw caps and PTFE-faced discs.

To a 1-ml Reactivial add 100 μl of plasma or serum. At the same time prepare reagent blank, control (QC) and standard vials. In the standard vials, place 10, 20, 40, 80 and 120 μl clonazepam working solution and evaporate to dryness at 40°C in a stream of dry nitrogen. To the blank and standard add 100 μl drug-free pooled human serum; to the control add 100 μl QC serum.

To each vial add 500 μl of working internal standard solution, cap securely, and vortex-mix for 60 sec; centrifuge at 2000 *g* for 1 min. Transfer the supernatant to another 1-ml Reactivial and evaporate the organic phase to approximately 50 μl at 40°C under a stream of nitrogen. Vortex each vial for 10 sec.

Gas—liquid chromatography

Gas—liquid chromatographic (GLC) analysis is performed using a Varian Aerograph Series 1440 gas chromatograph equipped with a ^{63}Ni electron-capture detector (8.5 mCi) and a 0.9 m × 2 mm I.D. glass column packed with GP 2% SP-2510DA on 100—120 mesh Supelcoport, with a 5-cm pre-column of 3% SP-2250DA on 100—120 mesh Supelcoport as previously described [36]. The instrument is operated isothermally with the oven, detector and injection port temperatures at 260°C, 300°C and 280°C, respectively. The carrier gas (nitrogen) flow-rate is 40 ml/min. Gas lines are fitted with filters containing molecular sieves (15 Å) and the electrometer range is 10^{-10} A/mV with attenuation of 4. The chromatogram is recorded on a Linear 361 recorder set at 1 mV full scale.

Inject 5 μl of each resulting solution into the chromatograph. Under the above conditions, the retention time for the internal standard is 3.20 min and for clonazepam 8.30 min. The ratio of peak heights of clonazepam standard to methyl-clonazepam is calculated and the value of QC and unknown specimens calculated by direct proportion.

RESULTS AND DISCUSSION

Selectivity and precision

Interference from drugs encountered as co-medication was studied. Phenobarbital, pentothal, carbamazepine, 5,5-diphenylhydantoin, di-*n*-propylacetate, ethosuximide, primidone, diazepam, nitrazepam, sulthiame, paracetamol, pseudoephedrine, chlorpheniramine, sodium cromoglycate, theophylline, salbutamol, declamethasone, prednisone, erythromycin, ampicillin, benzylpenicillin, gentamycin, amoxycillin and bactrim were dissolved in water at concentrations above their upper therapeutic limits. Extractions and GC determinations were performed as described. Only sulthiame was found to interfere with the analysis of clonazepam. It elutes as a broad overlapping peak with a retention time of 8.8 min and makes quantitation difficult. However, of

TABLE I

RETENTION TIMES FOR BENZODIAZEPINES

Benzodiazepine	Retention time (min)
Oxazepam	0.75
Diazepam	1.35
Flunitrazepam	1.80
Prazepam	2.25
Flurazepam	2.80
Nordiazepam	2.95
Methyl-clonazepam	3.20
Nitrazepam 7-amino metabolite	5.20
Nitrazepam	6.05
Clonazepam 7-amino metabolite	7.10
Clonazepam	8.30
Chlordiazepoxide	–
Nitrazepam 7-acetamido metabolite	15.75
Clonazepam 7-acetamido metabolite	20.50

the 350 patient samples analysed using the above procedure, only one has contained sulthiame as a co-medication. The pharmacologically inactive metabolites of clonazepam [9] and other benzodiazepines were all satisfactorily resolved (Table I). Plasma components do not interfere and neither the selectivity of a nitrogen–phosphorus detector [34] nor an extensive clean-up procedure is required [15]. A typical chromatogram of an extract of plasma is illustrated in Fig. 1.

The lower limit of detection is approximately 3 nmol/l. Over a period of 24 months, the between-run coefficient of variation of the assay for samples (n = 98) having a concentration of 100 nmol/l was 5.9%. Within-run precision was determined by analysis of ten extractions of drug-free plasma that had been supplemented to a concentration of 75, 125 and 200 nmol/l; coefficients of variation were 4.9%, 4.2% and 3.0%, respectively.

Comparison of column packings

Clonazepam, nitrazepam and desmethyldiazepam give very broad and poorly defined peaks by electron-capture GC on the phenyl silicone phases OV-1, OV-17 and OV-225 and special treatment (loading) of the column is required to deactivate sites before and during analyses. Peak area rather than peak height measurement is necessary to compensate for variability in peak shape. To evaluate the SP-2510DA phase and compare its performance with the above phases, 5 μl of a solution containing 20 nmol/l of the three benzodiazepines were injected into each of the four columns. With the phase SP-2510DA, electron-capture detector response, determined from peak area, and peak symmetry were substantially improved and more consistent. The more symmetrical peaks improve quantitation by peak height and peak area and with the improved detector response, from lower column adsorption, the sensitivity for analysing subtherapeutic concentrations is increased, particularly in capillary samples. Priming the column is not required. Clonazepam standards carried through the extraction procedure gave a linear response curve on the

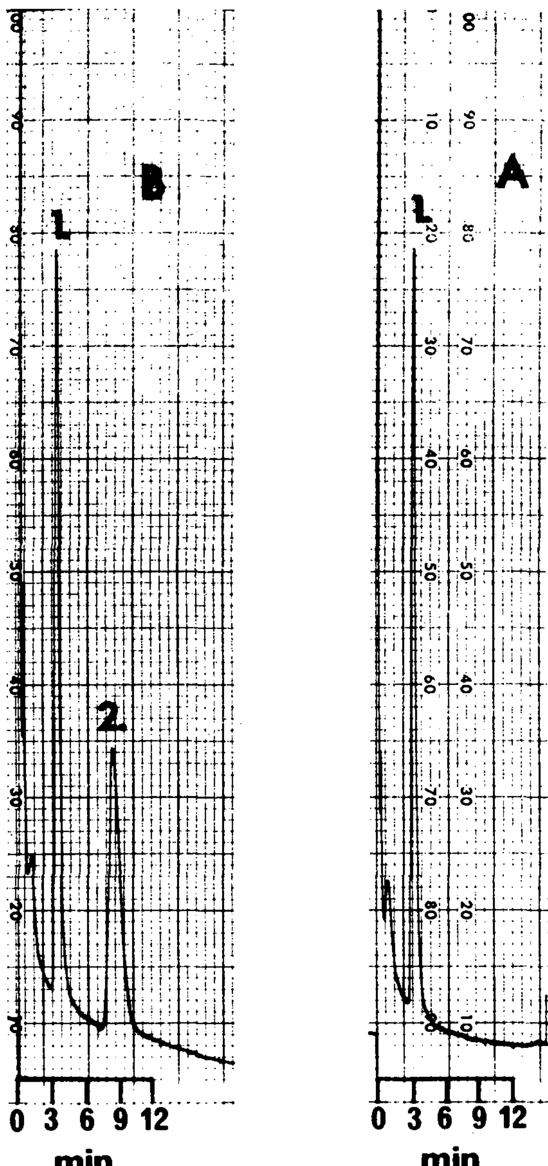

Fig. 1. Gas chromatograms of (A) extract of drug-free plasma, (B) extract of plasma sample containing clonazepam 100 nmol/l. Peaks: 1 = methyl-clonazepam (internal standard); 2 = clonazepam.

SP-2510DA phase in the range 5—900 nmol/l. The same standards showed linearity over a much smaller concentration 40—350 nmol/l on the other three phases. Calibration curves on liquid phases SP-2510DA and OV-17 are shown in Fig. 2.

Whilst the liquid phase SP-2510DA is not capable of adequately resolving all the benzodiazepines listed in Table I, when a short pre-column of SP-2250DA is added, elution of benzodiazepines which have similar retention times on the

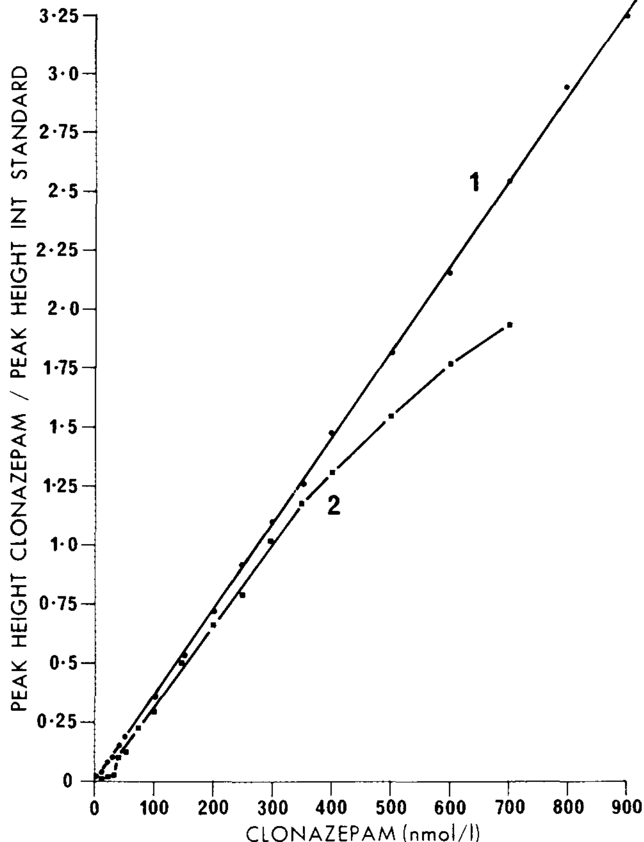

Fig. 2. Calibration curves of clonazepam relative to methyl-clonazepam, after extraction from plasma. Standards injected (1) onto SP-2510 DA-column with SP-2250DA pre-column and (2) onto OV-17 column.

single SP-2510DA phase is sufficiently retarded to give either complete separation, or separation with minimal overlap. In principle then, our assay procedure can be used to quantitate other benzodiazepines and we have, performing extractions and GC analysis as described above, also determined nitrazepam, diazepam and desmethyldiazepam levels.

The column packing deteriorates after approximately 350 injections of plasma extracts, resulting in loss of sensitivity and alteration of peak shape. Original performance is restored by repacking the 5-cm pre-column; the glass wool barrier between the two phases enables rapid and consistent replacement.

Extraction conditions

Various solvents were examined, together with extraction time and pH. Extractants chosen for investigation were immiscible with and less dense than plasma to provide for efficient removal of the solvent after extraction, had poor electron-capture response and dissolved clonazepam. They included diethyl ether, ethyl acetate, *n*-butyl acetate, ethyl acetate—cyclohexane (4:1, v/v) and methyl acetate—cyclohexane (7:3, v/v). All solvents extracted

clonazepam with greater than 95% efficiency at a plasma to solvent ratio of 1:5 and at the normal pH of plasma, and no significant variations in the recovery of clonazepam occurred if mixing was continued for 60 sec or longer. Ethyl acetate—cyclohexane (4:1, v/v) gave least interference of plasma components and emulsion free extracts. Similar recoveries were obtained with and without buffer (pH 3—9). For this reason a buffer solution was not included in the extraction, but may be necessary if the pH of the plasma sample were to greatly exceed normal values (e.g. in some post mortem specimens).

Practical applications

Fig. 3 shows the wide range in drug concentrations in plasma derived from patients of all ages who have been treated with clonazepam for the control of seizures, and the variability in plasma levels in different people taking comparable amounts of the drug. Because of a large number of variables such as patient compliance and sampling times not being specifically controlled, no attempt has been made to define the linear regression correlation between the daily dosage and the clonazepam plasma concentration.

Clonazepam levels obtained from paired serum and plasma samples are shown in Fig. 4. Correlation based on a least-squares linear regression formula gives a line of best fit with a slope of 1.042 and a Y-intercept of -1.419 nmol/l. The correlation coefficient is 0.990 ($n = 19$), thus clonazepam analysis on plasma and serum samples would appear to be interchangeable.

The small amount of plasma required (50—100 μl) allows clonazepam measurement in neonates and children using capillary blood samples. Adsorption losses, a factor regarded sufficiently serious by Parry and Ferry [22] to warrant the silanisation of glassware, are minimised by eliminating evaporation

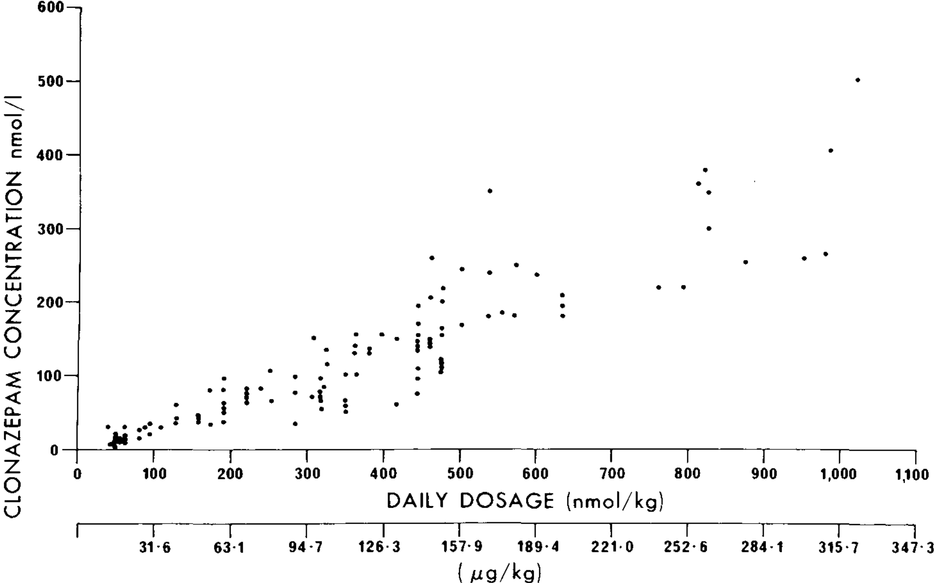

Fig. 3. Relationship between clonazepam plasma levels and the dose administered in 110 patients on chronic oral medication with clonazepam.

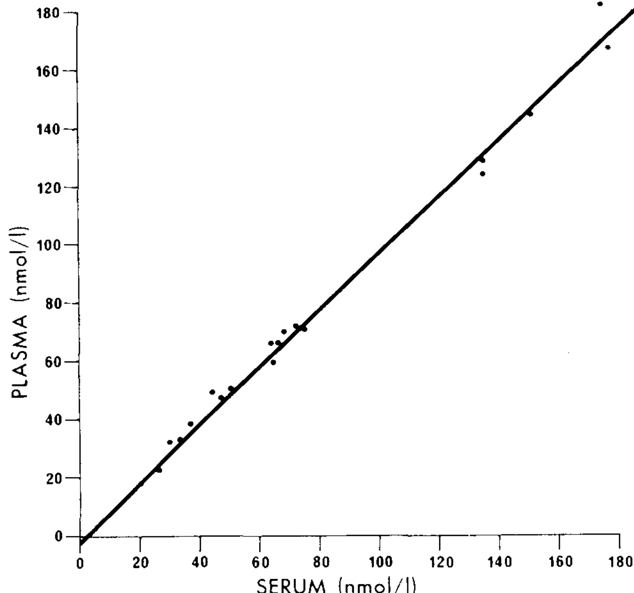

Fig. 4. Relationship between plasma and serum levels of clonazepam.

to dryness of the final extract and by conducting all manipulations quickly and conveniently in small vials. The internal standard, methyl-clonazepam is employed as it is a homologue of clonazepam and is not used as a drug.

In conclusion we believe that the procedure described offers significant improvement in peak shape, electron-capture detector response and linearity over other methods which measure the unchanged drug and in this regard is comparable to methods involving either derivatisation or acid hydrolysis. The selectivity of the column system enables other benzodiazepines to be well resolved and quantitated. With its simplicity, rapidity, sensitivity and small volume of plasma or serum required, clonazepam levels can be routinely monitored as frequently as other antiepileptic drugs.

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ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF 4-AMINOPYRIDINE IN SERUM

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SUMMARY

An assay for the quantitative estimation of 4-aminopyridine in biological fluids has been developed using 2-aminopyridine as internal standard and ion-pair reversed-phase (C_{18}) high-performance liquid chromatography with detection at 263 nm. A 7.5% solution of acetonitrile in water containing tetrabutylammonium iodide and sodium heptanesulfonate buffered at pH 3.0 provided excellent separation of the analytes from each other and from an interfering peak that was occasionally observed in the outdated human sera used in these studies. Sensitivity, specificity, precision, accuracy and reproducibility all were judged sufficient for the routine use of this assay for pharmacokinetic and pharmacodynamic studies.

INTRODUCTION

The actions of 4-aminopyridine (4-AP) on the processes responsible for transmitter release at autonomic ganglia [1, 2], neuroeffector junctions [3—8], and the neuromuscular junctions in skeletal muscle [9—12] are well documented. Recent studies [13, 14] have shown that 4-AP exerts its action on evoked transmitter release by enhancing dramatically the amount of calcium ions which enter the nerve terminal at the time of depolarization, an event on which release of all neurotransmitters is dependent. This drug has found useful clinical applications in reversing the effects of non-depolarizing neuromuscular blocking agents [15] and in the treatment of myasthenia gravis [16], the Eaton--Lambert myasthenie syndrome [17], and botulism [18].

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Furthermore, it has been demonstrated that 4-AP antagonizes the neuromuscular blockade produced by most antibiotics [19, 20].

In view of the basic and clinical pharmacological importance of 4-AP, we have initiated studies designed to characterize pharmacokinetic and pharmacodynamic properties and the metabolic fate of this prototype drug. In this regard we have focussed our attention on the development of an adequately sensitive, specific and rigorous assay that will provide reliable quantitative estimations of this drug in biological fluids. Although gas–liquid chromatography (GLC) [21, 22] and high-performance liquid chromatography (HPLC) [23] methods for 4-AP have been reported, these methods in general do not provide the accuracy and sensitivity that we anticipate will be required for our work. More recently more sophisticated GLC [24, 25] and HPLC [26] procedures have appeared in the literature. Based on a comparison of the results reported in this paper, we submit that the ion-pair based HPLC procedure described here is the method of choice for the quantitative analysis of 4-AP in biological fluids.

EXPERIMENTAL

Chemicals and reagents

The 4-AP was purchased from Aldrich (Milwaukee, WI, U.S.A.) and was recrystallized from acetonitrile. The internal standard 2-aminopyridine (2-AP) was obtained from Aldrich (Gold Label 99+%) and was used without further purification. The acetonitrile was of chromatographic purity and was obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). The water used for all solutions and the mobile phase was deionized and purified by the Milli-Q-System (Millipore, Bedford, MA, U.S.A.). Analytical grade dichloromethane, methanol, trifluoroacetic acid, and tetrabutylammonium iodide were obtained from Aldrich. Sodium heptanesulfonate was obtained from Eastman-Kodak (Rochester, NY, U.S.A.). Sodium dihydrogen phosphate and phosphoric acid were Baker analyzed reagents (J.T. Baker, Phillipsburg, NJ, U.S.A.).

Stock solutions

Stock solutions of 4-AP (4.92 mg per 100 ml) and 2-AP (4.97 mg per 100 ml) were prepared in water. Storage of these solutions at 4°C did not result in detectable decomposition. All chromatographic analyses were performed by diluting stock solutions or dissolving residues of sample extracts in 10% acetonitrile.

Chromatography

The high-performance liquid chromatograph consisted of a Beckman (Fullerton, CA, U.S.A.) Model 100A solvent metering system, an Altex (Berkeley, CA, U.S.A.) Model 155 variable-wavelength detector (detection at 263 nm, 0.01 a.u.f.s.), an Altex Series 210 sample injection valve with a 50-μl loop, and a Soltec (Encino, CA, U.S.A.) Model 233 linear recorder (input 0.1 V and 1.0 V).

A prepacked Ultrasphere C_{18} reversed-phase column (250 × 4.6 mm I.D.; particle size 5 μm; Altex) was used. In order to avoid contamination of the

analytical column, a pre-column (50 × 3.2 mm I.D.) tapfilled with Vydac RP (particle size 30–44 μm; Altex) was placed between the injector and the analytical column. The mobile phase consisted of a mixture of 0.015 *M* sodium heptanesulfonate, 0.002 *M* tetrabutylammonium iodide, and 0.01 *M* phosphate buffer (pH 3.0; 0.01 *M* sodium dihydrogen phosphate + 0.01 *M* phosphoric acid) in acetonitrile—water (7.5:92.5, v/v). The mobile phase was degassed by ultrasonic vibration prior to the addition of the ion-pair reagents and then filtered through a 0.45-μm filter. The assays were performed at ambient temperature with a flow-rate of 1 ml/min, generating a pressure of about 160 bar.

Preparation of sample

To a PTFE-lined screw-cap culture tube (150 × 16 mm) were added 1.0 ml of serum and 2.485 μg of 2-AP dissolved in 0.1 ml water. The samples were made alkaline (pH 12) with 20 μl of 2 *N* sodium hydroxide solution and extracted with dichloromethane (10 ml) by gently rotating the tubes on a rotary disc for 30 min. After centrifugation for 5 min at 3000 *g*, the upper (aqueous) layer was carefully removed with the aid of a Pasteur pipet. The organic layer was pipetted into a PTFE-lined screw-cap conical centrifuge tube (134 × 17 mm) to which was added 0.1 ml of 2 *M* trifluoroacetic acid in methanol. After vortex-mixing, the solvent was evaporated to dryness under a stream of nitrogen at 40°C using an N-Evap apparatus (Organomation, Northborough, MA, U.S.A.). The residue was dissolved in 0.1 ml of 10% acetonitrile by ultrasonification for 5 min. After centrifugation (2000 *g*, 1 min) a 50-μl aliquot of the solution was subjected to the liquid chromatographic analysis.

Quantitation

The procedure was standardized by analyzing drug-free serum samples spiked with known amounts of 4-AP. Peak height ratios of 4-AP vs. the internal standard 2-AP were used to establish calibration curves.

RESULTS AND DISCUSSION

Chromatographic conditions

Since 4-AP is a strong base (pK_a of 9.2 [27]) and is water soluble, both reversed-phase and ion-pair based chromatographic procedures should provide the resolving power to develop a quantitative assay. Previous reports and our own experience revealed that reversed-phase chromatography using methanol—acetic acid [23] or acetonitrile—methanol—ammonium carbonate [26] as the mobile phase gave tailing peaks. The problem may be explained by ion-exchange or absorption reactions of ionized 4-AP with non-bonded silanols on the stationary phase which may behave as a weak ion-exchange resin. Increasing the pH of the mobile phase to suppress ionization would be likely to improve the reversed-phase chromatographic characteristics of 4-AP. Unfortunately, silica based reversed-phase columns deteriorate when exposed to solutions at pH values greater than 7.4 [28].

Ion-pair chromatography techniques have provided excellent separations of amines and quaternary ammonium compounds without column degrada-

tion [29–31]. Reversed-phase ion-pair chromatography also allows one to separate both ionized and un-ionized compounds in biological samples under the same chromatographic conditions. These properties suggested to us that a reversed-phase ion-pair chromatographic method might offer a promising approach to the quantitative analysis of 4-AP. The discussion which follows summarizes our efforts to optimize the parameters of an ion-pair based HPLC assay for 4-AP on a C_{18} reversed-phase column.

Optimization of pH

The pH was an important parameter to evaluate in the development of this assay since the pH of the mobile phase will dictate the concentration of the ionic form of an organic base such as 4-AP. The pH of the mobile phase can be safely varied from 2 to 7.4 for use with an alkyl-bonded silica column. At pH 7.4, 4-AP is essentially completely protonated. The internal standard 2-AP has a pK_a of 6.9 [27] and will be fully ionized at pH values below 5.0. Therefore, the influence of the pH on chromatographic behavior was investigated in the pH range from 2 to 6. The results are summarized in Fig. 1 and

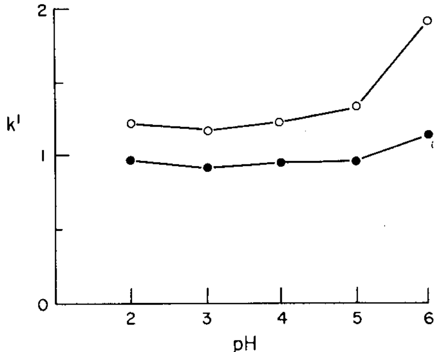

Fig. 1. Effect of pH on capacity ratio (k') of 4-AP (•——•) and 2-AP (○——○). Mobile phase (0.02 *M* sodium heptanesulfonate, 0.002 *M* tetrabutylammonium iodide, and 0.01 *M* phosphate buffer) in acetonitrile—water (10:90, v/v).

TABLE I

EFFECT OF pH ON CHROMATOGRAPHIC BEHAVIOR

pH	Peak asymmetry factor*		Plate count**	
	2-AP	4-AP	2-AP	4-AP
2	1.0	1.0	4800	9300
3	1.0	1.0	5300	8600
4	1.3	1.0	5600	7500
5	2.3	1.6	2900	4400
6	13.0	2.0	670	5300

*At 10% of peak height.

**$N = 5.54 \cdot \left(\frac{\text{retention time}}{W_{1/2}}\right)^2$.

Table I. At pH > 5, the peak asymmetry factor increased. Excellent resolution and column efficiency as indicated in the plate count values (Table I) were achieved at pH ⩽ 4. The pH chosen for this assay was 3.0.

Hydrophobic cation

It is known that the addition of a hydrophobic cation such as tetramethylammonium has a pronounced effect on the peak shape in the separation of cationic compounds [29—32]. Presumably the cation competitively inhibits the partitioning of the sample cation in nonbonded silanols on the stationary phase. This results in a repulsion or a decreased availability of binding sites for sample cation interactions. In the present work, the addition of tetrabutylammonium iodide had a pronounced effect on the peak shape. Alterations in the concentration of tetrabutylammonium iodide also effected the retention volume with increasing concentrations causing decreases in the retention volume (Fig. 2). Similar results have been reported for the acetylcholinesterase inhibitors neostigmine and pyridostigmine [32]. The time to achieve equilibration of the column also could be regulated by changing the concentration of tetrabutylammonium iodide (Table II). The higher the concentration the shorter the time required to achieve initial equilibrium conditions. In view of these results 2 mM tetrabutylammonium iodide was employed in the analysis of 4-AP.

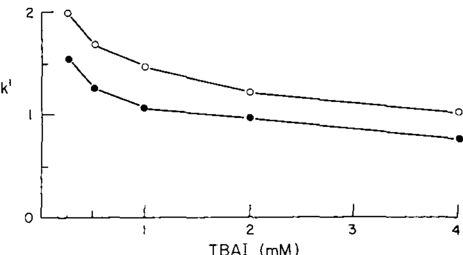

Fig. 2. Effect of tetrabutylammonium iodide (TBAI) concentration on capacity ratio (k') of 4-AP (•——•) and 2-AP (○——○). Mobile phase (0.01 M phosphate buffer, pH 3.0, and 0.02 M sodium heptanesulfonate) in acetonitrile—water (10:90, v/v).

TABLE II

EQUILIBRATION VOLUME VS. TETRABUTYLAMMONIUM IODIDE (TBAI) CONCENTRATION

Concentration TBAI (mM)	Column volumes to achieve equilibration
0.25	166 (690 ml)
0.5	90 (370 ml)
1.0	50 (210 ml)
2.0	30 (120 ml)
4.0	20 (85 ml)

Counter ion

The capacity ratio (k') can be varied by the choice of the counter ion. For protonated bases, alkylsulfonate (RSO_3^-) derivatives can be used. The following three sodium alkylsulfonates were examined: sodium butanesulfonate ($C_4H_9SO_3Na$), sodium pentanesulfonate ($C_5H_{11}SO_3Na$), and sodium heptanesulfonate ($C_7H_{15}SO_3Na$). Sodium butanesulfonate gave a rather low retention volume for 4-AP and 2-AP. An increase in the size of the counter ion increased the capacity ratio. Sodium heptanesulfonate gave the best peak characteristics and was selected for this assay.

The final regulation of the retention time was made by changing the concentration of the counter ion in the mobile phase. Fig. 3 shows the relationship between the counter-ion concentration and retention time. At higher concentrations ($\geqslant$ 35 mM) the k' vs. sodium heptanesulfonate concentration curve plateaued and the peak shapes deteriorated dramatically. Therefore 20 mM sodium heptanesulfonate proved to be satisfactory for the chromatographic separation of the pure analytes.

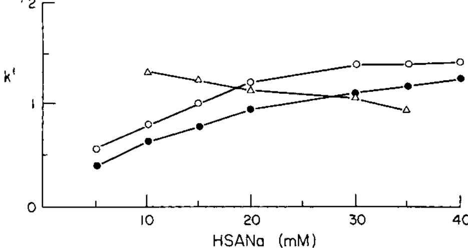

Fig. 3. Effect of sodium heptanesulfonate (HSANa) concentration on capacity ratio (k') of human serum interference (△——△). The corresponding curves for 4-AP (●——●) and 2-AP (○——○) are included. Mobile phase (0.01 M phosphate buffer, pH 3.0, and 0.002 M tetrabutylammonium iodide) in acetonitrile—water (10:90, v/v).

Organic solvent

The most commonly employed solvent combinations for reversed-phase ion-pair chromatography are water—methanol and water—acetonitrile. It is known that acetonitrile offers better column efficiencies due to its lower viscosity [28] and therefore we choose water—acetonitrile for our studies. The retention time could be regulated by changing the acetonitrile concentration. When the acetonitrile concentration exceeded 15%, a loss of resolution between 4-AP and 2-AP resulted. Moreover, 4-AP eluted near the solvent peak: 15% acetonitrile, $k' = 0.59$; 20% acetonitrile, $k' = 0.37$. For the pure analytes 5—10% acetonitrile provided satisfactory resolution although the retention volume was significantly increased at the lower concentration.

Detection

In the mobile solvent used in the assay, 4-AP and 2-AP give absorption maxima at 263 nm and 229 nm, respectively, values which are essentially identical to those published in the literature [33, 34]. Molar absorptivities are 16,900 for 4-AP and 9200 for 2-AP. In order to obtain greater sensitivity for 4-AP, the wavelength chosen for the assay was 263 nm. The lower limit

of detection for 4-AP was found to be 0.25 ng injected (signal-to-noise ratio $\geqslant 4$).

Chromatograms

Chromatography of blank serum extracts showed several peaks on the HPLC tracing other than those from the analytes. Occasionally human sera displayed a large peak (I, Fig. 4) that interfered with the analytes. The concentration of sodium heptanesulfonate in the mobile phase could be used to improve the separation of analytes from the interfering peak (Fig. 3). Use of 15 m*M* sodium heptanesulfonate provided the optimum separation. The final regulation of the retention volume was made by changing the concentration of acetonitrile. In order to optimize both resolution and elution time the amount of acetonitrile in the mobile phase was fixed at 7.5%. Fig. 4 shows chromatograms for blank sera and sera containing 98.4 ng/ml of 4-AP and 2.5 μg/ml of 2-AP. The retention times for 4-AP and 2-AP are 4.0 and 4.7 min, respectively.

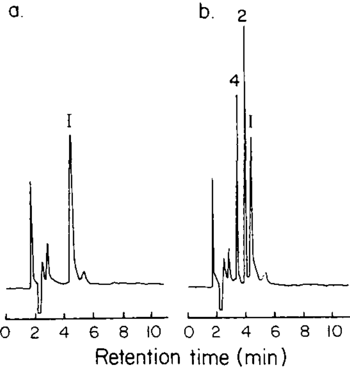

Fig. 4. HPLC tracings of extracts from (a) blank and (b) spiked human serum samples. Mobile phase (0.01 *M* phosphate buffer, pH 3.0, 0.015 *M* sodium heptanesulfonate, and 0.002 *M* tetrabutylammonium iodide) in acetonitrile—water (7.5:92.5, v/v). Peaks: (2) 2-AP, 2.5 μg/ml, (4) 4-AP, 98.4 ng/ml, (I) interference.

Recovery

The absolute analytical recoveries from human serum of 4-AP and internal standard were estimated by comparing the peak heights obtained from the injection of known quantities of the analytes with peak heights obtained from the injection of extracts of serum samples spiked with the analytes. Preliminary experiments revealed that analyses of spiked serum or aqueous samples afforded low recoveries and irreproducible results unless trifluoroacetic acid was added to the extraction solvent. These results may be explained by losses due to the volatility of the aminopyridines, especially 2-AP, during the evaporation. In attempting to overcome this problem, Uges and Bouma [26] added pentanol to prevent evaporation of aminopyridines. In our hands however, this procedure did not provide reproducible recoveries. Hengen and Hengen [35] reported that evaporation of the volatile alkaloid nicotine was prevented by the addition of hydrochloric acid to form the corresponding nonvolatile

hydrochloride salt. Since trifluoroacetic acid is volatile and easily removed by evaporation after salt formation, it was decided to add trifluoroactic acid to the extracts. The recovery of the aminopyridines was checked by adding different concentrations of trifluoroacetic acid to aqueous extract samples (196.8 ng 4-AP and 4.97 μg 2-AP per ml water). At a concentration of trifluoroacetic acid $\leqslant$ 20 μmole recoveries improved but still were not quantitative. Addition of 200 μmole of trifluoroacetic acid to serum extracts increased extraction reproducibility and recovery to a satisfactory level (Table III).

TABLE III

ABSOLUTE ANALYTICAL RECOVERY (n = 8)

Substance	Concentration in serum (ng/ml)	x (%)	S.D. (%)	C.V. (%)
4-AP	492.0	67.81	1.55	2.29
	196.8	66.09	2.72	4.12
	98.4	67.78	1.87	2.76
	49.2	66.38	1.48	2.23
	24.6	67.47	2.21	3.28
	9.84	64.08	3.13	4.88
	4.92	64.34	3.53	5.49
	1.968	66.79	4.21	6.30
2-AP	2485.0	93.20	1.36	1.46

TABLE IV

ACCURACY

4-AP (ng/ml)	Peak height ratio Mean ± S.D. (n = 4)	C.V. (%)	Expected ratio	Relative error (%)
492.0	3.6523 ± 0.0951	2.60	3.6488	0.10
196.8	1.4499 ± 0.0272	1.88	1.4584	−0.58
98.4	0.7288 ± 0.0207	2.84	0.7283	0.07
49.2	0.3570 ± 0.0066	1.85	0.3632	1.88
24.6	0.1873 ± 0.0047	2.51	0.1807	3.65
9.84	0.0712 ± 0.0013	1.83	0.0712	0.00
4.92	0.0358 ± 0.0014	3.91	0.0347	3.17
1.968	0.0138 ± 0.0008	5.80	0.0128	7.81

Calibration curve

The calibration curve was obtained by plotting the peak height ratios of 4-AP to internal standard using drug-free pooled human sera containing 4-AP in the range of 2—492 ng/ml. A linear relationship was observed over this range ($y = 0.00742x - 0.00183$, $r = 1.0000$). The lowest quantifiable level of drug was 1 ng/ml serum (signal-to-noise ratio > 3).

Accuracy

In amounts of 2—492 ng 4-AP was added to 1.0-ml aliquots of pooled human sera and the samples were analyzed by the present method. The results presented in Table IV show that the estimated amounts of 4-AP added were in good agreement with the actual amounts.

Specificity

In order to assess the potential application of this assay to problems involving multiple-drug regimens, sera containing pancuronium, pyridostigmine, and neostigmine, each at a concentration of 1 μg/ml serum, were examined. Pancuronium could not be detected under the conditions of this analysis. Retention times for pyridostigmine and neostigmine were 3.7 and 6.2 min, respectively. Furthermore, these quaternary ammonium compounds were not extracted efficiently and did not interfere with the analysis of 4-AP and 2-AP.

Reproducibility

Within-day precision of the assay was determined by performing ten replicate analyses on aliquots of a drug-free serum sample to which 4-AP was added to give a concentration of 98.4 ng/ml. The coefficient of variation was 2.84%. Day-to-day precision was estimated for the same test material over a period of ten working days. The coefficient of variation was 2.50%. The results demonstrate excellent reproducibility.

The assay method developed allows the simple, sensitive, rapid, and selective determination of 4-AP in human serum with good accuracy and precision. The method will be used to perform pharmacokinetic and metabolic studies of 4-AP.

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QUANTITATION OF 6-MERCAPTOPURINE IN BIOLOGIC FLUIDS USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY: A SELECTIVE AND NOVEL PROCEDURE

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SUMMARY

A simple, selective, sensitive and rapid procedure is described for the quantitation of 6-mercaptopurine (6-MP) in biological fluids. A sensitivity of at least 5 ng/ml is easily achieved in plasma on a reversed-phase octadecylsilane (C_{18}) column using a high-performance liquid chromatography system following an initial protein precipitation and a clean-up step. Mean extractability of the drug from plasma following this procedure is greater than 98% and the overall coefficient of variation for the assay is below 6%. Plasma levels of 6-MP were measured in a rhesus monkey for 12 h following an intravenous administration of a single bolus dose (4 mg/kg) of 6-MP.

INTRODUCTION

Immunosuppressive agents are used to control the rejection reaction caused by antigenic differences that remain after tissue typing and donor—recipient matching. The antimetabolite azathioprine (AZA) is one of the most important immunosuppressive agents [1]. AZA is cleaved in the body to 6-mercaptopurine (6-MP) and methylnitroimidazole [2]. It is the biologically active forms of 6-MP that cause inhibition of protein and nucleic acid synthesis [3]. However, a significant amount of attention is being diverted lately to the use of 6-MP as a chemotherapeutic agent in the maintenance therapy of rapidly fatal forms of leukemia characterized by replacement of bone marrow by primitive or blast cells, e.g. acute lymphoblastic leukemia (ALL). Following standard therapy treatments and the resulting prolongation of life, an increasing incidence of leukemic infiltration of the central nervous system (CNS) is observed.

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In order to evaluate the use of 6-MP in meningeal leukemias, an exploratory study was conducted in monkeys to quantitate the partitioning of drug across the blood—brain barrier. Fast disappearance from plasma and slow partitioning of a polar drug, e.g. 6-MP, would tend to make its concentration in cerebrospinal fluid (CSF) and plasma very low. In order to define the disposition profile of 6-MP in biologic fluids a selective, sensitive and rapid assay procedure was desirable.

A review of the published literature revealed several procedures for quantitating 6-MP in serum, plasma and/or urine. Bailey et al. [4] quantitated 6-MP by gas chromatography of samples derivatized with tetramethylammonium hydroxide (TMAH) reagent while Finkel [5] measured fluorescence of purine-6-sulphonate, an oxidized product of 6-MP. Rosenfeld et al. [6] employed gas chromatography—mass spectrometry for determination of 6-MP following its derivatization. None of these methods had the desired sensitivity or any significant reproducibility data. Some procedures had analytic recoveries as low as 18% [6].

A procedure reported by Maddocks [7] in 1979 for simultaneous determination of 6-MP and AZA involved a lengthy extraction following the derivatization with phenylmercuric nitrate and subsequent measurement of fluorescence. Lin et al. [8] recently reported a procedure for the quantitation of 6-MP in plasma following AZA administration but gave no data on the specificity and sensitivity of the assay.

Ding and Benet [9] recently reported a high-performance liquid chromatographic (HPLC) procedure for quantitating both 6-MP and AZA with an extraction recovery of approximately 12% for 6 MP. Attempts to reproduce or achieve the desired and/or reported sensitivity proved unsuccessful using their methodology. Most of the HPLC analyses of thiopurines have been from tissue extracts [10, 11]. An ion-pair reagent heptanesulfonic acid was successfully employed by Day et al. [12] to determine 6-MP in plasma by reversed-phase ion-pair chromatography. The assay was not sufficiently sensitive to quantitate 6-MP levels in CSF following its intravenous administration or vice versa. This paper presents a rapid, selective and sensitive method for quantitative determination of 6-MP in biological fluids by HPLC.

EXPERIMENTAL

Materials

6-MP was obtained from Burroughs Wellcome (Research Triangle Park, NC, U.S.A.). 6-Thioguanine (TG) was procured from the National Cancer Institute (National Institutes of Health, Bethesda, MD, U.S.A.) and 6-thiouric acid (TU) was generously supplied by Dr. Elion of Burroughs Wellcome. Dithiothreitol (DTT, Cleland's Reagent) was obtained from Eastman-Kodak (Rochester, NY, U.S.A.) and stored at refrigeration temperature. Glacial acetic acid was obtained from Fisher Scientific (Silver Spring, MD, U.S.A.). Spectral grade dichloromethane (J.T. Baker, Phillipsburg, NJ, U.S.A.) was employed in the extraction. Both methanol and acetonitrile (Burdick and Jackson Labs., Muskegon, MI, U.S.A.) used in the assay were HPLC grade. Water used was double distilled in a glass still. All other chemicals were reagent grade.

All HPLC solvents were passed through a 0.45-μm filter (Millipore, Bedford, MA, U.S.A.) prior to use and degassed. Stock solutions of 6-MP, TG, TU were made in water and kept protected from light by aluminum foil. All solutions were stored at 4°C and showed insignificant degradation over a period of two months.

Methods

Plasma (1 ml) was placed in an 8-ml screw cap disposable glass vial (Fisher Scientific). To this were added 80 μl of a 10 μg/ml solution of TG as an internal standard and 10 μl of DTT (1 *M*). The glass vial was then vortexed for 10 sec and 2 ml of acetonitrile were added using a Repipet® dispenser (Lab-industries, Berkeley, CA, U.S.A.). Each vial was then vortexed for 30 sec and centrifuged for 5 min at 2000 *g*. The supernate was then decanted into another 8-ml disposable screw cap glass vial. Using a Repipet, 2 ml of dichloromethane were then added to each vial. The vials were then shaken for 5 min on an automatic reciprocating shaker (Eberbach, Ann Arbor, MI, U.S.A.) and centrifuged for 5 min at 2000 *g*. From the top aqueous layer, 750 μl were removed into a disposable 12 × 75 mm borosilicate culture tube and evaporated under a gentle stream of nitrogen at 37°C. The residue was reconstituted with 150 μl of distilled water and vortexed for 1 min. A 15-μl aliquot of the sample was injected into the HPLC system.

The chromatographic analysis of the samples was performed using a Spectra-Physics liquid chromatograph Model 3500B (Spectra-Physics, Santa Clara, CA, U.S.A.) equipped with a Schoeffel variable-wavelength UV Spectroflow monitor Model SF 770 (Schoeffel Instrument, Westwood, NJ, U.S.A.) for detection at 322 nm. The analysis was performed on a 25 cm × 4.6 mm I.D. Altex Ultrasphere Octadecylsilane (ODS) analytical column with a 5-μm particle size (Beckman Instruments, Berkeley, CA, U.S.A.). Preceding the analytical column was a 7 cm × 2.2 mm I.D. guard column packed with Co:Pell ODS (30–38 μm) particles (Whatman, Clifton, NJ, U.S.A.). A mobile phase of acetonitrile—acetic acid—water (3.5:0.2:96.3) at a flow-rate of 1.4 ml/min was used as the eluent. The back pressure ranged from 152 to 186 bars. The eluent was degassed prior to the HPLC run. Retention times for TU (chief metabolite of 6-MP), 6-MP, internal standard (TG), and DTT under these conditions were 3.8, 4.8, 6.5, and 13.8 min, respectively. A fresh standard curve for 6-MP in plasma was obtained with each batch of samples. Standard curves for 6-MP were almost identical when prepared in plasma or water. The concentration of 6-MP was estimated by means of the ratio of its peak height to the peak height of the internal standard as compared with a simultaneously run standard curve. Analytic recovery of 6-MP was determined by comparison of direct injection of standard aqueous solution with the injection of the same standards run through the assay procedure. No interfering peaks were observed in the chromatograms for the monkey plasma samples.

RESULTS AND DISCUSSION

Typical HPLC tracings of plasma samples with or without drug are shown in Fig. 1. Sharp symmetrical peaks are shown for TU, 6-MP, TG, and DTT. As is

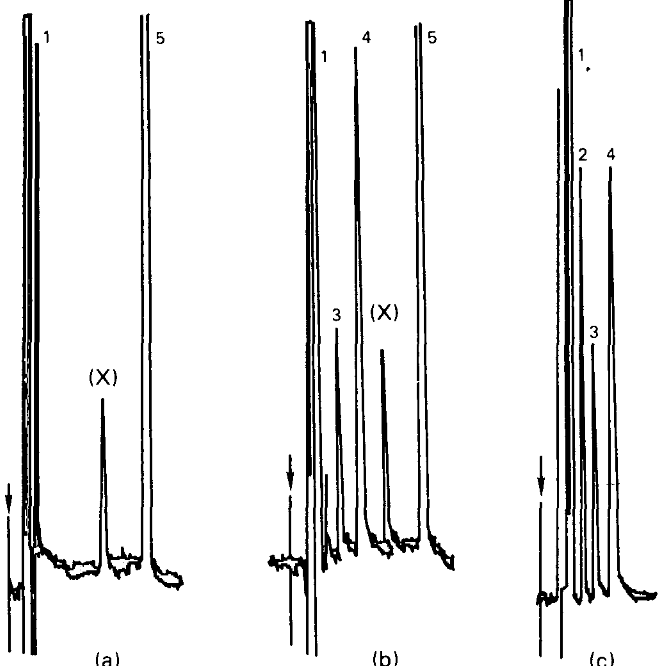

Fig. 1. Representative HPLC tracings of monkey plasma samples run through the assay procedure. Peaks: (retention times in the parentheses), (a) Blank plasma; (b) 8-h sample with 6-MP; (c) an aqueous mixture of TU, 6-MP and TG. 1, solvent front; 2, TU (3.8 min); 3, 6-MP (4.8 min); 4, TG (6.5 min); 5, DTT (13.8 min); X, peak from plasma.

often found with reversed-phase liquid chromatography, the compounds elute in order of decreasing polarity; DTT being the least polar, elutes last; whereas, the chief metabolite (TU) formed following oxidation of 6-MP by the enzyme xanthine oxidase, elutes first.

Fig. 2 shows a typical mean standard curve (n = 8) in plasma over a concentration range of 10—100 ng/ml parent drug (6-MP) over a 2-month period, where peak height ratio is plotted as a function of 6-MP concentration. Linearity in standard curves of 6-MP was established over an extended range of up to 10 μg/ml in human plasma. No attempt was made to quantitate the metabolite levels in plasma. However, as can be easily seen from Fig. 1c a clean separation was obtained between the parent compound 6-MP and its most predominant metabolite (TU) peak. Other polar metabolites of drug probably elute with the solvent front. Further studies are nor in progress to evaluate and describe the disposition of 6-MP in a subhuman primate following intravenous dosing.

The sensitivity of the assay for a 15-μl injection is at least 5 ng/ml for 6-MP. This was determined by observing the lowest concentration that maintained an arbitrary signal-to-noise ratio of 3. The assay sensitivity can be increased at least 2—8-fold by reducing the reconstitution volume to 100 μl and injecting up to 90 μl on the column. Table I shows the reproducibility of the assay at five concentrations. Each plotted value represents the mean of eight determinations

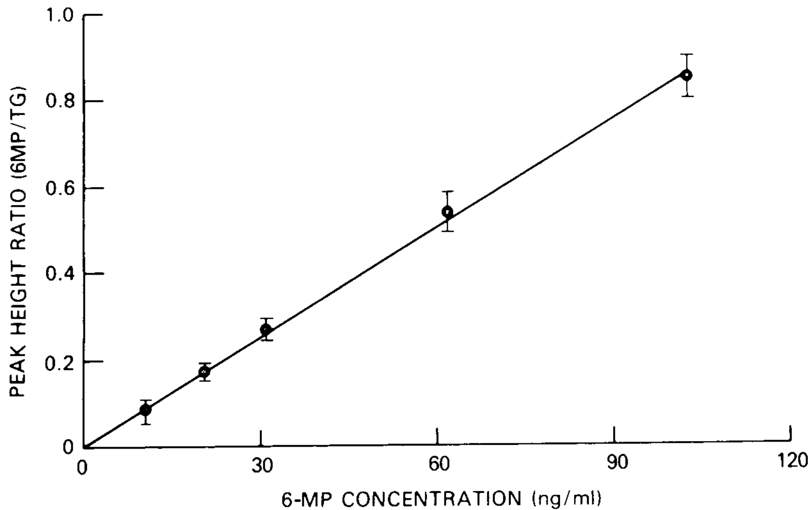

Fig. 2. Mean standard curve for 6-mercaptopurine in plasma over a 2-month period. Each point is a mean (± S.D.) of eight determinations.

TABLE I

ASSAY REPRODUCIBILITY OF EIGHT REPLICATES OVER A TWO-MONTH PERIOD FROM PLASMA

Actual concentration of standards (ng/ml)	Concentration of 6-MP		
	Mean	S.D.	C.V. (%)
10.4*	10.2	1.030	10.10
20.8	20.1	0.960	4.78
30.1	31.0	1.376	4.43
62.0	64.4	3.022	4.69
103.0	101.8	1.645	1.62
Mean			5.12

*n = 7 in this case.

over a 2-month period. Assay efficiency or recovery data are shown in Table II. The percent recovery of 6-MP was essentially complete and there was no statistically significant difference ($p>0.1$) in the recovery as a function of concentration.

Table I clearly shows that the mean coefficient of variation over the entire range is under 6%. One probable reason for the high variation observed in the low-concentration standard (Table I) could be that it was exposed to light at room temperature once for 1—2 h. Other evaluations of the variation associated with plasma standards containing 6-MP from 2 to 10 ng/ml, determined by injecting larger volumes, have shown the coefficients of variation to be about 6% for the 10 ng/ml and about 10% for 2 ng/ml sample.

The interday variability coefficients evaluated by comparing the peak height ratio of 6-MP to internal standard over a period of one week for two standard

TABLE II

ANALYTICAL RECOVERY DATA ON STANDARD SOLUTIONS OF 6-MP ASSAYED THREE TIMES

Concentration of standard (ng/ml)	Recovery (%)	
	Mean	S.D.
46.2	95.5	2.98
97.9	100.9	2.53
146.1	102.1	2.57
199.8	109.5	3.45
252.8	102.2	3.10
Mean*	102.0	
S.D.	4.99	

*n = 15.

plasma solutions, with concentrations representing each end of the standard curve, were 6.7% (n = 9) for 9.27 ng/ml and 4.0% (n = 12) for 97.9 ng/ml standard. Intraday coefficient of variation evaluated for one plasma standard (46.2 ng/ml) was less than 1.0% (n = 6).

In a study published earlier, Ding and Benet [9] reported no data on the reproducibility of their assay. With an overall extraction recovery of 12%, the coefficient of variation associated with the quantitation of 6-MP in biological fluids could be significantly high. However, as the data in Table I and Table II show, the assay procedure described here is sensitive and reproducible, the only step requiring some significant amount of time being the evaporation of the aqueous phase. Time required for the evaporation can be further shortened by using an Evapo-Mix (Buchler Instruments, Fort Lee, NJ, U.S.A.) if so desired.

The assay reported here has been successfully employed for the quantitation of 6-MP in plasma following an intravenous administration of a single bolus dose (4 mg/kg) to a monkey. Fig. 3 shows the plasma decay of 6-MP in a monkey which appears to follow a multicompartment open body model. The terminal half-life of the log-linear phase computed by linear regression analysis appears to be approximately 1.9 h.

HPLC analyses of the plasma samples revealed no interfering peaks in the chromatograms. Insignificant amount of metabolite (TU) was detected in the analysis of a few plasma samples. Thiouric acid, being even more polar than the parent compound, is eliminated even faster than 6-MP. Some other metabolites of 6-MP which are even more water soluble and are present in insignificant amounts in plasma probably elute with the solvent front and no attempt was made to separate them. Several drugs that might be administered concurrently with 6-MP were chromatographed to check for possible interference in the assay procedure. No interference was observed with methotrexate, AZA, caffeine, 5-fluorouracil, prednisone, theophylline, vinblastine and vincristine, and only cytarabine (ARA-C) showed a possible interference with the internal standard peak. However, the peak was rather small and broad at therapeutic drug concentration.

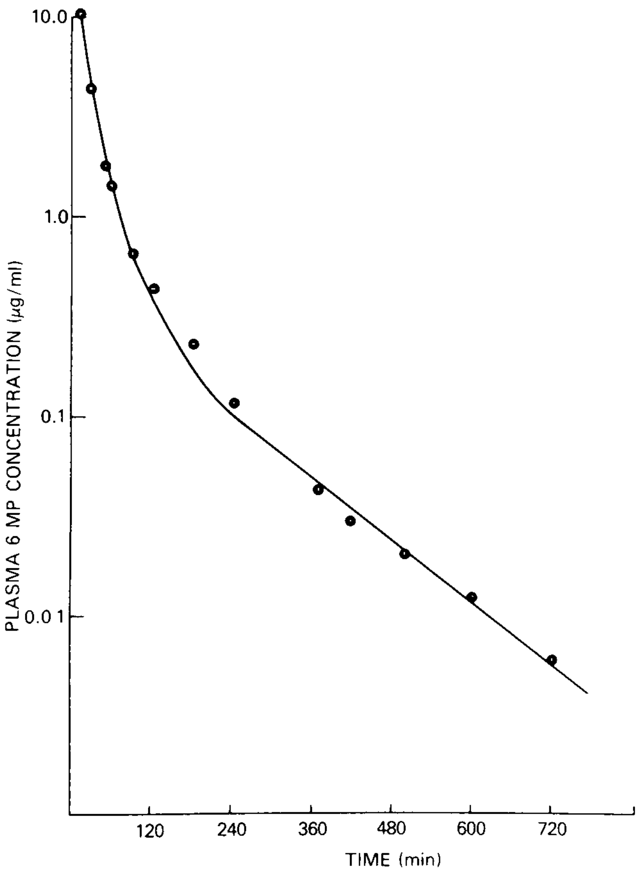

Fig. 3. Plasma concentrations of 6-MP observed in monkey 0-268 over a 12-h period following a single intravenous bolus dose (4 mg/kg).

As has been suggested in earlier reports [4, 9], it was deemed necessary to add sulfhydryl protecting reagent 1,4-dithiothreitol (DTT, Cleland's Reagent) immediately to all plasma standards, aqueous standards and monkey plasma samples to stabilize unsubstituted thiols. Ten microliters of DTT (1 *M* solution) were added per 1 ml of the sample resulting in a final concentration of 10 m*M* of DTT. Exclusion of DTT resulted in a lower peak height ratio (6-MP/TG) due to decomposition of drug during the extraction procedure. Bailey et al. [4] have also observed and reported such drug decomposition of 6-MP in serum.

We did not make any attempt to extract the drug into an organic phase, as earlier reports [6, 9] have shown the poor extractability of 6-MP. Excellent recovery and extractability can therefore be achieved by keeping the drug in the aqueous phase. Initial precipitation of plasma sample with acetonitrile removes most of the possible interferences from proteins and subsequent addition of dichloromethane selectively removes acetonitrile from the above solution leaving the drug in the desired aqueous phase.

This procedure basically takes advantage of the negligible solubility of 6-MP

in organic solvents, its small size and can therefore be suitably applied to other water soluble, low molecular weight compounds. It was also noted that longer column equilibration was required for TG when starting up the HPLC system. In order to avoid such delays, the mobile phase can be recirculated overnight through the system.

The assay reported here has been successfully employed for the quantitation of 6-MP in plasma following an intravenous administration of a single bolus dose (4 mg/kg) to a monkey. Fig. 3 shows the plasma decay of 6-MP in a monkey which appears to follow a multicompartment open body model. The terminal half-life of the log-linear phase computed by linear regression analysis appears to be approximately 1.9 h.

Further studies to elucidate the pharmacokinetics of 6-MP using this simple, precise and rapid assay procedure are presently underway in subhuman primates in our laboratories. Because of its simplicity and precision the assay lends itself to therapeutic drug monitoring as well. The assay has been successfully employed in quantitating drug levels in certain leukemic patients, following oral and/or intravenous 6-MP administration.

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CHROMBIO. 1259

RAPID ASSAY FOR PLASMA CHLORAMBUCIL AND PHENYL ACETIC MUSTARD USING REVERSED-PHASE LIQUID CHROMATOGRAPHY

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(First received December 30th, 1981; revised manuscript received February 12th, 1982)

SUMMARY

A rapid assay for chlorambucil, a drug used for the treatment of chronic lymphocytic leukemia, and its major metabolite is described. Chromatographic behaviour of the two compounds on two different reversed-phase columns is discussed, as well as the kinetics of their hydrolysis in aqueous medium. The developed analysis can be applied to the determination of the plasma levels of the drug and its metabolite. No sample preparation is required and the spectrophotometric detection affords the sensitivity in the picomole range. Total analysis time is between 10 and 15 min.

INTRODUCTION

Chlorambucil (Leukeran®) is a nitrogen mustard derivative administered in the treatment of chronic lymphocytic leukemia (CLL), ovarian and breast carcinomas, malignant lymphomas and Hodgkin's disease [1—3]. The structure of this drug (Fig. 1), in which the reactive bis(2-chloroethyl)amino group is substituted *para* to the carboxylic acid group on the aromatic ring, ensures the penetration of the alkylating function through cellular membranes [4].

The various analytical procedures reported for the quantitative assay of chlorambucil include the colorimetric determination of 4-(*p*-nitrobenzyl)pyridine derivatives, UV spectrophotometric, and chlorine titrimetric methods [2]. However, these procedures do not provide the sensitivity and accuracy needed to study the pharmacokinetics of the drug or its metabolism in humans [2]. Whereas the tandem operation of gas chromatography (GC) and mass spectrometry (MS), per se, satisfies such analytical criteria, the need for an ethyl acetate extraction procedure prior to the GC—MS assay limits the possible advantages

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CHLORAMBUCIL (LEUKERAN ^{R})

$HOOC-CH_2-CH_2-CH_2-C_6H_4-N(CH_2-CH_2-Cl)(CH_2-CH_2-Cl)$

4 [4-bis(2-chloroethyl) aminophenyl] butyric acid

PHENYL ACETIC MUSTARD (metabolite)

$HOOC-CH_2-C_6H_4-N(CH_2-CH_2-Cl)(CH_2-CH_2-CH)$

2 [4-bis(2-chloroethyl) aminophenyl] acetic acid

Fig. 1. Structures of chlorambucil and phenyl acetic mustard metabolite.

of the method: this extraction requires the use of a large volume of blood [5]; more importantly, chlorambucil itself is unstable in the ethyl acetate medium [2].

Currently available high-performance liquid chromatographic (HPLC) assays also necessitate several extraction steps [4], which impoverishes the recovery of the compounds of interest, and lack the sensitivity needed for the quantification of the drug or its main metabolite, phenyl acetic mustard (Fig. 1), which precludes their pharmacokinetic study [1].

Described in this paper is a reversed-phase liquid chromatographic assay for the sensitive and accurate quantification of the drug and its metabolite in plasma. The blood fluid is directly injected into the chromatographic system. Sample clean-up is achieved by means of a guard column and the recovery of the two mustards is high. The stability of the drug in aqueous medium and the kinetics of its hydrolysis are also discussed.

EXPERIMENTAL

Apparatus

A Waters ALC 204 liquid chromatograph (Waters Assoc., Milford, MA, U.S.A.) was used in the course of this study. Simultaneous monitoring of the UV-absorbing compounds at 254 nm and 280 nm was accomplished by means

of a Waters 440 dual-wavelength detector and the signals were recorded on a dual-pen recorder (Omniscribe, Houston Instrument, Austin, TX, U.S.A.). Chromatographic solutes were characterized by their stopped-flow UV spectra, obtained with a Schoeffel SF 770 Spectroflow Monitor, equipped with an MM 700 Memory Module and an SFA 339 Wavelength Drive, all from Kratos (Schoeffel Instrument Division, Westwood, NJ, U.S.A.). Simultaneous fluorometric detection was achieved using an SF 970 fluorescence monitor (Kratos, SID) set at an excitation wavelength of 285 nm and a 320-nm emission cut-off filter. Areas of the peaks detected at 254 nm were computed by a Hewlett-Packard 3380A integrator (Avondale, PA, U.S.A.).

Centrifugation of blood samples was performed with a Dynac centrifuge (Clay-Adams, Parsippany, NJ, U.S.A.). A Vortex Genie K 550-G (Scientific Industries, Springfield, MA, U.S.A.) was used to vortex the samples prior to injection. Eppendorf pipettes (Brinkman Instruments, Westbury, NY, U.S.A.) were also utilized for the sequential dilution of the reference solutions.

Columns

A Partisil PXS-10/25 ODS column (25 cm × 4.6 mm, 10 μm average particle size, Whatman, Clifton, NJ, U.S.A.) and a guard column (5 cm × 3.9 mm), packed with pellicular Co:Pell ODS (octadecyl groups chemically bonded to 30—32 μm glass beads, Whatman), were used. For faster elution of the drug and its metabolite, the Partisil ODS column was substituted with a Chromegabond MC-18 (15 cm × 4.6 mm, 5 μm average particle size, ES Industries, Marlton, NJ, U.S.A.).

Chemicals

Potassium dihydrogen phosphate (KH_2PO_4), HPLC grade, was purchased from Fisher Scientific Company (Fair Lawn, NJ, U.S.A.) and solutions were prepared at a 0.02 *M* concentration with doubly-distilled, deionized water. The aqueous buffer was filtered through a 0.45-μm pore-size Millipore membrane filter (Millipore, Bedford, MA, U.S.A.) and degassed under vacuum prior to use. The HPLC grade methanol was purchased from Fisher Scientific. The methanol—buffer mixtures were degassed by purging with helium.

Chlorambucil {4[4-bis(2-chloroethyl)aminophenyl] butyric acid} was obtained from Burroughs-Wellcome (Research Triangle Park, NC, U.S.A.) and the phenyl acetic mustard {2[4-bis(2-chloroethyl)aminophenyl] acetic acid} from the Institute of Cancer Research of the Royal Cancer Hospital (Sutton, Great Britain). Since the drug and its metabolite decompose rapidly in water at room temperature, care was taken to chill the glassware as well as the water in which the reference compounds were dissolved, by means of a dry-ice—acetone bath. All reference solution vials were kept on ice during use.

Chromatographic conditions

The chromatographic conditions were optimized for a selective analysis of chlorambucil and its phenyl acetic mustard in the presence of other plasma constituents. The eluent used with the Partisil PXS 10/25 ODS column was a solution (50:50, v/v) of anhydrous methanol—0.02 *M* KH_2PO_4 (natural pH). The temperature was ambient and the flow-rate 1.5 ml/min.

These conditions were slightly modified for faster elution of the drug and its major metabolite on a Chromegabond MC-18 column. The methanol–buffer eluent was a 55:45 mixture and the flow-rate 1.0 ml/min.

Samples

The developed method was tested in the analysis of heparinized plasma samples, obtained from patients on a chlorambucil regimen. The plasma specimens were prepared at the New York University School of Medicine (New York, NY, U.S.A.). Control samples from healthy individuals were obtained from the University of Rhode Island Health Services.

Identification of chlorambucil and its phenyl acetic mustard metabolite

Initial assignment of solute identities in CLL plasma was based on retention times and co-chromatography with the reference compounds. In addition, the ratios of peak heights recorded at 280 nm and 254 nm (Table I), the fluorometric selectivity and stopped-flow UV spectra enabled further characterization of the chromatographic eluites.

TABLE I

SPECTROSCOPIC DATA FOR CHLORAMBUCIL, ITS PHENYL ACETIC MUSTARD METABOLITE AND THEIR HYDROXYLATED DERIVATIVES

	Peak height ratio 280/254 nm	Fluorescence intensity
Chlorambucil	0.095	none
Monohydroxy derivative	0.125	slight
Dihydroxy derivative	0.136	moderate
Phenyl acetic mustard	0.150	none
Monohydroxy derivative	0.214	slight
Dihydroxy derivative	0.250	moderate

RESULTS

Chromatographic assay

Using a Partisil PXS 10/25 ODS (10 μm), the drug and its metabolite were resolved from other plasma constituents. This is illustrated with the chromatogram of a plasma sample to which reference solutions of chlorambucil and phenyl acetic mustard were added (Fig. 2). The blank plasma chromatogram showed no interference which corresponded to the retention of chlorambucil or its metabolite.

The calibration curves for these compounds were linear over the concentration range of interest and the correlation coefficients for chlorambucil and the phenyl acetic mustard were 0.99 and 0.98, respectively. The lower detection limits for the drug and its major metabolite in plasma were 11.9 and 2.07 pmole, respectively. Within-day and day-to-day coefficients of variation for retention times were 1.0% and 1.2%, respectively.

A chromatogram of a plasma specimen, obtained from a CLL patient about

an hour after ingestion of the drug (2 mg Leukeran® tablets from Burroughs-Wellcome), is exemplified in Fig. 3. As little as 10 μl of the blood fluid could be used to detect and quantify the drug and its metabolite.

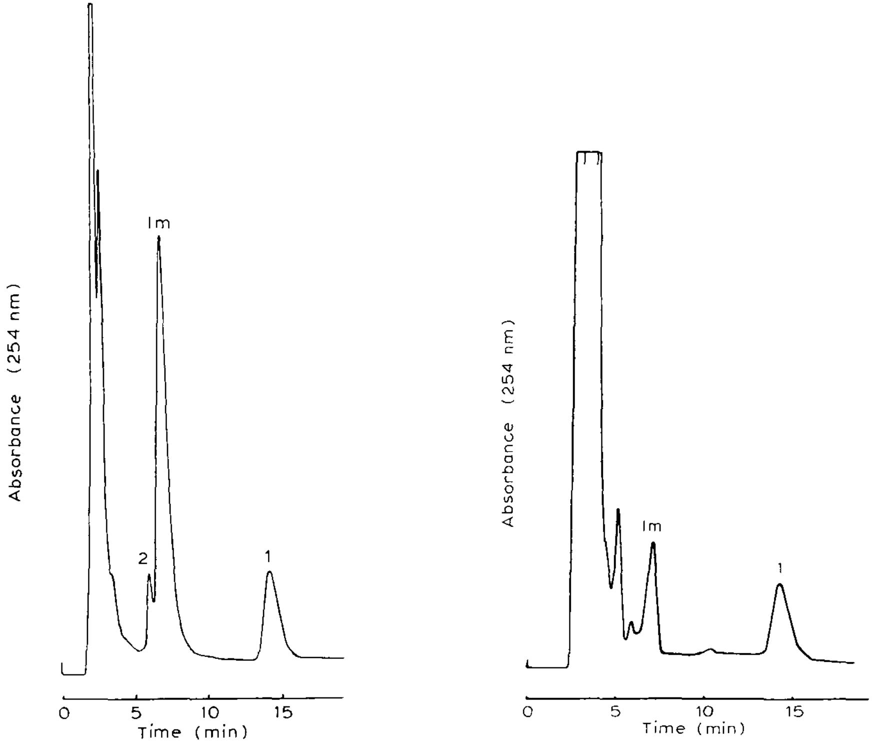

Fig. 2. Chromatogram of a plasma sample spiked with chlorambucil (1), phenyl acetic mustard (1m) and monohydroxy-chlorambucil derivative (2). Volume of plasma injected, 20 μl. Chromatographic conditions: column, Partisil PXS 10/25 ODS (10 μm); eluent, methanol—0.02 M KH_2PO_4, (1 : 1, v/v); flow-rate, 1.5 ml/min; temperature, ambient.

Fig. 3. Chromatogram of a plasma specimen obtained from a CLL patient on chlorambucil regimen. Volume of plasma injected, 30 μl. Chromatographic conditions as in Fig. 2. Peaks: 1 corresponds to 42.9 pmole of chlorambucil; 1m, phenyl acetic mustard.

A faster chromatographic analysis was possible using a shorter column, Chromegabond MC-18, with a slight modification of the eluent composition. The average particle diameter of the packing material was 5 μm and thus, adequate resolution of the early eluting solutes was maintained (Fig. 4).

Since chlorambucil is more stable in plasma than in water or ethyl acetate [2], the ethyl acetate extraction procedure was avoided and the removal of proteins and possible interferences was achieved by means of a guard column installed before the analytical column.

Recoveries were determined by adding known volumes of reference solutions to an aliquot of the drug-free plasma. Samples were assayed in triplicate and the average recoveries for different dilutions of the reference compounds were slightly higher than 100% (Table II). This could be attributed to the greater

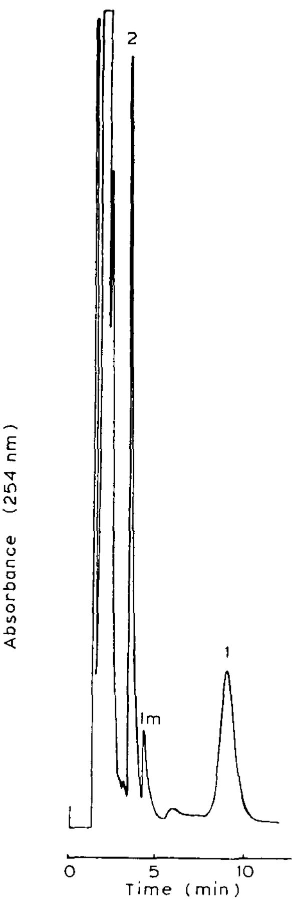

Fig. 4. Chromatogram of a plasma sample spiked with chlorambucil (1), phenyl acetic mustard (1m), and monohydroxy-chlorambucil derivative (2). Volume of plasma injected, 20 μl. Chromatographic conditions: column, Chromegabond MC-18 (5 μm); eluent, 45% of 0.02 M KH_2PO_4—methanol; flow-rate, 1.0 ml/min; temperature, ambient.

stability of the drug and its metabolite in plasma than in aqueous medium.

Hydrolysis of chlorambucil

In neutral or basic solutions, chlorambucil is rapidly hydrolyzed into its mono- and dihydroxy derivatives; the latter compound lacks antitumor activity. This decomposition, followed chromatographically as illustrated in Fig. 5, is noticably slower at 4°C than at 25.5°C.

The decomposition of chlorambucil in aqueous solution follows a first-order reaction rate (Fig. 6). Our results support the unimolecular nucleophilic substitution scheme devised by Owen et al. [6], in which the rate-limiting step is the ionization of one chlorine together with the formation of a cyclic ethyleneimmonium ion.

The chromatograms show that as the concentration of chlorambucil decreases, the concentration of the monohydroxy derivative increases initially and sub-

TABLE II

AVERAGE RECOVERIES FOR DIFFERENT DILUTIONS OF CHLORAMBUCIL AND ITS PHENYL ACETIC MUSTARD METABOLITE

	Concentration of reference solution (*M*)	Recovery (%)
Chlorambucil	$6.57 \cdot 10^{-4}$	106
	$1.32 \cdot 10^{-4}$	103
	$2.63 \cdot 10^{-5}$	104
Phenyl acetic mustard	$5.21 \cdot 10^{-4}$	98.4
	$1.04 \cdot 10^{-4}$	100
	$4.17 \cdot 10^{-6}$	106

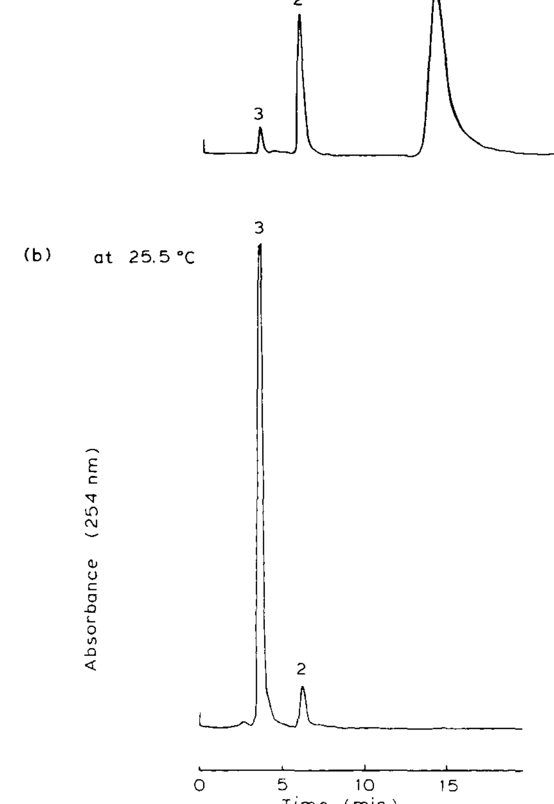

Fig. 5. Chromatograms illustrating the extent of hydrolysis of a reference solution of chlorambucil stored for 7 h at 4°C (a) and 25.5°C (b). Peaks: 1, chlorambucil; 2, monohydroxy-chlorambucil derivative; 3, dihydroxy-chlorambucil derivative. Chromatographic conditions as in Fig. 2.

sequently decreases due to its further conversion to the dihydroxylated form (Fig. 6). Since the rates of these reactions are temperature-dependent, the de-

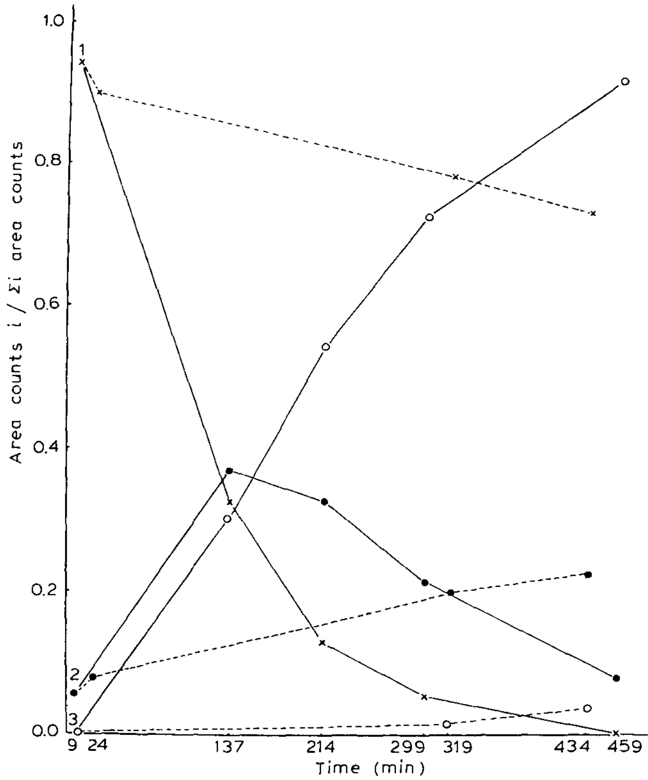

Fig. 6. Kinetic study of the decomposition of chlorambucil (1) into its monohydroxy (2) and dihydroxy (3) derivatives in aqueous medium at 4°C (— — —) and 25.5°C (———).

composition of chlorambucil and its phenyl acetic mustard metabolite can be minimized by chilling the reference solutions. This caution is particularly important in the determination of calibration curves for both the drug and its principal metabolite.

CONCLUSION

An efficient assay for the detection of chlorambucil and its major metabolite in plasma was developed using reversed-phase liquid chromatography. This method is rapid and does not involve any sample preparation. The quantification of picomole amounts of both the drug and its phenyl acetic mustard can be achieved. Recoveries are high, thus permitting accurate assessment of the pharmacokinetics of elimination of chlorambucil from the blood fluid. This is particularly useful in view of the side effects of the drug such as bone marrow suppression [7, 8], fibrosis, increased susceptibility to infections, tuberculosis [9], and seizures in children [10].

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CHROMBIO. 1264

SIMULTANEOUS DETERMINATION OF AMITRIPTYLINE, NORTRIPTYLINE AND THEIR RESPECTIVE ISOMERIC 10-HYDROXY METABOLITES IN PLASMA BY LIQUID CHROMATOGRAPHY

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SUMMARY

An ion-pair reversed-phase liquid chromatographic method for the determination of the tricyclic antidepressant amitriptyline, its demethylated metabolite nortriptyline, and their respective *cis*- and *trans*-hydroxylated metabolites in plasma is presented. After extraction from 1 ml of plasma, the reconstituted residue was chromatographed on a trimethylsilyl packed column using a mobile phase of acetonitrile and acetate buffer with sodium heptanesulfonate and triethylamine. Recovery of the drugs and its metabolites from plasma ranged from 56 to 99%. The method is suitable for determining plasma concentrations as low as 5 ng/ml (C.V. < 9%) for all six compounds.

Plasma concentrations of amitriptyline and its metabolites from eleven different patients are presented.

INTRODUCTION

It has been repeatedly demonstrated that individuals treated with a fixed dose of antidepressant medication have a 10—20 fold variation in their steady state plasma concentration and that this variation is genetically determined [1]. The investigation of plasma drug concentrations in a variety of studies has produced a wealth of important information on the pharmacokinetics and pharmacodynamics of these drugs but there remains a great deal of controversy in the relevance of routine plasma level monitoring of the tricyclic antidepressant drugs in clinical practice [2]. Brodie [3] in 1964 suggested that if one was unable to demonstrate clear relationships between the plasma concentration of the parent drug and its pharmacological activity then an investigation of the metabolites of that drug was indicated. Christiansen and Gram [4] demonstrated that there were hydroxylated metabolites present in the central nervous system in an acute overdose case and several studies have demonstrated

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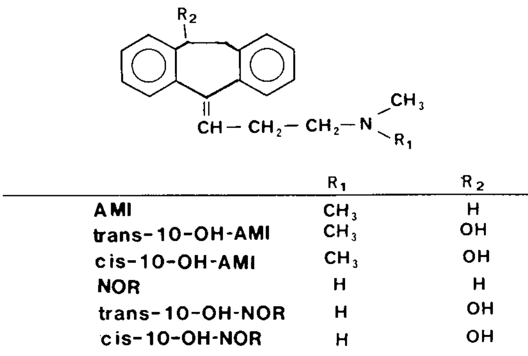
considerable quantities of unconjugated and conjugated hydroxy metabolites of tricyclic antidepressants in the plasma of treated patients [5—12]. It is now known that the hydroxy metabolites have strong cardiovascular activity [13], and are essentially equipotent to the parent compound in terms of blockade of norepinephrine and serotonin receptors in rat brain slices and isolated synaptosomal fractions [14—17]. The specificity of the respective hydroxylated metabolite is maintained, e.g. 2-hydroxydesmethylimipramine is 100 times more potent in inhibiting norepinephrine than serotonin accumulation. Finally, these metabolites are present in the plasma in the same order of magnitude as the primary and secondary amines [9, 11].



	R1	R2
AMI	CH3	H
trans-10-OH-AMI	CH3	OH
cis-10-OH-AMI	CH3	OH
NOR	H	H
trans-10-OH-NOR	H	OH
cis-10-OH-NOR	H	OH

Fig. 1. Chemical structures of amitriptyline (AMI) and metabolites.

Clearly, therefore, there is a need for adequate analytical methodology for the quantitation of the hydroxy metabolites of these psychotropic drugs. A method for the analysis of amitriptyline (AMI) and its hydroxylated metabolites (for structures see Fig. 1) using gas chromatography—mass spectrometry (GC—MS) was recently published by Garland et al. [18] but this procedure carefully dehydrated the hydroxylated compounds to form the 10,11-diene before GC—MS; thus the differentiation between the *E* and *Z* (*trans* and *cis*) forms of the 10-hydroxylated metabolites were obscured. A similar procedure has been recommended by Alvan et al. [7] for the drug nortriptyline (NOR) on the basis that the *E* and *Z* enantiomers were equipotent in terms of their blockade of norepinephrine, a point recently emphasized by Bertilsson et al. [9]. Despite these rather convincing data it appears to us that as the mode of action of these tricyclic antidepressant drugs is again being questioned that we should, if possible, develop a technique capable of separating these enantiomers.

Various methods have been reported for the analysis of AMI and metabolites and have been reviewed recently [19]. Liquid chromatography (LC) has, thus far, been the only method available for the separation of the *cis—trans* isomers of the 10-hydroxylated metabolites of AMI and NOR. However, the LC methods available lack either sensitivity, complete resolution of all metabolites, or many interfering and/or unidentified peaks [20, 21].

Therefore, we have developed a convenient method for the determination of AMI, NOR and their respective isomeric 10-hydroxylated metabolites in plasma using reversed-phase ion-pair LC.

EXPERIMENTAL

Reagents

Acetic acid, sodium acetate, sodium hydroxide and hydrochloric acid were all analytical reagent grade. Sodium heptanesulfonate (Eastman-Kodak, Rochester, NY, U.S.A.), triethylamine (Aldrich, Milwaukee, WI, U.S.A.) and isoamyl alcohol (Sigma, St. Louis, MO, U.S.A.) were all used as received. Acetonitrile was HPLC—UV grade from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.) and *n*-heptane was obtained from Fisher Scientific (Fairlawn, NJ, U.S.A.). Distilled water was passed through a Milli-Q Water Purification System (Millipore, Bedford, MA, U.S.A.) before use.

Apparatus

Chromatography was performed using a Model 6000A solvent delivery pump, a U6K injector or WISP 710A automatic sampler and a Model 440 UV absorbance detector at 254 nm (Waters Assoc., Milford, MA, U.S.A.). The separations were achieved with a 25 cm × 4.6 mm I.D. LC-1 (5 μm) particle size column (Supelco, Bellefonte, PA, U.S.A.). Chromatograms were recorded on a Model B5217-5 Ombiscribe recorder (Houston Instruments, Austin, TX, U.S.A.) and the data processed by a PDP 11/34 data acquisition system (Digital Equipment, Maynard, MA, U.S.A.). The detector signal output to the PDP 11/34 was amplified 50 × by an operational amplifier circuit.

Standards

Stock solutions of 1 mg/ml of each of the *cis* and *trans* isomers of the 10-hydroxyamitriptyline and 10-hydroxynortriptyline (H. Lundbeck and Co., Copenhagen, Denmark) were prepared as the free base in 0.01 *N* hydrochloric acid. Working solutions of 1 ng/μl were subsequently prepared. Amitriptyline · HCl (Merck, Sharp and Dohme, West Point, PA, U.S.A.) and nortriptyline · HCl (Eli Lilly, Indianapolis, IN, U.S.A.), stock solutions of 1 mg/ml were prepared in 0.1 *N* hydrochloric acid. The stock solutions were diluted with 0.01 *N* hydrochloric acid to give working solutions of 2 ng/ml. Loxapine succinate (Lederle Labs., Pearl River, NY, U.S.A.) stock solutions of 1 mg/ml in 0.1 *N* hydrochloric acid were prepared, then further diluted to 1 ng/ml in 0.01 *N* hydrochloric acid for use as the internal standard.

Standard curves in plasma were prepared containing five concentrations of spiked samples: 25, 50, 100, 200 and 400 ng/ml of amitriptyline and nortriptyline and 12.5, 25, 50, 100 and 200 ng/ml of each of the 10-hydroxy isomers of amitriptyline and nortriptyline. Each set of standards included a blank.

Extraction

To 1 ml of plasma standard or unknown sample, 100 μl (100 ng) of internal standard loxapine and 0.5 ml of 0.5 *N* sodium hydroxide were added to specially washed glassware. Eight millilitres of 1.5% (v/v) isoamyl alcohol in *n*-heptane were added and the mixture was shaken for 15 min and centrifuged at 1500 *g* for 10 min. The organic layer was then transferred to a 15-ml tapered centrifuge tube containing 1.2 ml 0.1 *N* hydrochloric acid. After mixing for 10

TABLE I

RECOVERY OF AMITRIPTYLINE AND METABOLITES FROM 1 ml PLASMA (n = 7)

	5 ng		25 ng		50 ng		200 ng		400 ng	
	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)
trans-10-OH-NOR	56	10.4	66	4.7			58	5.4		
trans-10-OH-AMI	84	7.0	89	4.8			91	5.8		
cis-10-OH-NOR	65	7.6	79	9.0			66	5.8		
cis-10-OH-AMI	94	4.9	91	5.4			92	4.3		
NOR	63	9.0			79	5.1			78	5.0
AMI	94	4.2			99	5.2			91	3.5

TABLE II

WITHIN-RUN PRECISION OF ASSAY BASED UPON PEAK-HEIGHT RATIOS AT VARIOUS CONCENTRATIONS (n = 5)

	5 ng/ml		25 ng/ml		50 ng/ml		100 ng/ml		200 ng/ml		400 ng/ml	
	Pk.ht. ratio	C.V. (%)	Pk.ht. ratio	C.V. (%)	Pk.ht. ratio	C.V. (%)	Pk.ht. ratio	C.V. (%)	Pk.ht. ratio	C.V. (%)	Pk.ht. ratio	C.V. (%)
trans-10-OH-NOR	0.10	8.9	0.35	6.7	0.95	0.8	2.01	6.4	4.08	7.3		
trans-10-OH-AMI	0.06	8.8	0.27	6.7	0.61	1.5	1.30	6.2	2.65	6.9		
cis-10-OH-NOR	0.08	6.9	0.30	9.4	0.65	3.0	1.37	7.0	2.77	6.7		
cis-10-OH-AMI	0.08	6.3	0.38	7.5	0.80	1.8	1.67	6.3	3.37	6.3		
NOR	0.05	6.4	0.24	3.3	0.38	6.4	0.89	6.4			3.40	6.2
AMI	0.04	7.3	0.20	5.0	0.35	1.9	0.78	6.9			3.05	5.6

min and centrifuging at 1500 *g* for 10 min, the organic layer was aspirated, the aqueous portion transferred to a 3-ml tapered glass-stoppered minicentrifuge tube and neutralized with 0.5 ml 0.5 *N* sodium hydroxide. One half (0.5) millilitre 1.5% isoamyl alcohol in *n*-heptane was added and the tube stoppered, shaken for 10 min and centrifuged for 5 min at 1500 *g*. The lower aqueous layer was discarded and the organic layer transferred to a 1.0-ml Reactivial (Pierce, Rockford, IL, U.S.A.). The contents were evaporated to dryness in a Model SVC-100 M Speed Vac Concentrator (Savant Instruments, Hicksville, NY, U.S.A.) at 45°C. The extract was then reconstituted with 100 µl of mobile phase, capped and mixed.

Chromatographic conditions

The mobile phase consisted of 0.1 *M* acetate buffer (pH 4.2)—acetonitrile (70:30) with 0.005 *M* heptanesulfonate and 0.01 *M* triethylamine added. The mixture was filtered and degassed prior to use. The flow-rate was 1.8—2.0 ml/min and the temperature ambient. The effluent was monitored through a UV detector at 254 nm.

Quantitation

All determinations were performed by calculating the peak height and/or area ratios of each compound to the internal standard. A linear regression analysis for each of the standard curves was performed by a computer program resulting in the calculation of slope, *x*-intercept, correlation coefficient, and standard errror of this estimate.

RESULTS AND DISCUSSION

The report describes the simultaneous determination of AMI and five metabolites by liquid chromatography with UV detection. This method is able to separate and quantitate the *cis* and *trans* isomers of 10-OH-AMI and 10-OH-NOR. A sample chromatogram appears in Fig. 2. Peak symmetry was enhanced by the addition of triethylamine. Otherwise, peak tailing and broadening occurs as well as loss of resolution among the *cis—trans*-hydroxy metabolites of AMI and NOR. Fig. 3 illustrates a chromatogram of a drug-free patient plasma. No interfering endogenous peaks were detected. This is a result of careful sample clean-up and the selectivity of this column.

The absolute recovery for all the compounds was checked at the sensitivity limit (5 ng/ml), at midpoint, and at the upper limit of linearity. The results are shown in Table I.

The precision of the reported procedures was determined by spiking 1-ml plasma aliquots of drug-free plasma with various concentrations of the 10-hydroxy metabolites of AMI and NOR and of AMI and NOR with the internal standard. The samples were processed as described. The resulting coefficients of variation for each compound appear in Table II. Day-to-day reproducibility for the method was assessed by analysis of the peak height ratios of each compound generated at each concentration point on the calibration curve. These results appear in Table III.

TABLE III

DAY-TO-DAY PRECISION OF THE ASSAY FOR AMI AND METABOLITES AT ALL CONCENTRATION POINTS ON THE LINEAR REGRESSION CURVE (n = 5)

	12.5 ng/ml		25 ng/ml		50 ng/ml		100 ng/ml		200 ng/ml		400 ng/ml	
	Pk.ht. ratio	C.V. (%)	Pk.ht. ratio	C.V. (%)	Pk.ht. ratio	C.V. (%)	Pk.ht. ratio	C.V. (%)	Pk.ht. ratio	C.V. (%)	Pk.ht. ratio	C.V. (%)
trans-10-OH-NOR	0.21	7.4	0.38	6.0	0.77	3.3	1.57	0.8	3.39	4.6		
trans-10-OH-AMI	0.13	12.9	0.28	8.7	0.55	5.2	1.15	5.7	2.43	5.8		
cis-10-OH-NOR	0.17	5.9	0.31	13.0	0.61	6.0	1.19	5.0	2.51	4.0		
cis-10-OH-AMI	0.18	7.3	0.36	7.7	0.72	3.5	1.49	3.1	3.12	5.2		
NOR			0.20	12.8	0.38	13.1	0.79	8.2	1.56	9.0	3.28	6.5
AMI			0.18	7.3	0.36	5.8	0.70	5.0	1.39	5.2	2.78	4.5

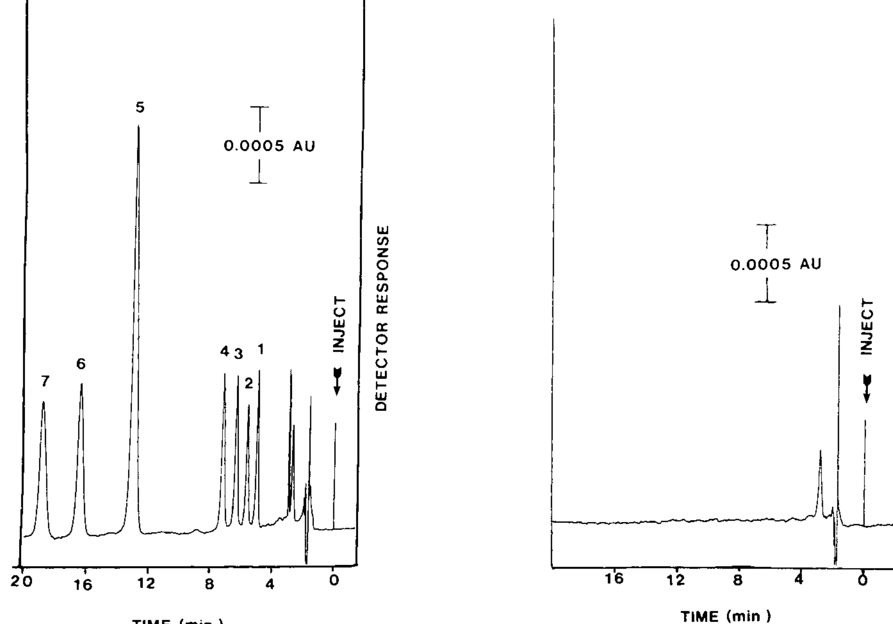


Fig. 2. Sample chromatogram of 1 ml plasma containing (1) *trans*-10-OH-NOR 25 ng, (2) *trans*-10-OH-AMI 25 ng, (3) *cis*-10-OH-NOR 25 ng, (4) *cis*-10-OH-AMI 25 ng, (5) loxapine 100 ng, (6) NOR 50 ng, and (7) AMI 50 ng. Chromatographic conditions as described in text. Entire reconstituted extract was injected.

Fig. 3. Sample chromatogram of 1 ml plasma blank. Entire reconstituted extract was injected.

Although loxapine was chosen as the internal standard for this method, several other readily available compounds such as doxepin, desmethyldoxepin or desmethylimipramine are among a few alternatives that were found to be suitable.

Because there are seven peaks of importance in this determination, the possibility of an interfering peak from another compound co-eluting is obviously greater than for a determination of one or two compounds. Probably the most frequently found interfering peak results from the presence of the benzodiazepines and their metabolites. For example, plasma from a patient receiving Limbitrol® (a combination drug containing chlordiazepoxide and amitriptyline) could not be analyzed for the 10-hydroxy metabolites of AMI because chlordiazepoxide and its three major metabolites (N-desmethylchlordiazepoxide, demoxapam, and desmethyldiazepam) co-extract, have similar retention times to the 10-hydroxy metabolites and are readily detected at 254 nm. A prior knowledge of the patient medication profile may help to eliminate these interferences by minor adjustments in the chromatographic conditions.

The detection limit for AMI and all of its metabolites in this assay is ca. 5 ng/ml. The detector response remained linear from 5 ng to about 500 ng, far exceeding the normal therapeutic range for these compounds.

The question of the stability of the 10-hydroxymetabolites of AMI and NOR

during the sample processing was resolved by subjecting 100 ng of each isomer of both hydroxy metabolites to separate extractions. The purpose was to determine if there was any conversion of the geometric isomers from one to another. Only one peak occurred from each hydroxy compound after routine processing thus indicating no conversion.

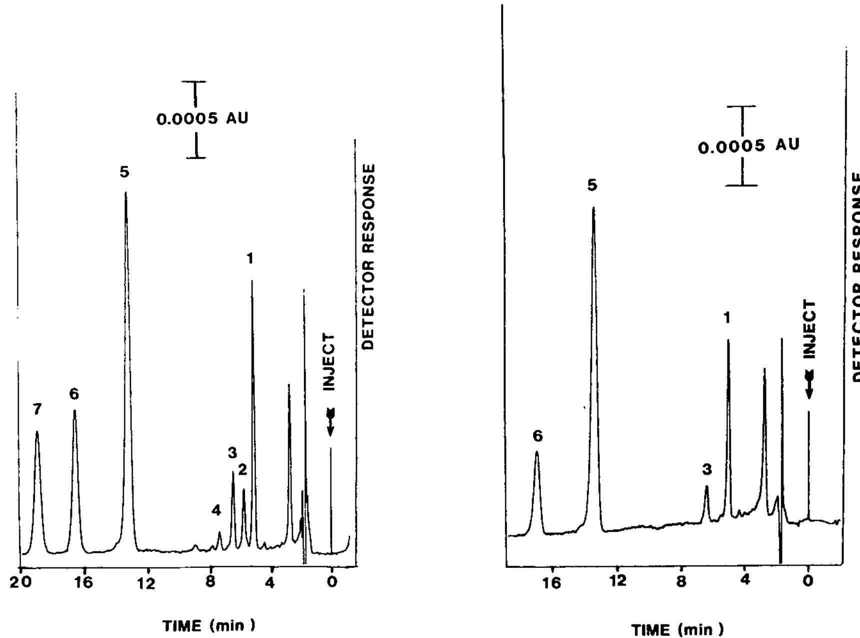


Fig. 4. Sample chromatogram of a 1-ml plasma sample from a patient receiving AMI 150 mg daily for 6 weeks. Concentrations were found to be (1) *trans*-10-OH-NOR 46 ng, (2) *trans*-10-OH-AMI 13 ng, (3) *cis*-10-OH-NOR 17 ng, (4) *cis*-10-OH-AMI trace (<5 ng/ml), (5) loxapine 100 ng, (6) NOR 51 ng, and (7) AMI, 51 ng. Chromatographic conditions as in Fig. 2.

Fig. 5. Sample chromatogram of a 1-ml plasma sample from a patient receiving NOR 75 mg daily for 2 days. Levels were found to be (1) *trans*-10-OH NOR 37 ng, (3) *cis*-10-OH NOR 11 ng, (5) loxapine 100 ng, and (6) NOR 40 ng. Chromatographic conditions as in Fig. 2.

A chromatogram of a plasma sample of an actual steady state patient receiving AMI is shown in Fig. 4. The concentration of *trans*-10-OH-NOR exceeds the concentration of both isomers of OH—AMI metabolites as well as the *cis* isomers of OH-NOR. This is generally the case as shown in Table IV. The concentrations of *trans*-10-OH-AMI are usually low in comparison with the *trans*-10-OH-NOR. The *cis*-10-OH-AMI isomer is found in even lower concentrations than the *trans* isomer or is not detected. This is in agreement with previous published data [9, 18, 20, 22]. Fig. 5 illustrates that patients receiving NOR only, can also be monitored for the two hydroxy isomers of NOR as well as the parent compound.

Since it is known that the unconjugated hydroxy metabolites of AMI and NOR are pharmacologically active, their measurement in plasma should be included in

TABLE IV

PLASMA LEVELS (ng/ml) OF AMI AND METABOLITES IN ELEVEN DIFFERENT PATIENTS RECEIVING AMI OR NOR

Subject	AMI	NOR	*trans*-10-OH-AMI	*cis*-10-OH-AMI	*trans*-10-OH-NOR	*cis*-10-OH-NOR
1	85	98	T**	ND***	25	14
2	80	48	20	ND	42	9
3	40	19	17	5	25	7
4	51	51	13	T	46	17
5*		159			93	31
6	163	143	37	10	231	62
7	65	114	13	ND	161	35
8	231	251	53	13	293	83
9	48	36	22	ND	64	7
10*		50			56	17
11*		51			43	15

*Patient receiving NOR only.
**T = Level < 5 ng/ml.
***ND = No detectable levels.

the evaluation of clinical response vs. plasma concentrations. This method permits the clinician to determine the individual patient's metabolic profile with respect to AMI and/or NOR hydroxylation.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF PIPOTIAZINE IN HUMAN PLASMA AND URINE

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SUMMARY

A high-performance liquid chromatographic method has been developed for the determination of pipotiazine in human plasma and urine. After selective extraction, pipotiazine and the internal standard (7-methoxypipotiazine) are chromatographed on a column packed with Spherosil XOA 600 (5 μm) using a 7:3 (v/v) mixture of diisopropyl ether—isooctane (1:1, v/v) + 0.2% triethylamine and diisopropyl ether—methanol (1:1, v/v) + 0.2% triethylamine + 2.6% water. The eluted compounds are measured by fluorescence detection. The sensitivity of the method was established at 0.25 ng/ml pipotiazine in plasma and 2 ng/ml pipotiazine in urine (C.V. < 5%). The method has been successfully applied to a pharmacokinetic study following a single oral administration of 10 mg of pipotiazine.

INTRODUCTION

Pipotiazine, 10-{3-[4-(2-hydroxyethyl)-1-piperidinyl]propyl}-N,N-dimethyl-10H-phenothiazine-2-sulphonamide (Fig. 1,I), is a polyvalent neuroleptic according to Deniker's classification [1]. Therapeutically it is available in the form of the hydrochloride, for oral use to produce rapid action (Piportil®) and as the undecylenic ester (Piportil M_2®) or palmitic ester (Piportil L_4®), all from Laboratoires Specia, Paris, France, for use via the intramuscular route to produce a long-lasting action.

The scarcity of pharmacokinetic data concerning this drug in man is attributable to the lack of analytical techniques of sufficient sensitivity and specificity to be able to assay pipotiazine in biological media, notably in the blood or urine of patients under treatment with the drug.

Hitherto, only two methods have been available. The first [2] is based on tritium-labelling of the molecule, and is of limited application; the second [3]

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Fig. 1. Structures of pipotiazine (I), and 7-methoxypipotiazine (II; internal standard).

is a gas chromatographic method which is sufficiently sensitive to assay pipotiazine during a course of repeated administration of the long-acting esters.

In this paper, we suggest a high-performance liquid chromatographic assay technique with a sensitivity and selectivity which enable it to monitor the pharmacokinetics of the unchanged pipotiazine both after a single dose and during a course of treatment using usual therapeutic dose schedules for both the oral form and the long-acting injectable form. An example of the application of this technique in a pharmacokinetic study in the healthy subject after the administration of 10 mg of pipotiazine is presented.

EXPERIMENTAL

Chemical and reagents

Apart from pipotiazine itself (I) and 7-methoxypipotiazine (II) as an internal standard (Fig. 1), the following compounds which are known as the three main metabolites of pipotiazine in urine were used in this study to check the selectivity of the technique: pipotiazine sulfoxide, pipotiazine N-oxide and 7-hydroxypipotiazine. All the standard solutions were made up in dilute hydrochloric acid (10^{-3} *M*) and kept at 4°C away from direct light. Such solutions are stable for at least a month.

The pH 10 buffer (Titrisol; Merck, Darmstadt, G.F.R.), four times the usual strength, was chosen according to the best yields of extraction. The solvents — *n*-heptane, dichloromethane, diethyl ether and isoamyl alcohol — were all of analytical quality.

The mobile phase for the chromatography was a mixture of the isohydric solvents A and B (7:3, v/v) suggested by Thomas et al. [4], having the follow-

ing compositions. Solvent A: diisopropyl ether (Chromosol; SDS, Peypin, France), 50 volumes, isooctane (Chromosol; SDS), 50 volumes; triethylamine (Merck), 0.2%. Solvent B: diisopropyl ether (Chromosol; SDS), 50 volumes; methanol (Prolabo, Paris, France), 50 volumes; triethylamine (Merck), 0.2%; water, 2.6%.

Apparatus

The high-performance liquid chromatographic apparatus used comprised a Chromatem 380 pump, a Touzart et Matignon pulsation damper, a Waters automatic injector (WISP 710 B), a Schoeffel spectrofluorimeter (F 5970) and a Kipp and Zonen (BD 8) recorder. The column (10 cm × 4.6 mm I.D.) was prepared with Spherosil (XOA 600 5 μm; Prolabo) under a pressure of 400 bars.

The mass spectrometer used to check the selectivity of the assay was a Finnigan 4000 coupled with a gas chromatographic unit and computerised.

Extraction procedure

Plasma. A 2-ml sample of plasma and 0.5 ml of the aqueous solution of the internal standard, containing either 20 ng or 10 ng of 7-methoxypipotiazine depending on the concentration to be determined, are measured into a 20-ml conical centrifuge tube fitted with a ground-glass stopper and containing 1 ml of pH 10 buffer and 10 ml of diethyl ether—dichloromethane (2:1, v/v). The stoppered tube was shaken for 15 min on an Infors shaker, and then centrifuged at 3000 *g* for 10 min. The organic phase was transferred to a 10-ml conical glass tube and then evaporated to dryness under a stream of nitrogen at 40°C.

Urine. A 2-ml sample of urine, diluted if necessary, was treated as above, but in this case the solvent mixture used was *n*-heptane—isoamyl alcohol (95:5, v/v). The organic phase was transferred to a centrifuge tube containing 1 ml of 0.1 *N* sodium hydroxide solution. The tube was stoppered, shaken for 1 min and centrifuged as before; the organic phase was evaporated to dryness under a stream of nitrogen at a temperature of 40°C.

CHROMATOGRAPHY

The evaporation residues were dissolved in 200 μl of the mobile phase and placed on the revolving platform of the automatic injector set for injections of 50—200 μl depending on the concentrations of pipotiazine expected. Figs. 2 and 3 show some examples of chromatograms obtained with a flow-rate of 1 ml/min, a recorder chart-speed of 2 mm/min, and wavelengths of 270 nm for excitation and 470 nm for emission. The choice of wavelengths is based on the UV spectral data of pipotiazine and the fluorescence maximum. The capacity ratio values were 7.5 for pipotiazine and 9 for the internal standard (retention times: 10.5 min, and 13.5 min, respectively). The average length of chromatographic run was 15 min. The threshold of detection estimated as twice the background was about 0.10 ng/ml (sensitivity of the detector: about 0.20 ng).

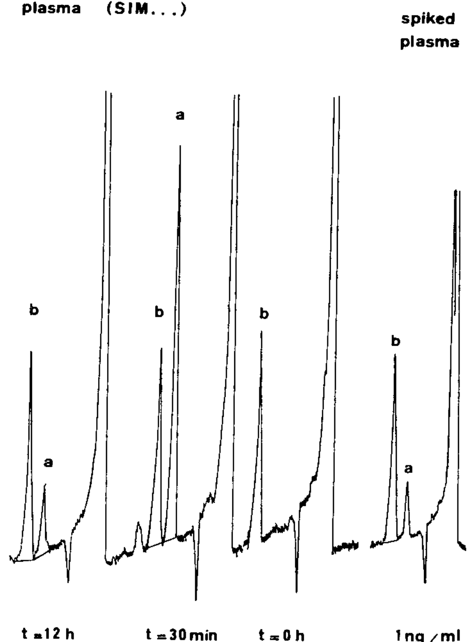


Fig. 2. Right: chromatogram obtained with plasma sample spiked with pipotiazine. Left: chromatograms of plasma sample extracts from a healthy subject (SIM) following oral administration of 10 mg of pipotiazine (sampling times: 0, 0.50, 12 h). Peaks: a = pipotiazine; b = internal standard.

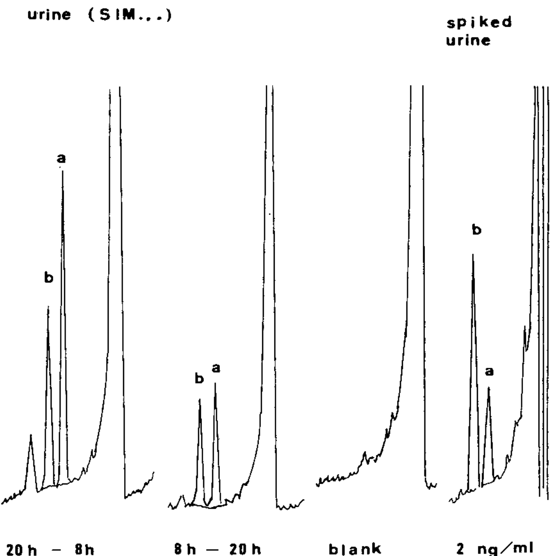


Fig. 3. Right: chromatogram obtained with urine sample spiked with pipotiazine and of a urine blank. Left: chromatograms of urine sample extracts from a healthy subject (SIM) following oral administration of 10 mg of pipotiazine (urine collected between 0 and 12 h, and between 12 and 24 h after administration). Peaks: a = pipotiazine; b = internal standard.

The plasma or urinary concentrations of pipotiazine were calculated from fluorimetric results, the appropriate peak height ratios being compared with a calibration curve.

The calibration curves for plasma and urine, calculated by linear regression from the data of six assays for each concentration of pipotiazine added, are as follows.

Plasma: $Y = -0.0030 + 0.2549X$, $r = 0.9982$
Confidence intervals at 95%: ordinate at the origin = -0.0030 ± 0.00431; slope = 0.2549 ± 0.0049.

Urine: $Y = -0.0346 + 0.2230X$, $r = 0.9989$
Confidence intervals at 95%: ordinate at the origin = -0.0346 ± 0.0543; slope = 0.2230 ± 0.0047.

Precision and accuracy of the method

The reliability characteristics of the method are given in Table I.

The scatter of concentration of pipotiazine used in constructing the calibration curves was evaluated by means of the method of variation coefficients. The sensitivity threshold of the method was established at 0.25 ng pipotiazine per ml of plasma; at this level, the variation coefficient is 11%. At higher plasma levels the coefficients are below 10%. Study of the scatter of urine determinations between 2 and 20 ng pipotiazine indicated that the variation coefficients were below 5%.

The average divergence between the amount of pipotiazine actually added

TABLE I

PRECISION AND RECOVERY IN THE DETERMINATION OF PIPOTIAZINE IN SPIKED HUMAN PLASMA AND URINE SAMPLES

Sample volume = 2 ml, for both urine and plasma. n = 6 in all cases.

Pipotiazine added (ng/ml)	Pipotiazine recovered (ng/ml)	S.D.	C.V. (%)	Absolute recovery (%)	Precision (%)
Plasma					
0.25	0.28	0.03	11.00	112.0	+12.0
0.50	0.53	0.02	4.04	106.0	+6.0
1.00	1.14	0.07	6.05	114.0	+14.0
2.00	2.03	0.04	1.91	101.5	+1.5
5.00	4.64	0.33	7.21	92.8	−7.2
10.0	10.11	0.15	1.46	101.1	+1.1
20.00	20.03	1.03	5.12	100.2	+0.2
Urine					
2.0	2.1	0.1	4.3	105	+5
5.0	4.9	0.0	0.9	98	−2
10.0	10.0	0.2	2.0	100	0
20.0	20.0	0.7	3.4	100	0

and the measurement obtained (accuracy) was 3.9 ± 7.3% for plasma and 1 ± 3% for urine. The average recovery of pipotiazine added was 104 ± 7% for plasma and 100 ± 3% for urine.

Selectivity

The selectivity of the method was established first of all with regard to the major known metabolites of pipotiazine in man (7-hydroxy, sulphoxide and N-oxide derivatives) [5]. In fact, as can be seen from the chromatogram (Fig. 4) obtained by direct injection of large amounts, greater than 1 μg, of these metabolites in 1 ml of the mixture of solvents A and B, the retention times of the metabolites (23.75 min, 67 min and 126.5 min, respectively) differed widely from those of pipotiazine and internal standard. Furthermore, under the conditions used for extraction of pipotiazine and internal standard from plasma and urine samples of a healthy subject, following oral administration of 10 mg of pipotiazine, no interference was observed. Therefore, because of the lack of interference with the assay of drug, injections were made every 15 min.

The selectivity of the method was also checked with regard both to minor metabolites and to unidentified impurities. With this aim, a urinary extract from a patient who had received a 10-mg oral dose of pipotiazine was tested on a mass spectrometer after chromatography. The experimental conditions were: electronic ionization 70 eV, temperature of the source 200°C, temperature of the probe 280°C. Comparison of the spectrum obtained with the reference spectrum (spectrum No. 143 An Pci) showed them to be identical and with the same *m/e* ratio: 142 (main peak), 170, 246, 345, 367, 475 (molecular peak).

Samples containing 3.25 ng/ml pipotiazine were kept away from the light at −20°C for three months; no degradation of the pipotiazine was observed.

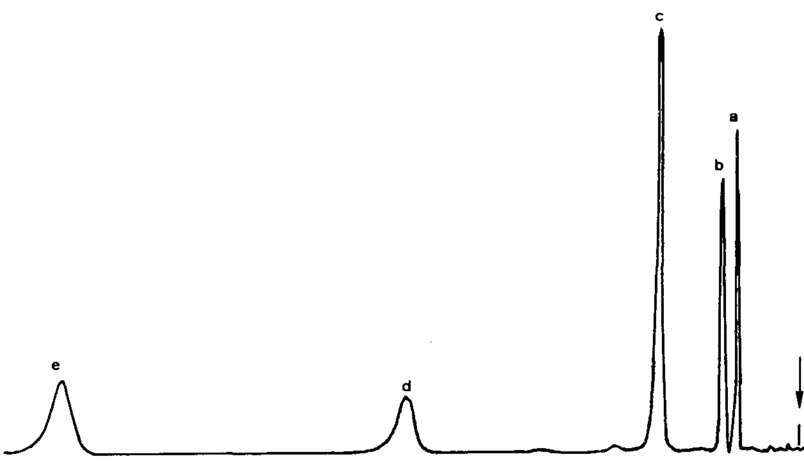


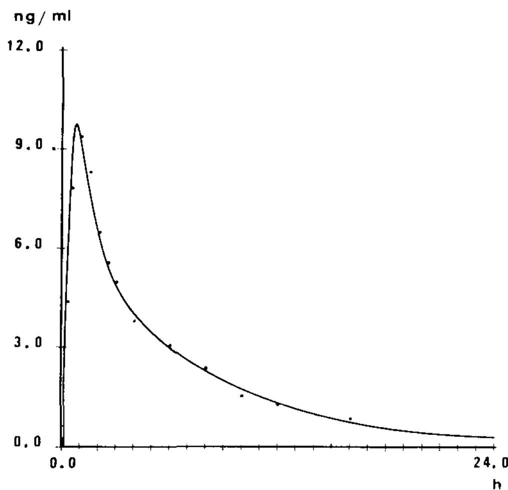
Fig. 4. Chromatogram of the major known metabolites of pipotiazine in man obtained by direct injection in the mixture of solvents A and B. Peaks: a = pipotiazine; b = internal standard; c = 7-hydroxypipotiazine; d = pipotiazine sulfoxide; e = pipotiazine N-oxide.

VALIDATION OF THE METHOD: PHARMACOKINETIC PROFILE IN MAN

Using the method suggested, the plasma levels and urinary excretion of pipotiazine were monitored in a healthy subject who had received 10 mg of Piportil® in solution form. Samples were taken at time 0 (just before administration of the drug), then at 0.25, 0.50, 0.75, 1.00, 2.00, 2.50, 3.00, 4.00, 6.00, 8.00, 10.00, 12.00, 16.00 and 24 h after ingestion of the solution. Blood samples (10 ml) were collected over heparin and, after centrifugation, the plasma was immediately frozen and stored at −20°C until analysis. Urines were collected during 24 h before, then between 0 and 12 h, and between 12 and 24 h, after administration of the drug. All determinations were performed in duplicate.

The chromatograms obtained from the plasma samples taken before, then 30 min and 12 h after administration are shown in Fig. 2; Fig. 3 shows those obtained from the urine samples collected before, then during, the two intervals after administration.

The curve of plasma concentration of unchanged pipotiazine versus time after a single oral administration of 10 mg of pipotiazine shown in Fig. 5 gives both plasma levels measured and the best fit to the experimental data [6, 7]. These data were recently checked in a pharmacokinetic study carried out on five healthy subjects who had also ingested 10 mg of pipotiazine [8]; the plasma concentration peak (about 10 ng/ml) achieved at 0.78 h after oral intake is followed by a biphasic decreasing curve ending approximately 24 h



equation of the best fitting curve :

$$C(t) = 6.6845 * \exp(-0.1362 * t) + 16.9342 * \exp(-1.3691 * t) - 23.6183 * \exp(-3.0353 * t)$$

lag t. = 0.15 h

Fig. 5. Pharmacokinetic profile of pipotiazine in a healthy subject treated with 10 mg of pipotiazine. (■) experimental data points; (——) simulated curve.

later. The amount of unchanged pipotiazine excreted in the urine is about 1% of the administered dose.

The proposed method for the high-performance liquid chromatographic assay of pipotiazine would therefore seem to be highly suitable to study clinical pharmacokinetics and bioavailability of Piportil® in its various presentations.

ACKNOWLEDGEMENTS

The authors express their gratitude to the clinical team (Professor Soulayrac, Hôpital Sainte Anne, Paris, France) responsible for the clinical assay. They also thank Mrs. Nicole Vivier for her technical assistance.

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Note

High-performance gel permeation chromatography of collagens

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Gel permeation chromatography appears to be a widely used tool in the characterization of different collagen polypeptide chains [1,2]. Considerable progress in the efficiency of separation of interstitial collagens was achieved by introducing reversed-phase high-performance liquid column chromatography (HPLC). Fallon et al. [3] used bonded cyanopropyl support columns for the separation of human type I, II and III collagens. In a previous report [4] we attempted to separate collagen type I and III polypeptide chains by means of HPLC using Separon HEMA 1000 Glc gel. Though a good quality of separations was achieved, the process was not governed solely by gel permeation as long as molecular entities of identical relative molecular mass were separated. This may be of advantage in the separation of certain collagen mixtures, but causes considerable difficulties when gel permeation separations are used for the investigation of complex mixtures of different collagen polypeptide chains and their fragments. We attempted, therefore, to abolish the secondary interactions during separation as much as possible and to establish a high efficiency procedure in which gel permeation would be the only mechanism involved.

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EXPERIMENTAL

Chromatographic techniques

Chromatography was carried out using a Pye-Unicam liquid chromatograph LC 20 equipped with UV detector LC 3 set at 230 nm. A stainless-steel column, 500 × 8 mm, packed with Separon HEMA 1000 Glc (12–17 μm; Laboratory Instrument Works, Prague, Czechoslovakia) was used. The apparatus was operated at a flow-rate of 1.5 ml/min (1.5 MPa overpressure). The sensitivity of the detector was set at 0.04, chart speed was 0.25 cm/min. The whole separation lasted less than 30 min. Several solvent systems were checked to obtain maximum separation according to relative molecular mass of individual polypeptide chains. Finally, isocratic elution with a solution containing 0.2 *M* NaCl—2*M* urea—0.05 *M* Tris · HCl buffer (pH 7.5) was used.

Collagen standards

Samples of individual types of collagen polypeptide chains were prepared by established methods from rat skin, bovine renal glomerular basement membrane and mouse EHS sarcoma. Detailed procedures can be found in the original literature listed in Table I.

In order to avoid problems arising from the high UV absorbancy of mercaptoethanol in UV light, S—S bonds were cleaved, where necessary, with concentrated formic acid, and to that 30% hydrogen peroxide was added to a final concentration of 2%. The reaction mixture was left for 2 h at room temperature and then loaded onto the column.

Individual isolated collagen polypeptide chains were checked for purity by their amino acid composition [10], sodium dodecyl sulphate (SDS)—polyacrylamide slab gel electrophoresis [11], and those originating from basement membranes also by immunoprecipitation test [12].

RESULTS AND DISCUSSION

The applicability of the present procedure is demonstrated by the following examples. Clear-cut separations are obtained with α-chain polymers (Fig. 1) and rapid information can be obtained about S—S bond cleavage in collagen type III (Fig. 2). The separation efficiency is sufficient to distinguish between α_1 (IV) and α_2 (IV) collagen polypeptide chains, a result that has not before been visualized by gel permeation chromatography (Fig. 3).

A general picture of the retention times of individual collagen species and some of their fragments is given in Table I. The retention times decrease with increasing relative molecular mass; however, the decrease is not strictly linear in the logarithmic scale as would be expected (Fig. 4). No separation of collagen polypeptide chains of identical relative molecular mass but originating from different collagen types was observed. It has been communicated before that sorption plays an important role in high-efficiency gel permeation chromatography [4]. Indeed, in our previous communications we have observed the separation of, for example, α_1 (I) and α_1 (III) on the same sorbent that has been used in the present experiments when isocratic elution with 0.05 *M* Tris · HCl, pH 7.5 (2 *M* with respect to urea) was used. This was ascribed either to differences in

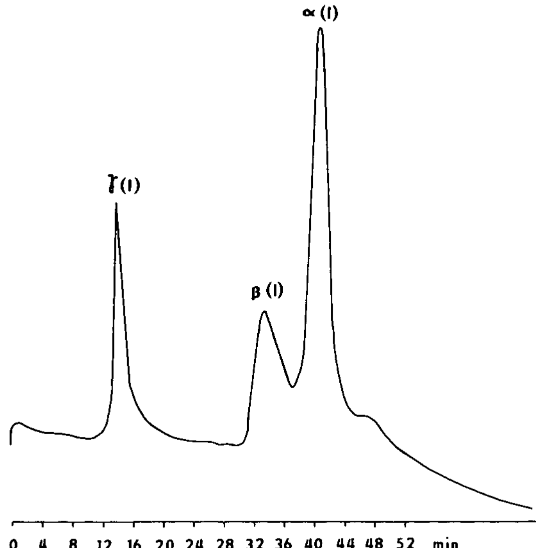

Fig. 1. HPLC separation of collagen type I α-chain polymers.

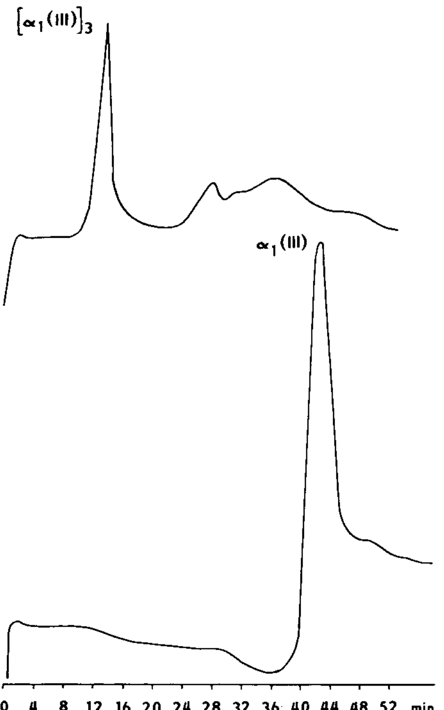

Fig. 2. Chromatographic behaviour of collagen type III preparation before (upper panel) and after (lower panel) S—S bond cleavage.

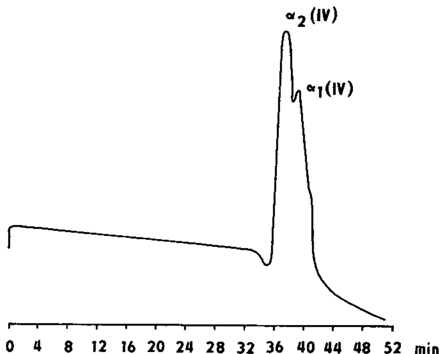

Fig. 3. Separation of α_1 (IV) and α_2 (IV) collagen polypeptide chains.

TABLE I

RETENTION TIMES OF DIFFERENT COLLAGEN CHAINS, THEIR POLYMERS AND FRAGMENTS

Retention times represent data obtained from five independent runs of the same preparation. The α_1(I) polypeptide chain served as internal standard.

	Type of collagen chain	Relative molecular mass	Retention time (min)	Principle of preparation	Literature reference (description of the preparation procedure)
1	α_1(I), α_1(II), α_1(III)	100,000	42.0	Rat skin, cartilage and calf skin, limited pepsin digestion	2
2	α_1(IV)	140,000	38.5	Mouse tumor and human placenta, limited pepsin digestion	5
3	α_2(IV)	160,000	37.0		
4	β(I)	200,000	34.0	Human placenta, limited pepsin digestion	6
5	γ(I)	300,000	14.0		
6	$[\alpha_1(III)]_3$	300,000	14.0		1
7	α_1(IV) BM	160,000	37.0	EHS tumor, limited pepsin digestion	7
8	α_2(IV) BM	180,000	34.5		
9	C_1 fragment	120,000 (110,000 to 140,000)	41.0	Minces of whole placental tissues, limited pepsin digestion	8
10	C fragment	95,000	43.0		
11	50 K fragment	50,000	50.0		
12	α_{1-3}(V)	110,000	41.5	Human placenta, limited pepsin digestion	9
13	7 S	360,000	4.0	Mouse tumor basement membrane, limited pepsin digestion	6
14	7 S coll	225,000	29.0		

hydrodynamic volumes of matching collagen polypeptide chains originating from different species [1], or adsorption and/or partition interactions of the separated protein molecules [2], or, finally, to the possible weak affinity of

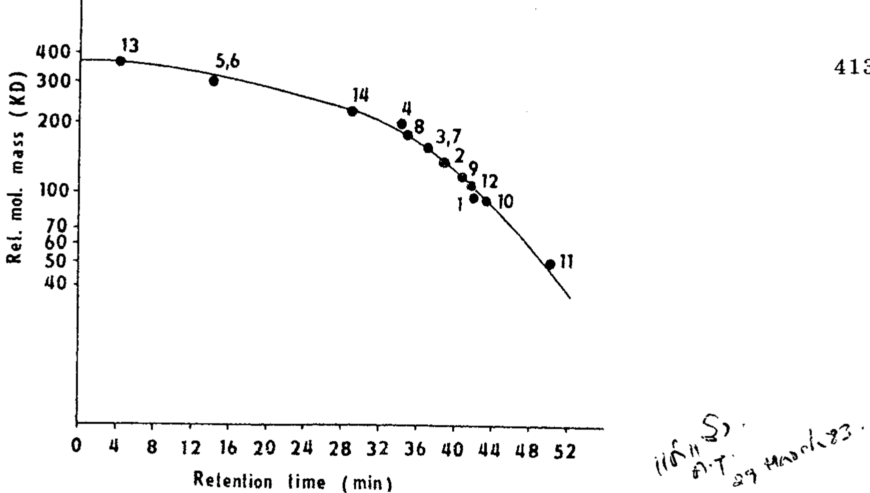

Fig. 4. Retention time vs. relative molecular mass relation for different polypeptide chains of the collagen family. Numbers correspond to listing of individual polypeptides in Table I.

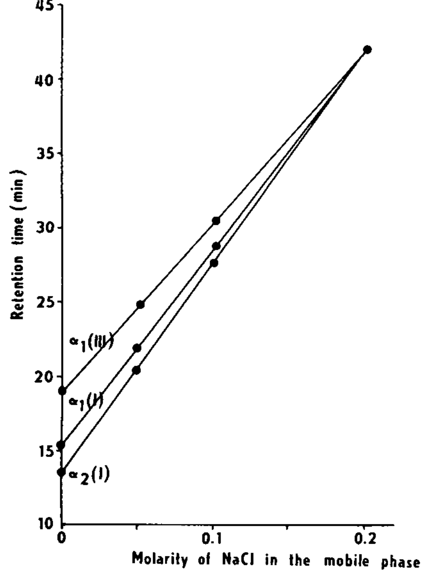

Fig. 5. Changes in retention of α_1(I), α_2(I) and α_1(III) with increasing NaCl molarity in the mobile phase. (Note that the three separated collagen α-chains are of identical relative molecular mass.)

collagen chains to the glucose-coated macroporous adsorbent causing respective retention differences of otherwise similar molecules.

It is now evidenced (Fig. 5) that an increase in ionic strength of the eluant is capable of completely abolishing the interspecies differences at least between collagen I, III, IV and V. The same result can be achieved either by adding sodium chloride to the eluting solvent or by increasing the concentration of the Tris buffer to 0.5 *M*. The separation conditions can be selected in such a way that gel permeation is the only mechanism governing the separation. Still the non-linearity of the retention time vs. log mol. wt. relation indicates that some other effects, though minimalized, still persist throughout the separation effected on Separon HEMA 1000 Glc.

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Note

Rapid and sensitive sialidase assay by high-performance liquid chromatography and its application to detection of sialidase in human urine

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(First received January 19th, 1982; revised manuscript received February 15th, 1982)

Sialidases are widely distributed in microorganisms and animal tissues [1]. The enzyme activity is assayed by measuring sialic acid or aglycone released from the substrate. Sialic acid is determined by the thiobarbituric acid assay [2, 3] or the more sensitive fluorometric thiobarbituric acid assay, which can detect 30 pmol of sialic acid [4]. The method used for determining aglycone depends on the substrate. 4-Methylumbelliferone N-acetylneuraminic acid ketoside has been used as a substrate for a convenient fluorometric assay of the aglycone [5]. However, the activity of sialidase may be affected by the aglycone, 4-methylumbelliferone, which is very bulky and hydrophobic. Modified natural substrates with tritium-labeled aglycones have also been used, and the aglycones released were separated from unhydrolyzed substrates by anion-exchange resin chromatography or thin-layer chromatography and then measured by scintillation spectrometry [6, 7]. Although the radiometric assay can detect amounts of product in the order of 10 pmol [7], the method for separating aglycones from unhydrolyzed substrates is time-consuming and special cautions are necessary on handling radioactive materials. Recently, Koseki et al. [8] used a fluorogenic substrate, α-D-N-acetylneuraminyl-β-D-galactopyranosyl-(1→4)-1-deoxy-1-[(2-pyridyl)-amino]-D-glucitol, which was derived from sialyllactose, for a fluorometric sialidase assay in which the fluorescent aglycone released was separated from the enzymatic reaction mixture by paper electrophoresis and extracted from the paper for quantitative measurement. The substrate is easier to handle than radioactive substrates, but the assay is not so sensitive and is time-consuming. The present report describes an improvement of the method of Koseki et al. using high-performance liquid chromatography (HPLC). Using this method, we detected sialidase for the first time in human urine.

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MATERIALS AND METHODS

Materials

Sialidase from *Clostridium perfringens* (0.5 units/mg) was purchased from P-L Biochemicals (Milwaukee, WI, U.S.A.). N-Acetylneuramin-lactose, consisting of 85% N-acetylneuraminyl-(2→3)-lactose and 15% N-acetylneuraminyl-(2→6)-lactose, was obtained from Sigma (St. Louis, MO, U.S.A.). The substrate in the sialidase assay, α-D-N-acetylneuraminyl-(2→3)-β-D-galactopyranosyl-(1→4)-1-deoxy-1-[(2-pyridyl)amino]-D-glucitol (PA-sialyllactose), was prepared by a slight modification of the method of Koseki et al. [8]. N-Acetylneuramin-lactose (17 mg) was dissolved in 0.7 ml of 2-aminopyridine reagent (prepared by mixing 830 mg of 2-aminopyridine, 320 mg of sodium cyanoborohydride, 0.37 ml of acetic acid, 3 ml of N,N′-dimethylformamide, and 0.3 ml of water), and then the mixture was heated at 80°C for 1.5 h. Water (4 ml) was then added and the mixture was applied to a Sephadex G-15 column (212 × 1.9 cm) equilibrated with 0.01 *M* ammonium bicarbonate and eluted with the same solution. The elution was monitored by the anthrone—sulfuric acid method [9] and by measuring the absorbance at 320 nm due to the pyridylamino derivative of the sugar. The 2→6 isomer of the pyridylamino derivative was eluted slightly faster than the 2→3 isomer. A chromatographically pure substrate was obtained by further purification of the substrate by HPLC in the manner described under "Sialidase assay". β-D-Galactopyranosyl-(1→4)-1-deoxy-1-[(2-pyridyl)amino]-D-glucitol (PA-lactose) and 1-deoxy-1-[(2-pyridyl)amino]-D-glucitol (PA-glucose) were prepared from lactose and glucose, respectively, by the method used for PA-sialyllactose.

Sialidase assay

A mixture of 15 μl of 0.5 m*M* PA-sialyllactose in 0.1 *M* sodium acetate buffer (pH 5.0) and 30 μl of sialidase preparation was incubated at 37°C for an appropriate period. Then 20 μl of the mixture were subjected to HPLC analysis to determine the amount of PA-lactose released by sialidase. The chromatograph used was a Gaschro-Kogyo Model 570B equipped with a stainless-steel column (300 × 4 mm) packed with TSK-Gel LS 410 (5 μm, C_{18}; Toyo-Soda Co, Tokyo, Japan). The detector was an Hitachi fluorescence spectrophotometer, Model 650-10M. The wavelengths of excitation and emission were 320 nm and 400 nm, respectively. Elution was carried out with 0.1 *M* acetic acid at a flow-rate of 1.6 ml/min at room temperature. The amount of PA-lactose released was calculated from its peak area on the chromatogram.

RESULTS AND DISCUSSION

Separation of PA-lactose and PA-sialyllactose by HPLC

PA-lactose and PA-sialyllactose were separated by HPLC under the conditions described under Materials and methods. As shown in Fig. 1, the separation of these substances was complete within 5 min. The elution position of PA-glucose completely overlapped that of PA-lactose under the conditions used. Therefore, sialidase activity can be determined even if the sample con-

tains β-galactosidase. It is possible to determine as little as 0.2 pmol of PA-lactose by this method.

Determination of sialidase

To examine the availability of the present method, we incubated PA-sialyllactose (0.167 m*M*) with various amounts of sialidase (27-133 ng/ml) from *Cl. perfringens* in 0.1 *M* sodium acetate buffer (pH 5.0) at 37°C for 15 min, and then analyzed the reaction mixtures by HPLC as described under Materials and methods. The amount of PA-lactose released was proportional to the amount of the enzyme [PA-lactose released (pmol/ml) = 0.556 × sialidase concentration (ng/ml)]. The sensitivity of the present method for sialidase assay was about two orders of magnitude greater than those of other methods so far reported.

The K_m value of the sialidase for PA-sialyllactose at pH 5.0 and 37°C was calculated to be 50 μM from plots of $\frac{s}{v} - s$ [10]. The enzyme concentration was 0.96 μg/ml, and the substrate concentration was varied from 15 to 74 μM. It is noteworthy that the K_m value was much lower than that for sialyllactose, which was reported to be 2.4 m*M* [11].

Sialidase activity in human urine

This sensitive method for sialidase assay was applied to the detection of sialidase activity in human urine. Urine from an adult person was dialyzed

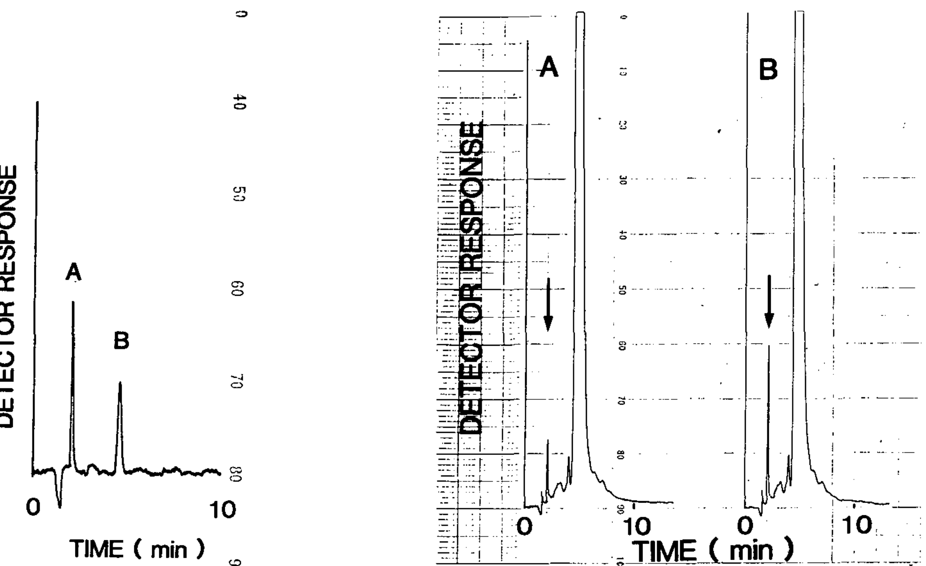

Fig. 1. Separation of PA-lactose and PA-sialyllactose by HPLC. HPLC was carried out as described under Materials and methods. Peaks: (A) PA-lactose (0.6 pmol); (B) PA-sialyllactose (0.6 pmol).

Fig. 2. Chromatogram of the digest of PA-sialyllactose by urine. PA-sialyllactose (0.167 m*M*) was incubated with dialyzed urine in 0.1 *M* sodium acetate buffer (pH 5.0) at 37°C for 2 h. The reaction mixture was analyzed as described under Materials and methods. (A) Substrate + urine heated at 100°C for 10 min (control). (B) Substrate + urine. The arrows show the elution position of PA-lactose.

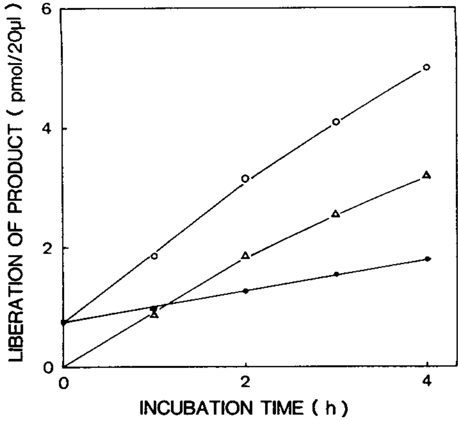

Fig. 3. Time course of hydrolysis of PA-sialyllactose by sialidase in urine. (•; *A*) PA-sialyllactose + urine heated at 100°C for 10 min. (○; *B*) PA-sialyllactose + urine. (△), *B* − *A*.

against 0.1 *M* sodium acetate buffer (pH 5.0) containing 0.0001% sodium ampicillin. The precipitate produced during dialysis was removed by centrifugation and the supernatant was used as enzyme solution. PA-sialyllactose was incubated with the dialyzed urine and the reaction mixture was analyzed as described under Materials and methods. The chromatogram of the reaction mixture after incubation for 2 h is shown in Fig. 2. The time course of the enzymatic reaction is shown in Fig. 3. Control experiments should be carried out because PA-lactose was produced during incubation of the substrate alone for many hours, as shown in Figs. 2A and 3. The decrease in activity during incubation for a long time is probably due to inactivation of the enzyme. The above result indicates the existence of sialidase in human urine, and illustrates the availability of the assay system.

The present assay using HPLC is more rapid and convenient and also more sensitive (1000-fold) than the assay method reported by Koseki et al. [8]. The sialidase assay for urine may be useful for diagnosis and investigation of the diseases associated with the enzyme, for example, sialidosis.

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Note

Improved method for the evaluation of 3-hydroxyproline and 4-hydroxyproline in the urine

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(First received January 12th, 1982; revised manuscript received February 8th, 1982)

The hydroxyprolines (Hyp) are amino acids characteristic of collagen. The most abundant isomer, 4-Hyp, is found in all the types of collagens whereas a position isomer, 3-Hyp, absent from most collagens, exists in minor quantities in type I collagen and in higher amounts in basement membrane collagen [1] or, as recently described, in a non-basement membrane collagen found in the renal cortex [2]. 3-Hyp constitutes a marker of basement membranes or kidney collagens. The reader is referred to general reviews such as ref. 3 for a description of the various types of collagens.

We described several years ago a specific method for the determination of 3-Hyp which necessitated two preliminary steps of ion exchange followed by amino acid chromatography on a Beckman amino acid analyzer [4]. This technique permitted us to gather more than 300 results from normal and pathological urines and to describe an increase of urinary 3-Hyp with a significant decrease in the molar ratio 4-Hyp/3-Hyp in all the cases of polycystic kidney disease which were studied [5]. Unfortunately, this technique was time-consuming and tedious.

In a previous paper [6], we introduced a general technique sensitive to the picomole level for the evaluation of 4-Hyp and 3-Hyp in collagen hydrolysates or in cell cultures. In this paper, we describe an adaptation of this method to urine. It is ten times faster than the former method, far more sensitive, and gives results in excellent correlation with it.

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The new analytical procedure comprises preliminary separation of the hydroxyprolines from some interfering organic substances by fast chromatography through a column of Biorex 70 resin, the removal of inorganic salts by Dowex 50W-X2 chromatography, and fluorometric detection comprising derivatization with the fluorophore 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) and thin-layer chromatographic separation of the fluorescent derivatives of Hyp on silica-gel plates, followed by quantitation with a recording spectrofluorometer.

MATERIALS AND METHODS

The usual reagents, all of the analytical grade, were purchased from Prolabo, Paris, France. Silica-gel 60 plates (ref. 5721) without fluorescent indicator were obtained from Merck (Darmstadt, G.F.R.), and NBD-Cl was from Aldrich-Europe (Beerse, Belgium). Biorex 70 and Dowex 50-X2 resins were purchased from Bio-Rad Labs. (Richmond, CA, U.S.A.). Ammonium formate was obtained from Sigma (St. Louis, MO, U.S.A.) and standard amino acids from Calbiochem (Los Angeles, CA, U.S.A.), except 3-Hyp which was prepared in the laboratory [7, 8].

Prior to analysis, the samples of urine are stored at —80° C and deproteinized by heating at 100°C for 2 min and centrifuging at 1500 *g* for 10 min. We verified the absence of any Hyp in the sediment. One millilitre of supernatant is mixed with 1 ml of 12 *M* hydrochloric acid and hydrolysed in a sealed tube for 18 h at 105°C. The hydrolysates are twice evaporated to dryness under vacuum and the residue is dissolved in 1 ml of 0.01 *M* ammonium formate buffer, pH 6.4. This solution is centrifuged and 200 μl of the clear supernatant are loaded on the top of the column of resin Biorex 70 prepared as follows.

Ion-exchange chromatography on Biorex 70

A 3 × 0.5 cm column of Biorex 70, 50—100 mesh (total capacity 3.3 mequiv.) is equilibrated in the Na^+ form by passage of 5 ml of 0.5 *M* sodium hydroxide. The resin is washed with 10 ml of distilled water and then with 20 ml of 0.1 *M* phosphate buffer, pH 6.4. Finally, the column is washed with 5 ml of 0.01 *M* ammonium formate solution. The sample of urinary hydrolysate is deposited at the top and the resin is eluted with 5 ml of the same buffer. In these conditions, the neutral and acidic amino acids are not bound to the resin and appear in the first 3 ml of effluent.

Ion-exchange chromatography on Dowex 50W-X2

A 3 × 0.5 cm column of the resin Dowex 50W-X2, 50—100 mesh (total capacity 1.2 mequiv.), is equilibrated in the H^+ form by passage of 20 ml of 2 *M* hydrochloric acid followed by distilled water until the pH of the effluent is neutral. The first 3 ml of effluent of the first column are acidified to pH 5.0 by addition of 100 μl of 0.1 *M* formic acid and this solution is passed through the Dowex 50 column. After the fixation of amino acids, the column is washed with 10 ml of distilled water. Then, the amino acids are eluted with 10 ml of a triethylamine—ethanol—water (20:40:40, v/v) solution. The eluate is

evaporated to dryness under a stream of nitrogen and the residue dissolved in 0.1 ml of distilled water.

Derivatization and thin-layer chromatography

To the previous solution are added 0.1 ml of a 3.0 *M* solution of triethylamine in ethanol and 0.1 ml of a 0.03 *M* solution of NBD in ethanol. The mixture is decanted and left in the dark in an incubator for 30 min at 65°C.

The NBD-amino acids are separated by thin-layer chromatography on silicagel plates. For the evaluation of 3-Hyp, 5-μl samples of the unknown derivatized solutions are spotted in triplicate on the starting line 1 cm from the inferior edge of the plate. Standards of NBD-3-Hyp prepared simultaneously are deposited on both sides of the unknown sample in the range 20—100 pmoles. After drying the spots under a stream of nitrogen, the plate is predeveloped in methanol to obtain a thinner linear spot. This predevelopment permits better separations. It is stopped when the spots are located 2 cm from the lower edge. Prior to development, the plates are activated by heating at 65°C for 10 min. They are developed in a glass tank previously saturated with the solvent chloroform—acetone—methanol—tributylamine (60:20:5:15, v/v). After 1 h (when the front has moved up to 12 cm from the starting line), the plates are dried in an oven at 65°C for 5 min. They are stored in the dark up to the time of the fluorometric evaluation.

When 4-Hyp is to be measured, the same derivatized sample may be used, but 4-Hyp is far more concentrated in the urine than 3-Hyp so the sample must be diluted by adding 19 volumes of a 50% ethanol solution to 1 volume of the derivatized solution prior to thin-layer chromatography. Also, the separation of 4-Hyp is improved by the use of a different solvent for developing the thin-layer plates, i.e. acetone—toluene—methanol—triethylamine (40:40:15:5, v/v).

The preliminary treatment by Biorex 70 and Dowex 50 allows the perfect separation of spots of 3-Hyp or of 4-Hyp from any interfering amino acid. We checked the identity of the spots by supplementing the urine with standards of 4-Hyp or of 3-Hyp.

The plate fluorescence is recorded with a Farrand spectrofluorometer Model Mark I equipped with a thin-layer-plate recording attachment. The excitation wavelength is set at 340 nm with an additional violet filter absorbing the light over 500 nm and a slit of 1 cm length and 0.5 nm width. The emitted light is read at 525 nm with an additional yellow filter to absorb radiation under 450 nm. For the calculation of fluorescence, it is necessary to measure the width of the Hyp peak at its half-height and its height from the baseline, in order to calculate the surface. When the deposit is rigorously standardized, the measurement of the height furnishes a reproducible value of the concentration. There is a linear relationship between the surface of the peak and the amount of Hyp deposited on the plate in the range 1—100 pmoles [6]. The correlation with the Beckman column chromatography method was checked by linear regression analysis.

RESULTS AND DISCUSSION

In this paper, we present an adaptation to urine of a general method for the

evaluation of the isomers of Hyp which was described more completely elsewhere [6].

Chromatography on Biorex 70 is necessary for the elimination of basic amino acids such as histidine and methylhistidine which migrate with the same mobility as 4-Hyp on thin-layer plates. Chromatography on Dowex 50W-X2 permits the elimination of inorganic ions which interfere with the final thin-layer chromatography. Fig. 1A shows that a known amount of 3-Hyp is quantitatively eluted in the first 3 ml of the effluent from the Biorex 70 column, and Fig. 1B shows that 3-Hyp is completely eluted from Dowex 50W-X2 by 10 ml of triethylamine—ethanol solution.

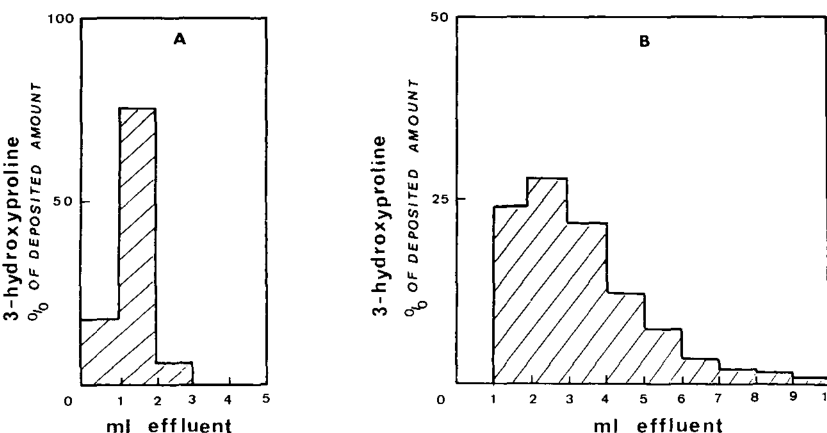

Fig. 1. Recovery of standard 3-Hyp from the preliminary stages of chromatography. (A) Column of Biorex 70 (Na^+). Elution by 0.01 M ammonium formate, pH. 6.6. (B) Column of Dowex 50W-X2 (H^+). Elution by 10 ml of triethylamine—ethanol—water (20:40:40, v/v).

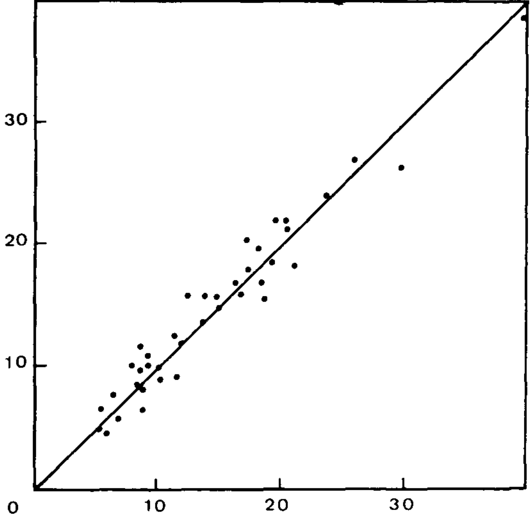

Fig. 2. Correlations of the concentrations of 3-Hyp measured in 39 human urines by ion-exchange chromatography (former method) and thin-layer chromatography (present method). $y = 0.97x + 0.36$, $r = 0.97$, $n = 39$.

In twenty experiments in which known amounts of 3-Hyp were successively chromatographed on the two columns, the final recovery was 99 ± 1.5% (mean ± 1 S.D.). Thirty-nine urine samples from normal or pathological subjects were analyzed in parallel by the former and the newer methods. As shown in Fig. 2, an excellent correlation was found, with $r = 0.97$, demonstrating that both methods may be used with the same reference values. Figs. 3 and 4 show the records of chromatograms used for the evaluation of 3-Hyp and 4-Hyp, respectively. These records demonstrate that the separation of 3-Hyp is complete with the first solvent and that of 4-Hyp with the second solvent. The two isomers would not be completely separated from interfering amino acids by a single solvent. This difficulty has no practical significance because the high concentration of 4-Hyp in the urine compared to 3-Hyp would necessitate in any case separate thin-layer chromatography of the two isomers to be performed.

The interest of the new technique is its speed and sensitivity. Ten evaluations can be performed within a day. The method is sensitive to the picomole level, which means that under the conditions described it is a hundred times more sensitive than the former method.

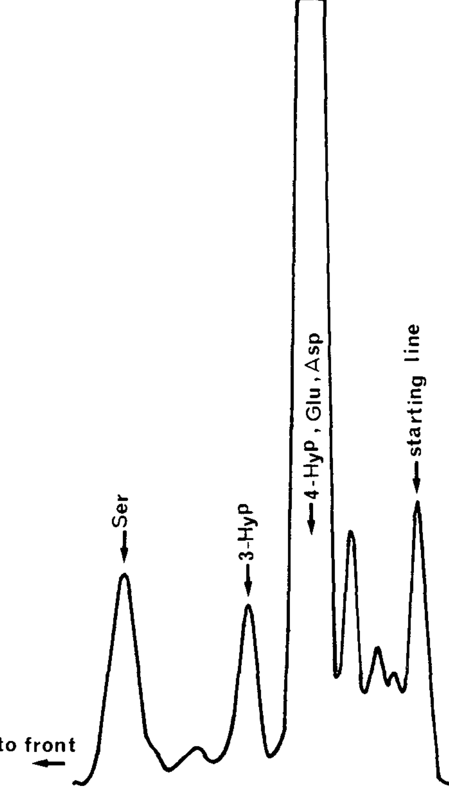

Fig. 3. Scan of thin-layer chromatogram showing the peak of NBD-3-Hyp from an hydrolysate of urine. Solvent: chloroform—acetone—methanol—tributylamine (60:20:5:15, v/v). Development for 1 h at 20°C. Spectrofluorometer Farrand Mark I, sensitivity 0.03, scan speed 150 mm/min.

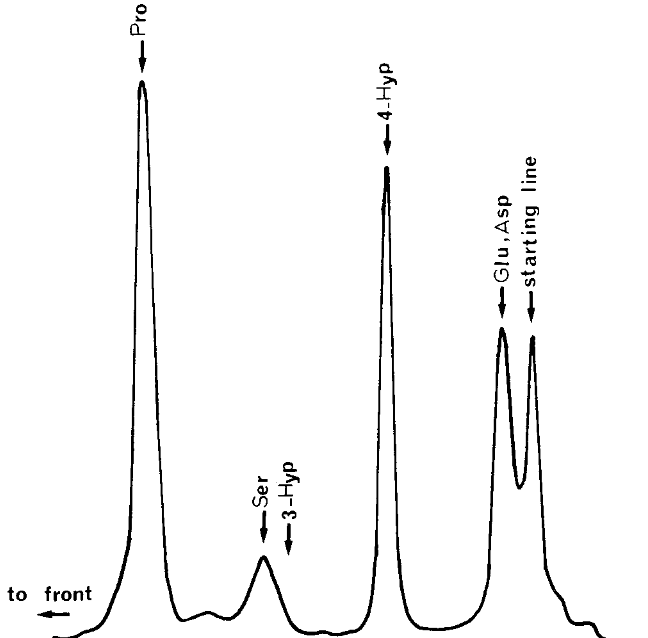

Fig. 4. Scanning of thin-layer chromatogram showing the peak of NBD-4-Hyp from an hydrolysate of urine. Solvent: acetone—toluene—methanol—triethylamine (40:40:15:5). Scanning conditions as in Fig. 3.

TABLE I

REFERENCE VALUES FOR THE CONCENTRATIONS OF 3-Hyp AND 4-Hyp IN HUMAN URINE

Values are the mean of 20 determinations ± 1 S.D.

	3-Hyp (μmol per 24 h)	4-Hyp (μmol per 24 h)	Ratio 4-Hyp/3-Hyp
Adults	12.5 ± 3.5	225.7 ± 61.5	18.0 ± 1.7
Children	6.0 ± 4.8	237.0 ± 201	39.0 ± 7.3
Teenagers	15.2 ± 6.4	517.0 ± 108	34.5 ± 7.5

Up to now, two methods for the evaluation of 3-Hyp in the urine have been described: one by Adams et al. [9] and the other by our group [4]. Both papers furnished nearly identical reference values (Table I). Both methods are tedious which prevents them from being used routinely. The present technique is fast enough for routine use. It permits not only the measurement of 3-Hyp but also of 4-Hyp with excellent precision. It must be emphasized that the amounts of 4-Hyp found with this technique are always 15% lower than the values given by the conventional techniques using chloramine T oxidation and coupling with *p*-methylaminobenzaldehyde (data not shown). This latter technique is probably less specific than the fluorometric one.

The semiological usefulness of urinary 3-Hyp evaluation firstly resides in the constancy of its increase in cases of polycystic kidney disease [10], while the

ratio 4-Hyp/3-Hyp is decreased. A second application of this evaluation lies in the fact that 3-Hyp exists only in the triple helical part of definitive interstitial collagen, not in the procollagen N-terminal extensions, while 4-Hyp exists in the N-terminal extension as well as in collagen itself. This provides a means of differentiating between increases in urinary 4-Hyp due to pure catabolism of collagen (4-Hyp and 3-Hyp are increased in parallel) and increases in urinary 4-Hyp due to an enhancement of collagen biosynthesis (4-Hyp increases because it comes from the N-terminal extension of procollagen, while 3-Hyp remains at the usual level).

ACKNOWLEDGEMENTS

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Note

Determination of morphine, morphine-3-glucuronide and (tentatively) morphine-6-glucuronide in plasma and urine using ion-pair high-performance liquid chromatography

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(First received November 26th, 1981; revised manuscript received February 22nd, 1982)

Morphine is metabolized in man primarily through conjugation with uridine diphosphoglucuronic acid in the 3-position. This conjugate is the major metabolite of morphine in several mammals [1] and it accounts for about 54—74% of the excretion products in man [2]. A minor portion is glucuronidated in the 6-position or demethylated to normorphine [3]. While investigating the clinical pharmacology of morphine in cancer patients with severe pain [4], a need arose to develop a simple analytical method for morphine and its metabolites in plasma and urine. We now present such a method for the simultaneous analysis of morphine, morphine-3-glucuronide and normorphine by high-performance liquid chromatography (HPLC) using ion-pair formation. A metabolite which is tentatively identified as the morphine-6-glucuronide is also coanalyzed in this method. The chromatographic system may also be used for the analysis of the morphine congeners codeine, ethylmorphine and heroin (diamorphine).

EXPERIMENTAL

Materials

Morphine-3-glucuronide and normorphine were generously supplied by the National Institute of Drug Abuse, Bethesda, MD, U.S.A. 1-Dodecyl sulphate (sodium salt) was chromatography grade (Regis Chemicals, Morton Grove, IL, U.S.A.). Acetonitrile was HPLC grade. All other chemicals were analytical reagents. The water used was glass-distilled.

Before use the Sep-Pak C_{18} cartridges (Waters Assoc., Milford, MA, U.S.A.) were treated with 5 ml of methanol, 3 ml of a 10% acetonitrile solution in 10 m*M* phosphate buffer (pH 2.1), and 5 ml of water.

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Apparatus

The chromatographic equipment consisted of a Constametric I pump (Laboratory Data Control, Riviera Beach, FL, U.S.A.), a 7120 injector (Rheodyne, Berkeley, CA, U.S.A.) with a 400-μl loop, an Ultrasphere ODS 150 × 4.6 mm reversed-phase column (5-μm particles; Altex, Berkeley, CA, U.S.A.) and a Spectromonitor III variable-wavelength UV detector (Laboratory Data Control).

Chromatographic conditions

The eluent was a 10 m*M* sodium dihydrogen phosphate buffer pH 2.1 (adjusted with phosphoric acid) containing 1 m*M* dodecyl sulphate and 26% acetonitrile. The flow-rate was 1.5 ml/min. The temperature was ambient and the detector wavelength was set at 210 nm.

Sample purification

One millilitre of plasma or urine (the urine samples normally had to be diluted) was mixed with 3 ml of 0.5 *M* ammonium sulphate adjusted to pH 9.3 with ammonia. This sample was passed through the Sep-Pak C_{18} cartridge. The cartridge was washed with 20 ml of 5 m*M* ammonium sulphate adjusted to pH 9.3 with ammonia, and 0.5 ml of distilled water. Morphine and its metabolites were eluted with 3.0 ml of a 10% acetonitrile solution in 10 m*M* phosphate buffer pH 2.1. The eluate was mixed with 3 ml of the 0.5 *M* ammonium buffer and treated on a second Sep-Pak C_{18} cartridge in the same way as on the first one. Part of the eluate (400 μl) was injected on the column.

RESULTS AND DISCUSSION

The separation of opium alkaloids by reversed-phase ion-pair HPLC has been described earlier [5,6]. A method similar to that used by Kubiak and Munson [6], but using 0.1 *M* phosphate buffer pH 3.3, 36% acetonitrile, and 5 m*M* dodecyl sulphate as pairing ion, was initially tested in our laboratory. This system provided a good separation of morphine from its congeners (Table I).

TABLE I

RETENTION TIMES FOR OPIUM ALKALOIDS

For conditions, see text.

Compound	Retention time (min)
Morphine	3.5
Codeine	5.8
Ethylmorphine	8.9
Heroin (diamorphine)	18.7

The detector wavelength was set at 210 nm to obtain the best possible response for morphine. For the determination of morphine-3-glucuronide it was necessary to decrease the acetonitrile concentration to 26% to improve the separation. The pH was adjusted to 2.1 in order to suppress the ionization of the glucuronic acid group ($pK_a \approx 3.2$). The separation between morphine-3-

glucuronide and impurities was improved by changing the dodecyl sulphate concentration to 1 m*M*.

Since morphine-3-glucuronide is a very polar compound, conventional organic extraction from an aqueous solution is difficult. We have found the Sep-Pak C_{18} cartridge to be a convenient tool for the purification of plasma and urine samples. A second Sep-Pak purification step was found to further exclude impurities. This was particularly important for the analysis of low morphine concentrations. Eighty-four per cent of morphine at a concentration of 100 ng/ml and 90% of the morphine-3-glucuronide at a concentration of 1000 ng/ml were recovered after the two-step purification on the Sep-Pak cartridges.

Standard curves were obtained by analysis of plasma spiked with morphine and morphine-3-glucuronide. The concentration range was 20—100 ng/ml for morphine, and 200—1000 ng/ml for morphine-3-glucuronide. The peak areas (peak height × peak width at half height) in mm^2 at 0.01 absorbance units full scale (a.u.f.s.) on the recorder were determined. The standard curves were linear for both morphine ($Y = 1.310X - 0.084$; $r = 0.9996$) and morphine-3-glucuronide ($Y = 0.992X + 0.740$; $r = 0.9999$). The coefficient of variation for the analysis of morphine in plasma was 7.6% at a level of 22 ng/ml ($n = 6$) and 3.8% at a level of 223 ng/ml ($n = 5$). The coefficient of variation for the analysis of morphine-3-glucuronide in plasma was 1.3% and 2.7% at a level of 200 and 2.100 ng/ml, respectively ($n = 5$). Minimum detectable amounts for morphine and morphine-3-glucuronide were 5 ng/ml. Chromatograms of blank plasma and of plasma spiked with morphine-3-glucuronide, normorphine and morphine are shown in Fig. 1. Chromatograms of plasma and urine from morphine-treated cancer patients are shown in Figs. 2 and 3.

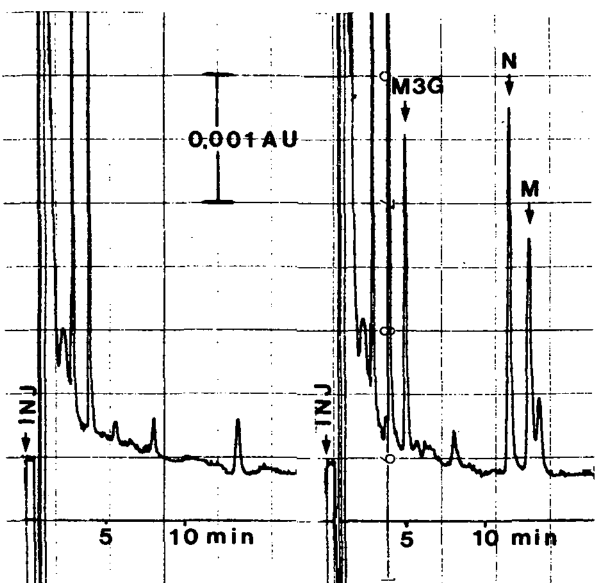

Fig. 1. Chromatograms of blank plasma, and of blank plasma spiked with about 100 ng/ml each of morphine-3-glucuronide (M3G), normorphine (N) and morphine (M).

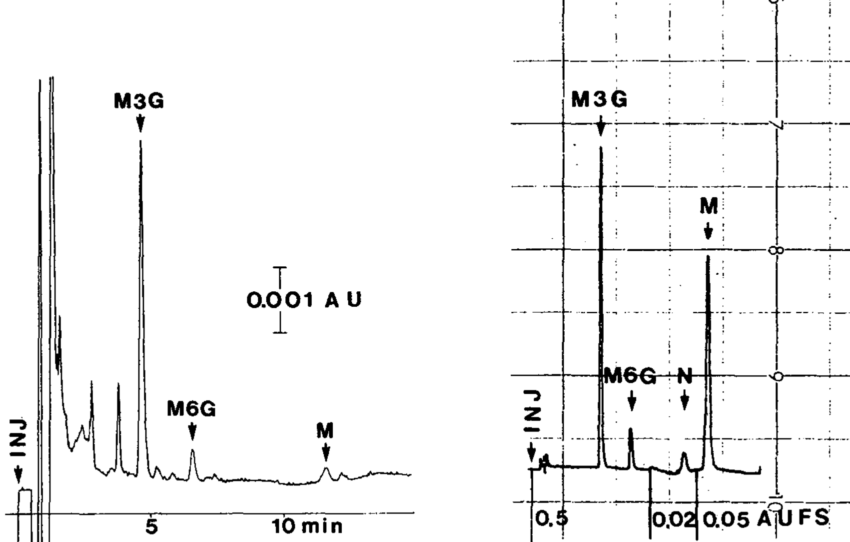

Fig. 2. Chromatogram of plasma from a morphine-treated cancer patient, with 244 ng/ml morphine-3-glucuronide (M3G), morphine-6-glucuronide (M6G), and 9 ng/ml morphine (M).

Fig. 3. Chromatogram of urine from a morphine-treated cancer patient, with morphine-3-glucuronide (M3G), morphine-6-glucuronide (M6G), normorphine (N) and morphine (M). Note the different sensitivities, indicated by absorbance units full scale (AUFS).

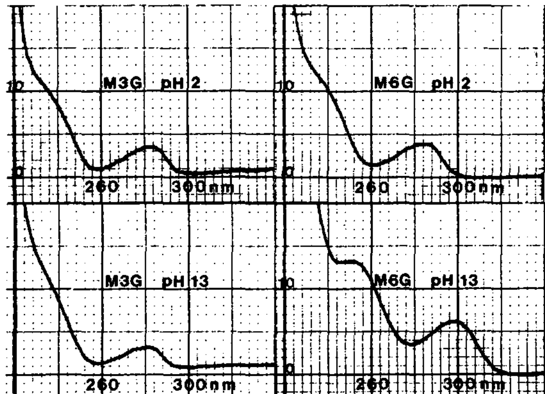

Fig. 4. UV spectra of morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) in acid and basic solution. A bathochromic shift is observed for morphine-6-glucuronide.

In chromatograms from human plasma and urine, a peak with an area of 5—20% of that of morphine-3-glucuronide was consistently found (see Figs. 2 and 3). This peak was tentatively identified as morphine-6-glucuronide in the following manner:

(A) A urine sample with high concentration of this substance was injected on the chromatograph, and the peak eluate was collected. The UV-absorbance curve of this fraction was similar to that of morphine-3-glucuronide. After

alkalinization it showed the bathochromic shift typical of a free phenolic group (Fig. 4) [3].

(B) After treatment of the collected fraction with 1 *M* hydrochloric acid for 1 h at 100°C, the peak area of the assumed morphine-6-glucuronide decreased by 20%. Concomitantly, a morphine peak appeared in the chromatogram. When the morphine-3-glucuronide fraction was treated in the same way, 87% of this metabolite was hydrolysed to morphine.

(C) To a diluted urine sample, containing about 80 μg/ml morphine-3-glucuronide and about 15 μg/ml morphine-6-glucuronide (assuming the same molar absorbance as for morphine-3-glucuronide) in a 0.1 *M* acetate buffer pH 5.5, 10% β-glucuronidase—arylsulphatase from *Helix pomatia* (Boehringer, Mannheim, G.F.R.) was added. The mixture was incubated for 24 h at 37°C. The morphine-3-glucuronide peak disappeared completely, and the peak area of morphine-6-glucuronide decreased by 74%. The morphine peak increased accordingly.

(D) When human liver microsomes (protein concentrations 4.5 mg/ml incubate) were incubated with 0.14 m*M* morphine and 13.5 m*M* uridine diphosphoglucuronic acid in a Tris—HCl buffer pH 8.7 [7], morphine-3-glucuronide and the assumed morphine-6-glucuronide were biosynthesized in about the same proportions as found in plasma.

Our method has been used in studies on morphine kinetics in cancer patients. No interfering peaks from concomitant drug therapy have been observed. However, in smoking patients, a tailing peak which precedes the tentative morphine-6-glucuronide peak might cause problems in the quantitation of

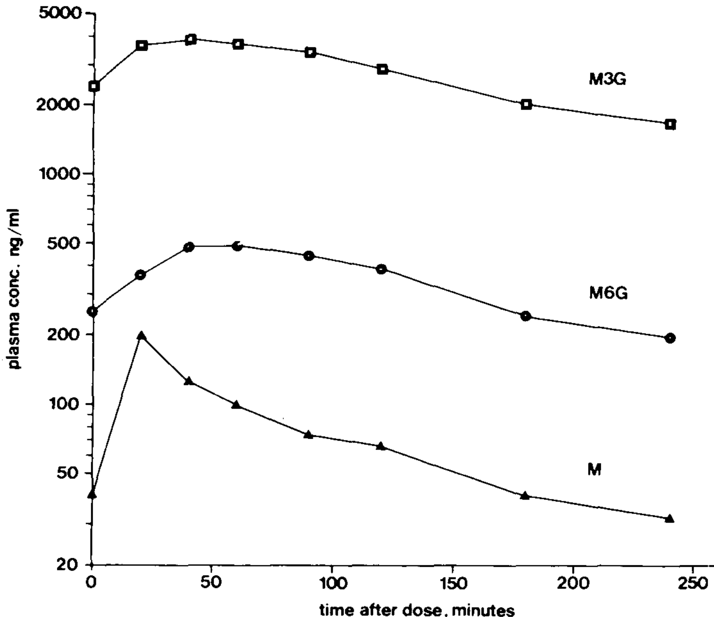

Fig. 5. Concentrations of morphine-3-glucuronide (M3G), morphine-6-glucuronide (M6G) and morphine (M) in plasma from a cancer patient treated with 100 mg of morphine hydrochloride orally at a regular 4-h dose interval.

low concentrations of this metabolite. Fig. 5 shows the plasma concentration of morphine and its glucuronide metabolites in a representative patient.

ACKNOWLEDGEMENTS

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Note

Simultaneous high-performance liquid chromatographic assay for quinidine, disopyramide and the mono-N-dealkylated metabolite of disopyramide

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It is of considerable use in the management of patients with disturbances of cardiac rhythm to have the means to monitor the serum concentration of the drug [1]. Although quinidine has been available for many years it has been shown that its pharmacokinetics vary considerably between individual patients and there is an advantage in individualising the dose of the drug based on serum concentrations measured at steady-state. This also applies to disopyramide which has become available more recently. The therapeutic drug monitoring of disopyramide is made more complex because one of its metabolites has pharmacological actions similar to the parent drug [2, 3]. The method described in this paper allows the simultaneous measurement of quinidine, disopyramide and its mono-N-dealkylated metabolite (MND) by a rapid, accurate and reproducible method suitable for use in a routine therapeutic drug assay laboratory.

EXPERIMENTAL

Materials

Disopyramide [4-diisopropylamino-2-phenyl-2-(2-pyridyl)-butyramide], mono-N-dealkylated disopyramide [4-isopropylamino-2-phenyl-2-(2-pyridyl)-butyramide] and the internal standard *p*-chlorodisopyramide [4-diisopropyl-amine-2-*p*-chlorophenyl-2-(2-pyridyl)-butyramide] were supplied by Roussel (Castle Hill, Australia). Quinidine was obtained from ICN K+K Labs. (Cleveland, OH, U.S.A.). Lignocaine and tocainide were from Astra Chemicals (North Ryde, Australia) and procainamide was from E.R. Squibb and Son (Melbourne,

*Lions Kidney and Medical Research Foundation Scientist.

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Australia). Mexilitine was obtained from Boehringer Ingelheim, Artarmon, Australia). Dichloromethane used for extraction and acetonitrile used in the mobile phase were both high-performance liquid chromatographic (HPLC) grade solvents obtained from Waters Assoc. (Eagle Farm, Australia). All other chemicals were analytical grade products available commercially.

Sample extraction

To each 12-ml glass culture tube, 500 μl of sample or standard were added. This was made alkaline by the addition of 50 μl of 5 *M* sodium hydroxide. Before sealing the tubes with PTFE-lined screw caps, 5 ml of dichloromethane, containing 2 mg/l *p*-chlorodisopyramide as internal standard, were added. The tubes were then mixed on a rotary mixer at 32 rpm for 15 min. After centrifugation at 600 *g* for 3 min, the upper, aqueous layer was aspirated and 3 ml of the remaining organic layer were transferred into tapered centrifuge tubes. The dichloromethane was evaporated using a stream of air and the residue re-dissolved in 100 μl of the mobile phase, from which 20 μl were injected onto the column.

Chromatography

The HPLC system consisted of a Waters Model 6000A solvent delivery system (Waters Assoc., Milford, MA, U.S.A.) with a 20-μl fixed-volume loop injector (Rheodyne, Berkeley, CA, U.S.A.). Eluent was monitored continuously for absorbance changes at 254 and 313 nm using a dual-channel Model 440 UV detector (Waters Assoc.,), the output of which was recorded by a dual-pen Omniscribe recorder (Houston Instrument, Austin, TX, U.S.A.). A Brownlee Labs. Spheri-5 RP-8 column (Activon Scientific Products, Granville, Australia) was used (particle size of the sorbent on these columns is 5 μm).

Samples were eluted isocratically at 2.5 ml/min using 40% acetonitrile (v/v) in 0.05 *M* sodium dihydrogen orthophosphate buffer (pH 3.00). The column was maintained at room temperature (ca. 21°C). Standard curves were prepared using blank plasma spiked with all three components to be assayed. Concentration ranges for disopyramide (0.8—8.1 mg/l) and quinidine (0.9—9.3 mg/l) cover their therapeutic ranges and the concentration range of MND covers the maximum expected in patients having therapeutic concentrations of the parent drug (0.4—3.5 mg/l). The ratios of the peak heights of the drug to internal standard were plotted against concentration to obtain the standard curves. Peak heights for quinidine were determined at 313 nm while those of disopyramide, MND and the internal standard were measured at 254 nm.

Possible interference by other antiarrhythmic drugs was evaluated by chromatographing lignocaine, procainamide, tocainide and mexilitine also.

RESULTS AND DISCUSSION

Using the conditions described, adequate separation of MND, quinidine, disopyramide and *p*-chlorodisopyramide was obtained (Fig. 1) with retention times of 2.2, 3.1, 4.2 and 7.2 min, respectively (Table I). Table I also shows the retention time of procainamide, tocainide, lignocaine and mexilitine to be 1.8,

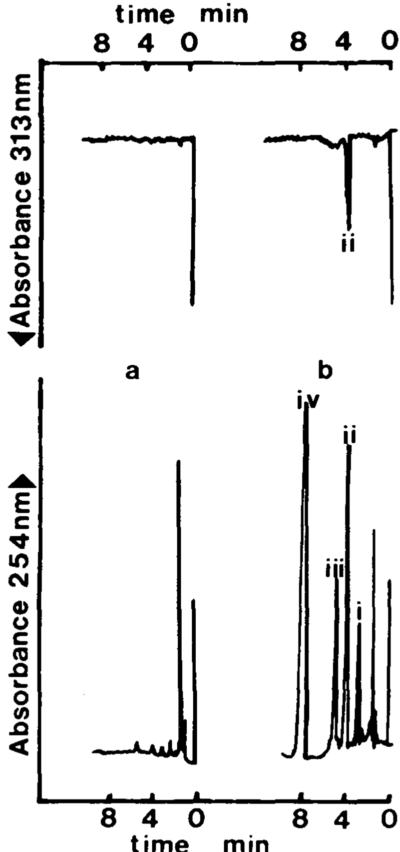

Fig. 1. HPLC tracing obtained using drug-free plasma (a) before spiking (extraction without internal standard), (b) after spiking with (i) mono-N-dealkylated disopyramide (ii) quinidine, (iii) disopyramide (extraction with internal standard iv).

TABLE I

RETENTION TIMES OF VARIOUS COMPOUNDS AND THEIR ABSORBANCE RATIOS

	Retention time (min)	Absorbance ratio (313 nm/254 nm)
Procainamide	1.8	0.69
Tocainide	2.0	N.D.*
MND	2.2	N.D.
Quinidine	3.1	0.28
Lignocaine	3.3	N.D.
Mexilitine	3.6	N.D.
Disopyramide	4.2	N.D.
p-Chlorodisopyramide	7.2	N.D.

*N.D., compound not detected at 313 nm.

TABLE II

EXTRACTION EFFICIENCY (n = 9)

Extraction efficiency expressed as percent of peak height of standard solutions prepared using mobile phase as solvent.

	$\bar{x}$ ± S.D.	μg/ml
MND	97.5 ± 6.1	3.45
Quinidine	94.8 ± 6.2	9.33
Disopyramide	77.5 ± 6.1	8.08

2.0, 3.3 and 3.6 min, respectively. These retention times indicate the lack of interference from these drugs.

Both efficiency and reproducibility of the extraction were acceptable (Table II). Standard curves using single assays of spiked plasma standards were prepared. These curves had the following parameters: MND, $y = 0.212x - 0.016$, $r = 0.999$; quinidine, $y = 0.128x - 0.010$, $r = 0.999$; and disopyramide $y = 0.106x - 0.0068$, $r = 0.999$. To assess the precision of the method a further nine samples from each spiked plasma standard were assayed as unknowns (Table III). The largest coefficients of variation were 5.5, 5.0 and 3.3% for disopyramide, MND and quinidine, respectively.

The reproducibility, precision and accuracy of this method are suitable for use both in pharmacokinetic studies and routine therapeutic drug monitoring. It is an example of how HPLC methods can be adapted to simultaneously measure multiple drugs and their metabolites often providing considerable

TABLE III

PRECISION OF ANALYSIS (n = 9)

	$\bar{x}$ ± S.D. (μg/ml)	C.V. (%)
MND*	0.35 ± 0.02	5.0
	0.86 ± 0.02	2.5
	1.75 ± 0.02	1.1
	3.46 ± 0.06	1.8
Quinidine**	0.97 ± 0.03	3.3
	2.3 ± 0.04	1.6
	4.7 ± 0.07	1.4
	9.3 ± 0.18	2.0
Disopyramide*	0.87 ± 0.05	5.5
	2.0 ± 0.06	2.8
	4.0 ± 0.15	3.6
	7.9 ± 0.35	4.4

*Analyte measured at 254 nm.
**Analyte measured at 313 nm.

advantages over alternative assays such as enzymeimmunoassay and radioimmunoassay. Simultaneous determination of a drug and its metabolite may allow better correlation of serum concentrations with drug effect and toxicity as has been suggested for the tricyclic antidepressants and procainamide [4—6].

This method is an improvement on a previously published method [1] because smaller samples of only 500 μl are acceptable, and the mono-N-dealkylated metabolite is measured simultaneously.

Since this method has been available in our therapeutic drug assay laboratory no interference from other drugs has been encountered, nor have any of the blank plasma samples contained any interfering compounds.

ACKNOWLEDGEMENTS

Financial support from the Lions Kidney and Medical Research Foundation is gratefully acknowledged. J.T.A. is a recipient of Grant No. 247 from the N.H. & M.R.C. of Australia.

We would also like to thank Dr. J. Cameron of Hoechst Roussel (Castle Hill, Australia) for assistance with equipment and provision of disopyramide, mono-N-dealkylated disopyramide and *p*-chlorodisopyramide.

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CHROMBIO. 1274

Note

High-performance liquid chromatography assay of acebutolol and two of its metabolites in plasma and urine

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(First received December 9th, 1981; revised manuscript received February 10th, 1982)

Acebutolol, a β-adrenergic receptor antagonist, is used in Europe and is undergoing clinical testing in the U.S.A. We report a high-performance liquid chromatography (HPLC) assay which has advantages over previously published assays [1,2] in increased sensitivity, simultaneous separate quantitation of acebutolol and two major metabolites, and applicability to both plasma and urine samples with minor variations in the sample processing.

EXPERIMENTAL

Materials

Acetonitrile and ethyl acetate were purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Water was distilled using an all-glass still. Acebutolol, (±)-1-(2-acetyl-4-*n*-butyramidophenoxy)-2-hydroxy-3-isopropylaminopropane); metabolite I, (±)-1-(2-acetyl-4-acetamidophenoxy)-2-hydroxy-3-isopropylamino-propane; metabolite II, (±)-1-(2-acetyl-4-aminophenoxy)-2-hydroxy-3-iso-propylaminopropane; and internal standard, (±)-1-(2-propyl-4-*n*-butyramido-phenoxy)-2-hydroxy-3-isopropylaminopropane were kindly supplied by Ives Labs. (New York, NY, U.S.A.) and May & Baker (Dagenham, Great Britain). All other chemicals were analytical reagent grade.

Sample preparation

For plasma samples, 1 ml of plasma is added to a test tube containing 1 μg of internal standard in 1 ml of 0.01 *M* phosphate buffer at pH 6.0 (KH_2PO_4—Na_2HPO_4). The phosphate buffer also contains appropriate amounts of acebutolol and metabolites where required for standard curve calibrators. After addition of 200 μl of 2 *M* sodium hydroxide and 10 ml of ethyl acetate,

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tubes are vortexed for 90 sec and centrifuged for 5 min at 500 *g*. The organic phase is transferred to a tapered test tube (a test tube with a capillary of 300 μl capacity fused to the bottom). A back-extraction is accomplished by addition of 150 μl of 0.005 *M* sulfuric acid, vortexing for 90 sec, immersing the tubes in a dry ice—acetone bath until the aqueous phase solidifies and then centrifuging for 5 min. The aqueous phase, by now thawed and approximately 250 μl, is transferred to disposable polyethylene limited-volume inserts (Brinkman, Westbury, NY, U.S.A.) for the injection vials of the HPLC automatic sampler.

Urine samples are processed similarly, with the following exceptions: 0.1 ml of urine and 4 μg of internal standard are used, extraction of urine is into 10 ml of diethyl ether instead of ethyl acetate, back-extraction uses 200 μl of sulfuric acid and the freezing step is omitted.

Chromatography

The apparatus used consists of a 6000A pump, with eluent flow-rate at 1.0 ml/min, a 710B automatic sample injector (both Waters Assoc., Milford, MA, U.S.A.), a VUV-10 ultraviolet detector set at 243 nm (Varian, Palo Alto, CA, U.S.A.) and an SP 4100 computing integrator (Spectra-Physics, Santa Clara, CA, U.S.A.). A 2-μm inline filter (Alltech, Deerfield, IL, U.S.A.) is used before the Spherisorb ODS 5-μm, 250 × 4.6 mm, column (Altex, Berkeley, CA, U.S.A.). The mobile phase consists of an aqueous solution containing 6% of a 0.1 *M* phosphate buffer at pH 4.0 (H_3PO_4—KH_2PO_4) filtered through a 0.45-μm filter (type HA, Millipore, Bedford, MA, U.S.A.) and 55% of acetonitrile. The operating pressure is approximately 90 bar. Injection volume varies from 10 to 150 μl.

Quantitation

Standard curves are run daily. For plasma samples, they include calibrators of acebutolol and both metabolites at 0, 20, 40, 60, 80, 100, 200, 500, 1000, 2000 and 3000 ng/ml. A 5000 ng/ml calibrator is also used when samples higher than 3000 ng/ml are encountered. The standard curve is divided into three ranges to avoid undue weighting by high standards. Within each range a least-squares regression fit of peak height ratio versus concentration is used to compute concentrations of samples within that range.

For plasma, the low range contains the calibrators up to 100 ng/ml and is used to quantitate samples containing 20—100 ng/ml; the interim range includes the calibrators from 80—1000 ng/ml and is used for samples from 100—1000 ng/ml; the high range has the calibrators over 500 ng/ml and is used for samples of 1000—5000 ng/ml. The urine standard curve consists of calibrators at 0, 1, 2, 4, 10, 20, 40, 100, 150, 200 and 250 μg/ml. It also is divided into three ranges; calibrators from 0—10 μg/ml are used for quantitating urine samples from 1—10 μg/ml, calibrators from 4—100 μg/ml for quantitating samples from 10—100 μg/ml, and calibrators from 40—250 μg/ml for quantitating samples from 100—250 μg/ml. (All ranges described are inclusive.)

RESULTS AND DISCUSSION

Linearity, precision and accuracy specifications for the assay of acebutolol

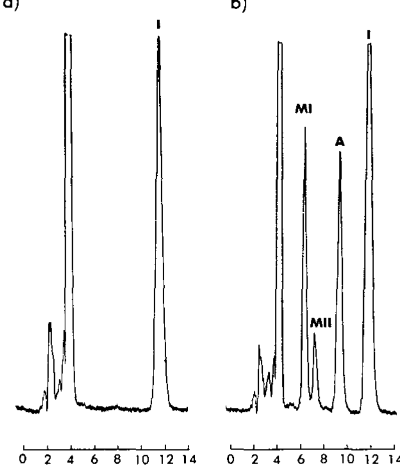

Fig. 1. Chromatograms from a subject (a) before and (b) 8 h after taking a 400-mg oral dose of acebutolol. Peaks: MI = metabolite I (6.6 min); MII = metabolite II (7.5 min); A = acebutolol (9.6 min); I = internal standard (12.0 min).

and both metabolites in plasma and in urine, are shown in Table I. Fig. 1 shows chromatograms resulting from plasma samples collected (a) before and (b) 8 h after a subject took a single oral dose of 400 mg of acebutolol. Fig. 2 shows (a) plasma concentrations and (b) urinary excretion rates in an individual following a single 400-mg oral acebutolol dose.

The modificiations in sample prepartion described for urine were found to be necessary for consistent assay, particularly amongst urines from different subjects. For reproducible urine assay also, we recommend use of one injection volume only, for all standards and samples within each concentration range. In practice, this may mean that scaling considerations necessitate some repeat injections when assaying samples of widely differing concentrations.

Meffin et al. [1] reported an HPLC assay for acebutolol and metabolite I

TABLE I

ASSAY PERFORMANCE SPECIFICATIONS

Type of sample	Assay range	Range linearity*			Concentration of samples spiked for precision and accuracy tests (μg/ml)	Precision**
		Acebutolol	Metabolite I	Metabolite II		Acebutolol
Plasma	Low	8.0±3.2 (27)	7.0±4.4 (27)	5.3±2.4 (7)	0.020	17 (16)
	Interim	3.7±2.2 (27)	4.2±2.5 (27)	3.6±1.3 (7)	0.120	5.1 (20)
	High	2.4±2.0 (23)	3.2±1.7 (23)	4.5±3.5 (3)	1.200	5.4 (19)
Urine	Low	6.0±2.2 (6)	5.2±3.6 (6)	6.0±1.4 (3)	5	3.0 (9)
	Interim	5.2±2.6 (6)	3.3±1.6 (6)	4.6±1.1 (3)	50	7.8 (9)
	High	3.5±2.5 (6)	2.4±1.1 (6)	1.4±0.5 (3)		

*Coefficient of variation of concentration—normalized peak height ratios (%), mean ± S.D. of n (in parentheses) standard curves.

**Coefficient of variation of n (in parentheses) determinations (%).

***Deviation of mean determination from concentration spiked (%); n as for precision.

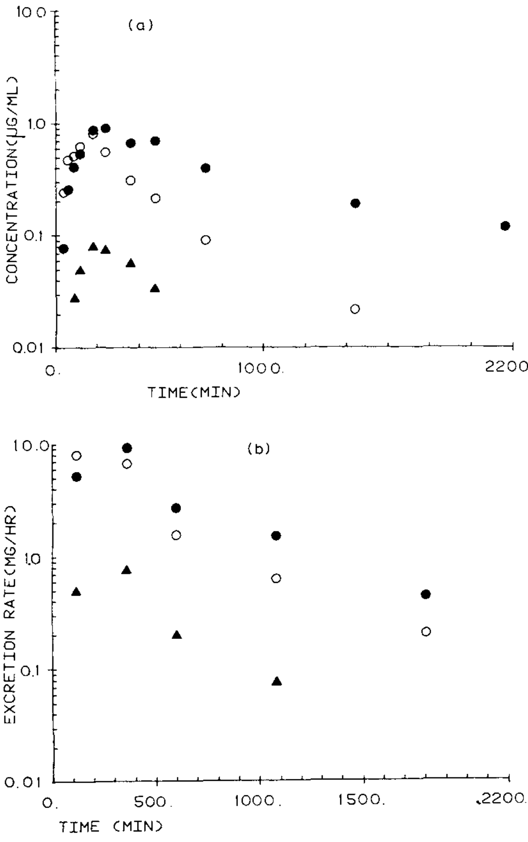

Fig. 2. (a) Plasma concentration of acebutolol and two major metabolites after a single oral dose of 400 mg and (b) urinary excretion rates for the same subject. (○) Acebutolol; (●) metabolite I; (▲) metabolite II.

		Accuracy***		
Metabolite I	Metabolite II	Acebutolol	Metabolite I	Metabolite II
15 (16)	11 (3)	−0.4	−6.5	−0.3
6.4 (20)	8.0 (7)	−1.5	−3.5	−1.0
9.6 (19)	12 (3)	+1.5	−6.5	−11
3.3 (9)	1.7 (3)	−0.6	−1.8	+2.2
9.2 (9)	6.9 (3)	−3.7	−5.1	−0.3

in blood, plasma and urine. They reported a sensitivity of 50 ng per sample but they did not give any accuracy specifications and gave precision specifications for blood only. Guentert et al. [2] reported a modified assay for these compounds in blood and plasma with a sensitivity of 50 ng/ml. With further modifications in chromatography and in sample processing we have extended the sensitivity of this assay to 20 ng/ml in plasma and have compiled specifications for assay of urine. Furthermore, we can simultaneously but separately measure a second major metabolite of acebutolol [3] in both plasma and urine. While sensitivity down to 5 ng/ml of acebutolol and metabolite I can be obtained with fluorescence rather than UV detection, as reported by Lefebvre et al. [4], simultaneous quantitation of the second metabolite, with very different fluorescence properties, is then impaired.

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Note

Improved acetaminophen assay sensitivity by modification of a high-performance liquid chromatography technique

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(First received December 9th, 1981; revised manuscript received February 10th, 1982)

Several assays for acetaminophen using high-performance liquid chromatography (HPLC) have been described. Some are suitable for analysis in pharmaceutical preparations, and others are suitable for analysis in biological fluids, but only at clinical to toxic concentrations of the drug [1—4]. This paper gives detailed performance specifications for a modification of the method of Fletterick et al. [1] which extends the assay sensitivity at least ten-fold to allow measurement of concentrations necessary for pharmacokinetic studies.

EXPERIMENTAL

Materials

Ethyl acetate, chloroform and heptane were from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Water was distilled from an all-glass still. Acetaminophen (4-acetaminophenol) was obtained from Sigma (St. Louis, MO, U.S.A.) and 3-acetaminophenol from Aldrich (Milwaukee, WI, U.S.A.). All other chemicals were of analytical reagent grade.

Sample preparation

One ml of plasma and 1 ml of 0.5 *M* phosphate buffer at pH 7.0 (KH_2PO_4—Na_2HPO_4) were added to test tubes containing 25 μg of 3-acetaminophenol (internal standard) and for standard curve calibrators, appropriate amounts of acetaminophen, in 550 μl of water. To each tube, 10 ml of ethyl acetate were added before vortexing for 1 min and then centrifuging for 5 min at 500 *g*. The organic phases were transferred to fresh test tubes and evaporated to dryness at 45°C under a nitrogen stream. These extracts were reconstituted in 200 μl of ethanol and vortexed for 30 sec before transfer to disposable poly-

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ethylene limited-volume inserts (Brinkman, Westbury, NY, U.S.A.) for the injection vials of the HPLC automatic sampler.

A slightly modified method for concentrations below 0.2 μg/ml uses 5 μg of internal standard, reconstitution in 50 μl of ethanol, and glass limited-volume inserts (Waters Assoc., Milford, MA, U.S.A.) for the automatic sampler vials.

Chromatography

The mobile phase was water-saturated chloroform—heptane—ethanol—acetic acid (225:700:75:1). Prior to use, it was degassed in an ultrasonic bath for 5 min. The column was washed with and stored in heptane when not in use. The chromatographic system consisted of a 6000A pump with eluent flow-rate at 1.6 ml/min, a 710B automatic sample injector (both Waters Assoc.), a UV-50 ultraviolet detector set at 248 nm (Varian, Palo Alto, CA, U.S.A.) and a SP4100 computing integrator (Spectra-Physics, Santa Clara, CA, U.S.A.). The column was Spherisorb SI, 5 μm, 250 × 4.6 mm (Altex, Berkeley, CA, U.S.A.). The operating pressure was about 60 bar. The injection volume was 25 μl.

Quantitation

The standard curve was computed from samples spiked with acetaminophen concentrations of 0, 0.2, 0.5, 1.0, 2.0, 5.0 and 10.0 μg/ml. An unweighted least-squares linear fit of peak area ratio versus concentration was used to estimate sample concentrations. Samples at 20 and 50 μg/ml have been run to confirm the linearity of the assay at higher concentrations. When samples between 0.1 and 0.2 μg/ml have been assayed, an additional standard at 0.1 μg/ml has been used. For samples containing well below 0.2 μg/ml, however, it is preferable to use the modified method described, which employs standards at 0, 0.02, 0.05, 0.1, 0.2 and 0.5 μg/ml of acetaminophen.

RESULTS

Table I shows linearity, precision and accuracy specifications for both of the procedures described. Fig. 1 shows chromatograms from plasma samples of a subject (a) before dosing and (b) 13 h after the last of a series of thirteen 6-hourly 650-mg oral doses of acetaminophen. These samples were processed by the normal range method and the chromatogram in Fig. 1b corresponds to a concentration of 0.17 μg/ml. Retention times for internal standard and acetaminophen are 6.2 and 8.8 min, respectively. Also shown in Fig. 1 are (c) a chromatogram from drug-free plasma processed without internal standard and (d) the same plasma spiked with 0.02 μg/ml of acetaminophen and processed with internal standard. Both of these latter samples were processed by the method for lower concentrations.

In drug-free plasma samples from twenty five different people, no peaks in the position of acetaminophen were observed at attenuations giving well defined, quantitative peaks from 0.1 μg/ml spiked samples. In addition, no interference with the assay was caused by aspirin or its metabolites in plasma after a 650-mg dose of aspirin, but higher levels of salicylate (>20 μg/ml) than encountered might interfere.

TABLE I

LINEARITY, PRECISION AND ACCURACY SPECIFICATIONS

Range*	Linearity**	Concentration of samples spiked for precision/bias tests*** (μg/ml)	Precision §	Bias §§
Normal	6.8 ± 1.8	0.2	8.1 (n=5, on 5 days)	−6.1 (n=5, on 5 days)
	Mean ± S.D.	1.0	3.6 (n=5, on 5 days)	−0.1 (n=5, on 5 days)
	(n=5 curves)	10.0	1.1 (n=5, on 5 days)	−0.0 (n=5, on 5 days)
Low	10.3	0.02	25.0 (n=4)	−6.3 (n=4)
	(n=1 curve)	0.05	8.6 (n=5)	−6.9 (n=5)
		0.1	5.2 (n=5)	+2.9 (n=5)

*Low range refers to the assay procedure modified for low concentrations.
**Coefficient of variation of concentration—normalized peak area ratios (%).
***Not standard curve calibrators but additional controls.
§ Coefficient of variation of determined concentration (%).
§§ Percent deviation of mean determination from amount spiked.

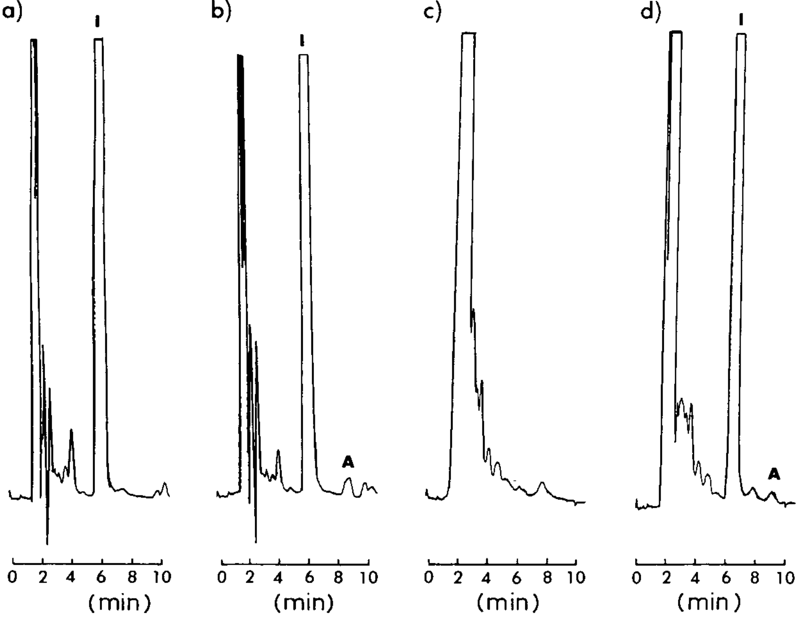

Fig. 1. Chromatograms from a subject (a) before dosing and (b) 13 h after the last of thirteen 6-hourly 650-mg acetaminophen doses, assayed by the first method described; and (c) from blank plasma without internal standard and (d) a 0.02 μg/ml spiked standard, assayed by the more sensitive method. The concentration of the sample yielding chromatogram (b) was 0.17 μg/ml. Peaks: I = 3-acetaminophenol, the internal standard; A = acetaminophen.

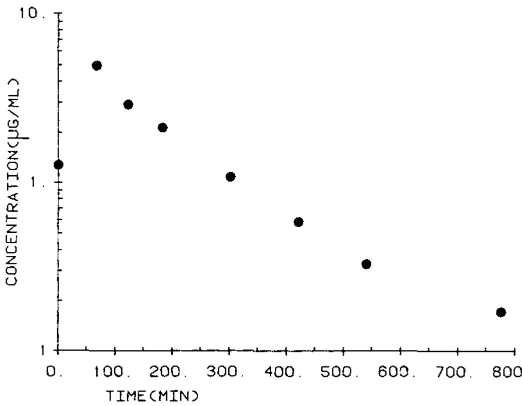

Fig. 2. Time course of plasma acetaminophen concentrations for 13 h following the last of thirteen 6-hourly 650-mg acetaminophen doses.

Fig. 2 shows a profile of plasma concentrations during the 13 h following the last acetaminophen dose referred to above. All samples were assayed by the normal range method.

DISCUSSION

The assay of Gotelli et al. [2] is sensitive enough to monitor acetaminophen at therapeutic levels. However, while the authors state that standards ranging from 0.5—400 µg/ml gave a linear response, no specifications are given relating to samples less than 5 µg/ml. Fletterick et al. [1] described their method also as being linear down to 0.5 µg/ml. However, the only specification including concentrations any lower than 5 µg/ml, is a correlation coefficient of 0.990 for samples ranging from 0.5—300 µg/ml when assayed by the HPLC method and an undescribed colorimetric method. Furthermore, there does appear to be significant HPLC interference from endogenous compounds, as Fletterick et al. [1] report that five pre-dose samples from five volunteers had a mean assayed value of 0.0 ± 0.1 µg/ml. At the time of submission of the current paper, another [5] appeared describing a reversed-phase method for which precision specifications were given for samples at 0.25 µg/ml and above, although no accuracy specifications were given. As Ameer et al. [5] stated, however, routine quantitation of plasma concentrations as low as 0.1—0.2 µg/ml is required for meaningful interpretation of single-dose acetaminophen pharmacokinetic studies.

We give detailed performance specifications for a method of acetaminophen assay which is rapid, convenient, selective, precise and more sensitive (0.05 µg/ml) than the other methods available [1—5]. The five-fold (or more) advantage in sensitivity makes this assay particularly attractive for pharmacokinetic studies. No loss in performance has been observed after well over 500 plasma assays, despite avoiding the time-consuming column regeneration procedures required by the assay according to Fletterick et al. [1].

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Note

High-performance liquid chromatographic analysis of propafenone in human plasma samples

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(Firest received December 3rd, 1981; revised manuscript received February 11th, 1982)

Propafenone [2′-(2-hydroxy-3-propylamino-propoxy)-3-phenylpropiophenone] is a new antiarrhythmic drug which is undergoing clinical testing in several countries. In vitro studies have demonstrated that propafenone depresses $\dot{V}_{max}$ of the action potential, but does not alter the resting membrane potential [1]. Electrophysiological studies have shown that it prolongs sinus node recovery time, and lengthens the effective refractory period of the atrium and AV node [2]. Clinically, propafenone has been reported to be useful in suppressing chronic recurrent supraventricular and ventricular tachycardias, tachyarrhythmias and ectopic beats [3, 4].

In order to design optimal protocols to evaluate the clinical efficacy of propafenone, a better understanding of the pharmacokinetics and pharmacodynamics is needed. At present very little is known about the disposition kinetics of this drug. Prior to initiating pharmacokinetic and pharmacodynamic studies of propafenone, we have developed a simple and rapid high-performance liquid chromatographic procedure for measuring propafenone concentrations in biological fluids. This method is described and discussed in this report.

EXPERIMENTAL

Chemicals and reagents

Propafenone hydrochloride, the internal standard (Fig. 1) and ^{14}C-labeled propafenone were obtained from Knoll (Ludwigshafen, G.F.R.). Glass distilled acetonitrile was obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.), AR grade heptane was purchased from Mallinckrodt (St. Louis, MO, U.S.A.), isoamyl alcohol from Eastman-Kodak (Rochester, NY, U.S.A.), and

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PROPAFENONE R = $-CH_2-CH_2-CH_3$

INTERNAL STANDARD R = $-CH_2-CH(CH_3)_2$

Fig. 1. Chemical structures of propafenone and the internal standard.

98% pure *n*-nonylamine from Aldrich (Milwaukee, WI, U.S.A.). All other chemicals and solvents were of reagent grade.

Instrumentation

A Waters Assoc. (Milford, MA, U.S.A.) M6000A Solvent Delivery System was used to pump the mobile phase through a Waters μBondapak CN column (30 cm × 3.9 mm); particle size was 10 μm. The flow-rate was adjusted to 1.6 ml/min which produced a precolumn pressure of 68 atm (1000 p.s.i.). The detector was a Schoeffel 770 UV—visible spectrophotometer with absorbance monitored at 209 nm.

Mobile phase

The mobile phase contained 25% acetonitrile and 75% 0.005 *M* potassium dihydrogen phosphate (pH 2.4). *n*-Nonylamine was added to the mixture to give a concentration of 0.02 *M*. The solution was filtered and degassed using vacuum.

Calibration standards

Calibration standards were prepared by adding weighed amounts of drug (reflecting amounts of free base) to distilled water. A few drops of 5 *N* hydrochloric acid (to a pH of 3.5) were added to ensure stability. These standards were prepared in appropriate dilutions to deliver between 10 and 250 ng of drug per 100 μl of solution. Calibration curves were prepared daily from these solutions. It was found that these solutions were stable, without observable decomposition, after being stored in the refrigerator for 9 months. Aliquots (100 μl) of each standard were pipetted into screw capped tubes. A 100-μl aliquot of internal standard (100 ng/100 μl) was added along with 0.5 ml of blank plasma. These samples were extracted along with the patient samples as described below.

Extraction procedure

Aliquots of 0.1—1 ml of patient plasma are added to screw-capped tubes along with 100 μl of the internal standard. To each tube are added 200 μl of 2 *N* sodium hydroxide (2/3 saturated with sodium chloride) and 2.0 ml of 1%

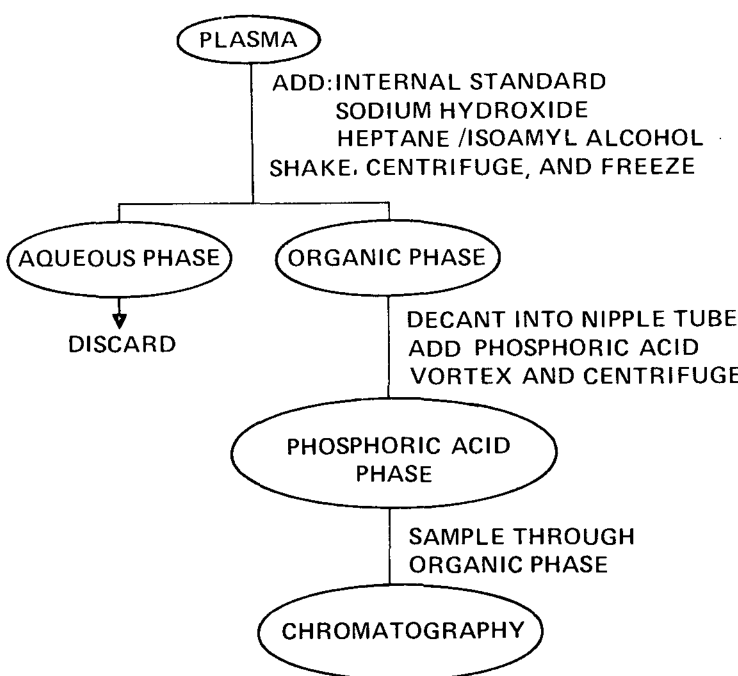

Fig. 2. Schematic outline of the sample preparation scheme used in the analysis of propafenone in plasma.

isoamyl alcohol in heptane. The tubes are capped and rocked on a Lab quake shaker for 10 min. After centrifugation to separate the organic and aqueous layers the tubes are placed briefly in a dry ice—acetone mixture to freeze the aqueous layer. The organic phase is then decanted into a clean nipple tube. To the nipple tube are added 200 μl of 0.2 *N* phosphoric acid. The tubes are then capped, shaken for 2 min on a Vortex mixer and centrifuged. A portion of the acid phase is then injected onto the column using a 50-μl syringe. This procedure is outlined schematically in Fig. 2.

RESULTS AND DISCUSSION

The retention times for propafenone and the internal standard are 5.9 and 7.0 min, respectively. The column temperature was not controlled, but generally stayed between 20°C and 28°C. Changes in temperature do influence the retention times, and this can be compensated for by changing the percentage of acetonitrile in the mobile phase.

Chromatograms of an extracted plasma sample from a healthy subject taking no medication and a sample from a patient who was being treated with propafenone are shown in Fig. 3. The patient was taking 150 mg of propafenone three times daily and the blood sample was drawn 1.5 h after a morning dose. The propafenone concentration at that time was 654 ng/ml. While no interferences have been observed in blank plasma samples from several sources, there was an unidentified peak eluting at 4 min in this patient's sample. This may be a metabolite of propafenone. This peak also has been observed in plas-

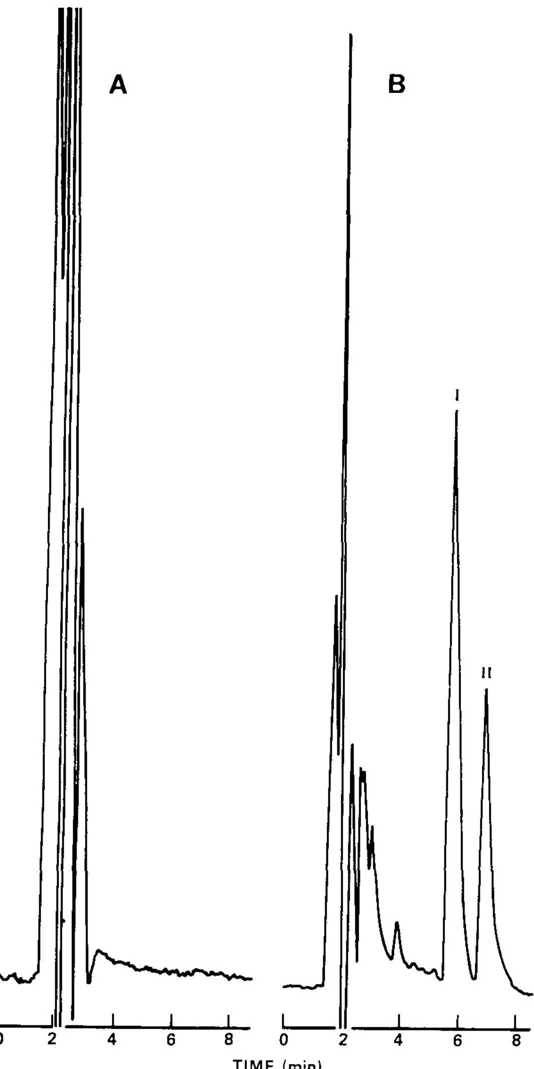

Fig. 3. Chromatograms of extracted patient plasma (A) not taking propafenone and (B) 1.5 h after taking 150 mg of propafenone orally. Peaks: I = propafenone; II = internal standard. The retention times of these two peaks are 5.9 and 7.0 min, respectively.

ma samples from other patients who were taking propafenone. The identity of the compound eluting at this time has not yet been determined. Another peak, eluting prior to propafenone, but with baseline separation, also has been noted in some patient samples. Whether this is also a metabolite of propafenone, or due to some other drug is not presently known.

Interference with this analysis by other drugs has been considered extensively, since patients taking antiarrhythmic drugs are often receiving numerous other medications. Most drugs studied do not interfere with this procedure, but a few interferences were observed. The following drugs do not interfere with

the analysis of propafenone: lidocaine, aldactone, warfarin, diltiazem, cimetidine, procainamide, atenolol, sotolol, quinidine, chlorothiazide, furosemide, digoxin and propranolol. The drugs which do interfere with the analysis of propafenone were nifedipine, verapamil and diazepam. However, it is not likely that the slow channel blockers, verapamil and nifedipine, would be administered concurrently with propafenone. The retention time of diazepam was 5.4 min, which was very close to that of propafenone. These two peaks can be further separated by slowing down the chromatography, but this greatly limits the utility of this method for handling large numbers of samples.

Calibration curves are linear up to 500 ng. The usual range employed has been 25—500 ng, since this includes the range of most of the samples analyzed to date. The use of less internal standard permits the analysis of concentrations as low as 5 ng/ml. The daily fluctuation in slope of the standard curve is slight; the coefficient of variation of the slope of five different standard curves was only 3.5%. The y-axis intercepts are not different from zero.

The efficiency of the extraction procedure was determined using [^{14}C] propafenone as tracer. It was found that 78% of the drug was extracted into the final acid phase, 18% was left in the plasma, 1% was in the organic phase and 3% was unaccounted for. The percent extracted was constant with a standard deviation of only 1.6% (n = 4).

Using labeled propafenone, the partitioning between red blood cells and plasma water was also examined. A plasma/blood concentration ratio of 1.12 was observed. This suggests that propafenone is bound to plasma proteins, and only the free drug partitions into the red cells.

To increase the range of analysis, it is often desirable to use varied volumes of patient plasma. Larger volumes may be employed for low-concentration samples and small volumes for high-concentration samples. Varied volumes can be employed if plasma volume does not affect the extraction of the drug and alter the relationship between the drug and internal standard. In order to test this, spiked plasma samples were analyzed. The extracted volumes of plasma ranged from 0.1 to 1.0 ml. The coefficient of variation was 1.5% indicating that sample size does not affect the relative extraction efficiences of propafenone and the internal standard.

The reproducibility of this procedure was evaluated by analyzing several samples at specific plasma concentrations (Table I). A range of 5—150 ng/ml was studied. The greatest standard deviation was observed with the 50 ng/ml samples, but this only amounted to 3.6 ng/ml. Despite the large coefficient of

TABLE I

REPRODUCIBILITY OF A GIVEN PLASMA CONCENTRATION

Concentration (ng/ml)	n	C.V. (%)
5	6	10.6
10	6	8.7
50	12	7.1
100	6	0.7
150	6	2.1

variation observed with the 5 ng/ml samples, this is only a standard deviation of 0.53 ng/ml. We have observed that this method is very stable and quite reproducible from day to day.

The method reported here has sufficient sensitivity for both therapeutic monitoring and pharmacokinetic studies. While it is specific for propafenone, three drug interferences have been identified. The method is relatively simple and rapid, allowing analysis of up to 40 samples daily.

ACKNOWLEDGEMENTS

The authors acknowledge the technical assistance of Sabra Abraham and the assistance of Glenda Rhodes with the preparation of this manuscript. They appreciate the significant efforts of Dr. Edward B. Kirsten, Knoll Pharmaceutical Company, Whippany, NJ, U.S.A. in helping them obtain propafenone hydrochloride, the internal standard and ^{14}C-labeled propafenone.

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Note

Specific and sensitive assay of celiprolol in blood, plasma and urine using high-performance liquid chromatography

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Celiprolol (Fig. 1) is a new β-adrenergic antagonist undergoing clinical trials in Europe, for use in hypertension and in angina pectoris. Little is known about its metabolism and assays to-date have been based upon radiotracer or other potentially non-specific or insensitive methods. A modification of previous high-performance liquid chromatographic (HPLC) assays for acebutolol [1—3] provides a convenient and specific assay for celiprolol in whole bood, plasma or urine, with excellent precision and accuracy specifications and with sufficient sensitivity (10 ng/ml in plasma) for pharmacokinetic studies. Quantitation of one and possibly even a second metabolite, appears possible with this method.

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Fig. 1. Molecular structures of (a) celiprolol and (b) acebutolol, the internal standard used.

EXPERIMENTAL

Materials

Acetonitrile and ethyl acetate were obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Water was distilled using an all-glass still. Fig. 1 shows the internal standard, acebutolol, (±)-1-(2-acetyl-4-*n*-butyramidophenoxy)-2-hydroxy-3-isopropylaminopropane (Ives Laboratories, New York, NY, U.S.A.) and celiprolol, N-4-(3-*tert.*-butylamino-2-hydroxypropoxy)-3-acetylphenyl-N′,N′-diethylurea (Chemie Linz, Linz, Austria). All other chemicals used were analytical reagent grade. Acebutolol and celiprolol solutions were made in 0.01 *M* phosphate buffer (KH_2PO_4/Na_2HPO_4) at pH 6.0. All concentrations and amounts of celiprolol in this paper refer to celiprolol · HCl equivalents.

Sample preparation

Blood samples were prepared by adding 1 ml of blood to glass test tubes in which had been placed 1 ml of 0.01 *M* phosphate buffer at pH 6.0 containing 1 μg of acebutolol and for standard curve calibrators, appropriate amounts of celiprolol. To this were added 2 ml of acetonitrile. After vortex-mixing the contents for 1 min, the tubes were centrifuged for 5 min at 500 *g*. The supernatants were decanted into test tubes and the volume reduced to approximately 1.5 ml at 40°C under a nitrogen stream. To these tubes, 200 μl of 2 *M* sodium hydroxide and 10 ml of ethyl acetate were added. The tubes were vortexed for 90 sec and centrifuged for 5 min, after which the organic phases were transferred to tapered extraction tubes (test tubes with capillaries of 300-μl capacity, fused to the bottom) and 150 μl of 0.01 *M* sulfuric acid were added. After capping and vortexing for 90 sec, the tubes were placed in a dry ice—acetone bath for 1 min to solidify the contents. They were then centrifuged for 5 min. The aqueous phases (approximately 250 μl), now thawed, were transferred to disposable polyethylene limited-volume inserts (Brinkmann, Westbury, NY, U.S.A.) for the injection vials of the HPLC automatic sampler.

Plasma samples were prepared by the same method, except that the aceto-

TABLE I

ASSAY PERFORMANCE SPECIFICATIONS

Type of sample	Assay range*	Range linearity: coefficient of variation of concentration-normalized peak height ratios (%)	Concentration of samples spiked** for precision and accuracy tests (ng/ml)	Number of samples	Precision: coefficient of variation of determinations (%)	Bias: deviation of mean from concentration spiked (%)
Blood	Low	4.8	20	5	9.1	−18
	Interim	4.5	100	5	2.1	−7.2
	High	3.1	1000	5	0.5	−1.7
Plasma	Low	13.9	10	6	28	+6.3
			20	6	8.9	−4.3
	Interim	2.1	100	6	1.5	−3.8
	High	6.6	1000	6	1.2	−2.7
Urine	Low	6.8	200	5	5.0	+23
	Interim	10.9	2000	5	3.7	−2.4
	High	11.4	20,000	5	5.4	+8.0

*Range defined in text under Quantitation.

**Not standard curve calibrators but samples run in addition.

nitrile protein-precipitation step and subsequent evaporation were omitted.

For urine samples, the plasma processing method was employed using only 200 μl of sample and 800 μl of phosphate buffer containing the appropriate standards (2 μg of acebutolol). Diethyl ether (10 ml) rather than ethyl acetate was the extracting solvent and the back-extraction was accomplished by adding 200 μl of 0.01 *M* sulfuric acid and omitting the freezing step.

Chromatography

The mobile phase was an aqueous solution containing 55% of acetonitrile and 6% of a 0.1 *M* phosphate buffer (H_3PO_4/KH_2PO_4) at pH 4.0, filtered through a 0.45-μm filter (type HA, Millipore, Bedford, MA, U.S.A.). The eluent was pumped at 1.0 ml/min at about 86 bar (Model 6000A pump, Waters Assoc., Milford, MA, U.S.A.), through a 2-μm inline filter (Alltech, Deerfield, IL, U.S.A.) before reaching a Spherisorb ODS 5 μm, 250 mm $\times$ 4.6 mm column (Altex, Berkeley, CA, U.S.A.). Samples were injected by automatic sample injector (Waters 710B). Injection volume was 150 μl for low- and intermediate-range samples and 40 μl for high-range samples. These ranges are defined below. Absorbance of the effluent was monitored at 237 nm (by either a VUV-10 or a UV-50 detector, Varian, Palo Alto, CA, U.S.A.). Peak heights were measured by a computing integrator (Spectra-Physics 4100, Santa Clara, CA, U.S.A.).

Quantitation

Standard curves for blood and plasma samples used calibrators with concentrations of 0, 10, 20, 50, 100, 200, 500, 1000, 2000, and 5000 ng/ml. To avoid undue weight being assigned to high-concentration points, the standard curves were divided into three ranges. For each range a least-squares linear regression was fitted to peak height ratio versus concentration. The low range contained the calibrators up to 100 ng/ml, the interim range calibrators between 100 and 1000 ng/ml and the high range calibrators between 500 and 5000 ng/ml. These three ranges were used to quantitate samples with 10—100 ng/ml, 100—1000 ng/ml and 1000—5000 ng/ml of celiprolol, respectively. The urine standard curve included calibrators at 0, 0.2, 0.5, 1, 2, 5, 10, 20, 50, 100 and 200 μg/ml and was also divided into three ranges. Calibrators from 0—2 μg/ml were used for quantitating urine samples from 0.2—2 μg/ml, calibrators from 1—20 μg/ml for quantitating samples from 2—20 μg/ml and calibrators from 10—200 μg/ml for quantitating samples from 20—200 μg/ml.

RESULTS AND DISCUSSION

Linearity, precision and accuracy specifications for the assay of celiprolol in blood, plasma or urine are shown in Table I. For the urine data, five different blank urines were used in order to assess any variability due to differences in urine composition.

Chromatograms arising from plasma and blood samples are almost identical in appearance. Fig. 2 shows chromatograms from a plasma sample and a blood sample taken before a celiprolol dose and from a blood sample taken after administration of celiprolol. A chromatogram from a pre-dose blood sample

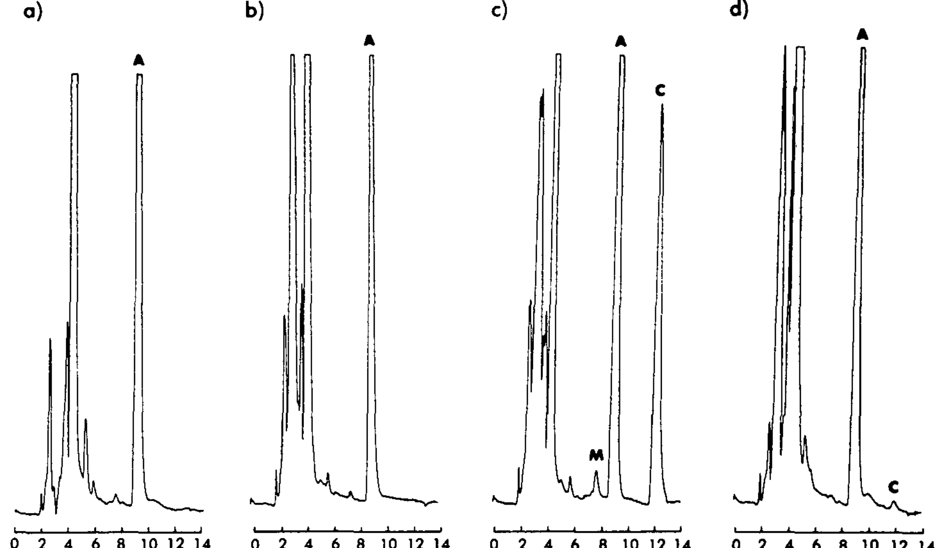

Fig. 2. Chromatograms from (a) a plasma sample and (b) a blood sample both taken before dosing, (c) a blood sample taken after administration of a 300-mg oral dose of celiprolol, (d) a pre-dose blood sample spiked with 10 ng/ml of celiprolol. Peaks: A, acebutolol, the internal standard; C, celiprolol; M, a possible metabolite of celiprolol.

spiked with 10 ng/ml of celiprolol (the lowest calibrator) is also shown. Retention times are 9.0 and 12.1 min for acebutolol and celiprolol, respectively. The peak at 7.8 min is designated M on the chromatogram from the post-dose blood sample. Because this peak is not noticeable in blank or spiked samples and because its amplitude from both plasma and blood rises and falls with celiprolol levels, this peak may represent a metabolite of celiprolol.

Fig. 3 shows chromatograms from urine samples collected before and after a dose of celiprolol and from a spiked pre-dose sample at 0.2 μg/ml which is the lowest calibrator. Peaks marked in the post-dose sample include the possible metabolite at 7.5 min (designated M), the internal standard at 8.7 min, celiprolol at 11.5 min, and a further peak at 8.1 min (designated N), not well resolved from the acebutolol peak, which may represent another metabolite of celiprolol.

Fig. 4 shows celiprolol concentrations in plasma and in blood of a healthy adult subject at various times after a single oral dose of 300 mg. Also shown are "relative" concentrations of the compound with a 7.8-min chromatographic retention time.

These "relative" concentrations were estimated using the peak height ratio for this compound and the relationship of concentration to peak height ratio for celiprolol. If the molecular weights and absorbance coefficients are indeed identical, the calculated concentrations of the potential metabolite would be an overestimate since there appears to be less peak spread associated with the earlier peak. Fig. 5 shows urinary excretion rate plots for celiprolol and the two potential metabolites, estimated similarly.

Previous studies with celiprolol have been based on radiolabelled drug or

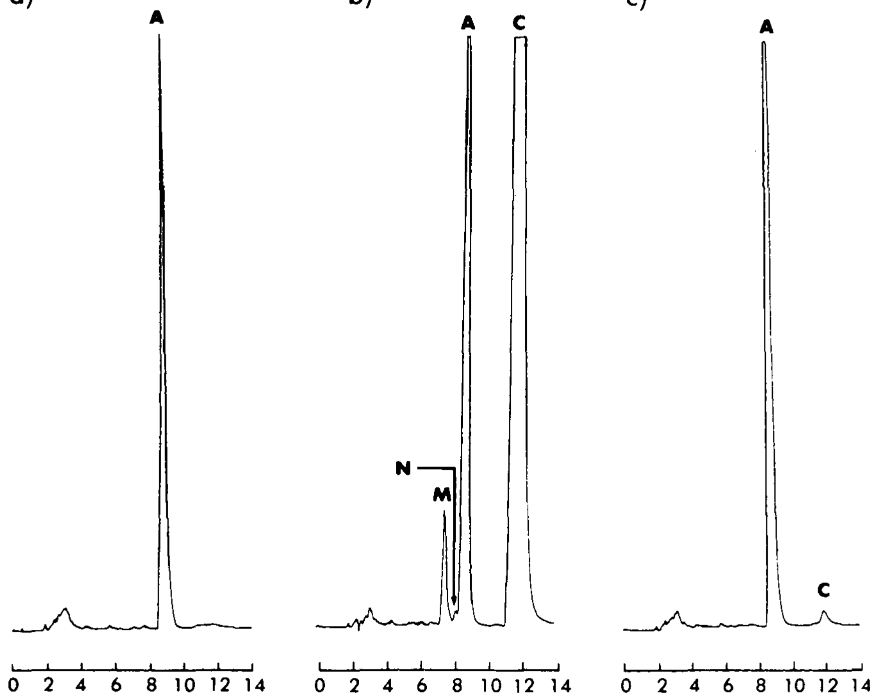

Fig. 3. Chromatograms from urine samples collected (a) prior to and (b) after administration of a 300-mg oral dose of celiprolol and (c) from a urine sample spiked with 0.2 μg/ml of celiprolol. Peaks: A, acebutolol, the internal standard; C, celiprolol; M, N, possible metabolites of celiprolol.

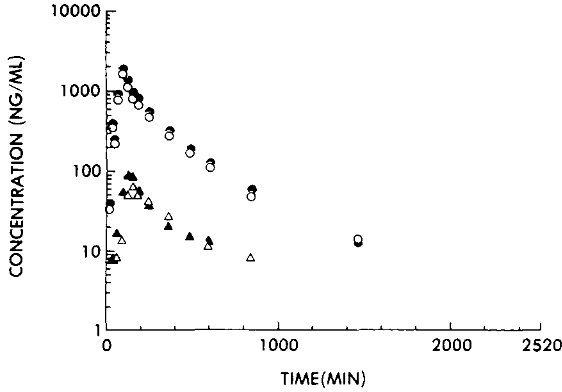

Fig. 4. Blood (•) and plasma (○) concentrations of celiprolol in a subject following an oral dose of 300 mg of celiprolol. Also shown are "concentrations" of a possible metabolite of celiprolol in blood (▲) and in plasma (△).

on nonspecific or insensitive assay procedures. Detailed specifications are given here of a specific and convenient HPLC assay for celiprolol in blood, plasma and urine. This assay has sufficient sensitivity, accuracy and precision to be of use in pharmacokinetic studies. In addition, it appears to quantitate a potential

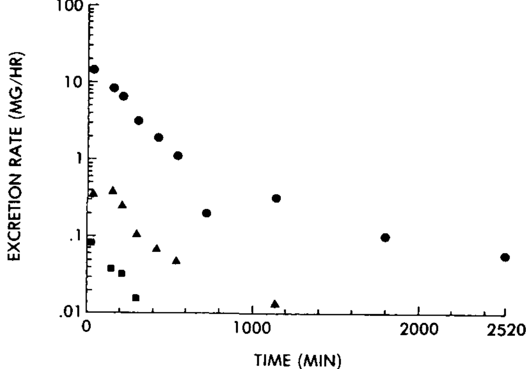

Fig. 5. Urinary excretion rate of a subject following an oral dose of 300 mg celiprolol. Shown are rates of celiprolol elimination (●) as well as "elimination" of two possible metabolites of celiprolol. These compounds correspond to the peaks designated M (▲) and N (■) in Fig. 3.

metabolite apparent in all three biological fluids and a second potential metabolite which was observed only in urine.

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We are grateful to Ms. Anita Jonda for management of the clinical study.

NOTE ADDED IN PROOF

Since submission of this paper, we have investigated the use of fluorescence detection with this assay. Using a fluorescence detector (Model 650-10LC; Perkin Elmer, Norwalk, CT, U.S.A.) set at 335 nm excitation wavelength and 472 nm emission wavelength (20-nm slit widths) and quinidine as an internal standard, the sensitivity of the technique described above can be extended down to 5 ng/ml allowing celiprolol plasma concentrations to be traced for an extra half-life. The following linearity specifications were obtained: coefficient of variation of concentration—normalized peak height ratios in the low range (calibrators at 0, 5, 8, 10, 20, 50 ng/ml) = 5.4 ± 1.5% (n = 3 standard curves on 3 days); in the intermediate range (calibrators as before) = 4.0 ± 0.6%; in the high range (calibrators as before) = 6.2 ± 1.3%. Interday bias/precision specifications (defined in Table I) for controls spiked at 5, 10, 100 and 1000 ng/ml were —2.8/4.1% (n = 4), +9.3/7.4% (n = 3), —2.0/5.2% (n = 3) and +1.9/5.2% (n = 3), respectively. The metabolite detected in plasma by ultraviolet monitoring can be similarly detected by fluorescence monitoring.

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Note

Fluorodensitometric determination of nadolol in plasma and urine

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Nadolol, *cis*-5-{3-[(1,1-dimethylethyl)amino]-2-hydroxypropoxy}-1,2,3,4-tetrahydro-2,3-naphthalenediol, is a long-acting β-adrenoceptor blocking drug [1,2]. Following the administration of therapeutic doses, plasma levels proved to be in the nanogram range [3]. To measure plasma levels of drugs for pharmacokinetic studies or drug monitoring, sensitive and rapid methods are required which allow analysis of many samples per day. Two methods for the analysis of nadolol in biological materials have been described up to now: a fluorimetric method following the oxidation and coupling of the resulting aldehyde with *o*-phenylenediamine [4] and a gas—liquid chromatographic (GLC) determination combined with mass spectrometric quantitation [5]. Both methods are rather laborious. A third assay, using high-performance liquid chromatography, described recently, requires electrochemical detection [6], the latter not being generally available. We describe here a densitometric method with fluorimetric quantitation which proved to be simple and rapid.

EXPERIMENTAL

Apparatus

A chromatogram spectrophotometer KM 3 from Carl Zeiss with a Perkin-Elmer recorder Model 56 was used.

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Standard and reagents

Nadolol was obtained from Heyden (Munich, G.F.R.). Standard solutions (10 mg of nadolol dissolved in 100 ml of methanol, and stock solution diluted 1:10) were stored at 4°C for one week. Solvents, chemicals (all analytical grade and used without further purification), and thin-layer plates (10 × 20 cm, coated with silica gel 60, without fluorescence indicator) were purchased from Merck (Darmstadt, G.F.R.).

Glassware

All glassware used in the extraction procedure (100 × 16 mm screw-capped test tubes, and plastic caps with Teflon linings) was washed with detergent solution, rinsed with tap water, then distilled water, and dried. Reagents were added with automatic pipettes.

Method

Extraction procedure for plasma. A 2-ml plasma sample was pipetted into a test tube, and 1.5 g of sodium chloride and 1 ml of 5 *N* sodium hydroxide were added. The mixture was shaken in a horizontal position. Then 5 ml of diethyl ether were added and the tubes shaken again for 10 min. Thereafter the samples were centrifuged at 2000 *g* to separate the layers. The organic phase was removed completely and transferred to another test tube. The solvent was concentrated to dryness at 40°C under an atmosphere of nitrogen. Together with twelve samples of unknown nadolol content, four blank plasma samples (2 ml) spiked with 60, 100, 200, and 600 ng of nadolol were processed.

Extraction procedure for urine. To 0.2 ml (or less) of urine 0.5 ml of 1 *N* hydrochloric acid was added. The acidic solution was pre-extracted with 5 ml of diethyl ether by shaking for 5 min. The layers were separated by centrifuging and the organic layer was discarded. To the aqueous layer 1 g of sodium chloride and 1 ml of 5 *N* sodium hydroxide were added. Extraction of nadolol and evaporation of the solvent were performed as described for plasma. Again, four samples of spiked blank urine (60, 200, 400, and 600 ng per 0.2 ml) were extracted together with twelve patient samples.

Chromatography. The residues were dissolved in 50 μl of methanol by shaking vigorously; 40 μl of the solution were spotted on a thin-layer plate (6 mm wide, Linomat III; Camag, Muttenz, Switzerland). On each plate were spotted twelve extracts of plasma or urine samples of unknown nadolol content and four extracts of spiked plasma. The thin-layer plates spotted with plasma extracts were developed in a saturated glass tank containing the solvent system chloroform—methanol—glacial acetic acid (75:20:5, v/v; R_F = 0.26). Urine extracts were chromatographed in chloroform—methanol—glacial acetic acid (60:35:10, v/v; R_F = 0.47). After developing for 8 cm the plates were air-dried and thereafter dipped into a solution of 4% nujol in cyclohexane. After another drying period of at least 20 min the plates were scanned.

Fluorescence measurement. Fluorimetric measurements were performed using the monochromator—sample mode of the scanner. Fluorescence of nadolol was excited with the 265-nm line of a medium pressure lamp ST 41. A 313-nm monochromatic filter served for the selection of the emitted fluores-

cent light (intensification ten times). The slit was 1 × 8 mm, and the plates were scanned at 100 mm/min. The peaks were registered on an interfaced recorder. The amount of nadolol in plasma and urine was determined by a calibration curve. Peak height was plotted versus nadolol content in spiked samples. The calibration curve was established for each plate.

Nadolol plasma levels and urinary excretion

Nadolol tablets containing 60 and 120 mg of the drug were administered to seven healthy subjects. Blood samples were drawn by venipuncture before the administration of the drug and up to 48 h afterwards. Plasma was separated by centrifugation. Urine was collected in definite intervals for 72 h. Plasma and urine specimens were stored frozen at −20°C until analysis.

RESULTS AND DISCUSSION

Excitation of nadolol on thin-layer chromatographic (TLC) plates with shortwave UV light (265 nm) resulted in the emission of a rather shortwave fluorescent light. A similar behaviour has been reported for atenolol [7,8]. The native fluorescence of nadolol was increased about twofold by spraying the plate with a mixture of 10% citric acid in water—ethylene glycol (1:1), and about fivefold by dipping into a solution of 4% nujol in cyclohexane. Fluorescence enhancement due to nujol is based upon the non-polarity and viscosity of the solvent [9]. Using the latter version the limit of detection of nadolol on TLC plates is in the order of 5 ng per spot. The calibration curve is linear up to 1400 ng per spot. Use of either peak height or peak area for the calibration curve proved to be of the same quality. As the correlation coefficient of both parameters exceeded 0.996, peak height was chosen for analysis as it is obtained more easily. Typical calibration curves are:

pure substance: $Y = 1.160 + 0.187X$, $r = 0.9999$

nadolol extracted from plasma: $Y = 1.386 + 0.088X$, $r = 0.9999$

nadolol extracted from urine: $Y = 2.736 + 0.118X$, $r = 0.9997$.

Extraction of nadolol from urine and plasma was performed by a method similar to that described by Ivashkiv [4], except that plasma samples were extracted by diethyl ether, too. Thus evaporation of butyl acetate, which has a relatively high boiling point leading to a prolonged evaporation time, was avoided. Following the extraction of nadolol from plasma and urine the recovery is incomplete (63% for plasma, 77% for urine samples). Therefore spiked plasma samples were carried through the analysis to avoid major fluctuations in the results due to day-to-day variation. Chromatography of nadolol extracted from plasma in the solvent system described by Dreyfuss et al. [10] leads to chromatograms free of interfering peaks (Fig. 1). Chromatography of urine extracts following the same extraction schedule gives an additional peak interfering with nadolol. This can be avoided by increasing the amounts of methanol and acetic acid in the chromatographic system together with a pre-extraction of the acidified urine specimens with ether.

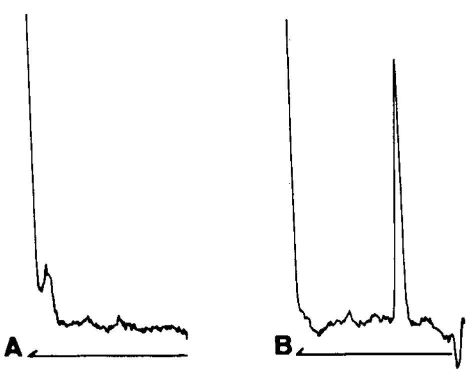

Fig. 1. Chromatograms of blank plasma (A) and of 2 ml of blank plasma spiked with 400 ng of nadolol (B). Analysis performed as described in the text. Direction of development = direction of the scan, as indicated by the arrows.

TABLE I

VARIATION COEFFICIENTS FOR NADOLOL DETERMINATIONS IN PLASMA AND URINE

Seven samples of each concentration were investigated.

Sample	Concentration* of nadolol	Variation coefficient (%)
Plasma	1000	3.9
	400	3.6
	200	3.8
	100	7.1
	50	4.0
Urine	1000	6.5
	50	7.5

*Plasma: ng per 2 ml. Urine: ng per 0.2 ml.

Recovery of nadolol from plasma was 62--64%, recovery from urine samples was 76--78%. Analyses were performed with five and two different concentrations for plasma and urine, respectively, on three different days. Reproducibility of the method proved to be sufficient for the determination of nadolol in biological fluids. The relative standard deviation was calculated from seven replicate analyses of relevant concentrations of nadolol in plasma and urine. The variation coefficient was usually less than 4% for plasma samples and about 7% for urine; for details see Table I. According to the evaluation of other beta-blockers by fluorodensitometry, the variation coefficient exceeds that of the GLC methods slightly [5,8,11].

Following the administration of nadolol to healthy volunteers (60 and 120 mg oral dose) plasma levels could be monitored for at least 12 h. Nadolol could be detected in urine specimens for 72 h. Representative plasma levels and urinary excretion rates ($\Delta U/\Delta t$ versus midpoint time) are shown in Fig. 2. Only unchanged nadolol was excreted in urine and faeces following the administration of [^{14}C]nadolol to hypertensive patients either intravenously or by

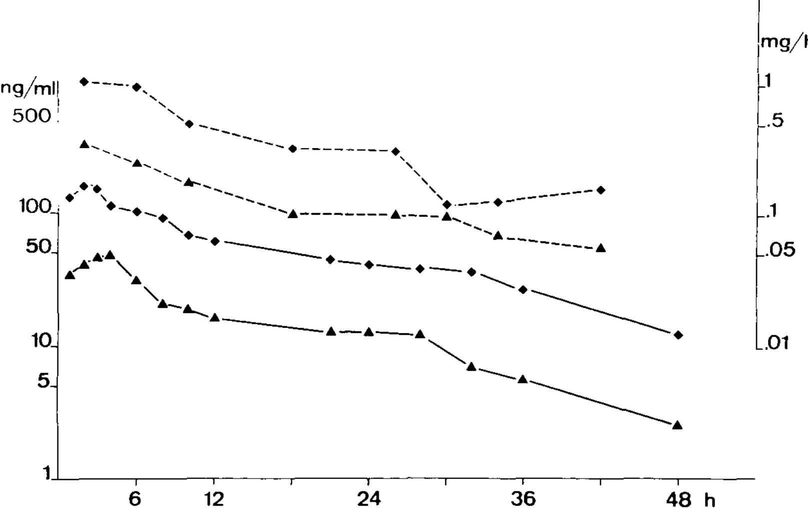

Fig. 2. Nadolol plasma levels (solid line) and urinary excretion rate ($\Delta U/\Delta t$ versus midpoint time; dotted line) of a healthy subject administered 60 mg (▲) and 120 mg (♦) orally.

the oral route [10]. Therefore, the interference of metabolites does not seem to decrease the specificity of the method described.

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Book Review

Immunochemical techniques for the identification and estimation of macromolecules (Laboratory Techniques in Biochemistry and Molecular Biology, edited by T.S. Work and E. Work, Vol. 1, Part III), by J. Clausen, Elsevier/North-Holland Biomedical Press, Amsterdam, New York, Oxford, 2nd (revised) ed., 1981, XIV + 387 pp., price Dfl. 170.00, US$ 72.25, ISBN 0-444-80245-2 (hardback); price Dfl. 61.00, US$ 26.00, ISBN 0-444-80244-4 (paperback).

This is the updated version of a popular book on immunochemical methods that appeared in 1969 and that became slightly obsolete as the result of the fast expanding field of immunochemistry. The volume is amazingly well assembled: it offers equally good reading for newcomers and experts in the field. After the general introduction, in which a classification of antigens is presented along with the present image of antigen—antibody reaction, the other chapters are devoted to immunodiffusion in gels, antisera, techniques of immunodiffusion, techniques of immunoelectrophoresis, visualization and interpretation of precipitates in gels, immunoadsorbent techniques, radioimmunochemical techniques, enzyme- and metalloimmunoassays, difficulties and artefacts arising in gel diffusion and in immunoelectrophoresis, immunochemical reactions in free buffer systems, immunofluorescence techniques, complement fixation test, application of lymphocytes for tracing antigens and, naturally, varia. But beyond this scope, the book also has Chapter 16 entitled Appendices, which was, without doubt, hard work to put together, but which doubles at the least the practical value of the volume. Here is a practical guide for most of the operations needed in immunochemical analyses: it starts with the preparation of Freud's adjuvant, offers a wide selection of staining procedures for immunoelectrophoresis, preparation of protein immunoadsorbents and finally deals with the separation of lymphocytes by affinity chromatography. Many other procedures are included, the enumeration of which is clearly beyond the scope of a short review.

Another positive feature of Clausen's book is the fact that complex immunological phenomena are explained as clearly as possible at the level of present knowledge — something that is far from being respected in other books devoted to immunochemistry. In the eyes of those involved in separation science the parts on immunoelectrophoretic methods and immunoaffinity methods will be welcomed; but readers will find here the proper value of separation procedures in immunology (and immunochemistry) as such.

In conclusion, the book is not only a well-presented work on immunochemical methods for the estimation and identification of macromolecules as advertised in its title, but it is pleasant reading as well. And last but not least: it will surely become a valuable handbook (a real and tiny one) in many protein laboratories.

Prague (Czechoslovakia) Z. DEYL

Related articles published in *Journal of Chromatography*, Vols. 238—241

Volume 238

Universal, multi-channel ultraviolet detection in the purity analysis of 2-ethyl-3-(4-hydroxybenzoyl)-indolizine by high-performance liquid chromatography
by B.F.H. Drenth, R.T. Ghijsen and R.A. de Zeeuw (Groningen, The Netherlands) (Received November 23rd, 1981) 113

Large injection volumes of derivatized iodothyronines in capillary gas chromatography
by J.A. Corkill and R.W. Giese (Boston, MA, U.S.A.) (Received November 16th, 1981) 133

Medium-pressure liquid chromatography of Leozym, a pectic enzyme preparation on ion-exchange derivatives of Spheron
by L'. Rexová-Benková and J. Omelková (Bratislava, Czechoslovakia) and O. Mikeš and J. Sedláčková (Prague, Czechoslovakia) (Received November 30th, 1981) 183

High-performance liquid chromatography of proteins: analytical applications
by K. Buchholz and B. Gödelmann (Frankfurt, G.F.R.) and I. Molnar (Berlin, G.F.R.) (Received November 10th, 1981) 193

Apparatus for the recovery of proteins from polyacrylamide gels during electrophoresis
by M. Faupel, M. de Gasparo and E. von Arx (Basle, Switzerland) (Received December 15th, 1981) 226

High-performance liquid chromatographic determination of phosphate esters of dexamethasone and prednisolone and their sulphite adducts
by J. Dijkstra (Venlo, The Netherlands) and D. Dekker (Zoetermeer, The Netherlands) (Received December 4th, 1981) 247

Ready separation of ergocornine, α- and β-ergocryptine by high-performance liquid chromatography
by B. Herényi and S. Görög (Budapest, Hungary) (Received December 9th, 1981) 250

Thin-layer chromatographic analysis of 1(1-phenylcyclohexyl)pyrrolidine in urine
by R.D. Budd (Downey, CA, U.S.A.) (Received November 20th, 1981) 261

Reverse salt gradient chromatography of tRNA on unsubstituted agarose. I. Variations in elution profile and evidence for two fractionation mechanisms
by M. Spencer and M.M. Binns (London, Great Britain) (Received November 3rd, 1981) 297

Reverse salt gradient chromatography of tRNA on unsubstituted agarose. II. Further study of binding mechanisms
by M. Spencer (London, Great Britain) (Received November 3rd, 1981) 307

Reverse salt gradient chromatography of tRNA on unsubstituted agarose. III. Physical and chemical properties of different batches of Sepharose 4B
by M. Spencer (London, Great Britain) (Received November 3rd, 1981) 317

Procedure for the chiral derivatization and chromatographic resolution of *R*-(+)- and *S*-(—)-propranolol
by J.A. Thompson (Boulder, CO, U.S.A.), J.L. Holtzman (Haverford, PA, U.S.A.), M. Tsuru (Minneapolis, MN, U.S.A.), C.L. Lerman (Haverford, PA, U.S.A.) and J.L. Holtzman (Minneapolis, MN, U.S.A.) (Received December 28th, 1981) 470

Adsorption chromatographic separation of testosterone-3-(O-carboxymethyl) oxime tyrosine methyl ester and its ^{125}I-labelled derivative
by G. Tóth (Budapest, Hungary) (Received December 22nd, 1981) 476

Separation of radiation and photo-induced 5,6-dihydrothymine derivatives by reversed-phase high-performance liquid chromatography
by J. Cadet, M. Berger and L. Voituriez (Grenoble, France) (Received December 2nd, 1981) . . 488

Determination of thiabendazole by ion-pair high-performance liquid chromatography
by B.R. Belinky (Cincinnati, OH, U.S.A.) (Received November 26th, 1981) 506

Determination of the γ-aminobutyric acid agonist 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol by high-performance liquid chromatography using UV or electrochemical detection
by S.M. Madsen (Valby/Copenhagen, Denmark) (Received November 27th, 1981) 509

Rapid extraction and measurement of morphine and opiate antagonists from rat brain using high-performance liquid chromatography and electrochemical detection
by R.B. Raffa, J.J. O'Neill and R.J. Tallarida (Philadelphia, PA, U.S.A.) (Received December 28th, 1981) . . . 515

Determination of morphine, diamorphine and their degradation products in pharmaceutical preparations by reversed-phase high-performance liquid chromatography
by I. Beaumont and T. Deeks (Birmingham, Great Britain) (Received November 19th, 1981) . . 520

Determination of emetine and cephaeline in Ipecac roots by high-performance liquid chromatography
by N.P. Sahu and S.B. Mahato (Calcutta, India) (Received December 15th, 1981) . . . 525

Volume 239

Multi-component analyses of human body fluids and tissues in health and disease using capillary gas chromatography—mass spectrometry and high-resolution two-dimensional electrophoresis
by E. Jellum (Oslo, Norway) . . . 29

Analysis of picomolar concentrations of 6-oxo-prostaglandin $F_{1\alpha}$ in biological fluids
by S.E. Barrow, K.A. Waddell, M. Ennis, C.T. Dollery and I.A. Blair (London, Great Britain) . . 71

Simultaneous analysis of imipramine and its metabolite desipramine in biological fluids
by J.C. Craig, L.D. Gruenke and T.-L. Nguyen (San Francisco, CA, U.S.A.) . . . 81

Measurement of endogenous leucine enkephalin in canine thalamus by high-performance liquid chromatography and field desorption mass spectrometry
by D.M. Desiderio and S. Yamada (Memphis, TN, U.S.A.) . . . 87

Long-term variation study of blood plasma levels of chloroform and related purgeable compounds
by C.D. Pfaffenberger and A.J. Peoples (Miami, FL, U.S.A.) . . . 217

Gas chromatographic and mass spectrometric studies on the metabolism and pharmacokinetics of Δ^1-tetrahydrocannabinol in the rabbit
by D.J. Harvey, J.T.A. Leuschner and W.D.M. Paton (Oxford, Great Britain) . . . 243

Unidimensional, sequential separation of PTH-amino acids by high-performance thin-layer chromatography
by S.A. Schuette and C.F. Poole (Detroit, MI, U.S.A.) . . . 251

Quantitative determination of lecithin and sphingomyelin at nanogram levels by high-performance thin-layer chromatography using fluorescence. Preliminary results
by L. Zhou, H. Shanfield and A. Zlatkis (Houston, TX, U.S.A.) . . . 259

Automated qualitative and quantitative metabolic profiling analysis of urinary steroids by a gas chromatography—mass spectrometry—data system
by J.J. Vrbanac, W.E. Braselton, Jr., J.F. Holland and C.C. Sweeley (East Lansing, MI, U.S.A.) . 265

Simultaneous determination of glyceryl trinitrate and its principal metabolites, 1,2- and 1,3-glyceryl dinitrate, in plasma by gas chromatography-negative ion chemical ionization-selected ion monitoring
by H. Miyazaki, M. Ishibashi, Y. Hashimoto, G. Idzu and Y. Furuta (Tokyo, Japan) . . . 277

Gas chromatographic—mass spectrometric analysis of endogenous levels of estradiol in plasma and in cytosol from rat uterus
by M. Tetsuo, H. Eriksson and J. Sjövall (Stockholm, Sweden) . . . 287

Compilation of gas chromatographic retention indices of 163 metabolically important organic acids, and their use in detection of patients with organic acidurias
by K. Tanaka and D.G. Hine (New Haven, CT, U.S.A.) . . . 301

Determination of tocainide in human plasma by gas chromatography with nitrogen-selective detection after Schiff base formation
by L. Johansson and J. Vessman (Mölndal, Sweden) . . . 323

Determination of picomole amounts of choline and acetylcholine in blood by gas chromatography—mass spectrometry equipped with a newly improved pyrolyzer
by Y. Hasegawa, M. Kunihara and Y. Maruyama (Gumma, Japan) . . . 335

Quantification of endogenous aliphatic alcohols in serum and urine
by H.M. Liebich, H.J. Buelow and R. Kallmayer (Tübingen, G.F.R.) . . . 343

High-performance displacement chromatography of corticosteroids. Scouting for displacer and analysis of the effluent by thin-layer chromatography
by H. Kalász and Cs. Horváth (New Haven, CT, U.S.A.) 423

Resolution of optical isomers of Dns-amino acids by high-performance liquid chromatography with L-histidine and its derivatives in the mobile phase
by S. Lam and A. Karmen (Bronx, NY, U.S.A.). 451

Total amino acid analysis using pre-column fluorescence derivatization
by H. Umagat, P. Kucera and L.-F. Wen (Nutley, NJ, U.S.A.). 463

A simple high-performance liquid chromatographic pre-column technique for investigation of drug metabolism in biological fluids
by W. Voelter and T. Kronbach (Tübingen, G.F.R.) and K. Zech and R. Huber (Konstanz, G.F.R.). 475

Hydrophobicity and chromatographic behaviour of aromatic acids found in urine
by T. Hanai and J. Hubert (Montréal, Canada) 527

High-performance liquid chromatography of human serum lipoproteins. Selective detection of triglycerides by enzymatic reaction
by I. Hara, K. Shiraishi and M. Okazaki (Ichikawashi, Japan) 549

Separation and determination of naproxen enantiomers in serum by high-performance liquid chromatography
by J. Goto, N. Goto and T. Nambara (Sendai, Japan) 559

Two-dimensional separation system for analysis of proteins employing isoelectric focusing and high-performance liquid chromatography
by K. Kojima, T. Manabe, T. Okuyama, T. Tomono, T. Suzuki and E. Tokunaga (Tokyo, Japan) 565

Characterization of bile acid methyl ester acetate derivatives of rat bile using solventless glass capillary gas chromatography and electron impact and ammonia chemical ionization mass spectrometry
by T. Murata, S. Takahashi, S. Ohnishi, K. Hosoi, T. Nakashima, Y. Ban and K. Kuriyama (Kyoto, Japan) 571

Determination of captopril and its disulphide in biological fluids
by Y. Matsuki, T. Ito, K. Fukuhara, T. Nakamura, M. Kimura and H. Ono (Kanagawa, Japan) and T. Nambara (Sendai, Japan) 585

Microdetermination of prostaglandins and thromboxane B_2 by gas chromatography using an electron-capture detector
by H. Miyazaki, M. Ishibashi, K. Yamashita and I. Ohguchi (Tokyo, Japan), H. Saitoh, H. Kurono and M. Shimono (Kyoto, Japan) and M. Katori (Kanagawa, Japan). 595

Determination of β-adrenergic blocking drugs as cyclic boronates by gas chromatography with nitrogen-selective detection
by T. Yamaguchi, Y. Morimoto, Y. Sekine and M. Hashimoto (Osaka, Japan) 609

Reversed-phase liquid chromatographic resolution of underivatized D,L-amino acids using chiral eluents
by N. Nimura, A. Toyama, Y. Kasahara and T. Kinoshita (Tokyo, Japan). 671

Determination of blood transketolase by high-performance liquid chromatography (a preliminary note)
by M. Kimura and Y. Itokawa (Kyoto, Japan) 707

Determination of prednisone and prednisolone in human serum by high-performance liquid chromatography — especially on impaired conversion of corticosteroids in patients with chronic liver disease
by T. Ui, M. Mitsunaga, T. Tanaka and M. Horiguchi (Tokyo, Japan) 711

Determination of 5′-nucleotidase activity in human erythrocytes and plasma using high-performance liquid chromatography
by T. Sakai, S. Yanagihara and K. Ushio (Tokyo, Japan) 717

Pre-column labelling for high-performance liquid chromatography of amino acids with 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole and its application to protein hydrolysates
by Y. Watanabe and K. Imai (Tokyo, Japan) 723

Analysis of body functions using a clinical liquid chromatograph
by H. Miyagi, J. Miura, Y. Takata, S. Kamitake, S. Ganno and H. Yamagata (Ibaraki, Japan). . . 733

Preparation of affinity adsorbents with Toyopearl gels
by I. Matsumoto, Y. Ito and N. Seno (Tokyo, Japan) 747

Adsorption chromatography of proteins on siliconized porous glass
by T. Mizutani and T. Narihara (Nagoya, Japan) 755

Separation and determination of bile acids by high-performance liquid chromatography using immobilized 3α-hydroxysteroid dehydrogenase and an electrochemical detector
by S. Kamada, M. Maeda, A. Tsuji and Y. Umezawa (Tokyo, Japan) and T. Kurahashi (Kyoto, Japan) 773

Volume 240

Studies on lectins. LIII. Affinity electrophoresis in the study of the effect of detergents on the interaction of lectins with carbohydrates
by L. Pechová, M. Tichá and J. Kocourek (Prague, Czechoslovakia) (Received December 17th, 1981). 43

Investigation of the effect of added organic amine on the chromatography of tricyclic antidepressant drugs using reversed-phase chromatography on octadecylsilica with sodium lauryl sulphate as pairing ion
by C.T. Hung, R.B. Taylor and N. Paterson (Aberdeen, Great Britain) (Received December 11th, 1981) 61

Evaluation of polyethylene glycol-HT as a stationary phase for capillary column gas—liquid chromatography of trimethylsilyl ethers of bile acid methyl esters
by N. Tanida, Y. Hikasa and T. Shimoyama (Hyogo, Japan) (Received December 9th, 1981) . . 75

Separation of digoxin, digitoxin and their potential metabolites, impurities or degradation products by high-performance liquid chromatography
by B. Desta, E. Kwong and K.M. McErlane (Vancouver, Canada) (Received December 21st, 1981). 137

Rapid method for purification of plasmid DNA and DNA fragments from DNA linkers using high-performance liquid chromatography on TSK-PW gel
by M.E. Himmel (Golden, CO, U.S.A.), P.J. Perna (Fort Collins, CO, U.S.A.) and M.W. McDonell (Boulder, CO, U.S.A.) (Received January 19th, 1982) 155

Separation of basic, hydrophilic peptides by reversed-phase ion-pair chromatography. I. Analytical applications with particular reference to a class of serine peptide substrates of cyclic AMP-stimulated protein kinase
by B. Fransson, U. Ragnarsson and Ö. Zetterqvist (Uppsala, Sweden) (Received January 4th, 1982). 165

Purification of human serum hyaluronidase using chromatofocusing
by M. Fenger (Næstved and Glostrup, Denmark) (Received December 30th, 1981) 173

Problems encountered during peptide derivatization for gas chromatographic—mass spectrometric analysis
by G.N. Jham (Viçosa, Brazil) (Received January 12th, 1982) 184

Liquid chromatographic determination of clorazepate decomposition rates
by C.R. Clark, W.R. Ravis, R. Dockens, J.M. Barksdale, H.S. Arrington and G.H. D'Andrea (Auburn University, AL, U.S.A.) (Received January 18th, 1982). 196

Determination of epichlorohydrin in blood by gas chromatography and selected ion monitoring
by L. De Petrocellis, M. Tortoreto, S. Paglialunga, R. Paesani, L. Airoldi, E.R. Castaneda and C. Pantarotto (Milan, Italy) (Received December 21st, 1981). 218

Dns derivatization of anabolic agents with high-performance liquid chromatographic separation and fluorescence detection
by A.T. Rhys Williams and S.A. Winfield (Beaconsfield, Great Britain) and R.C. Belloli (Fullerton, CA, U.S.A.) (Received October 19th, 1981) 224

Prediction of peptide retention times in reversed-phase high-performance liquid chromatography during linear gradient elution
by T. Sasagawa (Seattle, WA, U.S.A.), T. Okuyama (Tokyo, Japan) and D.C. Teller (Seattle, WA, U.S.A.) (Received January 20th, 1982) 329

Estimation of molecular weights of peptides by determination of height equivalent to a theoretical plate in size-exclusion chromatography
by Y. Shioya, H. Yoshida and T. Nakajima (Hiroshima, Japan) (Received January 11th, 1982) . 341

Application of fused-silica capillary gas chromatography to the analysis of underivatized drugs
by L.L. Plotczyk (Avondale, PA, U.S.A.) (Received February 1st, 1982) 349

Precision at the low picogram level in the analysis of derivatized iodothyronine standards by capillary gas chromatography with electron-capture detection
by J.A. Corkill, M. Joppich, A. Nazareth and R.W. Giese (Boston, MA, U.S.A.) (Received January 6th, 1982) 415

Detection and identification of volatile organic compounds in blood by headspace gas chromatography as an aid to the diagnosis of solvent abuse
by J.D. Ramsey and R.J. Flanagan (London, Great Britain) (Received January 19th, 1982) . . . 423

Analysis of insulin preparations by reversed-phase high-performance liquid chromatography
by L.F. Lloyd and P.H. Corran (London, Great Britain) (Received January 8th, 1982) 445

Quantitative high-performance liquid chromatographic determination of sulphur amino acids in protein hydrolysates
by L.N. Mackey and T.A. Beck (Cincinnati, OH, U.S.A.) (Received February 8th, 1982) 455

Quantitative determination of neutral lipids on chromarods
by E.R. Farnworth, B.K. Thompson and J.K.G. Kramer (Ottawa, Canada) (Received January 13th, 1982) 463

Use of high-performance liquid chromatography to quantitate thymine-containing pyrimidine dimers in DNA
by J.D. Love and E.C. Friedberg (Stanford, CA, U.S.A.) (Received December 24th, 1981) 475

High-performance liquid chromatographic separation of the diastereoisomers of propoxyphene. Determination of microquantities of *β-dl*-propoxyphene in commercial preparations of *α-d*-propoxyphene
by S. Barkan and I.W. Ainer (Washington, DC, U.S.A.) (Received January 27th, 1982) 547

Volume 241

Determination of apovincaminic acid in human plasma by gas—liquid chromatography
by M. Polgár and L. Vereczkey (Budapest, Hungary). 29

Investigation of the metabolites of tofizopam in man and animals by gas—liquid chromatography—mass spectrometry
by É. Tomori, G. Horváth, I. Elekes, T. Láng and J. Körösi (Budapest, Hungary) 89

Determination of apovincaminic acid in biological samples by high-performance liquid chromatography
by M. Kozma, P. Pudleiner and L. Vereczkey (Budapest, Hungary) 177

Bestimmung von Talinolol mit Hochleistungs-Flüssigkeits-Chromatographie in Humanplasma
von H. Pötter und M. Hülm (Radebeul, D.D.R.) und K. Richter (Dresden, D.D.R.) 189

Semi-preparative high-performance liquid chromatography in the isolation of derivatives of cardioactive steroidal lactones
by R. Megges and H. Grobe (Berlin-Buch, G.D.R.) 193

High-resolution gas chromatography of methylated ribonucleosides and hypermodified adenosines. Evaluation of trimethylsilyl derivatization and split and splitless operation modes
by T.R. Kemp, R. Andersen, J. Oh and T.H. Vaughn (Lexington, KY, U.S.A.) (Received February 20th, 1982) 325

Interactions between calmodulin and immobilized phenothiazines. Application to the purification of calmodulin from various tissues by affinity chromatography
by C. Rochette-Egly (Strasbourg, France), E. Boschetti (Villeneuve la Garenne, France) and P. Basset and J.-M. Egly (Strasbourg, France) (January 29th, 1982) 333

Amino acid analyses by high-performance liquid chromatography. An evaluation of the usefulness of pre-column Dns derivatization
by C. de Jong, G.J. Hughes, E. van Wieringen and K.J. Wilson (Zürich, Switzerland) (Received January 13th, 1982) 345

Amino acid microanalysis of proteins extracted from spots of fixed, stained, two-dimensional gels
by T. Manabe, O. Oda and T. Okuyama (Tokyo, Japan) (Received December 17th, 1981) 361

Affinity electrophoresis in an isotachophoretic discontinuous buffer system
by V. Hořejši, T.K. Datta and M. Tichá (Prague, Czechoslovakia) (Received January 25th, 1982). 395

Gas chromatographic assay of atropine and phenobarbital in pharmaceutical preparations containing Valeriana liquid extract
by P. Majlát (Budapest, Hungary) (Received January 28th, 1982) 399

Detection of binding between transfer RNA and RNA polymerase by high-performance liquid chromatography
by S. Amero (Morgantown, WV, U.S.A.) (Recieved February 3rd, 1982) 413

Separation of diacyl and plasmalogen phospholipids of rat brain synaptosomal membranes on chromarods
by M. Foot and M.T. Clandinin (Toronto, Canada) (Received January 29th, 1982) 428

Author Index

Ackerly, C.C.
— , Newman, R.A., Myers, C. and McCormack, J.J.
Liquid chromatographic separation and quantitation of 2-amino-1,3,4-thiadiazole (NSC-4728) from human and murine serum 175
Adam, M., see Deyl, Z. 409
Agris, P.F., see Gehrke, C.W. 297
Ahokas, J.T., see Norris, R.L.G. 433
Akagi, H., see Matsushima, E. 188
Alexander, N.M.
— and Neeley, W.E.
Chromatofocusing of human hemoglobins 137
Asada, H., see Niwa, T. 1
Aso, Y., see Noda, A. 345
Badcock, N.R.
— and Pollard, A.C.
Micro-determination of clonazepam in plasma or serum by electron-capture gas—liquid chromatography 353
Baker, J.K.
— , McChesney, J.D., Hufford, C.D. and Clark, A.M.
High-performance liquid chromatographic analysis of the metabolism of primaquine and the identification of a new mammalian metabolite 69
Bareggi, S.R., see Zecca, L. 168
Barfod, N.M.
High-performance liquid chromatographic purification of endogenous tumour cell growth inhibitory peptides 289
Bauer, L.A., see Watts, M.T. 79
Baughman, Jr., R.A., see Mico, B.A. 203
Bellon, G.
— , Bisker, A., Maquart, F.X., Thoanes, H. and Borel, J.P.
Improved method for the evaluation of 3-hydroxyproline and 4-hydroxyproline in the urine 420
Benet, L.Z., see Buskin, J.N. 454
— , see Mico, B.A. 203
Bieder, A., see Le Roux, Y. 401
Bisker, A., see Bellon, G. 420
Borel, J.P., see Bellon, G. 420
Boulton, A.A., see Davis, B.A. 219
Braughler, J.M., see McGinley, P.A. 29
Breckenridge, W.C., see Kuksis, A. 231
Bredenkamp, B.L.F.
— and Joubert, S.M.
The effect of ampholytes on ferritin isoelectric focussing patterns 327
Breithaupt, H.
— and Wilfling, M.
Determination of mexiletine in biological fluids by high-performance liquid chromatography 97
Brown, P.R., see Zakaria, M. 381
Bruckner, G., see Mai, J. 15
Buchwald, W.F., see Cone, E.J. 57
Buskin, J.N.
— , Upton, R.A., Jones, R.M. and Williams, R.L.
High-performance liquid chromatography assay of acebutolol and two of its metabolites in plasma and urine 438
— , Upton, R.A., Sörgel, F., Williams, R.L., Lang, E. and Benet, L.Z.
Specific and sensitive assay of celiprolol in blood, plasma and urine using high-performance liquid chromatography 454
— , Upton, R.A. and Williams, R.L.
Improved acetaminophen assay sensitivity by modification of a high-performance liquid chromatography technique 443
Caron, J.C.
— , Martin, B. and Shroot, B.
High-performance liquid chromatographic determination of urocanic acid isomers in biological samples 125
Castagnoli, Jr., N., see Shinohara, Y. 363
Chan, W.-Y., see Griesmann, G.E. 121
Chatterji, D.C., see Narang, P.K. 373
Choi, R.L.
— , Rosenberg, M., Grebow, P.E. and Huntley, T.E.
High-performance liquid chromatographic analysis of indapamide (RHC 2555) in urine, plasma and blood 181
Clark, A.M., see Baker, J.K. 69
Cone, E.J.
— , Gorodetzky, C.W., Yeh, S.Y., Darwin, W.D. and Buchwald, W.F.
Detection and measurement of opium

alkaloids and metabolites in urine of opium eaters by methane chemical ionization mass fragmentography 57
Cooper, T.B., see Suckow, R.F. 391
Darwin, W.D., see Cone, E.J. 57
Davis, B.A.
— , Durden, D.A. and Boulton, A.A. Plasma concentrations of *p*- and *m*-hydroxyphenylacetic acid and phenylacetic acid in humans. Gas chromatographic—high-resolution mass spectrometric analysis 219
De Jong, J.W., see Harmsen, E. 131
De Tombe, P.Ph., see Harmsen, E. 131
Deyl, Z.
— , Macek, K., Adam, M. and Horáková, M.
High-performance gel permeation chromatography of collagens 409
Dijkstra, D., see Feenstra, M.G.P. 271
Dring, L.G., see Gillet, G. 154
Durden, D.A., see Davis, B.A. 219
Feenstra, M.G.P.
— , Homan, J.W., Dijkstra, D., Mulder, T.B.A., Rollema, H., Westerink, B.H.C. and Horn, A.S.
Reversed-phase liquid chromatography with amperometric detection of lipophilic dopamine analogues and determination of brain and serum concentrations after sample clean-up on small Sephadex G-10 columns 271
Fornstedt, N.
A method for high-performance liquid chromatographic screening of UV-positive components in urine eluate from Sephadex G-10 and modifications for determination of urinary salicylic, salicyluric and gentisic acids 253
Fourtillan, J.B., see Lefebvre, M.A. 199
Fraisse-André, J., see Gillet, G. 154
Fremouw-Ottevangers, D.C., see Muskiet, F.A.J. 142
Furukawa, H., see Niwa, T. 1
Gaillot, J., see Le Roux, Y. 401
Geher, K., see Kuksis, A. 231
Gehrke, C.W.
— , Kuo, K.C., McCune, R.A., Gerhardt, K.O. and Agris, P.F.
Quantitative enzymatic hydrolysis of tRNAs. Reversed-phase high-performance liquid chromatography of tRNA nucleosides 297
Gerhardt, K.O., see Gehrke, C.W. 297
Gillet, G.
— , Fraisse-André, J., Lee, C.R., Dring, L.G. and Morselli, P.L.
Gas chromatographic method for the determination of progabide (SL 76.002) in biological fluids 154
Gorodetzky, C.W., see Cone, E.J. 57
Goswami, S.K., see Mai, J. 15
Grebow, P.E., see Choi, R.L. 181
Griesmann, G.E.
— , Chan, W.-Y. and Rennert, O.M.
Determination of γ-aminobutyric acid by reversed-phase high-performance liquid chromatography and pre-column labeling for fluorescence detection 121
Grupe, A.
— and Spiteller, G.
Unexpected metabolites produced from clomethiazole 335
Guadagni, L., see Zecca, L. 168
Halie, M.R., see Muskiet, F.A.J. 142
Hall, E.D., see McGinley, P.A. 29
Haraguchi, S.-I.
— , Terasawa, M., Toshima, H., Matsumoto, I., Kuhara, T. and Shinka, T.
Analysis of organic acids in the hearts of patients with idiopathic cardiomyopathy by gas chromatography—mass spectrometry 7
Harapat, S.R.
— and Kates, R.E.
High-performance liquid chromatographic analysis of propafenone in human plasma samples 448
Harmsen, E.
— , De Tombe, P.Ph. and De Jong, J.W.
Simultaneous determination of myocardial adenine nucleotides and creatine phosphate by high-performance liquid chromatography 131
Hayashi, C., see Yamaguchi, Y. 263
Hirata, M., see Noda, A. 345
Hirobe, M., see Matsumura, K. 148
Holliday, S.B., see Hoskins, J.A. 162
Homan, J.W., see Feenstra, M.G.P. 271
Horáková, M., see Deyl, Z. 409
Horn, A.S., see Feenstra, M.G.P. 271
Hoskins, J.A.
— and Holliday, S.B.
Determination of α-methyldopa and methyldopate in human breast milk and plasma by ion-exchange chromatography using electrochemical detection 162
Houston, J.B., see Taylor, G. 194
Hsu, K.-Y., see Noda, A. 345
Hufford, C.D., see Baker, J.K. 69
Huntley, T.E., see Choi, R.L. 181
Iguchi, S., see Noda, A. 345

Ikenaka, T., see Omichi, K. 415
Jakobs, U., see Krause, W. 37
Jannuzzo, M.G., see Moro, E. 207
Johnson, E.A.
Gel chromatography of heparin 216
Jones, R.M., see Buskin, J.N. 438
Jong, J.W. de, see Harmsen, E. 131
Joubert, S.M., see Bredenkamp, B.L.F. 327
Jozefczak, C.
— , Ktorza, N. and Uzan, A.
High-performance liquid chromatographic determination of indalpine, a new non-tricyclic antidepressant, in human plasma. Identification and simultaneous measurement of its major plasma metabolite 87
Julian, B., see Lefebvre, M.A. 199
Justová, V.
— , Wildtová, Z. and Stárka, L.
Separation of nanogram quantities of hydroxy metabolites of vitamin D_3 in plasma by thin-layer chromatography on silica gel 319
Kang-Lee, Y.A., see McKee, R.W. 309
Kates, R.E., see Harapat, S.R. 448
Kato, H., see Matsumura, K. 148
Kinsella, J.E., see Mai, J. 15
Krause, W.
— and Jakobs, U.
Determination of plasma levels of spirorenone, a new aldosterone antagonist, and one of its metabolites by high-performance liquid chromatography 37
Ktorza, N., see Jozefczak, C. 87
Kubo, O., see Matsumura, K. 148
Kuhara, T., see Haraguchi, S.-I. 7
Kuksis, A.
— , Myher, J.J., Geher, K., Breckenridge, W.C. and Little, J.A.
Lipid class and molecular species interrelationships among plasma lipoproteins of Type III and Type IV hyperlipemic subjects 231
Kuo, K.C., see Gehrke, C.W. 297
Lang, E., see Buskin, J.N. 454
Lee, C.R., see Gillet, G. 154
Lee, W.H., see Pieroni, J.P. 115
Lefebvre, M.A.
— , Julian, B. and Fourtillan, J.B.
High-performance liquid chromatographic assay for determination of a new β-blocking agent FM 24 199
Le Roux, Y.
— , Gaillot, J. and Bieder, A.
High-performance liquid chromatographic determination of pipotiazine in human plasma and urine 401
Little, J.A., see Kuksis, A. 231
McChesney, J.D., see Baker, J.K. 69
McCormack, J.J., see Ackerly, C.C. 175
McCune, R.A., see Gehrke, C.W. 297
Macek, K., see Deyl, Z. 409
McGinley, P.A.
— , Braughler, J.M. and Hall, E.D.
Determination of methylprednisolone in central nervous tissue and plasma using normal-phase high-performance liquid chromatography 29
McKee, R.W.
— , Kang-Lee, Y.A., Panaqua, M. and Swendseid, M.E.
Determination of nicotinamide and metabolic products in urine by high-performance liquid chromatography 309
Maeda, K., see Niwa, T. 1
Mai, J.
— , Goswami, S.K., Bruckner, G. and Kinsella, J.E.
Determination of prostaglandins and thromboxane as their pentafluorobenzyl-trimethylsilyl derivatives by electron-capture gas chromatography 15
Maquart, F.X., see Bellon, G. 420
Martin, B., see Caron, J.C. 125
Marunaka, T., see Matsushima, E. 188
Matsumoto, I., see Haraguchi, S.-I. 7
Matsumura, K.
— , Kubo, O., Tsukada, T., Nishide, K., Kato, H., Watanabe, K. and Hirobe, M.
Determination of tulobuterol in human serum by electron-capture gas—liquid chromatography 148
Matsushima, E.
— , Umeno, Y., Marunaka, T. and Akagi, H.
Determination of pentazirinocyclodiphosphathiazene in biological fluids by high-performance liquid chromatography 188
Matsuyama, K., see Noda, A. 345
Mico, B.A.
— , Baughman, Jr., R.A. and Benet, L.Z.
Determination of tiodazosin in plasma and whole blood by high-performance liquid chromatography 203
Miller, R.D., see Shinohara, Y. 363
Moro, E.
— , Jannuzzo, M.G., Ranghieri, M., Stegnjaich, S. and Valzelli, G.

Determination of 4'-epidoxorubicin and its 13-dihydro derivative in human plasma by high-performance liquid chromatography with fluorescence detection 207
Morselli, P.L., see Gillet, G. 154
Mulder, T.B.A., see Feenstra, M.G.P. 271
Muskiet, F.A.J.
— , Stratingh, C.M., Fremouw-Ottevangers, D.C. and Halie, M.R.
Mass spectrometric identification of isoputreanine, a metabolite of spermidine and/or spermine, in human urine 142
Mutschler, E., see Schäfer-Korting, M. 461
Myers, C., see Ackerly, C.C. 175
Myher, J.J., see Kuksis, A. 231
Narang, P.K.
— , Yeager, R. and Chatterji, D.C.
Quantitation of 6-mercaptopurine in biologic fluids using high-performance liquid chromatography: a selective and novel procedure 373
Neeley, W.E., see Alexander, N.M. 137
Newman, R.A., see Ackerly, C.C. 175
Nishide, K., see Matsumura, K. 148
Niwa, T.
— , Maeda, K., Asada, H., Shibata, M., Ohki, T., Saito, A. and Furukawa, H.
Gas chromatographic—mass spectrometric analysis of organic acids in renal tissue biopsy. Identification of 4-hydroxybutyric acid and 4-hydroxy-2-butenoic acid 1
Noda, A.
— , Hsu, K.-Y., Aso, Y., Matsuyama, K., Iguchi, S. and Hirata, M.
Studies of isoniazid metabolism in isolated rat hepatocytes by mass fragmentography 345
Norris, R.L.G.
— , Ahokas, J.T. and Ravenscroft, P.J.
Simultaneous high-performance liquid chromatographic assay for quinidine, disopyramide and the mono-N-dealkylated metabolite of disopyramide 433
Ohki, T., see Niwa, T. 1
Omichi, K.
— and Ikenaka, T.
Rapid and sensitive sialidase assay by high-performance liquid chromatography and its application to detection of sialidase in human urine 415
Panaqua, M., see McKee, R.W. 309
Pieroni, J.P.
— , Lee, W.H. and Wong, P.Y.-K.
Separation of six major prostacyclin metabolites by high-performance liquid chromatography 115
Pollard, A.C., see Badcock, N.R. 353
Raisys, V.A., see Watts, M.T. 79
Rane, A., see Svensson, J.-O. 427
Ranghieri, M., see Moro, E. 207
Ratge, D.
— and Wisser, H.
Urinary protein profiling by high-performance gel permeation chromatography 47
Ravenscroft, P.J., see Norris, R.L.G. 433
Rennert, O.M., see Griesmann, G.E. 121
Rollema, H., see Feenstra, M.G.P. 271
Rosenberg, M., see Choi, R.L. 181
Roux, Y. le, see Le Roux, Y. 401
Säwe, J., see Svensson, J.-O. 427
Saito, A., see Niwa, T. 1
Schäfer-Korting, M.
— and Mutschler, E.
Fluorodensitometric determination of nadolol in plasma and urine 461
Schlöbe, R.
— and Thijssen, H.H.W.
Quantitative thin-layer chromatography of trimethoprim and tetroxoprim using fluorescence densitometry 212
Shibata, M., see Niwa, T. 1
Shinka, T., see Haraguchi, S.-I. 7
Shinohara, Y.
— , Miller, R.D. and Castagnoli, Jr., N.
Ion-pair high-performance liquid chromatographic assay of 4-aminopyridine in serum 363
Shroot, B., see Caron, J.C. 125
Sjöqvist, F., see Svensson, J.-O. 427
Sörgel, F., see Buskin, J.N. 454
Spiteller, G., see Grupe, A. 335
Stárka, L., see Justová, V. 319
Stegnjaich, S., see Moro, E. 207
Stratingh, C.M., see Muskiet, F.A.J. 142
Suckow, R.F.
— and Cooper, T.B.
Simultaneous determination of amitriptyline, nortriptyline and their respective isomeric 10-hydroxy metabolites in plasma by liquid chromatography 391
Svensson, J.-O.
— , Rane, A., Säwe, J. and Sjöqvist, F.
Determination of morphine, morphine-3-glucuronide and (tentatively) morphine-6-glucuronide in plasma and urine using ion-pair high-performance liquid chromatography 427

Swendseid, M.E., see McKee, R.W. 309
Taylor, G.
— and Houston, J.B.
Simultaneous determination of promethazine and two of its circulating metabolites by high-performance liquid chromatography 194
Terasawa, M., see Haraguchi, S.-I. 7
Thijssen, H.H.W., see Schlöbe, R. 212
Thoanes, H., see Bellon, G. 420
Timm, U.
— and Weidekamm, E.
Determination of pyrimethamine in human plasma after administration of Fansidar or Fansidar—mefloquine by means of high-performance liquid chromatography with fluorescence detection 107
Tombe, P.Ph. de, see Harmsen, E. 131
Toshima, H., see Haraguchi, S.-I. 7
Tsukada, T., see Matsumura, K. 148
Umeno, Y., see Matsushima, E. 188
Upton, R.A., see Buskin, J.N. 438, 443, 454
Uzan, A., see Jozefczak, C. 87
Valzelli, G., see Moro, E. 207
Watanabe, K., see Matsumura, K. 148
Watts, M.T.
— , Raisys, V.A. and Bauer, L.A.
Determination of thiabendazole and 5-hydroxythiabendazole in human serum by fluorescence-detected high-performance liquid chromatography 79
Weidekamm, E., see Timm, U. 107
Westerink, B.H.C., see Feenstra, M.G.P. 271
Wildtová, Z., see Justová, V. 319
Wilfling, M., see Breithaupt, H. 97
Williams, R.L., see Buskin, J.N. 438, 443, 454
Wisser, H., see Ratge, D. 47
Wong, P.Y.-K., see Pieroni, J.P. 115
Yamaguchi, Y.
— and Hayashi, C.
Enzymatic detection of urinary conjugated steroids after gel chromatography 263
Yeager, R., see Narang, P.K. 373
Yeh, S.Y., see Cone, E.J. 57
Zakaria, M.
— and Brown, P.R.
Rapid assay for plasma chlorambucil and phenyl acetic mustard using reversed-phase liquid chromatography 381
Zecca, L.
— , Guadagni, L. and Bareggi, S.R.
Determination of sodium flavodate in body fluids by high-performance liquid chromatography. Application to clinical pharmacokinetic studies 168

Subject Index

Acebutolol
HPLC assay of acebutolol and two of its metabolites in plasma and urine 438
Acetaminophen
Improved acetaminophen assay sensitivity by modification of a HPLC technique 443
Adenine nucleotides
Simultaneous determination of myocardial adenine nucleotides and creatine phosphate by HPLC 131
Aldosterone antagonists
Determination of plasma levels of spirorenone, a new aldosterone antagonist, and one of its metabolites by HPLC 37
Alkaloids
Detection and measurement of opium alkaloids and metabolites in urine of opium eaters by methane chemical ionization mass fragmentography 57
Amino acids
Improved method for the evaluation of 3-hydroxyproline and 4-hydroxyproline in the urine 420
γ-Aminobutyric acid
Determination of γ-aminobutyric acid by reversed-phase HPLC and precolumn labeling for fluorescence detection 121
4-Aminopyridine
Ion-pair HPLC assay of 4-aminopyridine in serum 363
2-Amino-1,3,4-thiadiazole
LC separation and quantitation of 2-amino-1,3,4-thiadiazole (NSC-4728) from human and murine serum 175
Amitriptyline
Simultaneous determination of amitriptyline, nortriptyline and their respective isomeric 10-hydroxy metabolites in plasma by LC 391
Anthraquinone glycoside
Determination of 4'-epidoxorubicin and its 13-dihydro derivative in human plasma by HPLC with fluorescence detection 207
Celiprolol
Specific and sensitive assay of celiprolol in blood, plasma and urine using HPLC 454
Chlorambucil
Rapid assay for plasma chlorambucil and phenyl acetic mustard using reversed-phase LC 381
Clomethiazole
Unexpected metabolites produced from clomethiazole 335
Clonazepam
Micro-determination of clonazepam in plasma or serum by electron-capture GLC 353
Codeine
Detection and measurement of opium alkaloids and metabolites in urine of opium eaters by methane chemical ionization mass fragmentography 57
Collagens
High-performance gel permeation chromatography of collagens 409
Collagens
Improved method for the evaluation of 3-hydroxyproline and 4-hydroxyproline in the urine 420
Creatine phosphate
Simultaneous determination of myocardial adenine nucleotides and creatine phosphate by HPLC 131
Cyclodiphosphathiazenes
Determination of pentazirinocyclodiphosphathiazene in biological fluids by HPLC 188
Disopyramide
Simultaneous HPLC assay for quinidine, disopyramide and the mono-N-dealkylated metabolite of disopyramide 433
Dopamine analogues
Reversed-phase LC with amperometric detection of lipophilic dopamine analogues and determination of brain and serum concentrations after sample clean-up on small Sephadex G-10 columns 271
4'-Epidoxorubicin
Determination of 4'-epidoxorubicin and its 13-dihydro derivative in human plasma by HPLC with fluorescence detection 207

Fansidar
Determination of pyrimethamine in human plasma after administration of Fansidar or Fansidar—mefloquine by means of HPLC with fluorescence detection 107
Ferritin
The effect of ampholytes on ferritin isoelectric focussing patterns 327
Flavonoids
Determination of sodium flavodate in body fluids by HPLC. Application to clinical pharmacokinetic studies 168
FM 24
HPLC assay for determination of a new β-blocking agent FM 24 199
Gentisic acid
A method for HPLC screening of UV-positive components in urine eluate from Sephadex G-10 and modifications for determination of urinary salicylic, salicyluric and gentisic acids 253
Glucocorticoids
Determination of methylprednisolone in central nervous tissue and plasma using normal-phase HPLC 29
Hemoglobins
Chromatofocusing of human hemoglobins 137
Heparin
Gel chromatography of heparin 216
4-Hydroxy-2-butenoic acid
GC—MS analysis of organic acids in renal tissue biopsy. Identification of 4-hydroxybutyric acid and 4-hydroxy-2-butenoic acid 1
4-Hydroxybutyric acid
GC—MS analysis of organic acids in renal tissue biopsy. Identification of 4-hydroxybutyric acid and 4-hydroxy-2-butenoic acid 1
Hydroxyphenylacetic acids
Plasma concentrations of *p*- and *m*-hydroxyphenylacetic acid and phenylacetic acid in humans. GC—high-resolution MS analysis 219
Hydroxyprolines
Improved method for the evaluation of 3-hydroxyproline and 4-hydroxyproline in the urine 420
5-Hydroxythiabendazole
Determination of thiabendazole and 5-hydroxythiabendazole in human serum by fluorescence-detected HPLC 79
Indalpine
HPLC determination of indalpine, a new non-tricyclic antidepressant, in human plasma. Identification and simultaneous measurement of its major plasma metabolite 87
Indapamide
HPLC analysis of indapamide (RHC 2555) in urine, plasma and blood 181
Isoniazid
Studies of isoniazid metabolites in isolated rat hypatocytes by mass fragmentography 345
Isoputreanine
MS identification of isoputreanine, a metabolite of spermidine and/or spermine, in human urine 142
Lipids
Lipid class and molecular species interrelationships among plasma lipoproteins of Type III and Type IV hyperlipemic subjects 231
Lipoproteins
Lipid class and molecular species interrelationships among plasma lipoproteins of Type III and Type IV hyperlipemic subjects 231
Mefloquine
Determination of pyrimethamine in human plasma after administration of Fansidar or Fansidar—mefloquine by means of HPLC with fluorescence detection 107
6-Mercaptopurine
Quantitation of 6-mercaptopurine in biologic fluids using HPLC: a selective and novel procedure 373
α-Methyldopa
Determination of α-methyldopa and methyldopate in human breast milk and plasma by ion-exchange chromatography using electrochemical detection 162
Methyldopate
Determination of α-methyldopa and methyldopate in human breast milk and plasma by ion-exchange chromatography using electrochemical detection 162
Methylprednisolone
Determination of methylprednisolone in central nervous tissue and plasma using normal-phase HPLC 29
Mexiletine
Determination of mexiletine in biological fluids by HPLC 97

Morphine
Detection and measurement of opium alkaloids and metabolites in urine of opium eaters by methane chemical ionization mass fragmentography 57
Morphine
Determination of morphine, morphine-3-glucuronide and (tentatively) morphine-6-glucuronide in plasma and urine using ion-pair HPLC 427
Morphine glucuronides
Determination of morphine, morphine-3-glucuronide and (tentatively) morphine-6-glucuronide in plasma and urine using ion-pair HPLC 427
Nadolol
Fluorodensitometric determination of nadolol in plasma and urine 461
Nicotinamide
Determination of nicotinamide and metabolic products in urine by HPLC 309
Nortriptyline
Simultaneous determination of amitriptyline, nortriptyline and their respective isomeric 10-hydroxy metabolites in plasma by LC 391
Nucleotides
Simultaneous determination of myocardial adenine nucleotides and creatine phosphate by HPLC 131
Organic acids
Analysis of organic acids in the hearts of patients with isiopathic cardiomyopathy by GC—MS 7
Organic acids
GC—MS analysis of organic acids in renal tissue biopsy. Identification of 4-hydroxybutyric acid and 4-hydroxy-2-butenoic acid 1
Pentazirinocyclodiphosphathiazene
Determination of pentazirinocyclodiphosphathiazene in biological fluids by HPLC 188
Peptides
HPLC purification of endogenous tumour cell growth inhibitory peptides 289
Phenylacetic acid
Plasma concentrations of *p*- and *m*-hydroxyphenylacetic acid and phenylacetic acid in humans. GC—high-resolution MS analysis 219
Phenyl acetic mustard
Rapid assay for plasma chlorambucil and phenyl acetic mustard using reversed-phase LC 381
Pipotiazine
HPLC determination of pipotiazine in human plasma and urine 401
Polyamines
MS identification of isoputreanine, a metabolite of spermidine and/or spermine, in human urine 142
Polypeptides
High-performance gel permeation chromatography of collagens 409
Primaquine
HPLC analysis of the metabolism of primaquine and the identification of a new mammalian metabolite 69
Progabide
GC method for the determination of progabide (SL 76.002) in biological fluids 154
Promethazine
Simultaneous determination of promethazine and two of its circulating metabolites by HPLC 194
Propafenone
HPLC analysis of propafenone in human plasma samples 448
Prostacyclin
Separation of six major prostacyclin metabolites by HPLC 115
Prostaglandins
Determination of prostaglandins and thromboxane as their pentafluorobenzyl-trimethylsilyl derivatives by elelctron-capture GC 15
Prostaglandins
Separation of six major prostacyclin metabolites by HPLC 115
Proteins
Urinary protein profiling by high-performance gel permeation chromatography 47
Pyrimethamine
Determination of pyrimethamine in human plasma after administration of Fansidar or Fansidar—mefloquine by means of HPLC with fluorescence detection 107
Quinidine
Simultaneous HPLC assay for quinidine, disopyramide and the mono-N-dealkylated metabolite of disopyramide 433
Ribonucleic acids, transfer
Quantitative enzymatic hydrolysis of tRNAs. Reversed-phase HPLC of tRNA nucleosides 297
Salicylic acid
A method for HPLC screening of

UV-positive components in urine eluate from Sephadex G-10 and modifications for determination of urinary salicylic, salicyluric and gentisic acids 253
Salicyluric acid
A method for HPLC screening of UV-positive components in urine eluate from Sephadex G-10 and modifications for determination of urinary salicylic, salicyluric and gentisic acids 253
Sialidase
Rapid and sensitive sialidase assay by HPLC and its application to detection of sialidase in human urine 415
Sodium flavodate
Determination of sodium flavodate in body fluids by HPLC. Application to clinical pharmacokinetic studies 168
Spermidine
MS identification of isoputreanine, a metabolite of spermidine and/or spermine, in human urine 142
Spermine
MS identification of isoputreanine, a metabolite of spermidine and/or spermine, in human urine 142
Spirorenone
Determination of plasma levels of spirorenone, a new aldosterone antagonist, and one of its metabolites by HPLC 37
Steroids
Determination of methylprednisolone in central nervous tissue and plasma using normal-phase HPLC 29
Steroids
Determination of plasma levels of spirorenone, a new aldosterone antagonist, and one of its metabolites by HPLC 37
Steroid conjugates
Enzymatic detection of urinary conjugated steroids after gel chromatography 263
Tetroxoprim
Quantitative TLC of trimethoprim and tetroxoprim using fluorescence densitometry 212
Thiabendazole
Determination of thiabendazole and 5-hydroxythiabendazole in human serum by fluorescence-detected HPLC 79
Thromboxane
Determination of prostaglandins and thromboxane as their pentafluorobenzyl-trimethylsilyl derivatives by electron-capture GC 15
Tiodazosin
Determination of tiodazosin in plasma and whole blood by HPLC 203
Trimethoprim
Quantitative TLC of trimethoprim and tetroxoprim using fluorescence densitometry 212
tRNAs
Quantitative enzymatic hydrolysis of tRNAs. Reversed-phase HPLC of tRNA nucleosides 297
Tulobuterol
Determination of tulobuterol in human serum by electron-capture GLC 148
Urocanic acid
HPLC determination of urocanic acid isomers in biological samples 125
Vitamins
Determination of nicotinamide and metabolic products in urine by HPLC 309
Vitamin D_3
Separation of nanogram quantities of hydroxy metabolites of vitamin D_3 in plasma by TLC on silica gel 319

PUBLICATION SCHEDULE FOR 1982

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MONTH	J	F	M	A	M	J	J	A	S	O	N	D
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Electron Capture – Theory and Practice in Chromatography

edited by A. ZLATKIS, *Houston, TX, USA and* C.F. POOLE, *Detroit, MI, USA*

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This book provides the first comprehensive coverage of all aspects of the theory, design, operation and applications of the electron capture detector (ECD) from the chromatographer's point of view. In addition, an up-to-date look at the ancillary techniques of selective electron-capture sensitization, atmospheric pressure ionization and plasma chromatography has been included. ECD users will find the solutions to instrumental and technical problems which arise during practice particularly valuable. These have been derived from the experiences of the internationally distinguished team of authors.

Each chapter has been prepared by experts in their field and provides an in-depth coverage of its topic. The basic theory of the mechanisms of electron capture detection is included. Practical sections form the bulk of the book and are devoted to such topics as the construction and operating principles of the detector, including the establishment of instrument design criteria, and the different methods of derivatization. A more personal touch is provided by the inventor of the ECD, J.E. Lovelock, in his review of the development of the technique. Other chapters illustrate the importance of ECD in trace analysis in environmental and biomedical research. A unique feature is the extensive tabulation of all the pertinent data concerning the use of ECD in gas and liquid chromatography.

For those analytical chemists who use chromatography in their research, this book should become a standard text.

CONTENTS: Chapter 1. The electron-capture detector – A personal odyssey *(J.E. Lovelock).* 2. The design and operation of the electron-capture detector *(C.F. Poole and A. Zlatkis).* 3. Theory of electron capture *(W.E. Wentworth and E.C.M. Chen).* 4. Selective electron-capture sensitization *(F.C. Fehsenfeld, P.D. Goldan, M.P. Phillips and R.E. Sievers).* 5. Oxygen-doping of the carrier gas in electron-capture detection *(E.P. Grimsrud).* 6. Wide-range calibration of electron-capture detectors *(R.E. Kaiser and R.I. Rieder).* 7. Response of the electron-capture detector to compounds with natural electrophores *(J. Vessman).* 8. Sensitive derivatives for the determination of organic compounds by electron-capture gas chromatography *(C.F. Poole and A. Zlatkis).* 9. The detection of inorganic and organometallic compounds by electron-capture gas chromatography *(C.F. Poole and A. Zlatkis).* 10. Environmental applications of the electron-capture detector – pesticides *(W.P. Cochrane and R.B. Maybury).* 11. Environmental applications of the electron-capture detector – dioxins *(F. Bruner).* 12. The electron-capture detector as a monitor of halocarbons in the atmosphere *(P.G. Simmonds).* 13. Biomedical applications of the electron-capture detector *(J. Vessman).* 14. Negative ion atmospheric pressure ionization mass spectrometry and the electron-capture detector *(E.C. Horning, D.I. Carroll, I. Dzidic and R.N. Stillwell).* 15. Electron-capture process and ion mobility spectra in plasma chromatography *(F.W. Karasek and G.E. Spangler).* 16. The electron-capture detector as a detector in liquid chromatography *(U.A.Th. Brinkman).* Subject index.

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