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BIOMEDICAL APPLICATIONS

INDEX

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HYDROXYCARBOXYLIC AND OXOCARBOXYLIC ACIDS IN URINE: PRODUCTS FROM BRANCHED-CHAIN AMINO ACID DEGRADATION AND FROM KETOGENESIS

HARTMUT M. LIEBICH* and CLAUDIA FÖRST

Medizinische Universitätsklinik, D-7400 Tübingen (F.R.G.)

(Received February 1st, 1984)

SUMMARY

Hydroxy- and oxomonocarboxylic acids in urine of healthy individuals and of patients with diabetic ketoacidosis are analysed as methyl esters and methyl esters/O-methyloximes, respectively, by gas chromatography and gas chromatography—mass spectrometry. The derivatives are pre-fractionated by thin-layer chromatography.

The acids originate mainly from ketogenesis and from the metabolism of valine, leucine and isoleucine. The amino acid metabolites fall into three groups: the 2-oxocarboxylic acids (2-oxoisovaleric acid, 2-oxoisocaproic acid and 2-oxo-3-methylvaleric acid); the 2-hydroxycarboxylic acids (2-hydroxyisovaleric acid, 2-hydroxyisocaproic acid and 2-hydroxy-3-methylvaleric acid); and the 3-hydroxycarboxylic acids (3-hydroxyisobutyric acid, 3-hydroxyisovaleric acid, 3-hydroxy-2-ethylpropionic acid, *threo*-3-hydroxy-2-methylbutyric acid and *erythro*-3-hydroxy-2-methylbutyric acid). The *threo* form of 3-hydroxy-2-methylbutyric acid is the major constituent within the diastereomeric pair. Of the three groups of amino acid metabolites, the 3-hydroxycarboxylic acids in particular are elevated during ketoacidosis.

The characteristic general features of the mass spectrometric fragmentation of the derivatives of the identified components are systematically described. The discussion of the fragmentation includes constituents of low concentrations, such as 3-oxocaproic acid, 4-oxobutyric acid and 5-oxocaproic acid, which can be detected only when the pre-fractionation technique is applied.

INTRODUCTION

Hydroxy- and oxomonocarboxylic acids have been described as urinary excretion products in a number of congenital and acquired metabolic disorders; some have been found in normal urines too. 2-Oxocarboxylic acids and 2-hydroxycarboxylic acids were reported to be characteristic metabolites in maple syrup urine disease [1—3]; 3-oxocarboxylic acids and 3-hydroxycarboxylic acids have been described in propionic acidaemia [4, 5]; and several 3-

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hydroxycarboxylic acids in cases of ketoacidosis [6, 7]. Even though some of the acids have been reported as normal urinary constituents [8—10], they have been described only in small groups and under specific aspects. This paper deals with a systematic investigation of hydroxy- and oxomonocarboxylic acids in urine of healthy individuals and of patients with diabetic ketoacidosis, with the emphasis on those acids that originate from ketogenesis and from the metabolism of the branched-chain amino acids.

EXPERIMENTAL

Sample preparation

The sample preparation procedure included the reaction of the urine samples with O-methylhydroxylamine hydrochloride to form the O-methyloximes from the oxocarboxylic acids, the isolation of the organic acids by anion-exchange using Amberlyst A-26, the formation of the methyl esters with diazomethane, and the pre-fractionation of the derivatives by preparative thin-layer chromatography (TLC). The details of the method have been described [11]. Only the following modifications were employed. The urine sample (66 ml) was added slowly and under shaking to 134 ml of isopropanol, and any precipitated proteins were removed by centrifugation. The supernatant solution was added to 330 mg of O-methylhydroxylamine hydrochloride, kept at 65°C for 1 h and then applied in equal portions to two 25-cm long anion-exchange columns. For small samples (10 ml of urine), TLC plates with a 0.25-mm thick layer of silica gel were sufficient; the larger samples used in this study required a gel thickness of 2 mm. Instead of pre-fractionating the acid derivatives into the previously described four fractions, a total of eight fractions was obtained by dividing fraction 2 into two equally broad subfractions 2a and 2b, and fraction 3 into four equally broad subfractions 3a—d.

Gas chromatographic and mass spectrometric analysis

Fractions 2a, 2b and 3a—d were analysed by gas chromatography (GC) and gas chromatography—mass spectrometry (GC—MS). The GC separations were performed on a Model 3700 gas chromatograph with a flame-ionization detector (Varian, Darmstadt, F.R.G.) under the following conditions: 25 m × 0.2 mm I.D. fused-silica column, coated with OV-1701 (Scientific Glass Engineering, Weiterstadt, F.R.G.); carrier gas, nitrogen at 4 ml/min; column temperature, 40°C for 10 min, then programmed at 2°C/min; injector block temperature, 250°C; sample size, 1 μl at a splitting ratio of 1:20. Under the chosen conditions the derivatives of the hydroxycarboxylic and oxocarboxylic acids appeared in the early portions of the chromatograms (within 40 min).

For the GC—MS analyses a combination of a Model 2700 gas chromatograph, CH 5 mass spectrometer and Spectrosystem 100 MS computer (Varian-MAT, Bremen, F.R.G.) was used. The gas chromatograph and the mass spectrometer were interfaced by an open coupling system. By automatic repetitive scanning, the mass spectra were recorded over the mass range *m/e* 15—300 and stored on magnetic tape. Helium was used as the carrier gas. Otherwise, the same GC conditions were used as described for the GC separations. The MS conditions were as follows: ionization by electron impact; ionization energy,

70 eV; accelerating voltage, 3 kV; multiplier voltage, 2.25 kV; emission current, 300 μA; ion source temperature, 220°C; interface temperature, 220°C; resolution, 600.

Reference substances

The described hydroxycarboxylic and oxocarboxylic acids were identified on the basis of the mass spectra and the GC retention indices of the derivatives of the urinary components and of reference substances. The reference compounds were either purchased or synthesized. Glycolic acid, methyl lactate, sodium salt of 2-hydroxybutyric acid, 2-hydroxyisovaleric acid, 2-hydroxyisocaproic acid, 3-hydroxypropionic acid, pyruvic acid, 2-oxobutyric acid, 2-oxovaleric acid, 2-oxoisocaproic acid and 2-oxo-3-methylvaleric acid were purchased from Fluka (Neu-Ulm, F.R.G.), methyl 2-hydroxyisobutyrate and 5-oxocaproic acid from EGA-Chemie (Steinheim, F.R.G.), sodium salt of 2-hydroxy-3-methylvaleric acid, 2-oxoisovaleric acid and 4-oxobutyric acid from Sigma Chemie (Munich, F.R.G.), methyl 3-hydroxybutyrate and methyl 3-oxobutyrate from E. Merck (Darmstadt, F.R.G.). The identification of 3-hydroxyisobutyric acid and 3-hydroxyisovaleric acid was based on reference spectra of the methyl esters from the literature only [7, 12]. Methyl 3-hydroxy-2-ethylpropionate, methyl 3-hydroxy-2-methylbutyrate and methyl 3-oxocaproate were synthesized.

Methyl 3-hydroxy-2-ethylpropionate was synthesized by reduction of ethylmalonic acid with $LiAlH_4$, methylation of the resulting 3-hydroxy-2-ethylpropionic acid with diazomethane and purification of the methyl ester by preparative TLC.

Methyl 3-hydroxy-2-methylbutyrate (mixture of the two diastereomers) was prepared from methyl 3-oxo-2-methylbutyrate by reduction with $NaBH_4$ and purification on an Extrelut® column (Merck) using ethyl acetate as the eluent. By this method about equal amounts of the *threo* and *erythro* forms were obtained.

Methyl *erythro*-3-hydroxy-2-methylbutyrate was synthesized by reaction of the acetoxymercury adduct from tiglic acid with hydrogen sulphide [13].

Methyl 3-oxocaproate was prepared by ester condensation between methyl butyrate and methyl acetate, resulting in a mixture of methyl 3-oxobutanoate, methyl 3-oxocaproate, methyl 2-ethyl-3-oxobutanoate and methyl 2-ethyl-3-oxocaproate. This mixture was suitable to determine the mass spectrum and the retention behaviour of the 3-oxocaproic acid derivative.

All hydroxycarboxylic compounds were used in the form of their methyl esters, all oxocarboxylic compounds as methyl ester/O-methyloxime derivatives. The carbonyl functions were transformed into the O-methyloximes; free carboxyl groups were methylated according to the procedure described for the urinary constituents. Sodium salts were passed through a cation-exchange column filled with Dowex 50W-X8 (Serva) to form the free acids, which were then derivatized.

RESULTS AND DISCUSSION

The analysis of the hydroxy- and oxomonocarboxylic acids within the com-

plex mixture of organic acids in urine involves several considerable problems. Apart from 3-hydroxybutyric acid and 3-oxobutyric acid which are very major components in cases of increased ketogenesis, most of the other acids are (except in cases of some inborn errors of the metabolism of amino acids) excreted into the urine in low amounts. Their separation from the major components and from other organic acids is for some acids difficult to achieve by GC, unless

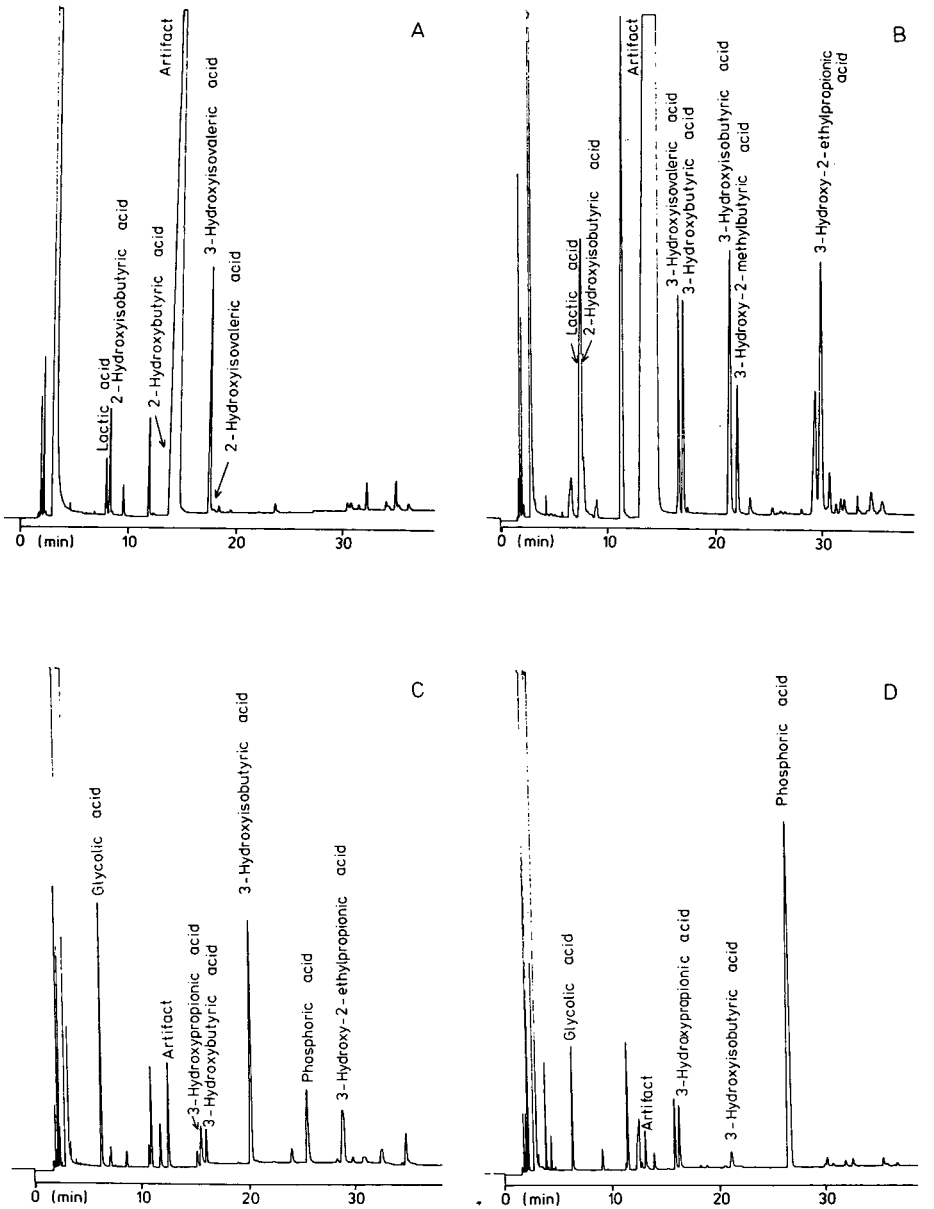


Fig. 1. Early portions of the gas chromatograms of fraction 3a (A), fraction 3b (B), fraction 3c (C) and fraction 3d (D) of the derivatives of the organic acids in urine of a normal individual.

a pre-fractionation procedure is applied to partially separate otherwise unresolved constituents. A second problem is encountered with respect to the oxocarboxylic acids. They require derivatization of the carbonyl group to prevent decomposition during the analytical procedure. The O-methyloxime derivatives have proved to be suitable, even though some of them give *syn—anti* isomeric peaks.

Pre-fractionations

By modifying the previously described TLC pre-fractionation method [11], fraction 2 containing the oxocarboxylic acids was further fractionated into 2a and 2b, and fraction 3 containing the hydroxycarboxylic acids, was divided into 3a—d. Proceeding from 2a to 3d, the substances show increasing polarity.

Fig. 1 exemplifies the early portions of the chromatograms of fractions 3a—d of the organic acids in a normal urine. These early portions contain the hydroxycarboxylic acids. Because of the limited separating efficiency of the TLC plate, most hydroxycarboxylic acids appear in more than one subfraction. Because this complicates quantitative determinations, the refined subfractionation may be omitted for the quantification of those constituents which are sufficiently separated. The least polar acids, such as lactic acid, 2-hydroxyisobutyric acid, 2-hydroxybutyric acid, 2-hydroxyisovaleric acid and 3-hydroxyisovaleric acid are part of fractions 3a and 3b, the more polar acids, such as glycolic acid

TABLE I

DISTRIBUTION OF THE OXOCARBOXYLIC AND HYDROXYCARBOXYLIC ACID DERIVATIVES IN THE TLC FRACTIONS

Substance	Fraction
Pyruvic acid	2a
2-Oxobutyric acid	2a
2-Oxoisovaleric acid	2a
2-Oxovaleric acid	2a
2-Oxo-3-methylvaleric acid	2a
2-Oxoisocaproic acid	2a
3-Oxobutyric acid	2a, 2b
3-Oxocaproic acid	2a
4-Oxobutyric acid	2b
5-Oxocaproic acid	2b
2-Hydroxyisobutyric acid	2b, 3a, 3b, (3c)
2-Hydroxyisovaleric acid	2b, 3a
2-Hydroxyisocaproic acid	2b
3-Hydroxyisovaleric acid	2b, 3a, 3b
Lactic acid	3a, 3b
2-Hydroxybutyric acid	3a
2-Hydroxy-3-methylvaleric acid	3a
3-Hydroxybutyric acid	3a, 3b, 3c, (3d)
3-Hydroxyisobutyric acid	3a, 3b, 3c, 3d
3-Hydroxy-2-methylbutyric acid	3a, 3b
3-Hydroxy-2-ethylpropionic acid	3a, 3b, 3c
Glycolic acid	3c, 3d
3-Hydroxypropionic acid	3d

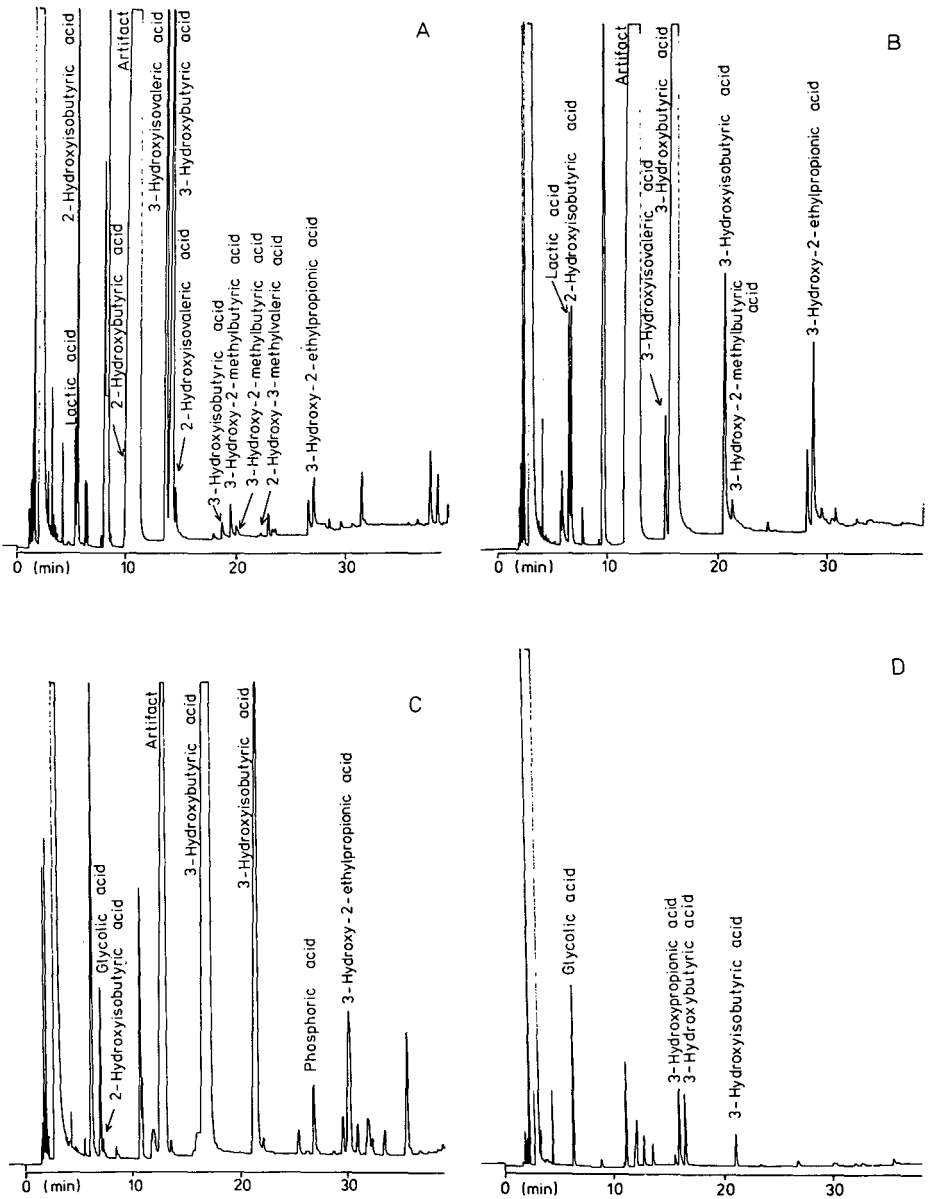


Fig. 2. Early portions of the gas chromatograms of fraction 3a (A), fraction 3b (B), fraction 3c (C) and fraction 3 d (D) of the derivatives of the organic acids in urine of a diabetic patient with ketoacidosis. The first peak of 3-hydroxy-2-methylbutyric acid in fraction 3a is the *threo* form, the second peak the *erythro* form.

and 3-hydroxypropionic acid, part of fractions 3c and 3d. 3-Hydroxybutyric acid has its maximum in 3b. The distribution of all of the hydroxycarboxylic acids in the different fractions is indicated in Table I. Fractions 3a and 3b, in some cases also 3c, contain an artifact which is introduced during the sample preparation, and which so far can not be eliminated. In the chromatograms it partially covers the peak of 2-hydroxybutyric acid.

The advantage of the subfractionation of fraction 3 becomes clear when the hydroxycarboxylic acids are investigated in urine of patients with ketoacidosis. Under this condition, 3-hydroxybutyric acid is by far the main hydroxy acid. Without subfractionation, it would cover 3-hydroxyisovaleric acid, 3-hydroxypropionic acid and 2-hydroxyisovaleric acid. Fig. 2 is an example of the hydroxycarboxylic acids in urine of a patient with ketoacidosis. When the ketoacidosis is very pronounced, the width of the 3-hydroxybutyric acid peak represents several minutes, even when the separating efficiency of the GC column is high and no peak tailing occurs (Fig. 3). Another advantage of the subfractionations is an enrichment effect for acids occurring in very low amounts, e.g. 2-hydroxy-3-methylvaleric acid in fraction 3a (Fig. 2) or *erythro*-3-hydroxy-2-methylbutyric acid which is the diastereomer of the diastereomeric pair of 3-hydroxy-2-methylbutyric acid that occurs in a lower concentration and which appears in some urines as a small peak after the major diastereomer, i.e. *threo*-3-hydroxy-2-methylbutyric acid (Fig. 2A).

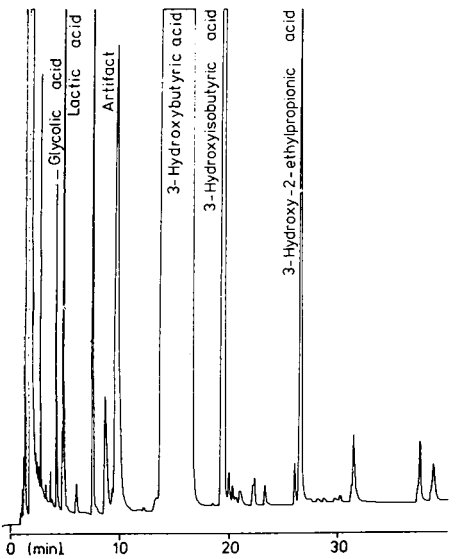


Fig. 3. Early portion of the gas chromatogram of fraction 3c of the derivatives of the organic acids in urine of a diabetic patient with ketoacidosis.

By subfractionating fraction 2 into 2a and 2b, most of the oxocarboxylic acids, except 3-oxobutyric acid, are enriched in 2a. By this procedure it is possible to detect 3-oxocarboxylic acids occurring in low concentrations, such as 3-oxocaproic acid in fraction 2a (Fig. 4) in cases of ketoacidosis. As a fraction slightly less polar than 3a, fraction 2b contains also portions of the least polar hydroxycarboxylic acids, i.e. 2-hydroxyisobutyric acid, 2-hydroxyisovaleric acid, 3-hydroxyisovaleric acid and 2-hydroxyisocaproic acid (Fig. 4).

Classification of the hydroxycarboxylic acids

Except for glycolic acid, 2-hydroxyisobutyric acid, 2-hydroxybutyric acid and 3-hydroxypropionic acid, whose formation is not completely clear, the bio-

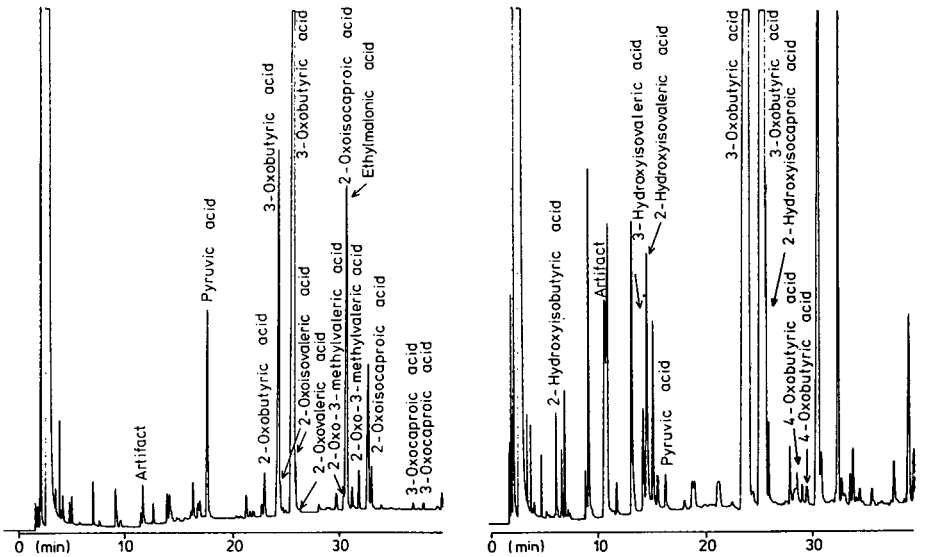
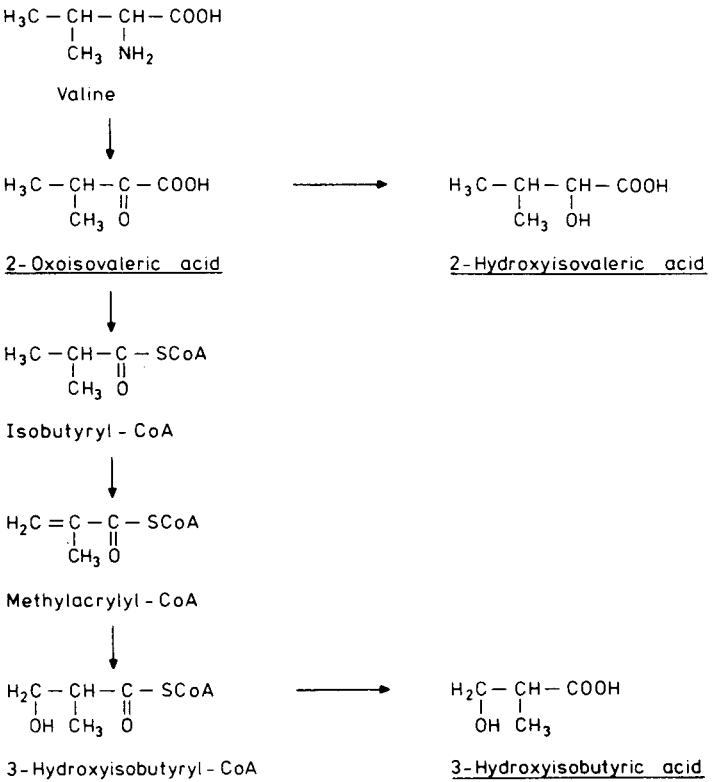
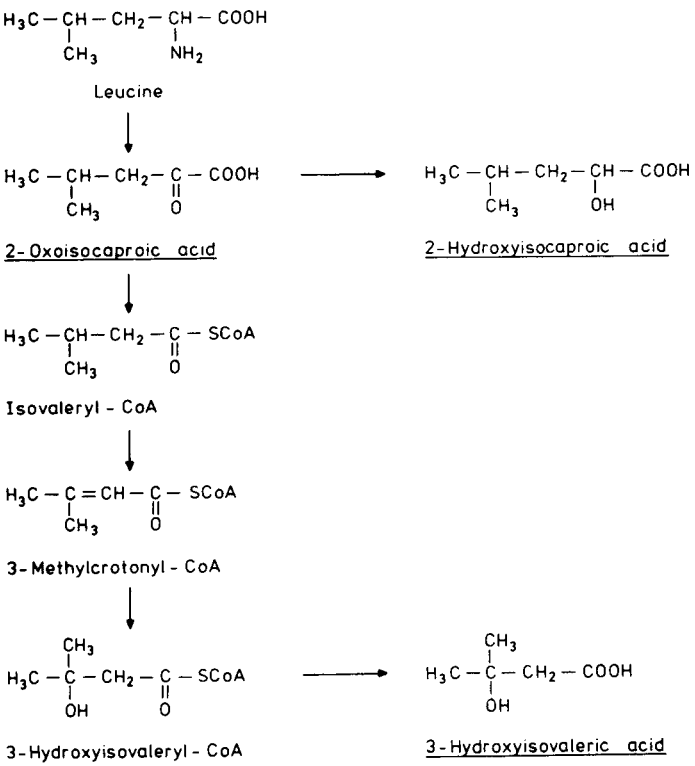


Fig. 4. Early portions of the gas chromatograms of fraction 2a (left) and 2b (right) of the derivatives of the organic acids in urine of a diabetic patient with ketoacidosis. The O-methyloxime derivatives of 2-oxoisovaleric acid, 2-oxoisocaproic acid, 2-oxo-3-methylvaleric acid, 3-oxobutyric acid, 3-oxocaproic acid and 4-oxobutyric acid occur as *syn—anti* isomers.

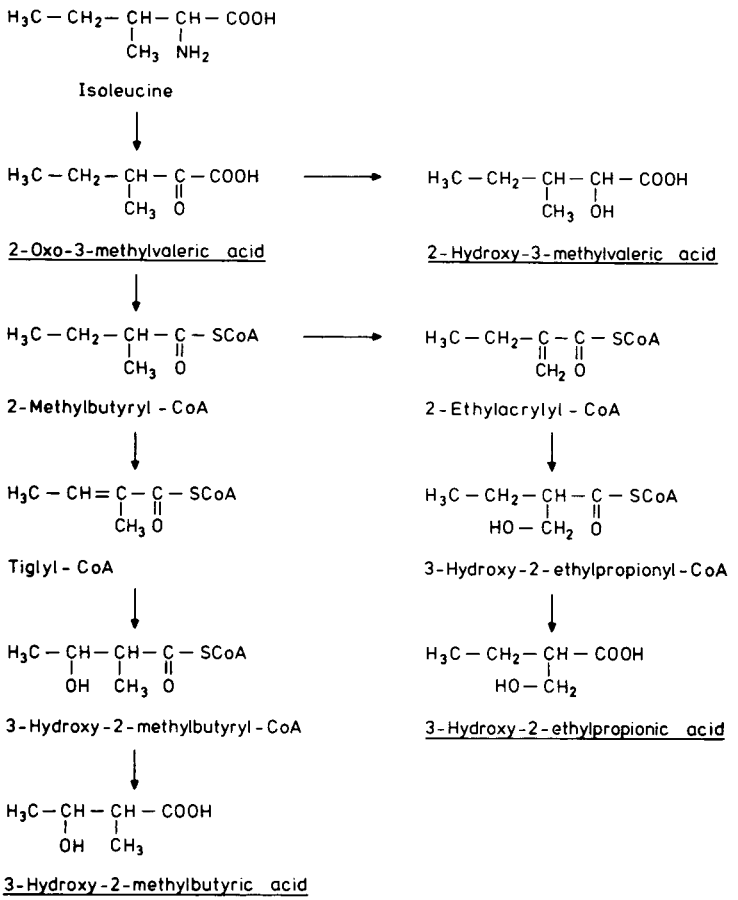


Scheme 1. Metabolism of valine. The underlined substances are found in urine.



Scheme 2. Metabolism of leucine. The underlined substances are found in urine.

chemical origin of the other hydroxycarboxylic acids is easily classified. Lactic acid is the reduction product of pyruvic acid and is formed under the conditions of anaerobic glycolysis. 3-Hydroxybutyric acid is generated by ketogenesis. All the other hydroxycarboxylic acids are metabolites of the branched-chain amino acids valine, leucine and isoleucine. 2-Hydroxyisovaleric acid, 2-hydroxyisocaproic acid and 2-hydroxy-3-methylvaleric acid are formed by reduction of 2-oxocarboxylic acids, which themselves result from the oxidative desamination of the amino acids valine, leucine and isoleucine, respectively (Schemes 1—3). 3-Hydroxycarboxylic acids are products from the amino acids as well, and are formed in a reaction including several steps. 3-Hydroxyisobutyric acid stems from valine (Scheme 1), 3-hydroxyisovaleric acid from leucine (Scheme 2) and 3-hydroxy-2-methylbutyric acid and 3-hydroxy-2-ethylpropionic acid from isoleucine (Scheme 3). The formation of two different 3-hydroxycarboxylic acids from isoleucine is possible because the elimination of hydrogen in 2-methylbutyryl-CoA (coenzyme A) and the subsequent addition of water can be orientated towards two sides (Scheme 3). In the metabolization of isoleucine both the *threo* and the *erythro* forms of 3-hydroxy-2-methylbutyric acid are produced. However, the major diastereomer is the *threo* form. This has been proved by preparing the reference compound *erythro*-3-hydroxy-2-methylbutyric acid by stereospecific synthesis.



Scheme 3. Metabolism of isoleucine. The underlined substances are found in urine.

Classification of the oxocarboxylic acids

Pyruvic acid originates from glucose and is produced in the glycolytic process. 2-Oxoisovaleric acid, 2-oxoisocaproic acid and 2-oxo-3-methylvaleric acid stem, as mentioned above, from the oxidative transamination of valine, leucine and isoleucine (Schemes 1—3). 3-Oxobutyric acid is, besides 3-hydroxybutyric acid, the main product of ketogenesis, which means that it is formed from acetyl-CoA according to the 3-hydroxy-3-methylglutaryl-CoA cycle. For the homologous 3-oxocarboxylic acid, i.e. 3-oxocaproic acid, the biochemical pathway is not clear. However, since 3-oxocaproic acid occurs in a detectable amount only in cases of ketoacidosis, a condensation mechanism analogous to the ketogenesis pathway can be assumed, with butyryl-CoA and acetyl-CoA as starters. Acetyl-CoA would act as the acidic compound to react with the thioester group of butyryl-CoA. The formation of 2-oxobutyric acid and 2-oxovaleric acid cannot be easily explained. The same holds true for 4-oxobutyric acid and 5-oxocaproic acid which are often found in urine of ketoacidotic patients. 4-Oxobutyric acid has been described as a metabolite of γ-aminobutyric acid in a patient with neurological abnormalities [14].

Mass spectrometric fragmentation and identification of the hydroxycarboxylic acid methyl esters

The mass spectrometric fragmentation of the methyl esters of the hydroxycarboxylic acids is characterized by a number of general features. Even though the spectra of several of these esters have been published, it appears useful to point out these features systematically. Table II shows the intensities of the fragments.

The molecular ion can be distinguished as a small peak only in the methyl esters of glycolic acid, lactic acid and 3-hydroxypropionic acid. In the substances with higher molecular weight it is completely missing. $(M-1)^+$ occurs with low intensity in the esters of 3-hydroxypropionic acid and 3-hydroxybutyric acid. It is absent in all the other compounds.

Several major fragment peaks originate from α-cleavage processes with respect to the C=O group of the carbomethoxy unit. In the spectra of the methyl esters of the 2-hydroxycarboxylic acids, the ion $(M-COOCH_3)^+$ reaches a high intensity; in some cases it is the base peak. In the 3-hydroxy compounds this fragment peak is very small. Instead, the formation of the ions $(M-OCH_3)^+$ and $(COOCH_3)^+$ is favoured in the fragmentation of the 3-hydroxycarboxylic acid methyl esters. These processes are, with the exception of lactic acid methyl ester, of little importance in the 2-hydroxy compounds. In the same context the ion $(M-H_3COH, -CH_3)^+$ can be seen. It is favoured in the 3-hydroxycarboxylic acid methyl esters, except in 3-hydroxypropionic acid, where no methyl group is available.

The loss of water, resulting in the ions $(M-H_2O)^+$ and $(M-CH_3, -H_2O)^+$ with relative intensities up to 18%, is observed only for the 3-hydroxycarboxylic acid methyl esters, apparently because of the easy formation of a C=C double bond conjugated with the C=O group. The olefinic ion $(M-COOCH_3, -H_2O)^+$ is more generally formed and occurs in a broad range of relative intensities in the 2-hydroxy and 3-hydroxy compounds.

The fragment ion *m/e* 43 is observed in all of the hydroxycarboxylic acid methyl esters and is in several cases the base peak. In the methyl esters of 2-hydroxyisovaleric acid and 2-hydroxyisocaproic acid, *m/e* 43 probably corresponds to the isopropyl ion; in lactic acid, 2-hydroxyisobutyric acid, 3-hydroxybutyric acid, 3-hydroxy-2-methylbutyric acid and 3-hydroxyisovaleric acid, *m/e* 43 is assumed to correspond to the acetyl ion.

Very indicative for the interpretation of the spectra of the hydroxycarboxylic acid methyl esters are ions from McLafferty rearrangement processes. The ion *m/e* 90 (fragment a in Scheme 4) is formed from the 2-hydroxycarboxylic acid methyl esters with a carbon chain of four or more C atoms. In the shorter-chain esters of glycolic acid, lactic acid and 2-hydroxyisobutyric acid, this process is not possible. For the 3-hydroxy compounds, two different McLafferty rearrangements are possible, one involving the hydrogen from an alkyl group (Scheme 5), the other the hydrogen from the OH group (Scheme 6). Both processes lead to the fragment b in Schemes 5 and 6, with the masses *m/e* 74, *m/e* 88 or *m/e* 102 depending on the structure. In the 3-hydroxycarboxylic acid methyl esters with carbon chains of four or more C atoms, both rearrangements are possible and cannot be differentiated from the mass spectra. In the esters with a straight chain of three carbon atoms, i.e. 3-hy-

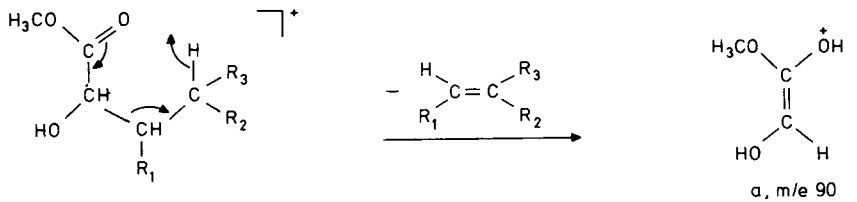
TABLE II

MASS SPECTROMETRIC FRAGMENTATION OF THE METHYL ESTERS OF HYDROXYCARBOXYLIC ACIDS

The values represent the relative intensities (%) of the fragments listed.

Substance	MW	M^+	$(M-1)^+$	$(M-COOCH_3)^+$	$(M-OCH_3)^+$	$(COOCH_3)^+$
Glycolic acid	90	8	–	100		20***
Lactic acid	104	2	–	100	1	53
2-Hydroxybutyric acid	118	–	–	100*	–	
2-Hydroxyisobutyric acid	118	–	–	100*	1	
2-Hydroxyisovaleric acid	132	–	–	100	–	7
2-Hydroxyisocaproic acid	146	–	–	43	–	10
2-Hydroxy-3-methylvaleric acid	146	–	–	65	–	7
3-Hydroxypropionic acid	104	2	6	14	81**	18
3-Hydroxybutyric acid	118	–	2		23	25*
3-Hydroxyisobutyric acid	118	–	–		40**	31*
3-Hydroxyisovaleric acid	132	–	–	12	8	80
3-Hydroxy-2-methylbutyric acid: *threo*	132	–	–	9	19	37
erythro		–	–	3	17	15
3-Hydroxy-2-ethylpropionic acid	132	–	–	8	19**	14

*Superimposition of $(M-COOCH_3)^+$ and $(COOCH_3)^+$.
**Superimposition of $(M-OCH_3)^+$ and $(M-HOCH_2)^+$.
***Superimposition of $(M-OCH_3)^+$ and $(COOCH_3)^+$.



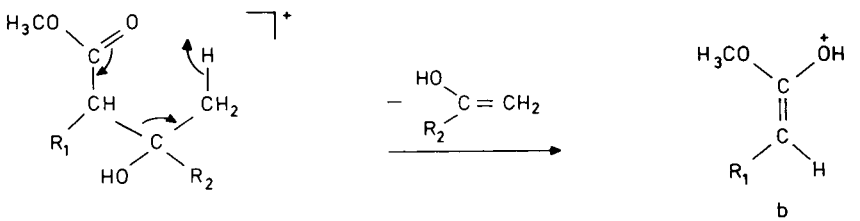
R_1	R_2	R_3	
H	H	H	2-Hydroxybutyric acid
CH_3	H	H	2-Hydroxyisovaleric acid
H	CH_3	CH_3	2-Hydroxyisocaproic acid
CH_3	H	CH_3	2-Hydroxy-3-methylvaleric acid

Scheme 4. McLafferty rearrangement of 2-hydroxycarboxylic acid methyl esters.

droxypropionic acid, 3-hydroxyisobutyric acid and 3-hydroxy-2-ethylpropionic acid, only the rearrangement involving the OH group is feasible (Scheme 6).

Because of its ethyl substituent, the spectrum of the methyl ester of 3-hydroxy-2-ethylpropionic acid shows some features that are not observed in the other substances. Most characteristic in the spectrum is the fragment

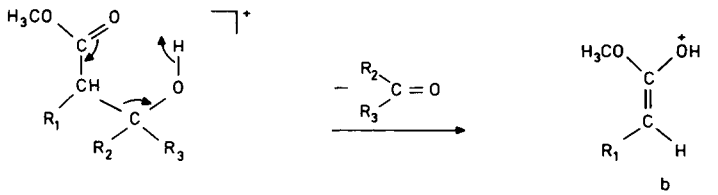
m/e 61	$(M-CH_3, -H_3COH)^+$	$(M-H_2O)^+$	$(M-CH_3, -H_2O)^+$	$(M-COOCH_3, -H_2O)^+$	*m/e* 43	$(M-CH_3)^+$	a	b
18	–	–	–	–	4	–	–	–
54	–	–	–	–	92	25	–	–
13	–	–	–	18	32	–	4	–
5	–	–	–	14	77	6	–	–
7	2	–	–	32	20	–	60	–
7	–	–	–	84	100	–	17	–
5	–	–	–	27	19	–	100	–
8	–	18	15	–	58	–	–	100
35	46	9	17	24	100	32	–	86
5	9	12	11	10	15	4		100
1	73	–	–	4	100	52	–	37
5	24	3	4	26	56	7	–	100
5	19	1	4	16	32	4	–	100
3	–	3	3	85	30	–	–	47



R_1	R_2	
H	H	3-Hydroxybutyric acid
H	CH_3	3-Hydroxyisovaleric acid
CH_3	H	3-Hydroxy-2-methylbutyric acid

Scheme 5. McLafferty rearrangement of 3-hydroxycarboxylic acid methyl esters involving an alkyl group.

group *m/e* 101, 102, 103, 104 and the ion *m/e* 87 (Fig. 5). The fragment *m/e* 101 can be explained by $(M-OCH_3)^+$ and $(M-HOCH_2)^+$, *m/e* 102 and *m/e* 104 by two McLafferty rearrangement processes, and *m/e* 103 by the loss of the ethyl group. The base peak *m/e* 87 results from the McLafferty product, *m/e* 102 by γ-cleavage (Scheme 7).



b

R_1	R_2	R_3	
H	H	H	3-Hydroxypropionic acid
C_2H_5	H	H	3-Hydroxy-2-ethylpropionic acid
CH_3	H	H	3-Hydroxyisobutyric acid
H	H	CH_3	3-Hydroxybutyric acid
H	CH_3	CH_3	3-Hydroxyisovaleric acid
CH_3	H	CH_3	3-Hydroxy-2-methylbutyric acid

Scheme 6. McLafferty rearrangement of 3-hydroxycarboxylic acid methyl esters involving the OH group.

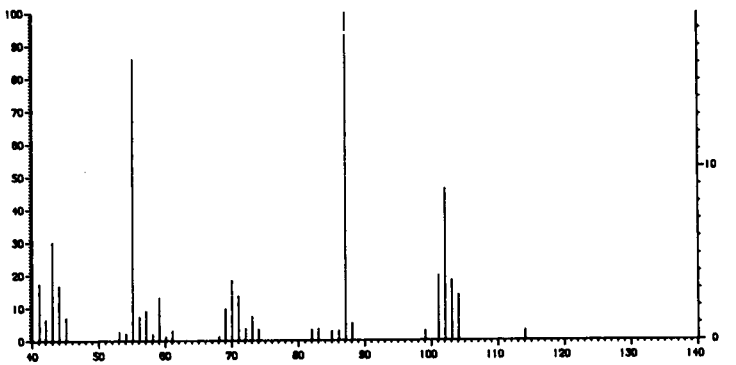
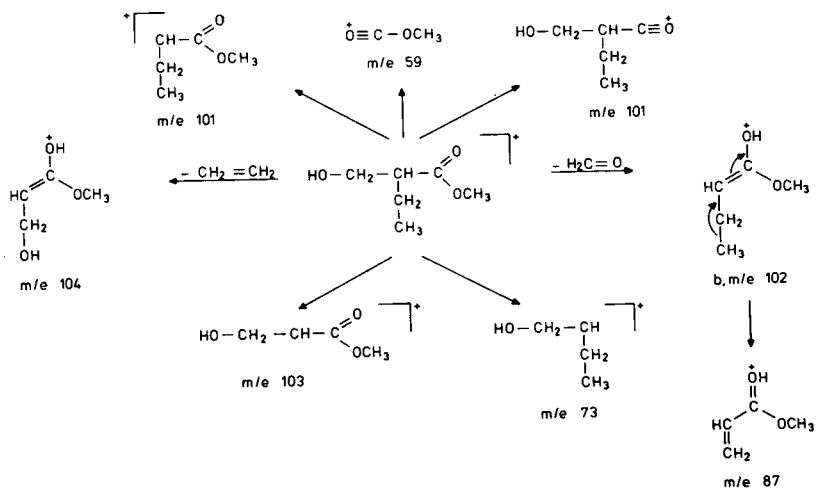


Fig. 5. Mass spectrum of the methyl ester of 3-hydroxy-2-ethylpropionic acid.



Scheme 7. Mass spectrometric fragmentation of the methyl ester of 3-hydroxy-2-ethylpropionic acid.

Mass spectrometric fragmentation and identification of the O-methyloxime derivatives of the oxocarboxylic acid methyl esters

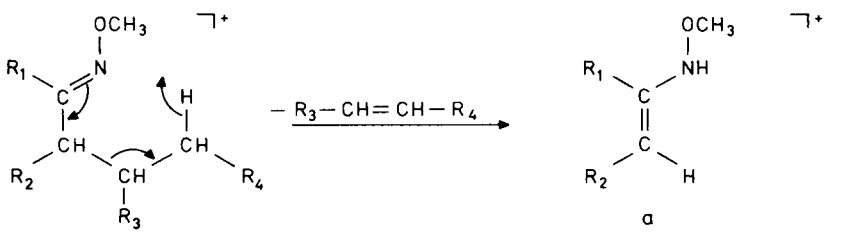
The mass spectrometric fragmentation of this group of substances has also some characteristic general features. As in the case of the hydroxy compounds, these characteristics are systematically described, even though some spectra are published. The relative intensities of the ions are listed in Table III. The *syn—anti* isomers of the O-methyloxime derivatives give very similar mass spectra. The intensities given in Table III refer to the first GC peak of the isomeric pair.

The intensity of the molecular ion decreases with increasing molecular weight. It is lower in the 2-oxo than in other oxocarboxylic acid derivatives.

Among the α-cleavage processes with respect to the C=O function of the carbomethoxy unit, the formation of $(M-COOCH_3)^+$ is strongly favoured in the 2-oxocarboxylic acid derivatives, and in some cases it leads to the base peak. It is also the main fragment in the spectrum of the derivative of 4-oxobutyric acid. The ions $(M-OCH_3)^+$ and $(COOCH_3)^+$ are generally produced from the oxocarboxylic acid derivatives. The formation of the ion $(M-OCH_3)^+$ may involve the carbomethoxy unit and the O-methyloxime group. Besides the methoxy group, in some instances the molecular ions lose methanol.

The ion $(M-CH_2COOCH_3)^+$ generated by a β-cleavage process, occurs with high abundance or even as the base peak in the mass spectra of the 3-oxocarboxylic acid derivatives. It is also formed when the O-methyloxime group is more distant from the carbomethoxy group, i.e. in the spectra of the 4-oxobutyric acid and 5-oxocaproic acid derivatives.

In the spectra of most of the oxocarboxylic acid derivatives, four ions are observed with the compositions $(M-H_3COH, -CH_3)^+$, $(M-H_3COH, -OCH_3)^+$, $(M-COOCH_3, -CH_3)^+$ and $(M-COOCH_3, -H_3COH)^+$. The relative intensities of these fragments vary strongly, the abundance of $(M-COOCH_3, -H_3COH)^+$ normally being pronounced.



R_1	R_2	R_3	R_4	
$COOCH_3$	H	H	H	2-Oxovaleric acid
$COOCH_3$	CH_3	H	H	2-Oxo-3-methylvaleric acid
$COOCH_3$	H	CH_3	H	2-Oxoisocaproic acid
$CH_2-COOCH_3$	H	H	H	3-Oxocaproic acid
CH_3	H	H	$COOCH_3$	5-Oxocaproic acid

Scheme 8. McLafferty rearrangement of oxocarboxylic acid derivatives involving the O-methyloxime group.

TABLE III

MASS SPECTROMETRIC FRAGMENTATION OF THE METHYL ESTERS OF OXOCARBOXYLIC ACIDS

The values represent the relative intensities (%) of the fragments listed.

Substance	MW	M^+	$(M-COOCH_3)^+$	$(M-OCH_3)^+$	$(M-CH_2COOCH_3)^+$	$(COOCH_3)^+$
Pyruvic acid	131	35	100	16	–	44
2-Oxobutyric acid	145	16	100	60	–	33
2-Oxovaleric acid	159	1	47	48	–	52
2-Oxoisovaleric acid	159	4	100	23	–	28
2-Oxo-3-methylvaleric acid	173	–	100	34	–	85
2-Oxoisocaproic acid	173	–	24	18	–	62
3-Oxobutyric acid	145	90	6	38	100	56
3-Oxocaproic acid	173	27	10	17	80	46
4-Oxobutyric acid	145	18	100	74	12	48
5-Oxocaproic acid	173	14	27	48	100	93

As in the mass spectra of the hydroxycarboxylic acid methyl esters, McLafferty ions are indicative for the fragmentation pattern of the oxocarboxylic acid derivatives. Two rearrangement processes are possible, one involving the O-methyloxime group, the other the carbomethoxy group. The first process leads to fragment a (Scheme 8). In the 2-oxocarboxylic acid derivatives, it requires a straight carbon chain of at least five atoms, and in 3-oxocarboxylic acid derivatives a chain of at least six carbon atoms. The second process results in fragment b, *m/e* 74, which corresponds to the regular McLafferty ion from methyl esters. In the 3-oxocarboxylic acid derivatives, a chain length of four or more carbon atoms is necessary for this fragmentation. The process is not possible in the 2-oxo compounds and the 4-oxobutyric acid derivative.

Comparison of the excretion of hydroxycarboxylic and oxocarboxylic acids in urine of normal individuals and diabetic patients with ketoacidosis

Ten of the thirteen hydroxymonocarboxylic acids described in Table I are regularly found in normal urine and can be recognized in Fig. 1. The other three substances, i.e. 2-hydroxyisovaleric acid, 2-hydroxyisocaproic acid and 2-hydroxy-3-methylvaleric acid, occur in very low amounts and are often below the detection limit. In the normal urine depicted in Fig. 1, of these three acids only 2-hydroxyisovaleric acid was identified. In healthy individuals, ketogenesis is reflected in the urinary excretion of 3-hydroxybutyric acid; the degradation of the branched-chain amino acids is shown in the excretion of a series of 3-hydroxycarboxylic acids, i.e. 3-hydroxyisobutyric acid, 3-hydroxyisovaleric acid, 3-hydroxy-2-methylbutyric acid and 3-hydroxy-2-ethylpropionic acid. The series of the 2-hydroxycarboxylic acids, which are the direct reduction products from the 2-oxocarboxylic acids, are of little importance in normals.

In diabetic patients with ketoacidosis, the urinary excretion of the ketone

$(M-H_3COH, -CH_3)^+$	$(M-H_3COH)^+$	$(M-H_3COH, -OCH_3)^+$	$(M-COOCH_3, -CH_3)^+$	$(M-COOCH_3, -H_3COH)^+$	a	b
–	3	–	1	–	–	–
–	20	2	10	18	–	–
3	1	11	–	45	100	–
2	3	6	–	33	–	–
8	–	20	20	72	34	–
2	–	12	73	46	46	–
3	78	25	3	71	–	58
100	18	10	7	27	20	35
–	70	80	2	91	–	–
17	–	8	22	22	36	24

body 3-hydroxybutyric acid is naturally increased as compared to normals. In accordance with elevated levels of the branched-chain amino acids, i.e. valine, leucine and isoleucine, in the case of diabetic ketoacidosis [15, 16], their metabolites are also increased. It is observed that the 3-hydroxy series in particular is affected. Considering the two possible metabolites of the 3-hydroxy series resulting from isoleucine, 3-hydroxy-2-ethylpropionic acid is the major product (Figs. 2 and 3). For 3-hydroxy-2-methylbutyric acid, which is the other metabolite from isoleucine, an elevation is not always found. The three components of the 2-hydroxy series of the amino acid metabolites are increased (Figs. 2 and 4). However, their absolute amounts remain low.

The excretion of oxocarboxylic acids in normal urine is very low. Only pyruvic acid is found in considerable but varying amounts [11]. In patients with ketoacidosis, a large quantity of the ketone body 3-oxobutyric acid is found in urine as a result of strong ketogenesis. All three 2-oxocarboxylic acids as metabolites of valine, leucine and isoleucine, are raised, too. However, as in the case of the 2-hydroxycarboxylic acids, their absolute quantities in urine are low as compared to serum [11]. Apparently the 2-oxocarboxylic acids are reutilized and, in contrast to the ketone bodies, the organism loses only small amounts of these constituents.

ACKNOWLEDGEMENT

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CHARACTERIZATION OF ARTEFACTS PRODUCED BY TREATMENT OF ORGANIC ACIDS WITH DIAZOMETHANE

SONJA BAUER, MANFRED NEUPERT and GERHARD SPITELLER*

Lehrstuhl für Organische Chemie I, Universität Bayreuth, Postfach 3008, Universitätsstrasse 30, D-8580 Bayreuth (F.R.G.)

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SUMMARY

When α,β-unsaturated acids and α-keto acids react with diazomethane not only are the corresponding methylates produced, but also diazomethane is added to the C=C double bond or to the oxo group. The gas chromatographic and mass spectral behaviour of these undesired products and some further artefacts produced in the hot inlet lines of a gas chromatograph are described. The mass spectra and retention indices allowed the structural assignment of several "unknown" compounds found previously in the methylated acid fraction of urine. A detailed analysis of the reaction of α-oxo acids with diazomethane revealed that, besides the already known oxirane methyl esters, homologous esters are also produced by an insertion reaction.

INTRODUCTION

Acidic compounds occurring in biological fluids are obtained either by extraction [1—3] or ion-exchange chromatography [4]. Their separation and characterization is possible after appropriate derivatization by glass capillary gas chromatography—mass spectrometry (GC—MS) [5, 6].

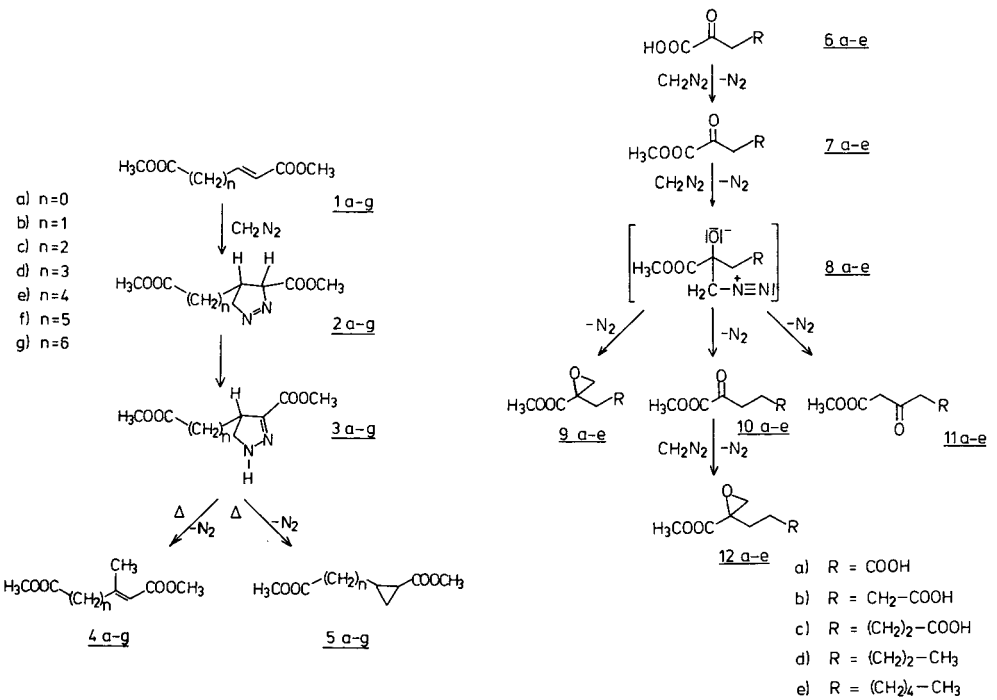
In most cases the acids are transformed into their trimethylsilylated derivatives, which show excellent gas chromatographic properties [7, 8]. Unfortunately, trimethylsilyl esters of acids are rather sensitive to hydrolysis [9]. Therefore any further chromatographic separation step such as liquid chromatography or thin-layer chromatography [10], necessary for the detection of trace compounds, is excluded.

In these cases, methylation of the acid fraction is preferred. Usually this transformation is achieved by treatment with ethereal diazomethane solution. Unfortunately diazomethane not only attacks the acidic hydrogens of acids,

phenols or enols as desired [11], but also adds to the double bonds of α,β-unsaturated esters (Scheme 1, 1a—g) [12] producing Δ^1-pyrazolines (2a—g) which rearrange to Δ^2-pyrazolines (3a—g) [13]. Δ^2-Pyrazolines may decompose in the injector of a gas chromatograph, sometimes producing several thermal reaction products [14] (Scheme 1).

Analogous to the α,β-unsaturated acids, α-oxo acids (Scheme 2, 6a—e) react with diazomethane not only by esterification. The expected methylates (7a—e) are only minor reaction products, because they react further to produce oxiranes (9a—e) [15] by addition of CH_2N_2 to the carbonyl function. This reaction occurs via the zwitterion (8a—e). It can be expected — analogous to the reaction of ketones with diazomethane [11] — that the zwitterion (8a—e) is able to stabilize itself either by elimination of N_2 and ring closure to form the oxirane ring, or by migration of one of the substituents to give the homologous methyl esters (10a—e) (Scheme 2), although migration products or derivatives thereof (12a—e) are not known from the literature [15].

It is rather difficult to recognize undesired reaction products as artefacts without knowledge of their retention indices and mass spectra. We recently reinvestigated the reaction of α,β-unsaturated acids and α-oxo acids with diazomethane to obtain information on the gas chromatographic behaviour and mass spectra of such degradation products to ensure their detection as artefacts in acid fractions of biological fluids and to allow the recognition of the original compounds. We also investigated whether a short reaction time with diazomethane might avoid undesired side-reactions.



Scheme 1. Formation of artefacts from α,β-unsaturated esters.

Scheme 2. Reaction of α-oxo acids with diazomethane.

EXPERIMENTAL

Instruments

A Carlo Erba Fractovap 4160 gas chromatograph was used under the following conditions: hydrogen flow-rate, 2 ml/min; 25-m thin-film glass capillary coated with OV-101; injector temperature, 275° C; oven temperature, 80—280° C; temperature programme, 5 min isothermal, then 2° C/min; flame-ionization detection (FID).

When a Carlo Erba Fractovap 2400T was used for preparative GC, the conditions were: injector temperature, 275° C; oven temperature, 80—260° C; temperature programme, 4° C/min; FID. Column: 1.5 m × 6 mm I.D. filled with 3% OV-17 on Chromosorb W AW DMCS.

For mass spectrometry, a Varian MAT 312 mass spectrometer was used with the following conditions: electron-impact (EI) ion source; electron energy, 70 eV; registration of the total ion current signal at 20 eV. The mass spectrometer was combined with a Varian gas chromatograph 3700, equipped with a 25-m thin-film glass capillary column coated with OV-101; the temperature programme was 5 min isothermal, then 2° C/min; helium flow-rate was 2 ml/min. The instrument was combined with a MAT 200 data system, using a PDP 11/34 computer.

^{1}H-NMR (proton nuclear magnetic resonance) measurements were carried out with a Bruker WM 250 instrument.

Materials

Fumaric acid, maleic acid, laevulinic acid and triphenylphosphine were obtained from E. Merck (Darmstadt, F.R.G.). Cyclopentanone, cyclohexanone, cyclopheptanone, cyclooctanone, glutaconic acid, 2-bromoacetic acid methyl ester, citraconic acid, mesaconic acid, 3-methylglutaconic acid dimethyl ester, 2-oxoglutaric acid, itaconic acid and 3-chloroperbenzoic acid were purchased from EGA-Chemie (Steinheim, F.R.G.). 2-Oxoadipinic acid and 2-oxohexanoic acid (sodium salt) were obtained from Sigma (Munich, F.R.G.) and 2-oxo-octanoic acid was supplied by Fluka (Neu-Ulm, F.R.G.).

Derivatization of α,β-unsaturated dioic acids and of 2-oxo acids

The conversion of an α,β-unsaturated dicarboxylic acid into its dimethyl ester by use of diazomethane is a fast reaction compared to the formation of pyrazolines. Therefore, with regard to the cyclo-addition of CH_2N_2 to α,β-unsaturated acids, it is irrelevant whether the carboxylic acid or the corresponding methyl ester is used.

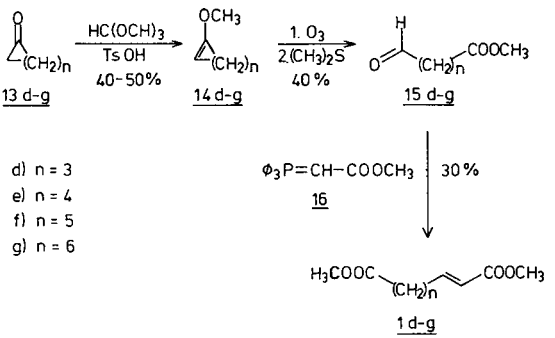
To 1 ml of an ethereal solution containing approximately 1 mg of α,β-unsaturated dioic acid or the corresponding dimethyl ester, or to 1 mg of 2-oxo acid, dissolved in 3 ml of methanol, ethereal diazomethane solution (5%) was added until the solution remained yellow. The excess diazomethane was evaporated by a nitrogen stream. The residue was evaporated to dryness in vacuo and dissolved in 100 μl of ethyl acetate. A 1-μl sample of this solution was injected into the gas chromatograph.

SYNTHESIS OF REFERENCE COMPOUNDS

α,β-Unsaturated dimethyl esters (1a—g)

1c was obtained by hydrolysis of 1,4-dicyanobutene-1 [16] and methylation of the resulting 2-hexenedioic acid by the method of Clinton and Laskowski [17].

The other α,β-unsaturated acids were prepared according to the synthetic approach of Gerlach et al. [18] (Scheme 3) starting from a cycloalkanone (Scheme 3, 13d—g) which was transformed to its methyl enol ether [14d—g). This was subjected to ozonolysis. The resulting ω-oxo ester (15d—g) (0.01 mol) was dissolved in 50 ml of toluene and a slight excess (molar ratio 1:1.1) of β-carbomethoxymethylenetriphenylphosphorane (16) [19] was added. After refluxing for 16 h, the deep yellow solution was cooled to room temperature and brought nearly to dryness in vacuo. Then 20 ml of *n*-hexane were added to the residue and the mixture was stirred at room temperature for 30 min. After filtration, the solvent was evaporated and the residue purified by vacuum destillation.

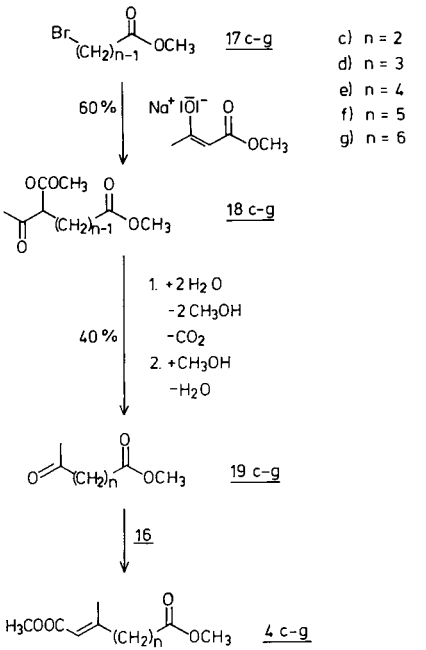


Scheme 3. Synthesis of α,β-unsaturated dimethyl esters.

Synthesis of 3-methylalkenedioic acid dimethyl esters (4c—g) (Scheme 4)

A solution of 0.05 mol of sodium hydride in 100 ml of dimethylformamide—toluene (1:1, v/v) was prepared at 0° C. To this solution 0.05 mol of methyl acetoacetate was slowly added within 20 min. Methyl ω-bromocarboxylate (Scheme 4, 17c—g) was added to the light yellow solution within 10 min. After refluxing for 5 h, the reaction mixture was cooled to room temperature. Then 10 ml of methanol were added. The mixture was stirred for a further 10 min. After addition of 50 ml of water, the mixture was extracted three times each with 50 ml of ether. The combined organic phases were dried over sodium sulphate; the solvent was evaporated and the residue purified by vacuum distillation. The resulting 2-acetylcarboxylates (18c—g) were heated with 30 ml of water to 230° C during 10 h [20]. This Meerwein hydrolysis gave the crude oxocarboxylic acids which were converted to the methyl esters (19c—g) by the method of Clinton and Laskowski [17].

These oxo esters (19c—g) were transformed into the 3-methylalkene dimethyl esters (4c—g) using sodium ylide (16) as described above, except that xylene was used instead of toluene to increase the reaction temperature. The resulting solution of 3-methylalkenedioic acid dimethyl ester (4c—g) was con-



Scheme 4. Synthesis of 3-methylalkenedioic acid dimethyl esters.

centrated to a volume of 5 ml; 50-μl portions were injected into the preparative gas chromatograph. *Z*- and *E*-isomers of the 3-methylalkene diesters (4c–g) were separated from each other, from the solvent and from impurities. The retention times, mass spectral and ^{1}H-NMR data are listed in Table I.

Δ^2-Pyrazolines (3a)

3,4-Dicarbomethoxy-Δ^2-pyrazoline (3a) was prepared according to the description of Roper and Ma [21]. 3-Carbomethoxy-Δ^2-pyrazoline-4-acetic acid methyl ester (3b) was prepared by the method of Birkhofer and Feldmann [22]. The mass spectral and NMR data are listed in Table I.

Cyclopropane derivatives (5a, 5b)

These compounds were synthesized from the methyl esters of acrylic acid, vinylacetic acid and allylacetic acid by addition of carbomethoxycarbene to the C=C double bond [23]. The carbon was generated from methyl diazoacetate by use of Phillips catalyst [24], using argon as inert gas. A 0.5-g amount of methylacrylate (methylvinylacetate or methylallylacetate, respectively) was dissolved in 10 ml of methylene chloride and immediately added to a suspension of 20 g of Phillips catalyst in 50 ml of methylene chloride. The reaction mixture was stirred at room temperature for 15 h. Then the solution was decanted and the catalyst was extracted with 100 ml of methylene chloride. The organic solutions were combined and concentrated to a volume of 1 ml. Then 2 ml of potassium hydroxide solution (10%) were added. After stirring for 10 h at 90°C, the mixture was cooled to room temperature. Then the reaction mixture was extracted twice with 2 ml of diethyl ether and the

TABLE I

SPECTROSCOPIC DATA OF THE SYNTHETIC 3-METHYLALKENEDIOIC ACID DIMETHYL ESTERS

RT = retention time (min), when a preparative gas chromatograph was used.

Compound	RT	RI	MS	NMR
3a		1506	186 (M^+, 2), 153 (4), 127 (42), 95 (100), 68 (10), 59 (30), 56 (9), 42 (15), 39 (10), 28 (12), 15 (50)	3.77 (s), 3.85 (s), 3.94 (dd, J_1 = 9.6 Hz, J_2 = 11.03 Hz), 4.10 (dd, J_1 = 9.6 Hz, J_2 = 11.03 Hz), 6.27 (s)
3b		1598	200 (M^+, 1), 185 (4), 169 (8), 141 (20), 140 (25), 139 (15), 127 (23), 113 (11), 112 (15), 109 (16), 108 (9), 95 (100), 81 (17), 74 (9), 59 (35), 43 (23), 42 (15), 39 (17), 28 (28), 15 (66)	2.5 (dd, J_1 = 10 Hz, J_2 = 16 Hz), 2.9 (dd, J_1 = 3 Hz, J_2 = 16 Hz), 3.40—3.55 (m), 3.58—3.70 (m), 3.70 (s), 3.82—3.91 (m), 3.84 (s), 6.35 (s)
3c		1720	214 (M^+, 1), 199 (5), 186 (17), 167 (24), 155 (48), 154 (13), 151 (20), 148 (10), 139 (31), 127 (13), 123 (22), 122 (26), 109 (24), 95 (100), 85 (10), 81 (36), 67 (18), 59 (47), 55 (25), 42 (24), 41 (26), 39 (29),	
3d		1830	228 (M^+, 3), 213 (3), 195 (16), 181 (18), 169 (54), 163 (15), 140 (16), 137 (26), 109 (45), 95 (100), 82 (26), 81 (40), 74 (16), 59 (57), 55 (35), 41 (42), 39 (29), 28 (41), 15 (87)	
3e		1923	242 (M^+, 4), 227 (4), 209 (19), 195 (14), 183 (33), 151 (15), 149 (14), 127 (18), 123 (22), 109 (18), 95 (100), 82 (41), 81 (25), 59 (48), 55 (32), 42 (28), 41 (27), 39 (21), 28 (37), 15 (63)	
4a, *Z*-isomer		1045	158 (M^+, 3), 127 (100), 126 (17), 99 (24), 98 (4), 68 (16), 59 (44), 39 (29), 29 (19), 15 (40)	1.98 (d, J = 1.6 Hz), 3.69 (s), 3.71 (s), 5.87 (q)
4a, *E*-isomer		1075	158 (M^+, 2), 127 (61), 126 (100), 99 (26), 98 (29), 68 (42), 59 (63), 39 (56), 29 (25), 15 (70)	2.30 (d, J = 1.6 Hz), 3.71 (s), 3.78 (s), 6.80 (q)

TABLE I (continued)

Compound	RT	RI	MS	NMR
4b, *Z*-isomer	17	1162	172 (M^+, 1), 141 (53), 140 (100), 113 (31), 112 (94), 109 (30), 108 (8), 97 (39), 85 (9), 82 (19), 81 (31), 59 (38), 55 (31) 53 (49), 41 (20), 39 (37), 27 (28), 15 (63)	1.98 (d), 3.69 (s), 3.71 (s), 3.75 (s), 5.86 (q)
4b, *E*-isomer	19	1198	172 (M^+, 2), 141 (32), 140 (100), 113 (44), 112 (87), 109 (19), 108 (21), 97 (26), 85 (10), 82 (20), 81 (28), 59 (42), 55 (36), 53 (38), 41 (18), 39 (37), 27 (28), 15 (64)	2.30 (d, J = 1.6 Hz), 3.71 (s), 3.78 (s), 5.80 (q, J = 1.6 Hz)
4c, *Z*-isomer	19	1278	186 (M^+, 2), 155 (43), 154 (100), 127 (31), 126 (37), 123 (17), 122 (51), 111 (19), 95 (94), 94 (40), 85 (42), 67 (62), 59 (32), 55 (22), 53 (16), 41 (43), 39 (38), 27 (24), 15 (60)	1.91 (d, J = 1.4 Hz), 2.47—2.52 (m), 2.88—2.95 (m), 368 (s), 5.72 (q, J = 1.4 Hz)
4c, *E*-isomer	21	1287	186 (M^+, 2), 155 (38), 154 (96), 127 (29), 126 (42), 123 (22), 122 (55), 111 (21), 95 (100), 94 (41), 85 (44), 67 (56), 59 (41), 55 (25), 53 (20), 41 (41), 39 (40), 27 (24), 15 (62)	2.18 (d, J = 1.4 Hz), 3.69 (s), 4.80—4.52 (m), 5.68 (q, J = 1.4 Hz)
4d, *Z*-isomer	22	1373	169 (44), 168 (96), 140 (22) 137 (28), 136 (57), 127 (19), 125 (20), 109 (100), 108 (45), 99 (22), 95 (77), 82 (26), 81 (65), 74 (17), 69 (18), 67 (48), 59 (46), 55 (41), 53 (31), 43 (32), 41 (50), 39 (52), 27 (23), 15 (71)	1.78—185 (m), 1.90 (d, J = 1.3 Hz), 2.37 (t, J = 7.6 Hz), 2.64—2.70 (m), 3.67 (s), 5.67 (q, J = 1.3 Hz)
4d, *E*-isomer		1420	169 (33), 168 (68), 140 (20), 137 (29), 136 (51), 127 (17), 125 (20), 109 (100), 108 (41), 99 (27), 95 (80), 82 (41), 81 (72), 74 (18), 69 (16), 67 (38), 59 (45), 55 (57), 53 (31), 43 (38), 41 (43), 39 (47), 29 (16), 27 (32), 15 (63)	1.78—1.90 (m), 2.16 (d, J = 1.2 Hz), 2.16—2.18 (m), 2.90—2.35 (m), 3.68 (s), 3.69 (s), 5.68 (q, J = 1.2 Hz)
4e, *Z*-isomer	24	1479	183 (30), 182 (49), 155 (13), 154 (23), 151 (18), 150 (62), 123 (25), 122 (51), 109 (26), 108 (28), 96 (23), 95 (100),	1.45—1.48 (m), 167—1.75 (m), 1.89 (d, J = 1.5 Hz), 2.30—2.40 (m), 2.60—2.67 (m), 3.67 (s), 5.68 (br)

(Continued on p. 250)

TABLE I (continued)

Compound	RT	RI	MS	NMR
			82 (25), 81 (32), 67 (36), 59 (38), 55 (31), 53 (19), 43 (18), 41 (37), 39 (33), 29 (38), 27 (19), 15 (56)	
4e, *E*-isomer	26	1530	183 (18), 182 (29), 154 (20), 155 (9), 151 (18), 150 (53), 123 (18), 122 (43), 109 (22), 108 (25), 96 (21), 95 (100), 82 (48), 81 (35), 67 (31), 59 (48), 55 (38), 53 (22), 43 (17), 41 (40), 39 (29), 29 (17), 27 (20), 15 (52)	1.23—1.31 (m), 1.45—1.70 (m), 2.12—2.20 (m), 2.15 (d, J = 1.5 Hz), 2.30—2.40 (m), 3.67 (s), 3.68 (s), 5.66 (br)
4f, *Z*-isomer	27	1571	197 (22), 196 (34), 168 (10), 164 (39), 137 (28), 136 (43), 114 (13), 109 (40), 108 (25), 96 (37), 95 (100), 83 (20), 82 (35), 81 (19), 67 (35), 59 (37), 55 (48), 43 (26), 41 (49), 39 (30), 29 (24), 27 (24), 15 (45)	1.33—1.56 (m), 1.60—1.70 (m), 1.88 (d, J = 1.3 Hz), 2.32 (t, J = 7.5 Hz), 2.62 (t, J = 7.5 Hz) 3.67 (s), 5.60 (br)
4f, *E*-isomer	28	1621	197 (23), 196 (36), 169 (14), 164 (40), 137 (30), 136 (39), 114 (12), 109 (41), 108 (25), 96 (34), 95 (100), 83 (20), 82 (71), 81 (20), 67 (34), 59 (46), 55 (49), 53 (19), 43 (18), 41 (45), 39 (32), 29 (20), 27 (22), 15 (53)	1.25—1.70 (m), 2.10—2.20 (m), 2.14 (d, J = 1.3 Hz), 2.32 (t, J = 7.6 Hz), 3.67 (s), 3.68 (s), 5.66 (q, J = 1.3 Hz)
4g, *Z*-isomer	29	1672	211 (16), 210 (22), 182 (15), 178 (18), 151 (22), 150 (33), 133 (21), 114 (18), 109 (38), 108 (27), 96 (33), 95 (100), 82 (37), 81 (21), 74 (17), 69 (32), 67 (37), 59 (34), 55 (40), 41 (53), 39 (21), 29 (16), 27 (15), 43 (16)	1.25 —1.69 (m), 1.88 (d, J = 1.3 Hz), 2.31 (t, J = 7.4 Hz), 2.61 (t, J = 7.5 Hz), 3.67 (s), 3.68 (s), 5.65 (br)
4g, *E*-isomer		1730	211 (17), 210 (19), 182 (13), 178 (17), 151 (23), 150 (31), 133 (22), 114 (23), 109 (42), 108 (32), 96 (34), 95 (89), 82 (100), 81 (28), 74 (28), 69 (40), 67 (39), 59 (50), 55 (63), 43 (23), 41 (66), 29 (25), 27 (29), 15 (54)	1.22—1.68 (m), 2.10—2.18 (m), 2.14 (d, J = 1.3 Hz), 2.25—2.35 (m), 3.67 (s), 3.69 (s), 5.66 (br)
5a, *Z*-isomer	16	1101	157 (M^+— 1, 2), 127 (100), 126 (15), 99 (38), 98 (33), 95 (7), 71 (20), 59 (37), 55 (14), 41 (28), 39 (31), 29 (10), 27 (15), 15 (58)	1.21—1.30 (m), 1.65—1.72 (m), 2.08 (dd, J_1 = 6.6 Hz, J_2 = 8.5 Hz), 3.70 (s)

TABLE I (continued)

Compound	RT	RI	MS	NMR
5a, *E*-isomer	14	1090	157 (M^+-1, 2), 127 (100), 126 (41), 99 (60), 98 (85), 95 (13), 85 (5), 83 (8), 71 (14), 59 (57), 55 (22), 41 (33), 39 (54), 29 (20), 27 (22), 15 (85)	1.42 –1.45 (m), 2.15–2.20 (m), 3.70 (s)
5b, *Z*-isomer	20	1175	141 (17), 140 (21), 113 (16), 112 (14), 109 (57), 108 (46), 99 (53), 98 (64), 81 (48), 71 (32), 59 (63), 55 (29), 53 (41), 41 (34), 39 (27), 27 (38), 15 (100)	0.97–1.02 (m), 1.10–1.19 (m), 1.55–1.60 (m), 2.30–2.54 (m), 3.68 (s), 3.69 (s)
5b, *E*-isomer	22	1190	141 (15), 140 (19), 113 (29), 112 (17), 109 (24), 108 (36), 99 (67), 98 (58), 81 (42), 71 (48), 59 (80), 55 (34), 53 (33), 27 (41), 15 (100)	0.80–1.86 (m), 1.23–1.31 (m), 1.46–1.50 (m), 1.62–1.80 (m), 3.70 (s), 3.69 (s)

organic solution was discarded. The aqueous phase was brought to pH 1 by adding concentrated hydrochloric acid and then extracted with 4 ml of diethyl ether. After methylation of the resulting organic solution by diazomethane, the ether was removed by a stream of nitrogen, and the residue redissolved in 2 ml of methanol; 50 μl of this solution were injected into the preparative gas chromatograph. Retention times, mass spectral and ^{1}H-NMR data are given in Table I.

2-(Carbomethoxy)-oxirane-acetic acid methyl ester (9a)

Seven grams (0.044 mol) of itaconic acid dimethyl ester, prepared from itaconic acid with methanol [17], were dissolved in 40 ml of methylene chloride; 9 g (85%) of 3-chloroperbenzoic acid, dissolved in 100 ml of methylene chloride, were slowly added with stirring and raising the temperature to 36° C. The solution was stirred a further 12 h at room temperature. Most of the 3-chlorobenzoic acid precipitated during this procedure. It was filtered off and the filtrate was washed three times each with 100 ml of 2 *M* sodium bicarbonate.

After evaporation of the solvent, 2 g of the residue were dissolved in 10 ml of cyclohexane and chromatographed on 180 g of silica gel (Fluka, 60) with cyclohexane–ethyl acetate (2:1) as solvent (^{1}H-NMR [25]).

2-(Carbomethoxy)-oxirane-propionic acid methyl ester (9b)

2-(Carbomethoxy)-oxirane-propionic acid methyl ester (9b) was prepared according to the procedure described for the synthesis of 9a, by treatment of 2-methyleneglutaric acid dimethyl ester [26] with 3-chloroperbenzoic acid (^{1}H-NMR [15]).

RESULTS

Reaction of α,β-unsaturated acids with diazomethane

The rate of production of pyrazolines by treatment of α,β-unsaturated

alkenedioic acids or their dimethyl esters (1a—g) is strongly dependent on the molecular structure: with maleic and fumaric acid Δ^2-pyrazoline formation (Scheme 1) is quantitatively complete within 30 sec. Higher homologues react much more slowly. If the reaction is stopped after 1 min, the production of pyrazolines can be nearly avoided. If a Δ^2-pyrazoline (3) is subjected to gas chromatography it does not form a sharp peak but a broad signal if OV-101 is used as stationary phase. Plotting the molecular weights of the homologues against their retention indices (RI) results in a straight line.

If the temperature of the injector is kept at about 280° C no significant decomposition of the pyrazolines (3a—g) is observed, but if the injector is heated to 320° C or more, the pyrazolines (3a—g) are partly decomposed producing the

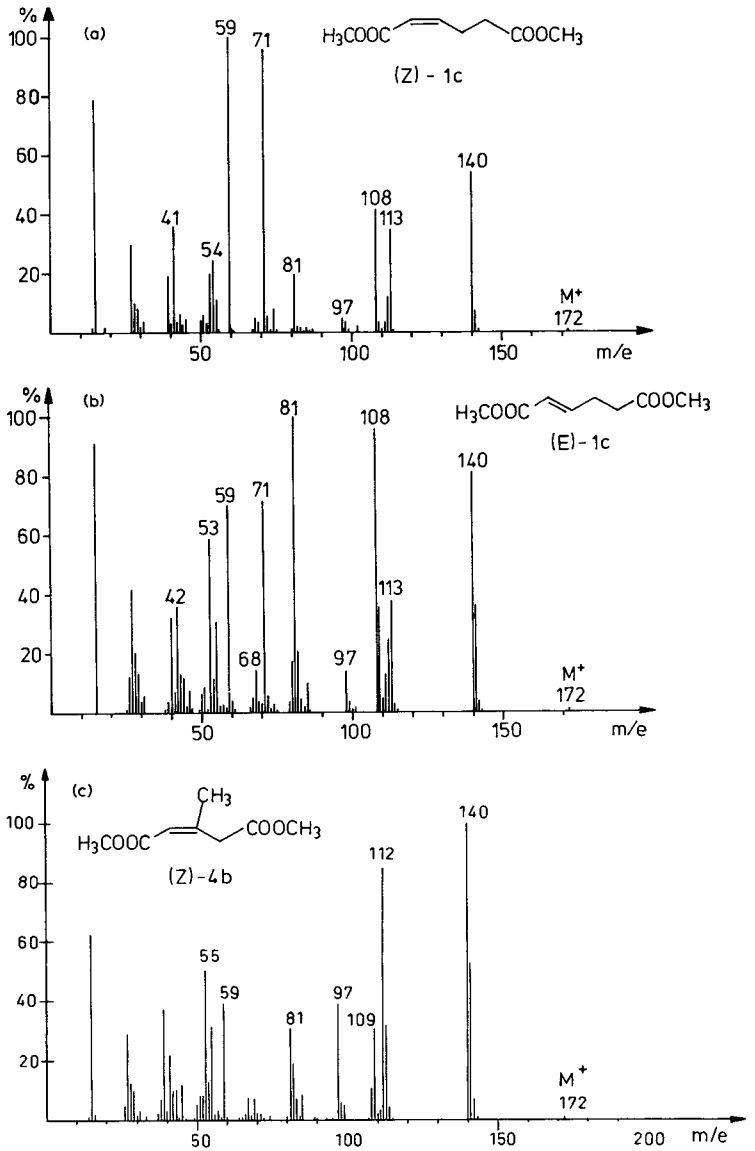


Fig. 1.

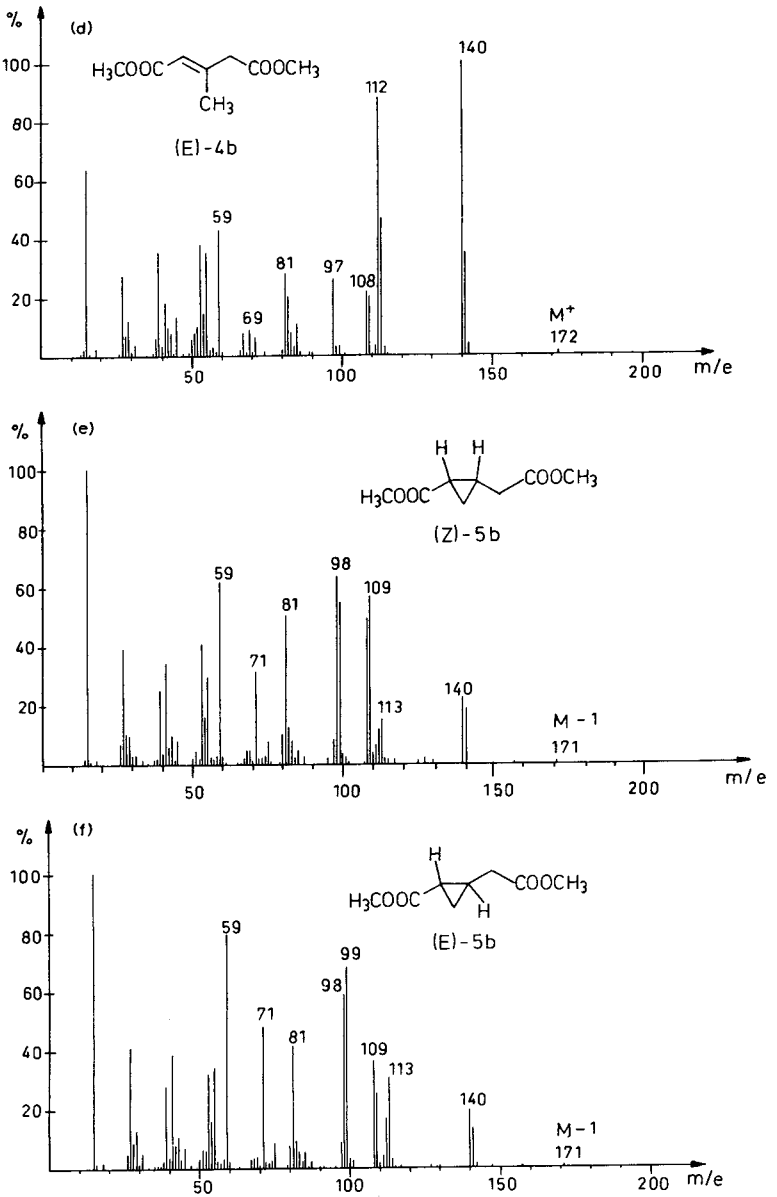


Fig. 1. Mass spectra of the methyl esters of hexenedioic acid (1c), 3-methylglutaconic acid (4b) and 1-carboxycyclopropane-2-acetic acid (5b) (both *Z*- and *E*-isomers).

methyl esters of 3-methylalkenedioic acids (4a—g) (*Z*- and *E*-isomers) as well as the corresponding cycloalkane derivatives (5a—g) (Scheme 1). The Δ^2-pyrazolines (3a—g) can be characterized not only by their RI values but also by their characteristic mass spectral fragmentation reactions. The most characteristic fragment in the mass spectrum of 3 is *m/z* 95. This ion is probably produced via the ion of *m/z* 127 by elimination of CH_3OH.

The *Z*- and *E*-isomers of 4 are well separated by gas chromatography (Table

I). Both isomers, and also their straight chain isomers, exhibit the same fragment ions with ions of $M^+ - 31$, $M^+ - 32$, $M^+ - 59$, $M^+ - 64$ and $M^+ - 73$ in their mass spectra, only differences in intensity being observed. Therefore identification requires a careful comparison with authentic compounds. Even the *Z*- and *E*-isomers can be distinguished if mass spectra and RI values are carefully compared.

Equally difficult is the differentiation between the isomeric straight-chain alkenedioic acid esters and cyclopropanedioic acid esters (5a—g) by comparison of their mass spectra alone. The mass spectra of the cyclopropane dioic acid methyl esters (5a—g) show the same main fragmentations as the mass spectra of the corresponding 3-methylalkenedioic acid dimethyl esters (4a—g).

The difficulties encountered in the identification of α,β-unsaturated esters (4a—g) and cyclopropanedioic acid dimethyl esters (5a—g) as well as *Z*- and *E*-isomers of 3-methylalkenedioic dimethyl esters (4a—g) by MS alone are demonstrated in Fig. 1.

Reactions of α-oxo acids with diazomethane

The GC analysis of the reaction products obtained from 2-oxoglutaric acid (6b) with diazomethane (Fig. 2) yielded as main product 2-(carbomethoxy)-oxirane-propionic acid methyl ester (9b) (88%); 2-oxoglutaric acid dimethyl

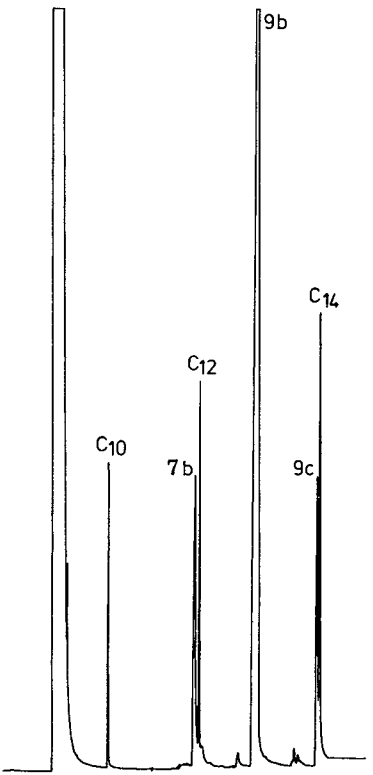


Fig. 2. Glass capillary gas chromatogram of the products formed by the reaction of 2-oxoglutaric acid with diazomethane.

TABLE II

REACTION PRODUCTS OF 2-OXO ACIDS WITH DIAZOMETHANE

Compound	RI	MS	%
9a	1180	174 (M^+, 0.5), 159 (1), 143 (11), 142 (6), 116 (5), 115 (100), 110 (18), 101 (10), 87 (8), 83 (17), 82 (2), 74 (15), 69 (3), 59 (34), 57 (35), 55 (9), 45 (48)	52
7b	1185	174 (M^+, 1), 143 (5), 142 (2), 116 (8), 115 (100), 87 (18), 59 (56), 55 (63), 45 (10), 43 (7)	5
9b	1292	188 (M^+, 0.5), 173 (2), 157 (4), 156 (3), 141 (11), 129 (34), 128 (20), 125 (30), 116 (6), 115 (98), 113 (10), 111 (8), 101 (12), 97 (18), 96 (9), 87 (18), 83 (8), 71 (7), 69 (21), 68 (7), 59 (54), 55 (94), 53 (6), 45 (100), 43 (16), 42 (20), 41 (21)	88
12b = 9c	1390	202 (M^+, 1), 172 (3), 171 (4), 170 (1), 155 (9), 152 (12), 143 (34), 142 (26), 139 (8), 129 (19), 128 (20), 124 (10), 115 (38), 111 (20), 101 (21), 97 (18), 83 (37), 81 (17), 71 (23), 69 (19), 59 (90), 55 (100), 45 (98), 43 (38), 42 (40), 41 (42)	5
7c	1281	188 (M^+, 1), 157 (10), 129 (78),101 (48), 97 (12), 59 (100), 55 (60), 42 (26), 41 (22)	4
12c	1497	216 (M^+, 4), 185 (6), 169 (16), 167 (10), 157 (39), 153 (11), 134 (36), 125 (27), 115 (64), 111 (43), 106 (20), 97 (27), 85 (30), 83 (50), 81 (24), 79 (39), 74 (22), 69 (20), 68 (28), 67 (30), 59 (90), 55 (100), 45 (97), 43 (88), 41 (63)	2
7d	1010	144 (M^+, 6), 102 (1), 85 (82), 59 (10), 57 (100), 55 (9), 41 (52)	2
9d	1083	158 (M^+, 1), 141 (2), 129 (4), 128 (1), 127 (1), 115 (44), 99 (10), 85 (38), 83 (12), 81 (14), 69 (8), 59 (18), 57 (33), 55 (18), 45 (49), 43 (100), 41 (34)	89
12d	1192	172 (M^+, 1), 143 (4), 141 (1), 129 (3), 125 (11), 115 (32), 99 (17), 95 (18), 87 (11), 85 (18), 83 (6), 81 (5), 71 (17), 69 (12), 59 (19), 55 (30), 45 (37), 43 (100), 41 (34)	9
7e	1195	172 (M^+, 1), 113 (50), 85 (20), 69 (5), 59 (7), 57 (14), 55 (10), 43 (100), 41 (21)	2
9e	1294	186 (M^+, 0.5), 155 (1), 153 (2), 125 (11), 117 (11), 115 (36), 113 (15), 109 (19), 101 (9), 85 (30), 69 (24), 67 (12), 59 (16), 55 (32), 45 (37), 43 (100), 41 (44)	90
12e	1392	200 (M^+, 2), 179 (1), 169 (1), 168 (1), 153 (4), 141 (6), 127 (8), 125 (15), 123 (18), 117 (19), 115 (43), 100 (13), 85 (38), 83 (12), 81 (11), 71 (11), 69 (15), 67 (20), 59 (22), 57 (43), 55 (61), 45 (42), 43 (100), 41 (56)	8

ester (7b) was produced only in 5% yield. A third peak in the gas chromatogram showed in its mass spectrum a parent ion peak at *m/z* 202 and key ions at mass *m/z* 45, *m/z* 55, *m/z* 59, *m/z* 101, and *m/z* 115, which suggested that its structure differed from the oxirane (*m/z* 188) (9b) by the presence of an additional methylene group. This assumption was confirmed by reacting 2-oxoglutaric acid (6b) with deuterodiazomethane [27], demonstrating the introduction of four C^2H_2 groups into the original molecule.

The ^{1}H-NMR spectrum of the isolated unknown compound of 202 molecular weight, revealed a pair of doublets at δ = 3.05 ppm and δ = 2.81 ppm (J = 6 Hz), assigned to an AB proton for oxirane ring protons, suggesting structure 9c for the compound.

To confirm this assumption 2-oxoadipinic acid (6c) was treated with diazomethane. The resulting main product 2-(carbomethoxy)-oxirane-butyric acid methyl ester (9c) showed the same mass spectrum and RI value as the by-product of the reaction of 2-oxoglutaric acid (6b) with diazomethane. Obviously, by migration of the zwitterion (8b), 2-oxoadipic acid methyl ester (7c = 10b) is formed which reacts immediately with diazomethane to give 12b = 9c as indicated in Scheme 2. The corresponding oxirane of 11b could not be detected.

All investigated compounds [2-oxoglutaric acid (6b), 2-oxoadipic acid (6c), 2-oxohexanoic acid (6d) and 2-oxooctanoic acid (6e)] showed an analogous behaviour on treatment with diazomethane (Table II).

Solvent effects

In general, the weak acid methanol increases the ratio of produced oxiranes [28] by increasing the polarization of diazomethane forming hydrogen bonds. Therefore we tried to see if the oxirane production could be avoided or at least reduced by the use of less polar solvents. The results of these experiments (Table III) demonstrate only negligible effects.

TABLE III

SOLVENT EFFECTS

Solvent	2-Oxoglutaric acid dimethyl ester (7b) (%)	2-(Carbomethoxy)-oxirane-propionic acid methyl ester (9b) (%)	2-(Carbomethoxy)-oxirane-butyric acid methyl ester (9c) (%)
Cyclohexane	48.2	51.5	0.3
Without solvent	59	41	—
Ether	55	45	—
Benzene	47.3	52.1	—
Ethyl acetate	53	47	—
Methanol	5	88	5

Reaction time

Reduction of the reaction time between acid and diazomethane from 10 min to 1 min (solvent methanol), increased the rate of dimethyl ester produced from 2-oxoglutaric acid (6b) from 5% to 59% and reduced the rate of oxirane

dimethyl ester (9b) production from 88% to 41%. Thus the production of oxirane can not be avoided but can be considerably reduced.

Mass spectra

In all oxiranes obtained from α-oxo methylates an intense ion is observed at *m/z* 115 ($C_5H_7O_3^+$) (Fig. 3) formed by β-cleavage from the molecule, a marked contrast to acyclic ethers in which α-cleavage is the predominant fission reaction [29]. The ion *m/z* 115 loses CH_3OH and CO to give the ion *m/z* 55 (base peak). Typical further key ions are found at *m/z* 45 ($C_2H_5O^+$), *m/z* 59 ($C_2H_3O_2^+$), *m/z* 83 ($C_4H_3O_2^+$) and *m/z* 101 ($C_4H_5O_3^+$) allowing firm identification of this class of compounds (Scheme 5).

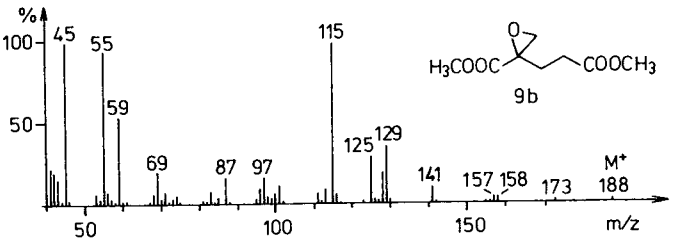
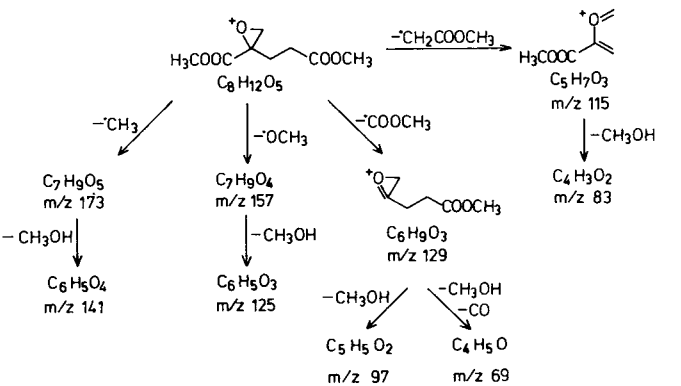


Fig. 3. Mass spectrum of 2-(carbomethoxy)-oxirane-propionic acid methyl ester (9b).



Scheme 5. Fission reactions of 2-(carbomethoxy)-oxirane-propionic acid methyl ester (9b).

Identification of "unknown" compounds in urine

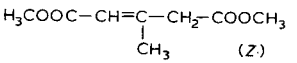
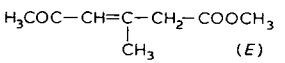
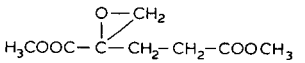
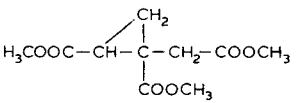
Some time ago our group published a paper [10] on the identification of acidic compounds in urine by glass capillary gas chromatography after treatment with diazomethane. Several of the peaks remained unknown since no comparison material was available. Some of these compounds have now been positively identified as artefacts from the compounds listed in Table IV.

DISCUSSION

It is not possible to stop the reaction of diazomethane with α,β-unsaturated acids or α-oxo acids at the step of the methyl ester, since these compounds

TABLE IV

COMPOUNDS POSITIVELY IDENTIFIED AS ARTEFACTS

Peak number in GC of urine (ref. 10)	RI	Structure	Artefact of
34	1163		
39	1195		
56	1291		2-Oxoglutaric acid
92	1428		Aconitic acid
98	1440	Isomers of 92	Aconitic acid
101	1445	Isomers of 92	Aconitic acid

react further to produce either mainly pyrazolines or oxiranes — even if very short reaction times are used.

The GC retention indices and mass spectral data of those reaction products are presented in this paper. These data are important in recognizing these compounds in complicated mixtures of acids obtained from biological fluids and in identifying the original compounds. Consequently a time-consuming structural elucidation is also avoided.

Due to the lack of key ions, characterization of them and decomposition products of Δ^2-pyrazolines is not possible from the mass spectra alone but requires also the measurement and comparison of RI values.

The accumulated data obtained from the most common α,β-unsaturated acids and α-oxo acids may also enable the detection of similar artefacts, not listed in the tables, by recognition of typical mass spectrometric key ions (for instance, ions of mass *m/z* 45, *m/z* 55, *m/z* 101 and *m/z* 115 for oxirane methyl esters, and ions of mass *m/z* 95 and *m/z* 127 for Δ^2-pyrazolines).

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CHROMBIO. 2143

DETERMINATION OF N-ACETYL-L-CYSTEINE IN BIOLOGICAL FLUIDS

HARTMUT FRANK* and DIETMAR THIEL

Institut für Toxikologie, Wilhelmstrasse 56, 7400 Tübingen 1 (F.R.G.)

and

KLAUS LANGER

Forschungsinstitut für Experimentelle Ernährung e.V., Langemarckplatz 5 1/2, 8520 Erlangen (F.R.G.)

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SUMMARY

For determination of a drug in biological fluids, accuracy and sensitivity of detection of the adopted method are decisive parameters, but practical aspects such as time and necessary instrumentation are equally important. In the case of N-acetyl-L-cysteine, sample pretreatment can be kept at a minimum if sufficiently selective modes of derivatization, chromatography and detection are employed. Its concentration in serum is between 1 and 20 μmol/l; for quantitative analysis capillary gas chromatography and mass fragmentographic detection are employed. For urine with its ten-fold higher concentration the preferred method is reversed-phase high-performance liquid chromatography after thiol-selective derivatization.

INTRODUCTION

N-Acetyl-L-cysteine, an endogenous product of cysteine metabolism [1], is of considerable therapeutic importance as a mucolytic drug in the treatment of bronchitis [2]; it also protects against the hepatotoxicity of high doses of *p*-acetylaminophenol [3]. For parenteral nutrition N-acetyl-L-cysteine may serve as a substitute for cysteine, offering the advantages of higher solubility and stability. In this context appropriate analytical methods are required for monitoring its bioavailability and pharmacokinetics. Gas chromatography and liquid chromatography are complementary methods for the quantitative determination of N-acetyl-L-cysteine in plasma and urine.

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EXPERIMENTAL

Chemicals

4-Chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) was from Serva (Heidelberg, F.R.G.). N,4-Dimethyl-N-nitrosobenzenesulfonamide was from E. Merck (Darmstadt, F.R.G.). Nucleosil C_{18}, 7 μm, was purchased from Macherey and Nagel (Düren, F.R.G.). All other chemicals of reagent grade were from Merck.

Buffers

EDTA—citrate: 29.4 g of sodum citrate and 744 mg of EDTA are dissolved in 200 ml of distilled water; the buffer is adjusted to pH 8.3 with sodium hydroxide.

Phosphate: 17.8 g of $Na_2HPO_4 \cdot 2H_2O$ are dissolved in 200 ml of distilled water; the buffer is adjusted to pH 8.0 with phosphoric acid.

Instrumentation for high-performance liquid chromatography (HPLC)

The liquid chromatograph is a modular instrument consisting of a Spectra-Physics pump Model 740 B, a pressure monitor Model 714, and a Rheodyne Injector Model 7105. The eluate is monitored at 470 nm with a spectrophotometer from ERC, Model 7210; the signals are registered with a Servogor recorder Model RE 571, and integrated with a Hewlett-Packard integrator Model HP 3390. The column, 30 cm × 4.6 mm, is filled with Nucleosil C_{18}, 7 μm, by slurry packing. During chromatography the column is immersed into a water bath wit a constant temperature of 30° C.

Derivatization and HPLC

The oxidized form N,N′-diacetylcystine must be reduced in order to determine the overall resorption of the administered compound; this is effected by the addition of 10 μl of a solution of dithioerythritol (145 mg in 10 ml water) and 100 μl of phosphate buffer to 250 μl of urine or deproteinized serum. An aliquot of 250 μl is removed after 30 min and mixed with 250 μl of EDTA—citrate buffer and 50 μl of a solution of NBD-Cl (100 mg in 100 ml of methanol). After 20 min the sample is ready for chromatography. Weighed standards are dissolved in water and treated analogously. Aliquots of 20 μl are injected for HPLC. The mobile phase is aqueous disodium hydrogen phosphate (0.5%)—acetonitrile (70:30, v/v); the flow-rate is 1.6 ml/min. More than 100 samples can be processed before a column needs to be repacked.

Sample preparation and gas chromatography (GC)

Blood serum (0.5 ml) is mixed with 20 μl of a solution of N-acetyl-D-cysteine (250 nmol/ml in distilled water). One drop of ethanethiol is added and after 5 min the protein is precipitated by the addition of 1 ml of acetone. After centrifugation the supernatant is adjusted to pH 9 with a drop of aqueous ammonia (25%) and extracted with 1 ml of hexane. The aqueous phase is then concentrated to about half of its initial volume with a stream of nitrogen and under moderate heating. The aqueous solution is transferred to an exchange column, Dowex 1-X8, OH^-, 50—100 mesh, 40 × 5 mm. After 10 min the column is washed with 4 ml of water, then with 5.0 ml of hydrochloric acid

(0.1 mol/l). The last 1.0 ml containing the N-acetylcysteine is collected in a Reacti-Vial®. The solution is dried in a vacuum centrifuge. Diazomethane in 0.9 ml of diethyl ether and 0.1 ml of methanol and a drop of ethanethiol are added to the dry residue and the sample is treated by sonication for 2 min. After 30 min at room temperature the solvent is removed with a nitrogen stream, the residue is dissolved in 10 μl of toluene under sonication and the sample is centrifuged; 1 μl (for urine samples 0.1 μl) is injected for gas chromatography—mass spectrometry (GC—MS).

GC—MS conditions

The instrument is a Finnigan 4021 with an Incos data system. The samples are injected in the splitless mode onto a glass capillary, 25 m × 0.25 mm, deactivated with diphenyltetramethyldisilazane according to the method of Grob et al. [4] and coated with Chirasil-Val; the split is opened after 0.7 min. The injector temperature is 250°C; the carrier gas is helium; the inlet pressure 100 kPa. The temperature programme is 0.7 min isothermal at 60°C, then at a rate of 40°C/min to 145°C, at a rate of 5°C/min to 175°C, and at a rate of 40°C/min to 200°C, isothermal for 6 min. Interface and ion-source temperatures are 250°C; electron-impact ionization, electron energy 70 eV; scans from *m/z* 100 to *m/z* 210 in 0.95 sec, bottom time 0.05 sec, electron multiplier 1.6 kV. Mass fragmentograms are constructed monitoring *m/z* 132, the base peak in the mass spectrum of N-acetyl-S-methylcysteine methyl ester. Also suitable is chemical ionization using isobutane and mass fragmentography of *m/z* 192, the quasimolecular ion.

RESULTS AND DISCUSSION

For pharmacokinetic studies non-selective methods have been employed, such as utilization of radioactive tracers [5] or determination of total thiol content [6]. However, to gain detailed insight into the metabolic fate of N-acetyl-L-cysteine, selective methods are required; more reliable is HPLC [7] or GC [1]. The HPLC method is based upon prechromatographic derivatization of the thiol group with different N-substituted maleimides forming fluorescent derivatives; the detection limit for the pure compound is in the femtomole range. A drawback of both published procedures is the relatively complicated isolation of N-acetyl-L-cysteine. In spite of their high sensitivity, they are apparently not suitable for analysis of serum; no quantitative data have been given although these values are of great importance for pharmacokinetic studies.

The goal of this investigation was to establish routine methods for the quantitative analysis of N-acetyl-L-cysteine in serum and urine in its therapeutic concentration range in a fast and uncomplicated manner. The choice of a method is not only determined by the sensitivity of the actual analytical step, but also by more practical aspects such as cost and time required for the procedure including sample pretreatment, and the limit of detection of the respective compound in an actual sample, not of the pure standard.

Determinations of N-acetyl-L-cysteine in serum were anticipated to be difficult, due to its low concentration and the small sample volumes available.

The therapeutic concentration range is about 1—30 μmol/l. In urine it is present in concentrations between 10 and 200 μmol/l; therefore, determination of N-acetyl-L-cysteine in urine should be relatively unproblematic, provided that potentially interfering substances are removed during the clean-up, separated by chromatography or selectively suppressed by the detection mode.

Conversion of N-acetyl-L-cysteine to the S-NBD derivative renders the sample pretreatment for HPLC very simple; for determinations in the relatively high concentration range typical for urine this derivative proved to be suitable. Reaction with NBD-Cl is fast, and the yields are reproducible and quantitative [8]. The derivatives are stable, which is especially important for routine analysis of large numbers of samples.

The chromatogram of a standard and a urine sample of a patient treated with N-acetyl-L-cysteine is shown in Fig. 1. The lower limit of photometric detection at 470 nm is about 5 μmol/l in urine; it is likely that employment of a fluorescence detector would increase the sensitivity. The calibration line is linear, with a slope of 1.06 cm peak height vs. 1 μmol/l and a correlation coefficient of 0.9996. The same slope is obtained for the oxidized form N,N'-diacetyl-L,L-cystine, indicating that reduction with dithioerythritol is quantitative. Appealing is the simplicity and reproducibility of the described procedure. Pretreatment of the sample is minimal, and reduction and derivatization take less than 1 h. This facilitates the analysis of large numbers of samples.

Analysis of sera of different patients showed that individually various contaminants interfere with the quantitative determination by HPLC. In this case we reverted to the method of enantioner labelling [9] in combination with capillary GC. A chromatogram of a standard is shown in Fig. 2a. Initially it was intended to employ a flame-ionization detector for GC, but with the adopted sample pretreatment the chromatograms were not sufficiently clean (not shown). An alkali flame-ionization detector with its high sensitivity and selectivity for nitrogen-containing compounds affords considerably less-complex

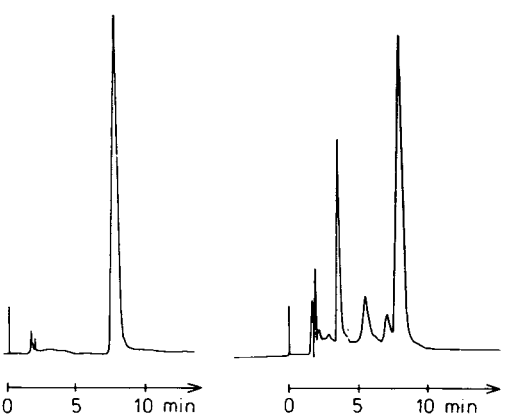

Fig. 1. HPLC of N-acetyl-S-NBD-cysteine on a column, 300 × 4.6 mm, packed with Nucleosil C_{18}, 7 μm. Mobile phase, aqueous disodium hydrogen phosphate (0.5%)—acetonitrile (70:30, v/v); flow-rate, 1.6 ml/min; photometric detection at 470 nm; injected amounts, 3.1 and 2.8 nmol. Left: aqueous solution of N-acetylcysteine, 153 μmol/l. Right: urine containing 140 μmol/l endogenous N-acetylcysteine.

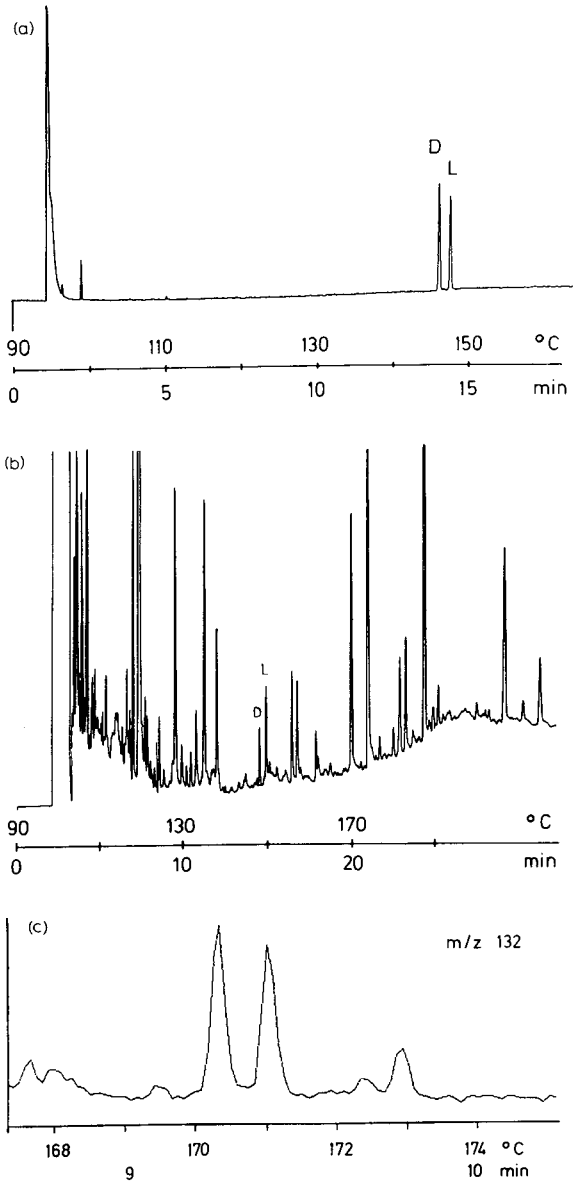

Fig. 2. (a) GC separation of the enantiomers (L and D) of N-acetyl-S-methylcysteine methyl ester on a capillary, 25 m × 0.25 mm, coated with Chirasil-Val; carrier gas is hydrogen, inlet pressure 50 kPa, temperature programme 4°C/min; split injection, split ratio 1:50; injector and detector temperature 250°C. (b) Determination of N-acetyl-L-cysteine by enantiomer labelling; separation on a glass capillary, 10.5 m × 0.1 mm, coated with Chirasil-Val; carrier gas hydrogen, inlet pressure 90 kPa; detection with a nitrogen-selective alkali flame-ionization detector. The concentration in this serum sample was determined as 12 μmol/l; amount after splitting 10 fmol. (c) Determination of N-acetyl-L-cysteine by enantiomer labelling and GC—MS (*m/z* 132) in a control serum spiked with 0.6 μmol/l N-acetyl-L-cysteine; 1.2 μmol/l N-acetyl-D-cysteine are added as internal standard.

chromatograms; however, for accurate quantitative analysis this still is deemed inappropriate due to the relatively noisy baseline and the appearance of a number of additional peaks (Fig. 2b). Logically, the next choice would have been a sulphur-selective flame-photometric detector, but we did not have such a device. Therefore, detection was done by mass fragmentography monitoring *m/z* 132 (Fig. 2c). The method is sufficiently sensitive to allow quantitative determinations in the concentration range expected for endogenous N-acetyl-L-cysteine, i.e. about 0.5 μmol/l. Control analyses were performed without addition of N-acetyl-D-cysteine to show that this isomer is not present endogenously.

A calibration graph was constructed with human blood serum spiked with increasing amounts of synthetic N-acetyl-L-cysteine. Between 0.5 and 30 μmol/l a straight line with a slope of 0.952, a correlation coefficient of 0.999 and an intercept of 0.43 μmol/l endogenous N-acetyl-L-cysteine were obtained. As the dosages applied in clinical studies lead to concentrations in serum of 1—20 μmol/l, the enantiomeric internal standard N-acetyl-D-cysteine is added to a concentration of 10 μmol/l. The great advantage of enantiomer labelling is that only sufficiently large signal-to-noise ratios of the peaks of both antipodes are required; the recovery over all steps of the analysis need not be known.

The described procedures have been employed in the assessment of the bioavailability of N-acetyl-L-cysteine. As an example the concentration—time curve in the blood of a male patient receiving an intravenous infusion of 1.6 mmol during the initial 6 h is shown in Fig. 3. The values determined with the less selective alkali flame-ionization detector are of similar magnitude.

The serum levels of N-acetyl-L-cysteine determined by HPLC are considerably lower. At first this was considered as an indication of binding to plasma proteins. As the clean-up for HPLC involves precipitation of plasma proteins, a significant portion of the drug would be removed with the precipitated protein. However, comparison of values obtained by enantiomer labelling with the addition of internal standard before and after precipitation of plasma

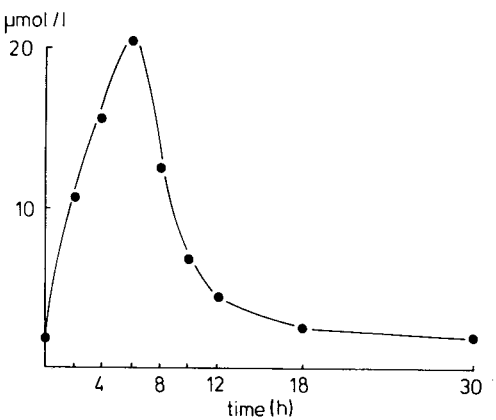

Fig. 3. Typical time dependence of the serum concentration of total N-acetyl-L-cysteine/cystine in a patient receiving 1.6 mmol of N-acetyl-L-cysteine by intravenous infusion during the initial 6 h.

proteins yielded identical values. The reason why HPLC affords lower values is currently being investigated.

In conclusion we want to emphasize that for routine determinations of drugs in biological fluids the method with the maximum sensitivity is not always the most suitable; in fact, the high selectivity of mass fragmentography, not so much its sensitivity, is crucial to achieve sufficiently accurate results. In addition, aspects such as time and cost are important from the point of view of practicability. The present study also shows that it may be necessary to employ different analytical procedures for the determination of the same compound in different types of samples.

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CHROMBIO. 2165

IDENTIFICATION OF DIETHYLENE GLYCOL IN SERA FROM EGYPTIAN CHILDREN BY FREQUENCY-PULSED ELECTRON-CAPTURE GAS—LIQUID CHROMATOGRAPHY

J.B. BROOKS

Biotechnology Branch, Division of Bacterial Diseases, Center for Infectious Diseases, Centers for Disease Control, Atlanta, GA 30333 (U.S.A.)

M.T. BASTA*

Biomedical Research Center for Infectious Diseases, P.O. Box 424, Cairo (Egypt)

C.C. ALLEY and J.S. HOLLER

Toxicology Branch, Clinical Chemistry Division, Center for Environmental Health, Centers for Disease Control, Atlanta, GA 30333 (U.S.A.)

and

A.M. EL KHOLY

Biomedical Research Center for Infectious Diseases, P.O. Box 424, Cairo (Egypt)

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SUMMARY

Sera taken from fifteen patients (from Kerdasa village near Cairo, Egypt) infected with *Schistosoma haematobium*, with eggs present in the urine, were studied by frequency-pulsed electron-capture gas—liquid chromatography (FPEC—GLC). Some of the patients were treated with metrifonate and again studied by FPEC—GLC. Diethylene glycol was detected in the sera of untreated patients infected with *S. haematobium*. This compound was identified by negative chemical ionization and electron-impact mass spectrometry. Initially we suspected that the build-up of diethylene glycol in these patients was caused by schistosomiasis infection. However, in a follow-up blind-coded study using FPEC—GLC, which included 37 sera from Kerdasa and Tamooh villages near Cairo, Egypt, we detected diethylene glycol in eleven samples, four of which were controls from the villages. These latter findings indicate that the source of diethylene glycol might be the environment or foodstuffs, but the specific source has not been determined. Regardless of the source, diethylene glycol could affect the health of these Egyptian children by causing a narcotic effect, increased bladder stones, and increased numbers of bladder tumours.

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INTRODUCTION

While conducting studies of sera from *Schistosoma*-infected children in Kerdasa, a village near Cairo, Egypt, we detected an unknown peak by frequency-pulsed electron-capture gas—liquid chromatography (FPEC—GLC). We thought this unknown peak might be associated with schistosomiasis. Schistosomiasis is a disease that is endemic and chronic in many areas of the world. Little is known about changes that take place in body metabolism or what products are produced in vivo by these organisms [1], but it is known that patients infected with *Schistosoma haematobium* have a higher incidence of bladder cancer, and that the disease seems to produce a narcotic effect on the host [1]. We decided to try and identify this unknown peak by gas chromatography—mass spectrometry (GC—MS).

FPEC—GLC is a selective sensitive analytical tool that has been used to detect changes in spent culture media [2—5] and from body fluids of diseased persons [5—10]. Mass spectrometry (MS) has been commonly used in combination with gas chromatography (GC) to identify unknown peaks detected by this technique. Identification by GC—MS of unknown peaks detected by FPEC—GLC analysis of body fluids is often hard to accomplish. The reasons for the difficulty lie in the fact that: FPEC—GLC often surpasses the detection limits of GC—MS, or that there is failure to detect a molecular ion in electron-impact spectra of fluorinated derivatives. The purposes of this study were as follows: (1) to find GC—MS conditions capable of obtaining electron-impact and chemical ionization spectra (both positive and negative) of the unidentified peak detected in the FPEC—GLC analysis of serum; (2) to identify the unknown compound, if possible; (3) to confirm identification by derivatization and analysis of the suspected compound by FPEC—GLC and GC—MS; and (4) to search the literature for information concerning the toxicity, physiological effects, and possible biosynthesis of the compound.

MATERIALS AND METHODS*

Serum specimens

The 1 ml of serum used for FPEC—GLC analysis in the study was serum left after routine tests had been performed. The first samples were collected from the village of Kerdasa. The control specimens were collected from employees at the Biomedical Research Center in Cairo. The samples were stored at −20°C until they were used. The median age, sex, and treatment regimen of the patients, and number of specimens used in the first study are given in Table I. The Biomedical Research Center for Infectious Diseases (BRCID) in Cairo, Egypt, confirmed *S. haematobium* infection by egg count in the urine. Following the first study, a second blind-coded study was conducted which involved FPEC—GLC analysis of sera from 37 additional children. The samples for the second study were taken from two villages near Cairo, those of Kerdasa and Tamooh. The samples consisted of fourteen control sera from the villages, nineteen sera

*Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or by the U.S. Department of Health and Human Services.

TABLE I

SUMMARY OF INFORMATION RELATING TO SPECIMENS USED IN THE FIRST FPEC—GLC STUDY

	No. of cases studied	Age range (years) (median)	Male:female	Egg count* (E.C.) high:mod.:low	No. of patients treated** (E.C. before treatment: E.C. after treatment)
S. haematobium	15	7—12 (11)	11:4	8:3.4	5 (1230:25), (500:32), (220:100), (188:15), (50:1)
Control	5	19—55 (27)	4:1	N/A	N/A

*For *S. haematobium*, high E.C. >400 per 10 ml of urine, moderate E.C. = 100—400 per 10 ml of urine, low E.C. <100 per 10 ml of urine.

**Sample taken from patient after two weeks of treatment with metrifonate (one course, 7.5 mg/kg)

from patients infected with *S. haematobium* and four sera from patients infected with *S. mansoni*. *S. mansoni* infection was confirmed by faecal egg count at the BRCID.

Extraction and derivatization procedures

Each serum specimen was placed in a 50-ml round-bottomed centrifuge tube with a Teflon-lined screw cap; then heptanoic acid (3.15 μmol in 0.1 ml distilled water, made basic to about pH 10 with sodium hydroxide to obtain solubility) and di-*n*-butylamine (1.19 μmol in 0.4 ml of distilled water made acidic to about pH 2 to increase solubility) were added to each sample as internal standards. Next, the samples were acidified (about pH 2) with 0.1 ml of 50% (v/v) sulfuric acid, mixed by shaking, and extracted with 20 ml of nanograde chloroform (Mallinckrodt) by shaking them for 5 min on a Burrell Wrist Action shaker at a setting of 10 to obtain carboxylic acids and alcohols. The residual aqueous phase was made basic (about pH 10) with 0.3 ml of 8 *M* sodium hydroxide and reextracted with 20 ml of chloroform, as described for the acidic extraction, to obtain the amines. The acidic chloroform extracts were derivatized with trichloroethanol—heptafluorobutyric anhydride (TCE—HFBA) to form TCE esters of carboxylic acids and HFBA esters of alcohol as described previously [2, 4]. The basic chloroform extracts containing amines and alcohols were derivatized with HFBA pyridine—ethanol to form amides as described previously [11]. A few HFBA derivatives of alcohols were prepared from pH 2 chloroform extracts using the procedure described [3]. After derivatization the samples were dissolved in 0.1 ml of xylene—ethanol (1:1). A 2-μl injection was used for analysis. The techniques for filling and cleaning the syringe have been described [4].

Apparatus

The derivatives were analysed on a Perkin-Elmer Model 3920 gas chromatograph equipped with dual 10 mCi ^{63}Ni frequency-pulsed electron-capture detectors. Two glass columns (7.3 m × 0.2 cm I.D.) packed with 3% OV-101 on 80—100 mesh Chromosorb W (HP AW DMCS) were used under conditions previously described [2, 10].

A Perkin-Elmer programmable processor (PEP-2) equipped with a Modular Software System (MS-16 revision B) accumulated data from the gas chromatograph, analysed the data according to a stored method, and prepared a report. An internal standard analysis was performed on the data by using heptanoic acid and di-*n*-butylamine, which were added as internal standards [2].

FPEC—GLC identification of the unknown peak 7 was made by comparing a known standard on an OV-101 packed column and on a 50-m OV-101 fused-silica capillary column (Perkin-Elmer) programmed as follows: isothermal at 100°C for 12 min, then 60°C/min to 265°C. We used a capillary column with a splitless injector. A 30-sec solvent vent was used, and helium was the carrier gas at a flow-rate of 3 ml/min. The recorder chart speed was 2.54 cm/min.

GC—MS analysis

A Finnigan 4023 gas chromatograph—mass spectrometer—data system (GC—MS—DS) equipped with a 25-m fused-silica column coated with OV-101 (Hewlett-Packard) was used. The capillary column was connected directly to the inlet of the MS source using a guide tube. The mass spectrometer was fitted with the pulsed positive ion/negative ion chemical ionization (PPINICI) accessory, allowing both positive and negative ion detection. The mass spectrometer was operated at an electron multiplier setting of 1200 V; the ionizing voltage was 70 V for all studies. We used helium as the carrier gas and methane (Matheson) as the reagent gas for chemical ionization with a source pressure at $1.2 \cdot 10^{-5}$ torr.

RESULTS

In the first study a compound was consistently detected by FPEC—GLC in the sera of fifteen untreated patients infected with *S. haematobium*, but this compound was not detected in the normal controls. As can be seen (Fig. 1A—C, peak 4) the compound was extractable under either acidic or basic conditions. It did not react with TCE, but did react with HFBA to form an electron-capturing derivative. The peak was found to be greatly reduced or eliminated after two weeks of effective therapy in five patients (Fig. 2A—C) and was not detected in the initial set of control specimens (Fig. 2D) which were obtained from laboratory personnel. The behaviour of the compound upon extraction and derivatization suggested that it contained hydroxyl group(s). The combined serum extracts from four different patients were necessary to obtain the electron-impact (EI) mass spectrum shown in Fig. 3. No molecular ion was observed for the derivative, but the observation of a fragment ion at *m/z* 213 is consistent with the presence of hydroxyl groups in the original compound. The other suggested structures for ions observed in the spectrum are those typically observed for fluorinated compounds [12]. No additional molecular weight information was obtained through positive chemical ionization. The negative chemical ionization (NCI), which is especially sensitive to halogens, was more sensitive than EI ionization by a factor of 5—10. The fluorinated derivatives react under NCI conditions through a dissociative resonance capture mechanism. Such a mechanism results in observed fragment ions $(M-20)^-$ and neutral HF. The fragment ion *m/z* 478 (Fig. 4) thus suggests a molecular weight of 498. The requirements of a 498 molecular weight and the hydroxyethylene structural unit suggested by *m/z* 241 in the EI spectrum limit the number of possible chemical structures. The analysis of the fluorinated derivative of diethylene glycol by co-chromatography on capillary and packed columns yielded identical retention times and mass spectra in EI and NCI to the un-

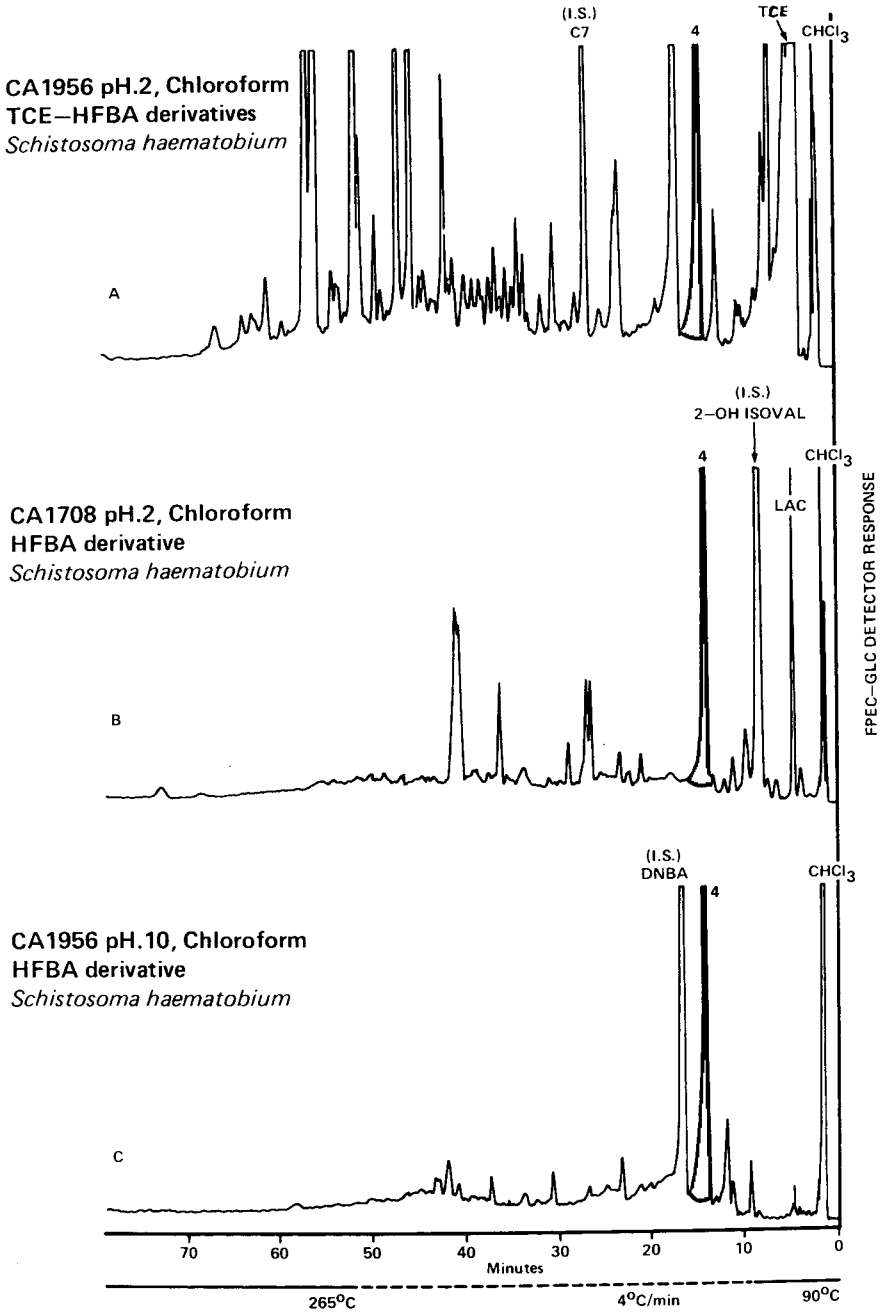

Fig. 1. FPEC–GLC chromatograms from serum analysed on OV-101 packed columns. The type of disease, extraction procedures, and type of extraction are indicated. TCE = trichloroethanol, HFBA = heptafluorobutyric anhydride, I.S. = internal standard, $CHCl_3$ = residual chloroform. The letter "C" before a number indicates a saturated straight-chain carboxylic acid with the number of the carbon atoms indicated by the number. The letter "i" indicates "iso" and the use of colon between two numbers indicates unsaturation. Peak 4 has been determined to be diethylene glycol. The samples were from Kerdasa village near Cairo, Egypt.

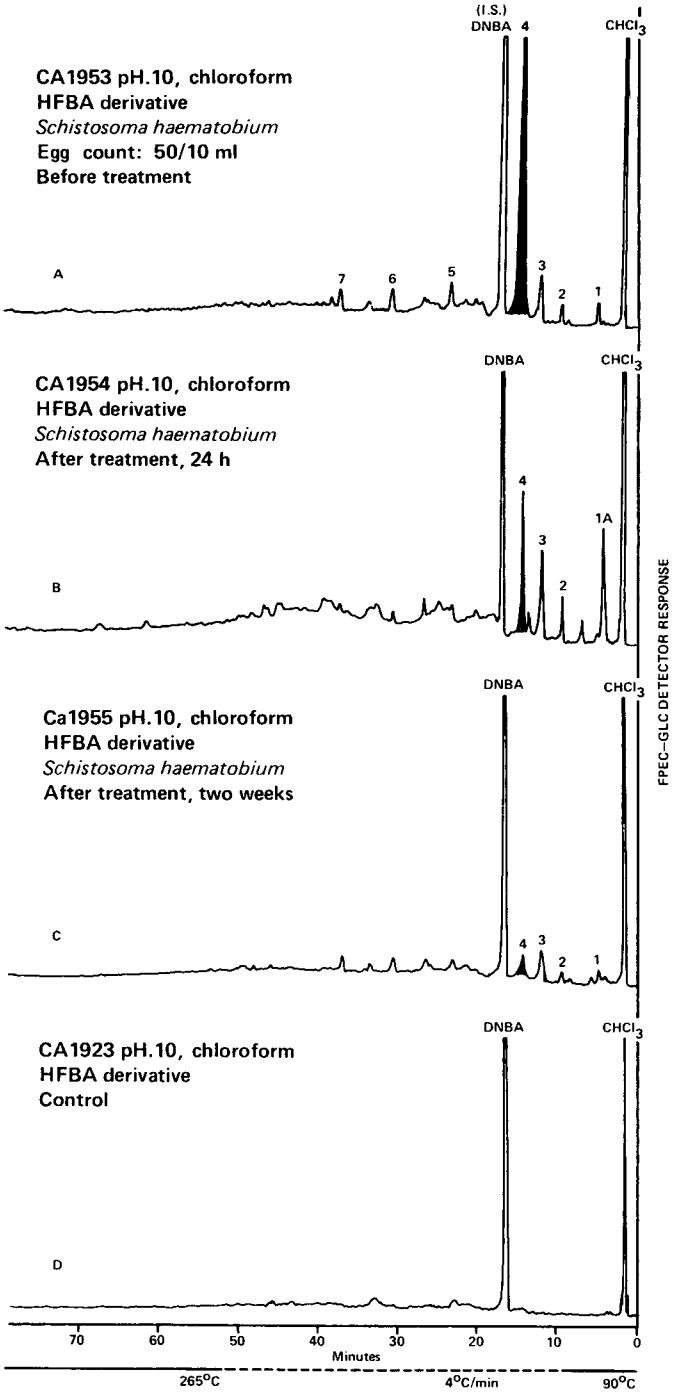

Fig. 2. FPEC—GLC chromatograms from sera analysed on OV-101 packed columns. The type of extraction and derivative are indicated. I.S. = internal standard, DNBA = di-*n*-butylamine, $CHCl_3$ = residual chloroform; a number or letter over a peak is an unidentified peak. The control serum was taken from laboratory personnel. Note that some diethylene glycol (peak 4) was carried over into the basic extraction.

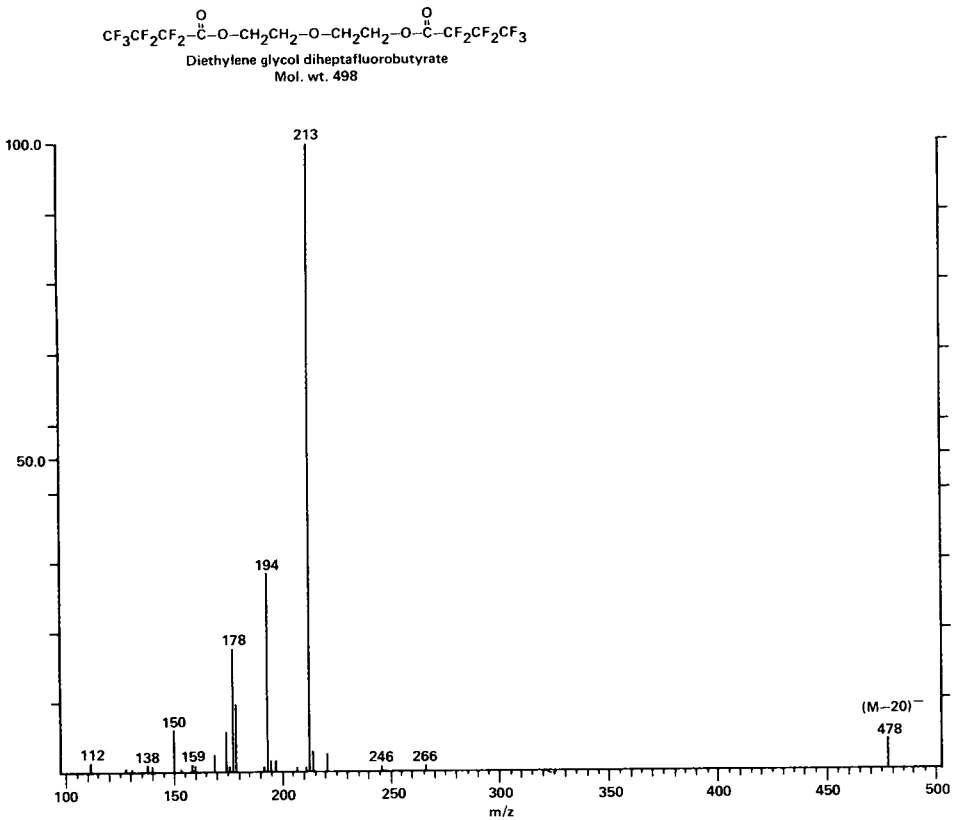

Fig. 3. Electron-impact mass spectrum of peak 4 (Fig. 1) obtained from an OV-101 fused-silica capillary column. Note that the spectrum is identical to the spectrum of authentic diethylene glycol diheptafluorobutyrate. Conditions are described in the text.

known gas chromatographic peak 4 (Fig. 1) and the unknown compound was identified as the HFBA diester of diethylene glycol.

In the second, blind-coded study diethylene glycol was detected by FPEC—GLC in about one fourth of the quantity detected in the first study, and then in only eleven of the 37 sera tested. Four of the samples containing diethylene glycol were controls, one was from a patient positive for *S. mansoni*, and the other six were from sera of patients infected with *S. haematobium*.

DISCUSSION

Our initial study results suggested the possibility that diethylene glycol might be a metabolic marker characteristic of infection with *S. haematobium*. The amounts present were about 11 nmol/ml of serum, a quantity that would require the ingestion of far greater amounts if the source was environmental or foodstuffs rather than that produced in the course of the infection. However, in our follow-up study in which 37 sera from Kerdasa and Tamooh were analysed, diethylene glycol was detected in moderate-to-trace amounts in eleven sera, four of which were from normal individuals and one of which was from a

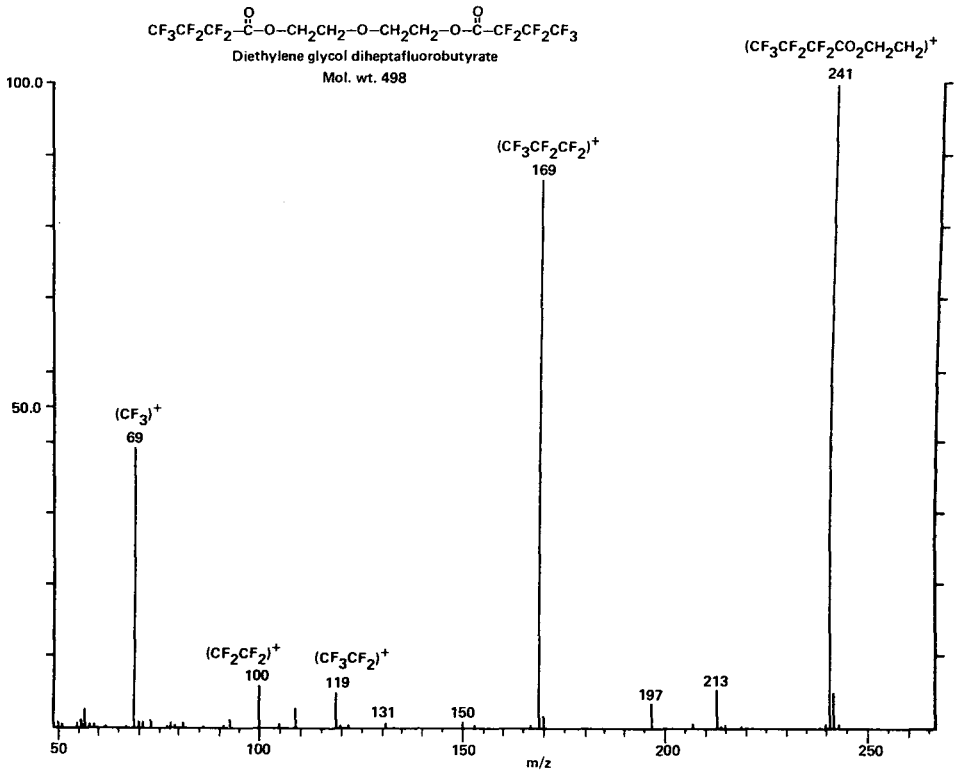

Fig. 4. Negative chemical ionization spectrum of peak 4 (Fig. 1) obtained from an OV-101 fused-silica capillary column. Note that the spectrum is identical to the spectrum of authentic diethylene glycol diheptafluorobutyrate.

person found to have a high *S. mansoni* egg count. We do not know of an environmental or food source by which these children could have been exposed to diethylene glycol. The median age of the patients was eleven years, which makes industrial exposure unlikely. Furthermore, serum levels of the compound decreased in amount in five patients following effective anti-schistosomal therapy, an observation which seems to indicate some relationship to the disease process. When patients did not respond to therapy, as indicated after two weeks by a consistant egg count, diethylene glycol was still present.

A computer search of the literature on biosynthesis, fermentation synthesis, and toxicity of diethylene glycol revealed that a great deal of research had been done on the toxicity of this compound, but we found no reports on its biosynthesis. Diethylene glycol is a central nervous system depressant, and a single lethal oral dose for man is approximately 1 ml/kg [13]. Based on a study [13] of 105 fatalities among 353 people who ingested diethylene glycol that contaminated a solution of sulphanilamide, symptoms included nausea, dizziness, and pain in the kidney region. Death resulted from renal failure [13]. A study conducted by Fitzhugh and Nelson [14] on rats showed that, at a 4% dietary level, there was depression of growth, formation of bladder stones, severe kidney damage, moderate liver damage, and frequent appearance of bladder tumours. Woo et al. [15] studied the volatile compounds present in the urine

of rats administered *p*-dioxane, an hepatic carcinogen in this species, and reported that the metabolite *p*-dioxan-2-one was detected. They also detected the same metabolite when diethylene glycol was administered to the rats.

Published data on the physiological effects of diethylene glycol showed similarities to published data on patients chronically infected with *S. haematobium*. The major correlations were that patients infected with *S. haematobium* experienced a narcotic effect, developed bladder stones, and showed increased incidence of bladder cancer [1].

In view of the facts that our second blind-coded study, involving two villages, revealed that diethylene glycol was found in some of the controls, was missing in some of the patients with schistosomiasis, and was detected in the serum of a patient infected with *S. mansoni*, it is likely that this compound is an industrial product that has become an environmental or food contaminant. Although no proof exists, clothing fabric treated with diethylene glycol or possibly corks in soft drink bottles could be a source of the compound. It has been two years since the study was completed and no known source has yet been identified. Whatever the specific source, the possibility exists that diethylene glycol may have an effect on the health of some exposed Egyptian children.

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SENSITIVE DETECTION OF AMINO ACIDS IN HUMAN SERUM AND DRIED BLOOD DISC OF 3 mm DIAMETER FOR DIAGNOSIS OF INBORN ERRORS OF METABOLISM

YOSHIHIKO WATANABE and KAZUHIRO IMAI*

Faculty of Pharmaceutical Sciences, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113 (Japan)

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SUMMARY

Sensitive high-performance liquid chromatographic determination of amino and imino acids in human serum (5 μl) and dried blood (2.6—2.8 μl) on a paper disc (3 mm diameter) of normal and abnormal newborns with inborn errors of metabolism (phenylketonuria, maple syrup urine disease and tyrosinosis) is described. Amino and imino acids in the biological specimens were extracted with ethanol and derivatized with 4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole at 60°C and at pH 8.0 for 1 min. The fluorescent derivatives were separated on μBondapak C_{18} and detected fluorometrically (530 nm/470 nm). The method was about one order of magnitude more sensitive than the similar method using *o*-phthalaldehyde. The amino acid contents obtained by the proposed method were comparable to those obtained by the amino acid analyser with use of *o*-phthalaldehyde.

INTRODUCTION

Various derivatives have been employed to enhance amino acid detection using either pre- or post-column techniques in high-performance liquid chromatography (HPLC). Post-column derivatization with ninhydrin is commonly adopted after separation of amino acids using ion-exchange chromatography [1]. Recently, *o*-phthalaldehyde (OPA) [2], a fluorogenic reagent for primary amines, has been used for such a purpose as a pre- or post-column derivatization reagent.

It has been revealed that 4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-F), a new fluorogenic reagent for both primary and secondary amines [3], is useful as a pre- and post-column derivatization reagent of amino and imino acids in HPLC [4—6].

The purpose of the present investigation was to develop a rapid and sensitive

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method for determination of amino and imino acids in human serum (5 μl) and dried blood on a paper disc (3 mm diameter) for diagnosis of inborn errors of metabolism.

EXPERIMENTAL

Reagents and chemicals

All chemicals were analytical reagent grade, unless otherwise noted. NBD-F was synthesized in our laboratory [7]. Standard amino acid solution was purchased from Ajinomoto (Tokyo, Japan). Dried blood on a paper disc from normal and abnormal newborns was kindly supplied by Dr. H. Naruse of the National Center for Nervous, Mental and Muscular Disorders (Tokyo, Japan). Methanol and tetrahydrofuran were of HPLC grade. Water was deionized and doubly distilled.

HPLC conditions and apparatus

An HPLC instrument (Waters Assoc., Milford, MA, U.S.A.) consisting of two Model 6000A pumps, a Model 600 solvent programmer and a U6K injector was used. All chromatographic runs were made at ambient temperature using a flow-rate of 2.0 ml/min. μBondapak C_{18} (30 cm × 3.9 mm I.D., 10 μm) (Waters Assoc.) was used. The column effluent was monitored by a Hitachi 650-10S spectrofluorometer (Hitachi Seisakusho, Tokyo, Japan) with an 18-μl flow-cell with excitation at 470 nm and emission at 530 nm, fitted with a Shimadzu R-1A integrator and a Hitachi 056 one-pen recorder.

The following three solvent systems were used: Solvent A, methanol—tetrahydrofuran—0.1 *M* phosphate buffer (pH 6.0, Na^+) (3.75:1.6:94.65); Solvent B, methanol—tetrahydrofuran—0.1 *M* phosphate buffer (pH 6.0, Na^+) (25:15:60); Solvent C, methanol—water (40:60). The elution programme was as follows: (1) isocratic elution by Solvent A for 24 min, (2) linear gradient from Solvent A to 100% Solvent B over 30 min, (3) isocratic elution by Solvent B for 6 min, and (4) isocratic elution by Solvent C for 12 min. All mobile phases were degassed daily with an ultrasonic bath under vacuum prior to use.

Extraction and derivatization procedure for serum samples

A 5-μl aliquot of serum and 15 μl of ϵ-aminocaproic acid solution (internal standard, 167 μM) were transferred to a 400-μl conical centrifuge tube. Then 50 μl of ethanol were added and the two phases were mixed thoroughly. The mixed solution was centrifuged at 8000 *g* for 5 min and 20 μl of the supernatant were transferred to a 500-μl reaction vial. 20 μl of 0.1 *M* borate buffer (pH 8.0, Na^+) and 12 μl of 83 m*M* NBD-F in ethanol were added and the mixture was heated at 60°C for 1 min. After cooling on ice—water, 150 μl of 0.5 m*M* hydrochloric acid were added to the reaction mixture, and 10 μl of the final solution were subjected to HPLC. Quantitation was made by calculation of the peak area ratio of each amino and imino acid to the internal standard.

Extraction and derivatization procedure for dried blood samples

One sheet of a paper disc (3 mm diameter) spotted with blood was soaked

in 40 μl of 70% ethanol containing 500 pmol of ϵ-aminocaproic acid and extracted at 5° C for 12 h. A 10-μl aliquot of the extract, 10 μl of 0.1 *M* borate buffer (pH 8.0, Na^+) and 6 μl of 83 m*M* NBD-F in ethanol were well mixed in a 500-μl reaction vial and heated at 60° C for 1 min. Then, 14 μl of 0.1 *M* hydrochloric acid were added to the reaction mixture, and 10 μl of the final solution were subjected to HPLC.

RESULTS AND DISCUSSION

Quantitative determination of amino acids in biological fluids usually involves separation by ion-exchange chromatography and detection by post-column derivatization with ninhydrin [1]. Substitution of ninhydrin by a fluorogenic reagent such as OPA [2] or fluorescamine [8], which react only with primary amines, has recently led to an increase in sensitivity of the method. Elimination of the post-column reactor yields a less expensive and more versatile system where high sensitivity can be achieved. Reversed-phase HPLC of amino acids of dansyl [9], phenylthiohydantoin [10] and OPA [11] derivatives has been performed.

NBD-F has been developed as a fluorogenic reagent for amines [3] with which it is more reactive than NBD-Cl [12]. Of particular importance is the fact that only one peak was observed for each amino and imino acid derivatized with NBD-F. Thus it has been used successfully for fluorometric determination of amino and imino acids and other amines with the pre- and post-column derivatization technique [4—6, 13]. Pre-column derivatization with NBD-F at pH 8.0 and at 60° C for 1 min and separation on HPLC afforded the detection limits of 10—100 fmol of amino and imino acids [4, 5]. Another advantage is that no interference from other compounds in biological samples would occur because the fluorescent excitation (470 nm) and emission (530 nm) of NBD-amino and -imino acid derivatives are in the long-wavelength region of the spectrum.

No significant change in the fluorescence intensities of NBD-amino and -imino acid derivatives, such as NBD-alanine [14], -glycine [15] and -proline [6] was observed during a 5-h period under dark conditions. Thus the average deviation of 10 pmol of each amino and imino acid was good (2.78%, $n = 5$). However, 30% of each derivative was degraded after 3 h exposure to indoor light, but NBD-Tyr, which is the most sensitive to light, completely disappeared under the same conditions.

In the present study, the application of the method to the analysis of amino and imino acids in biological fluids is demonstrated and discussed. Firstly, sera obtained from adults were subjected to amino acid analysis. Ethanol was chosen as deproteinizing agent instead of acidic agents such as perchloric and trichloroacetic acid, because it is preferable in the reaction of amino and imino acids with NBD-F, and its concentration was tentatively decided as 70% on the basis of the method of Ohura [16]. A chromatogram thus obtained is shown in Fig. 1.

In this experiment the same gradient elution system of 0.1 *M* sodium phosphate buffer (pH 6.0), methanol and tetrahydrofuran was adopted that had been used for hydrolysates of a few μg of proteins such as rabbit pyruvate

kinase M_1, rabbit aldolase A and papain [5]. The total analysis time for serum including the deproteinization and derivatization step is less than 80 min. All the amino and imino acid derivatives were well separated except that separations of serine (peak 3) from aspargine, glycine (peak 5) from glutamine, and threonine (peak 8) from taurine are difficult under the present conditions. When amino and imino acid standards were derivatized and chromatographed, the relationship obtained between peak area ratio to the internal standard (ϵ-aminocaproic acid) and amount of amino and imino acid was linear in the range 0.5—100 pmol.

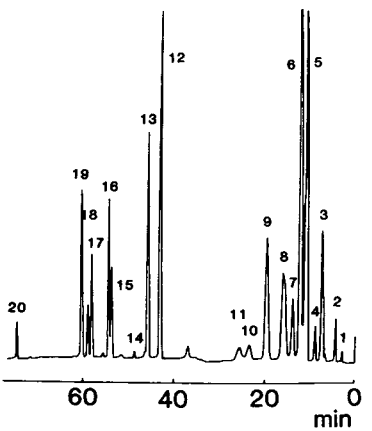

Fig. 1. Elution profile of a human serum. Procedures for deproteinization, derivatization and HPLC conditions are cited in the text. Peaks: 1 = NBD-Asp, 2 = NBD-Glu, 3 = NBD-Ser + NBD-Asn, 4 = NBD-His, 5 = NBD-Gly + NBD-Gln, 6 = NBD-OH, 7 = NBD-Arg, 8 = NBD-Thr + NBD-Tau, 9 = NBD-Ala, 10 = NBD-Pro, 11 = NBD-NH_2, 12 = NBD-ϵ-aminocaproic acid, 13 = NBD-Val, 14 = NBD-Met, 15 = NBD-Ile, 16 = NBD-Leu, 17 = NBD-Phe, 18 = NBD-Orn, 19 = NBD-Lys, 20 = NBD-Tyr.

TABLE I

AMINO AND IMINO ACID CONCENTRATIONS (μM) IN SERUM OF SEVEN NORMAL MALES

Amino acid	1	2	3	4	5	6	7
His	89	53	91	89	72	88	101
Arg	101	89	88	75	110	77	52
Ala	430	380	419	325	370	335	450
Pro	190	203	230	234	211	193	252
Val	221	250	240	237	252	205	232
Met	31	20	31	35	23	32	19
Ile	66	88	89	90	78	73	54
Leu	153	121	135	141	128	150	160
Phe	51	58	66	35	51	53	65
Orn	51	107	108	77	93	65	81
Lys	176	182	135	185	198	170	181
Tyr	45	53	40	72	38	40	63

Repetitive analysis of the same serum showed that the variations in area of all peaks compared to the area of the internal standard were small (coefficient of variation, C.V., is in the range 0.8—9.5%, n = 5). Thus the precision of the present method involving extraction and pre-column derivatization with NBD-F may be slightly inferior to that obtained by ion-exchange chromatography. Part of this deviation is due to pipetting errors that may occur during the extraction and derivatization procedure. The average recovery of amino and imino acids by addition of amino and imino acid standard solution to serum was 98.9% (n = 5).

The data obtained in this experiment (Table I) are comparable to those obtained using the other method [17]. The sensitivity of the method is about one order of magnitude higher than that described in a recent report [18] for 50 μl of serum using OPA as fluorogenic reagent.

Since this method allows the detection of amino and imino acids in extremely small amounts of serum, we tried to measure amino and imino acid contents in whole blood obtained from newborns.

A newborn blood sample (2.6—2.8 μl) applied on a paper disc (3 mm diameter) was extracted, as for serum, with 70% ethanol, derivatized with NBD-F and analysed by the proposed method. Fig. 2 shows a representative chromatogram of amino and imino acids in whole blood of a normal newborn. The variation in the ratio of peak area of each amino and imino acid to that of the internal standard was within C.V. = 8.4% (n = 4). A major part of this deviation might be caused by the sample preparation, especially by punching out of the paper.

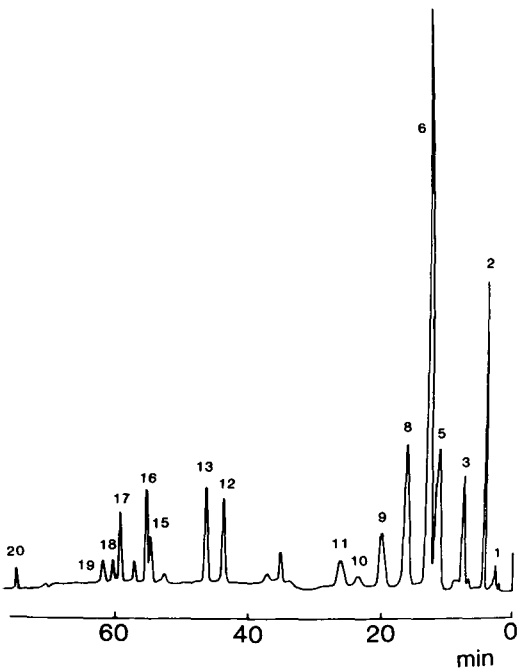

Fig. 2. Elution profile of a normal blood disc. Procedures for extraction, derivatization and HPLC conditions are cited in the text. Numbers are the same as in Fig. 1.

A summary of the results obtained from the whole blood of five normal newborns is made in Table II. The concentrations of each amino and imino acid in whole blood were comparable to the values reported by others using an amino acid analyser [19].

Fig. 3 shows chromatograms of amino and imino acid derivatives from blood of newborns with inborn errors of metabolism (phenylketonuria, maple syrup urine disease and tyrosinosis). Large peaks of amino acids [phenylalanine (peak 17) in Fig. 3a, valine (peak 13), isoleucine (peak 15) and leucine (peak 16) in Fig. 3b, and tyrosine (peak 20) in Fig. 3c] appear in these chromatograms. It is known that a large amount of alloisoleucine would arise in maple syrup urine disease, but in this system alloisoleucine and isoleucine co-eluted. The concentrations of amino and imino acids related to the diseases obtained by the proposed method and by an amino acid analyser using OPA as post-column derivatization reagent are shown in Table III, suggesting a good correlation between the two. In conclusion, the present method should be useful in the diagnosis of hereditary inborn errors of metabolism and would also be applicable to the detection of hyperprolinaemia [20].

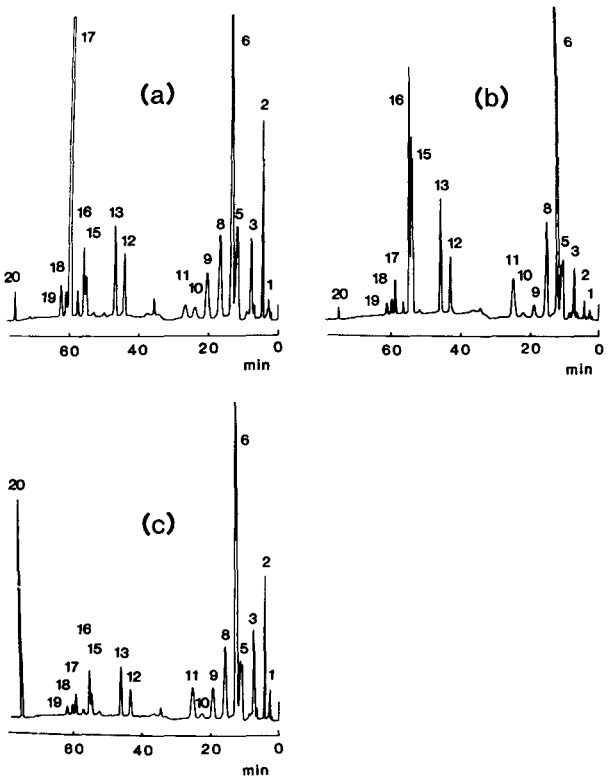

Fig. 3. Elution profiles of abnormal blood disc obtained from newborns with phenylketonuria (a), maple syrup urine disease (b) and tyrosinosis (c). Procedures for extraction, derivatization and HPLC conditions are cited in the text. Numbers are the same as in Fig. 1.

TABLE II

AMINO AND IMINO ACID CONCENTRATIONS (μM) IN DRIED BLOOD DISCS FROM FIVE NORMAL NEWBORNS

Amino acid	1	2	3	4	5
His	—	15	30	—	23
Ala	335	294	435	362	306
Pro	210	199	250	243	255
Val	208	270	190	185	235
Ile	95	83	70	85	65
Leu	126	154	160	129	177
Phe	51	33	55	80	71
Orn	46	59	76	110	57
Lys	164	188	130	139	155
Tyr	53	41	36	55	59

TABLE III

ABNORMAL AMINO ACID CONCENTRATIONS (μM) IN DRIED BLOOD DISCS OBTAINED FROM INBORN ERRORS OF METABOLISM: COMPARISON OF THE DATA FROM THE TWO METHODS

Disease	Amino acid	Sample	NBD-F*	OPA**
Phenylketonuria	Phe	1	2800	2720
		2	2100	2280
		3	1550	1510
Maple syrup urine disease	Val	4	400	420
		5	410	400
		6	380	360
	Leu	4	1200	1330
		5	1650	1690
		6	570	560
Tyrosinosis	Tyr	7	2010	2140
		8	930	960
		9	1200	1250

*Present method.
**Post-column derivatization with OPA.

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RAPID AUTOMATED ANALYSIS OF BIOGENIC AMINES AND THEIR METABOLITES USING REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

CHARLES F. SALLER* and ANDRE I. SALAMA

Stuart Pharmaceuticals, Division of ICI Americas, Inc., Wilmington, DE 19897 (U.S.A.)

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SUMMARY

A method is described for the rapid automated analysis of: dopamine and norepinephrine; the major dopamine metabolites 3,4-dihydroxyphenylacetic acid and homovanillic acid; and the indoles tryptophan, serotonin, and 5-hydroxyindoleacetic acid in less than 0.5 mg of brain tissue. Samples are deproteinized, injected directly onto a reversed-phase high-performance liquid chromatography column, and quantitated using an electrochemical detector with a glassy carbon electrode. High sample stability permits the use of an automatic sample injector at ambient temperatures. Depending upon the column particle size, sample run times are less than 7—12 min. Thus, over 50 duplicate samples can readily be measured in a single day with very little operator attention. The chromatographic system used also resolves epinephrine, and the catecholamine metabolites: 3-methoxytyramine, normetanephrine, and 3-methoxy-4-hydroxyphenylglycol; and with very little modification this assay also could be used to measure these compounds.

INTRODUCTION

A wide variety of fluorometric and radioenzymatic assays for the monoamines dopamine (DA), norepinephrine (NE), and serotonin (5-HT) and their metabolites exist [1—6]. These procedures are rapidly being replaced by methods which utilize high-performance liquid chromatography (HPLC) coupled with electrochemical detection [7—15]. However, most of these methods require some preliminary sample purification (e.g., ion-exchange chromatography or solvent extractions) and/or fairly long sample run times if both catecholamines (particularly NE) and indoles are to be resolved in the same system. Therefore, we have developed a rapid and highly sensitive assay for the measurement of the brain levels of the catecholamines DA and NE, the major DA metabolites 3,4-dihydroxyphenylacetic acid (DOPAC), and homo-

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vanillic acid (HVA), and the indoles tryptophan (Tryp), serotonin (5-HT), and 5-hydroxyindoleacetic acid (5-HIAA). In addition, the HPLC system used resolves epinephrine (E), the NE metabolites 3-hydroxy-4-methoxy-phenylglycol (MHPG) and normetanephrine (NMN), and the DA metabolite 3-methoxytyramine (3-MT) from all of the other compounds measured. Since MHPG exists primarily as a sulfate ester in rat brain [16] and the levels of NMN and 3-MT greatly increase upon decapitation [17, 18] we have not yet used the following procedures to routinely quantitate the tissue levels of these compounds.

Depending upon the particle size (3 or 5 μm) and size of the HPLC column used, the run time per sample is only 7 to 12 min. Moreover, no sample purification other than deproteinization and centrifugation is required prior to the HPLC analysis. Furthermore, sample stability has been optimized so that the entire procedure, after deproteinization, can be fully automated by using an automatic sample injector. Thus, over fifty samples can be analyzed in duplicate in a single day with less than 1 h of operator attention once the samples have been deproteinized.

MATERIALS AND METHODS

Tissue preparation

Frozen samples are weighed and transferred to polypropylene centrifuge tubes which are kept on dry ice. To 39 vols. of a buffer consisting of 7 vols. of 0.1 *M* monobasic sodium phosphate (adjusted to pH 4.0 using a saturated solution of citric acid) containing 1 m*M* disodium EDTA and 1 m*M* sodium octanesulfonic acid and 3 vols. of acetonitrile, one additional vol. of this buffer is added containing the internal standard, isoproterenol (ISO, 2 μg/ml). The solution containing ISO is stored at −20° C. Then, the samples are homogenized in 40 vols. (w/v) of this solution and centrifuged at 15,000 *g* for 20 min at 4° C. The supernatant is either injected immediately, kept at 4° C for up to 48 h, or stored at −20° C for longer periods. If samples are stored at −20° C, the centrifugation step is repeated.

Chromatography and detection

Aliquots (5—50 μl) of tissue samples or standards are injected onto a reversed-phase column using a WISP 710B automatic sample injector (Waters Assoc., Milford, MA, U.S.A.). Routinely, a 5-μm, 15 cm × 4.6 mm Ultrasphere ODS® column (Rainin Instruments, Woburn, MA, U.S.A.) is used. However, as is indicated below, a 3-μm, 7.5 cm × 4.6 mm Ultrasphere ODS column can be used with small sample volumes. To protect the analytical column from sample contaminants, a 4.0 cm × 4.6 mm Ultrasphere ODS precolumn is placed between the analytical column and the sample injector. All connections are made using the shortest possible lengths of stainless-steel tubing (1.58 mm O.D., 0.25 mm I.D.), to minimize extra-column band-broadening.

Slightly different mobile phases are used depending upon the particle size (3 or 5 μm) of the column used: 100 vols. of 0.1 *M* monobasic sodium phosphate containing 1 m*M* disodium EDTA and 1 m*M* sodium octanesulfonic acid are adjusted to pH 4.0 to 4.35 with a saturated citric acid solution, and

mixed with acetonitrile (8—11 vols.). Generally, satisfactory results were obtained at pH 4.0 using 10.5 vols. of acetonitrile with most of the analytical columns tested. These values for mobile phase composition are meant to serve as guidelines, since small changes in mobile phase composition were necessary for different columns and for columns that had been used for extended periods of time (see below for examples). Mobile phases are filtered through 0.2-μm nylon filters (Rainin Instruments), degassed under vacuum, and placed in a water bath at 35° C to keep them in a degassed state. All other components of the HPLC system are left at ambient temperature (22° C). Mobile phase flow-rate is maintained at 1 ml/min using a Beckman 112 pump (Beckman Instruments, Berkeley, CA, U.S.A.).

The effluent from the analytical column is passed through a TL-5 flow cell (glassy carbon electrode, 0.8 V; see below for details) of an LC-4A electrochemical detector (Bioanalytical Systems, W. Lafayette, IN, U.S.A.), and then to a waste receptacle or to a fraction collector, if desired. The electrical output of the detector is quantitated using a 3390A integrator (Hewlett-Packard, Avondale, PA, U.S.A.). Sample values are calculated relative to the peak height of the internal standard, ISO. A calibration table, used for this purpose by the integrator, is generated prior to each sample run by making at least four 5-μl injections (3-μm column) or four 20-μl injections (5-μm column) of homogenization buffer containing 0.5 ng each of DA, NE, 5-HT, DOPAC and 5-HIAA and 1.0 ng each of ISO, Tryp, HVA, and 3-MT. These calibration solutions are stable for several months at —20° C. To avoid long start-up times (particularly for the detector), all equipment is left running when samples are not being assayed and the mobile phase flow-rate is reduced to 0.1 ml/min.

All tubing, the injector, and the solvent pump are passivated; and the columns are washed according to the instructions provided with the electrochemical detector.

RESULTS AND DISCUSSION

Sample preparation and stability

A major advantage of this assay is the ease of sample preparation which reduces assay time and the possibilities for technical errors. A second advantage is the relatively high stability of the compounds measured in the buffer used for tissue homogenization. Thus, even at room temperature (22° C), all compounds assayed are completely stable for at least 24 h. In comparison, samples homogenized in 0.2 *M* perchloric acid are unstable at room temperature. In particular, 5-HT and 5-HIAA are rapidly destroyed.

High sample stability permits the use of an automatic sample injector at ambient temperatures, and therefore eliminates the need for tedious manual injections. Furthermore, tissue samples and standards stored in the homogenization buffer are stable for at least 48 h at 4° C and are stable for at least one week at —20° C. In addition, fairly large variations of the pH of this buffer do not apper to affect sample stability. Thus, tissue samples homogenized in buffers prepared as described above and adjusted to pH values from 3.0 to 5.0 were stable for at least 16 h at room temperature. A buffer pH of 4.0 was chosen because this buffer is similar in composition to the assay

mobile phase. Therefore, the solvent front observed after sample injections is minimal.

If less than 20 vols. of buffer are used for homogenization, sample deproteinization is not complete and some loss (5—10%) of 5-HT and 5-HIAA is observed after about 12 h at room temperature. Additional protein can be removed from tissue samples homogenized in 20 vols. or less of buffer, by storing the partially deproteinized samples at −20°C overnight, followed by centrifugation. However, the sensitivity of this assay is more than sufficient to quantitate each of the compounds measured in an extract derived from less than 0.5 mg of tissue. The use of dilute tissue extracts also ensures the complete recovery (>95% from whole brain) of ISO and all of the compounds measured by this assay. This was verified by adding 50 ng of ISO and each assay standard (NE, DA, 5-HT, etc.) to 1-ml aliquots of whole rat brain samples which were homogenized as described above. Then, the samples were centrifuged and injected into the HPLC system. Peak heights from sample aliquots with and without added standards were compared to obtain a measure of sample recoveries.

Chromatography

Shown in Figs. 1 and 2 are chromatograms of standards and tissue extracts obtained using a 5-μm Ultrasphere column. No additional peaks are observed beyond the peak corresponding to 5-HT. Similar chromatograms are obtained using 3-μm columns, except that the total sample run time is less than 7 min. Since the 3-μm column can accept only small sample volumes (about 5 μl), the 5-μm column is used routinely in most studies.

The capacity factors (k') of the compounds shown in Figs. 1 and 2 and several related compounds are listed in Table I for the 3- and 5-μm columns used. The monoamine precursors 3,4-dihydroxyphenylacetic acid (DOPA) and 5-hydroxytryptophan (5-HTP) are present in very small quantities in brain and are incompletely resolved from the solvent front (i.e., the initial large peak due

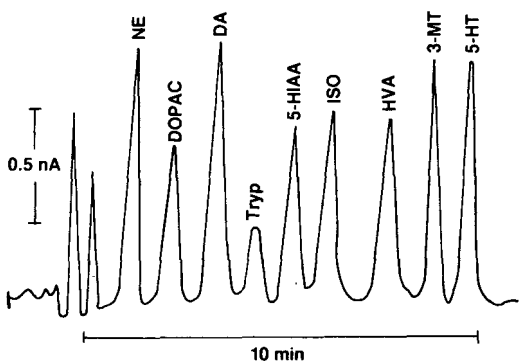

Fig. 1. Chromatogram of assay standards. A 20-μl aliquot of homogenization buffer (see Materials and methods) containing 0.5 ng each of DA, DOPAC, NE, 5-HT and 5-HIAA, and 1.0 ng each of Tryp, ISO, HVA and 3-MT was injected. Column: 5-μm Ultrasphere ODS; mobile phase: 100 vols. of 0.1 *M* monobasic sodium phosphate (adjusted to pH 4.35 with citric acid) containing 1 m*M* disodium EDTA and 0.75 m*M* sodium octanesulfonic acid, mixed with 10 vols. of acetonitrile; flow-rate: 1 ml/min; detector: 0.8 V vs. Ag/AgCl reference electrode.

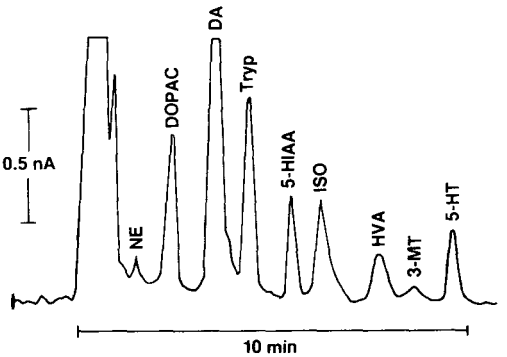

Fig. 2. Chromatogram of rat striatal tissue extract. Rats were decapitated and the striata were rapidly removed and frozen on dry ice. Then, frozen striata were homogenized in 40 vols. (w/v) of homogenization buffer (See Materials and methods) which contained 50 ng/ml of ISO, the internal standard. After centrifugation (15,000 *g* for 20 min at 4°C), a 20-µl aliquot of supernatant was injected and assayed using the conditions described in Fig. 1.

TABLE I

RETENTION (k') OF BIOGENIC AMINES, THEIR PRECURSORS, AND THEIR METABOLITES ON 3-µm (7.5 cm × 4.6 mm) AND 5-µm (15 cm × 4.6 mm) ULTRASPHERE ODS REVERSED-PHASE COLUMNS

Retention is expressed as k' values, which are the differences between the retention times of the compounds of interest and the retention time for an unretained compound (t_0) divided by t_0. Mobile phases: 3-µm column: 100 vols. of 0.1 *M* sodium phosphate buffer, pH 4.2, containing 1 m*M* disodium EDTA and 0.75 m*M* sodium octanesulfonic acid, and 9.5 vols. of acetonitrile; 5-µm column: 100 vols. of 0.1 *M* sodium phosphate buffer, pH 4.35, containing 1 m*M* disodium EDTA and 0.75 m*M* sodium octanesulfonic acid, and 10 vols. of acetonitrile. Buffer pH values were adjusted using a saturated solution of citric acid.

Compound	Retention (k')	
	3-µm Column	5-µm Column
DOMA	0.34	0.35
VMA	0.38	0.36
DOPA	0.39	0.42
DHPG	0.43	0.46
5-HTP	0.47	0.52
NE	0.51	0.59
MHPG	0.63	0.67
E	0.78	0.85
DOPAC	1.18	1.71
NMN	1.33	1.97
DA	1.52	2.14
Tryp	2.17	3.09
5-HIAA	2.80	3.43
HVA	3.38	4.95
3-MT	3.61	5.39
5-HT	4.06	5.79

to the injection of poorly retained tissue constituents and solvent). Tyrosine, the amino acid precursor of DOPA, is not detected at the electrode potentials used in this assay. The catecholamine metabolites 3,4-dihydroxyphenylglycol (DHPG), 3,4-dihydroxymandelic acid (DOMA), and vanillylmandelic acid (VMA) are also poorly resolved from the solvent front, and therefore do not interfere in this assay. Decreasing the pH value and/or acetonitrile concentration of the mobile phase might permit the resolution of these compounds (see below).

Although the k' values differ between the 3-μm and 5-μm columns, the relative positions of the compounds listed in Table I are almost identical ($r > 0.99$). However, minor differences in mobile phase composition have been found to be necessary to permit satisfactory compound resolution with these two types of columns. Likewise, depending upon the particle size and previous history of use of the HPLC column, minor variations in the composition of the mobile phase are necessary to adequately resolve all of the tissue components measured with minimal retention times. Therefore, the following data on mobile phase composition are presented both as a description of and as guide to the development of a suitable chromatographic system.

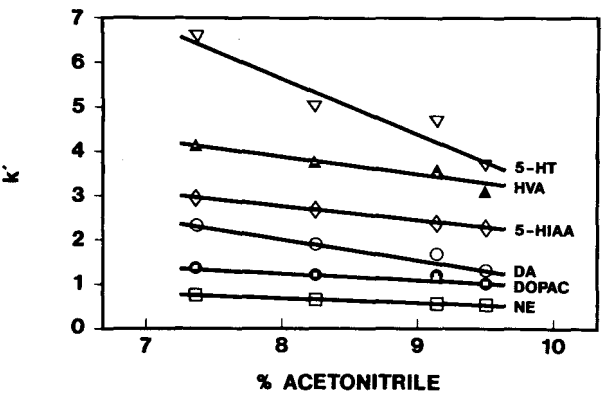

Fig. 3. Effect of acetonitrile concentration on retention (k'). Column: 3-μm Ultrasphere ODS; mobile phases: 100 vols. of 0.1 *M* monobasic sodium phosphate (adjusted to pH 4.2 with citric acid) containing 1 m*M* disodium EDTA and 0.75 m*M* sodium octanesulfonic acid, mixed with 8—10.5 vols. of acetonitrile; flow-rate: 1 ml/min.

The concentrations of acetonitrile and octanesulfonate ion, and buffer pH markedly affect the resolution and retention times of the compounds measured. In Fig. 3 the effects of acetonitrile concentration on the capacity factors for the amines DA, NE and 5-HT, and the acidic metabolites DOPAC, HVA, and 5-HIAA are shown. The capacity factors for all compounds decrease as the concentration of acetonitrile is increased. Increasing the mobile phase acetonitrile concentration decreases the k' values of the amines roughly in proportion to their elution order (i.e., the greatest effect is observed for 5-HT).

The retention times of the amines are also increased in the presence of anionic ion-pairing reagents such as sodium octanesulfonate. In agreement with others, preliminary investigations indicated that the k' values for the amines increase as the carbon chain and/or concentration of the ion-pairing

reagent are increased [7, 10, 12]. A concentration of 0.75 m*M* sodium octanesulfonate is sufficient for the resolution of all of the amines of interest. During the development of this assay, this concentration of sodium octanesulfonate was used. Since then, it was found that increasing the sulfonate concentration to 1 m*M* resulted in a better resolution of NE from the solvent front. This change, after minor adjustments in mobile phase composition, did not significantly affect the k' values of the other compounds of interest. Thus, increasing the sodium octanesulfonate to 1 m*M* concentration necessitated a 1—2% increase in the concentration of acetonitrile, so that the retention times of compounds with high k' values were not substantially increased. Likewise, minor corrections in mobile phase pH (0.1—0.3 pH units) were also necessary. These were accomplished as described in the following paragraph. In the absence of octanesulfonate ions, NE remains in the solvent front. It is important to note that considerable time (12—16 h) is necessary for equilibration of a column with a mobile phase containing an ion-pairing reagent, otherwise retention times may increase and peak heights may vary between sample runs [12]. The presence of octanesulfonate ions does not affect the retention times of the acidic compounds measured (DOPAC, HVA, 5-HIAA) or MHPG (a neutral compound).

Mobile phase pH greatly affects the k' values for the acidic compounds and Tryp (data not shown, see ref. 15), but not the retention of the amines (Fig. 4). The k' values for MHPG (a neutral compound) at different mobile phase pH values paralleled, but were slightly greater than those of NE. As mentioned previously, some differences in the characteristics of various columns may occur. Therefore, the construction of curves such as are shown in Figs. 3 and 4 may be necessary to ascertain what slight variations in the mobile phases described in Materials and methods may be necessary for the complete resolution of all compounds of interest. The data in Figs. 3 and 4 also indicate that once the composition of a mobile phase for use with a particular column is established, very careful control over the mobile phase acetonitrile concentration and buffer pH is necessary, otherwise large fluctuations in k' values will

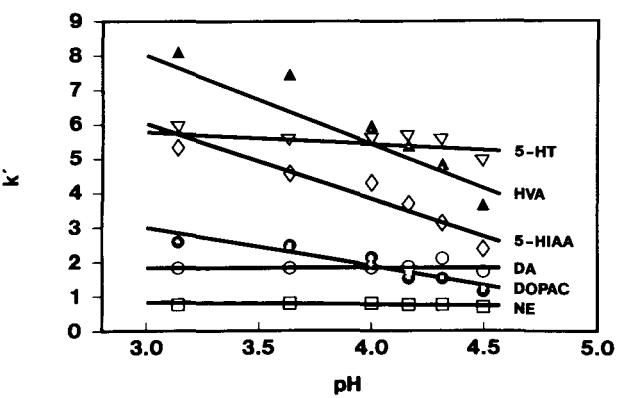

Fig. 4. Effect of pH on retention (k'). Column: 5-μm Ultrasphere ODS; mobile phases: 100 vols. of 0.1 *M* monobasic sodium phosphate (adjusted to pH values of 3.25 to 4.50 with citric acid) containing 1 m*M* disodium EDTA and 0.75 m*M* sodium octanesulfonic acid, mixed with 10 vols. of acetonitrile; flow-rate: 1 ml/min.

occur. Similarly, the concentration of octanesulfonate ions must be held constant within narrow limits.

Using the mobile phases and the sample preparation described above, analytical columns have been in use for several thousand sample injections. The use of a guard column greatly extends analytical column life. Moreover, guard columns have been in use for well over 3000 injections. Column back pressure is generally about 120 bars. Once column back pressure increases by over 30 bars, often all that is necessary to place the system back into service is to clean or replace the guard column frit which is closest to the sample injector. It is important that no voids in the column packing develop when the guard column frit is cleaned or replaced, since pressure changes may occur following sample injections and these pressure changes may results in spurious peaks on the sample chromatogram. Slowly raising and lowering mobile phase flow-rates is essential for maintaining the column packings. If column voids do occur, column performance can sometimes be returned to normal by filling the voids with a packing material similar to that already present in the column.

Electrochemical detection

The high sensitivity of this assay is largely due to the great sensitivity of electrochemical detection. Thus, 20—50 pg of DA, NE, 5-HT, DOPAC, and 5-HIAA and 50—150 pg of MNM, MHPG, 3-MT, HVA and Tryp can be easily detected in a 20-μl sample. The sensitivity of electrochemical detection depends partially on the background current (electrical noise) which is a function of the voltage applied to the electrode. Beyond 0.85 V, the background current increases tremendously (Fig. 5). Therefore, with this type of electrode and mobile phase, higher potentials are unfeasible. Lower background currents and somewhat higher sensitivity are possible using carbon paste electrodes. However, carbon paste electrodes are not very durable and require frequent repacking; whereas glassy carbon electrodes can be used continuously for several months with this system without resurfacing. In addition to electrode characteristics, the presence of EDTA in the mobile phase is useful in reducing background currents. In the absence of EDTA, background currents may double.

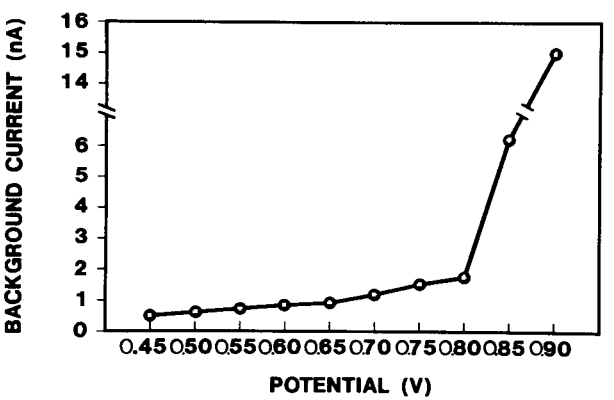

Fig. 5. Effect of electrode potential on background currents. Assay conditions were as described in Fig. 1. Background currents were measured once the currents had stabilized at each potential. Different electrodes give somewhat different responses.

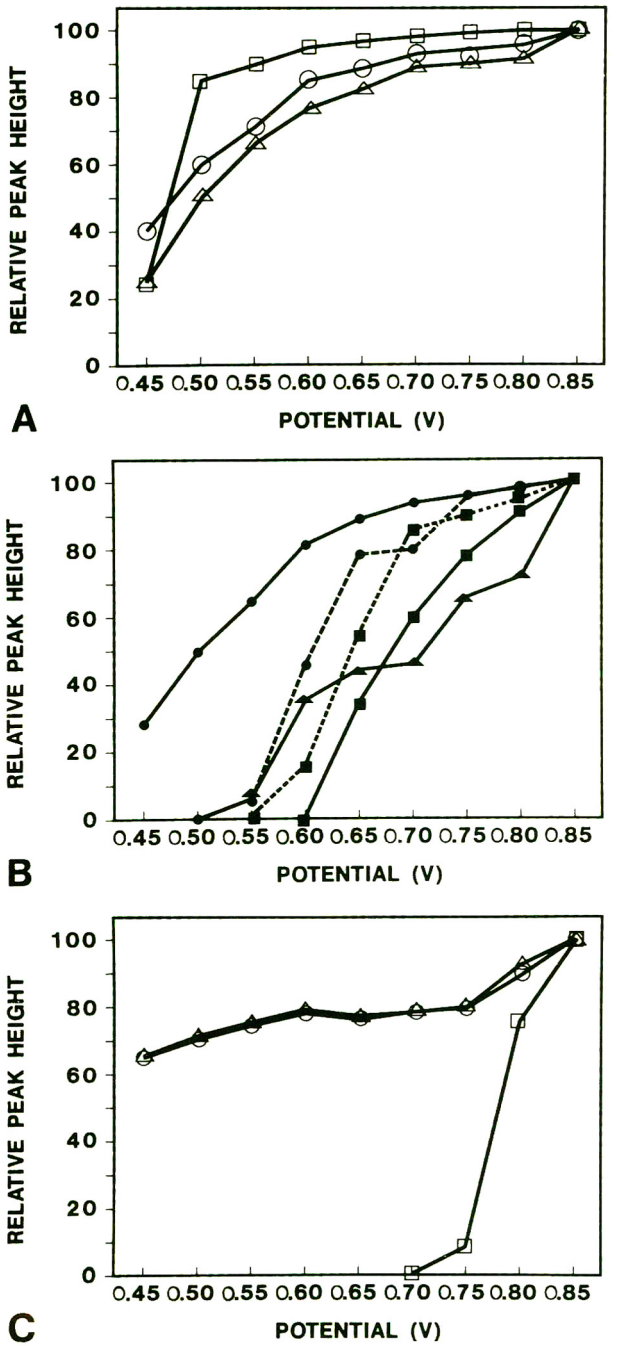

Fig. 6. Relative peak heights of: (A) the catecholamines DA (○) and NE (□), and the catecholamine-derivative ISO (△); (B) the catecholamine metabolites DOPAC (●—●), HVA, (▲—▲), MHPG (■—■), 3-MT (●- -●), and NMN (■- -■); and (C) the indoles Tryp (□), 5-HT (△), and 5-HIAA (○) as a function of electrode potential. The peak height observed at 0.85 V (vs. Ag/AgCl reference electrode) for each compound was set equal to 100. Assay conditions were as described in Fig. 1.

The magnitude of oxidation currents and, therefore, detector responses vary as a function of oxidation potential in a manner which is characteristic of the specific compounds measured by this system. The relative oxidation currents for some of the compounds which are resolved (detector response at 0.85 V for each compound set equal to 100) at various oxidation potentials are shown in Fig. 6. For the purpose of comparison, the peak heights of each compound in Fig. 6 relative to the internal standard ISO (set equal to 1) are given in Fig. 7. Because the detector response for each compound is near maximal and background currents are low (Fig. 5) and stable (i.e., almost no baseline drift over 24 h) at 0.8 V, this potential is generally used in the assay.

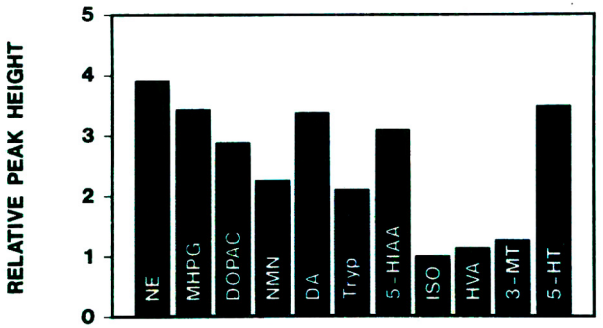

Fig. 7. Relative peak heights of equimolar amounts of the compounds in Fig. 6. Compounds are shown in the order of elution from the HPLC column and all peak height values are relative to ISO (set equal to 1). Chromatographic conditions were as described in Fig. 1. Detector: 0.85 V vs. Ag/AgCl reference electrode. Small differences in peak heights are observed depending upon the exact chromatographic conditions used.

The use of an internal standard, such as ISO, greatly contributes to the high reliability of this assay (see below). ISO does not overlap with any peaks produced by tissue constituents and is completely (> 95%) recovered from brain tissue homogenates. Since tissue values are calculated relative to the ISO peak, the effect of variations in detector sensitivity or injection volume are minimized. Thus, the coefficient of variation of samples injected over a 24-h period was less than 5% for each compound measured. The use of an internal standard also eliminates the need for repeated injections of reference samples and computer assisted manipulations of integrator data, such as are required by a recently published automated assay for some of the compounds measured by this assay [19].

The detection of NE with this assay poses a special problem. Unless the advantage of relatively short sample run times is compromised, the NE peak is near the tissue solvent front. The Hewlett-Packard integrator used in quantitating sample values can employ a tangent skimming procedure to calculate sample values from peaks which occur on the tail of the solvent front peak. However, the size and slope of the solvent front peak can vary with time, making this type of procedure unreliable. Fortuitously, the substances responsible for the solvent front of tissue samples are relatively unstable even at 4° C and the size of the solvent front greatly decreases with time. Since NE

and the other compounds measured by this assay are stable in the dark at room temperature (22°C) for at least 24 h, all that is generally necessary to reliably quantitate these compounds is to let tissue extracts sit in the dark for about 6–8 h at room temperature prior to analysis. This delay time may be programmed into the automatic sample injector.

The size of the solvent front from tissue samples may also be reduced substantially by decreasing the electrode potential of the electrochemical detector. For example, the size of the solvent front of tissue samples is much smaller at 0.65 V than at 0.80 V. Since the detector response is low for HVA and nonexistent for Tryp at 0.65 V (Fig. 6), the use of dual electrodes set at 0.65 V (or lower) and 0.8 V might be useful when low levels of NE and these compounds are to be measured in the same samples.

Verification of compound identity in tissue extracts

The identity of chromatographic peaks resulting from the analysis of brain extracts is demonstrated in several ways. First, there is a complete correspondence between the retention times of the tissue components and those of standards (Figs. 1 and 2). Second, varying the acetonitrile concentration and pH value of the mobile phase markedly alters the retention times for standards (Figs. 3 and 4) and tissue components in a similar manner. Third, the ratio of the peak heights for a compound at various oxidation potentials is relatively specific for that compound (Fig. 6), and the ratio of peak heights of standard and tissue samples measured at 0.8 V and 0.6 V are the same. For example, HVA is about twice as electrochemically active at 0.8 V as it is at 0.6 V, whereas Tryp is inactive at 0.6 V. An exact correspondence between the peak height ratios for standards and tissue extracts would be unlikely if a peak from the tissue extract is due to the oxidation of one or more compounds which differ from the standard. Fourth, the levels of the compounds measured vary in a predictable manner after various drug treatments. For example, reserpine, which disrupts biogenic amine storage, depletes tissue DA, NE, and 5-HT (Table II). Conversely, pargyline, a monoamine oxidase inhibitor, elevates tissue amine levels and decreases the levels of their deaminated metabolites: DOPAC, HVA and 5-HIAA (Table II). Likewise, although we have not routinely used this assay to measure 3-MT levels in brain tissues, the chromatographic peak corresponding to 3-MT increased when 3-MT catabolism was inhibited by

TABLE II

DA, 3-MT, DOPAC, HVA, NE, Tryp, 5-HT AND 5-HIAA CONCENTRATIONS IN RAT STRIATUM AFTER PARGYLINE AND RESERPINE

Rats were decapitated either 1 h or 20 h after receiving intraperitoneal injections of either pargyline or reserpine, respectively. Each value is the mean of six to eight duplicate determinations. The analytical conditions were as described in Figs. 1 and 2. All values for the drug-treated rats, except for Tryp and for 3-MT in the reserpine-treated rats, are significantly different from control values ($p < 0.05$, Newman–Keuls test). Values are expressed as ng/g ± S.E.M.

	DA	3-MT	DOPAC	HVA	NE	Tryp	5-HT	5-HIAA
Control	8701 ± 375	320 ± 27	1497 ± 7	1073 ± 54	108 ± 10	4621 ± 227	516 ± 27	738 ± 15
Reserpine (5 mg/kg)	6666 ± 307	327 ± 25	658 ± 40	738 ± 66	65 ± 8	4098 ± 222	470 ± 27	812 ± 22
Pargyline (50 mg/kg)	10,645 ± 1005	849 ± 136	271 ± 45	464 ± 62	136 ± 6	4506 ± 382	857 ± 61	484 ± 63

pargyline (Table II). The 3-MT levels in Table II are similar to those reported by others for striata taken from untreated decapitated rats [18, 20].

In summary, by a variety of criteria, this assay appears to adequately resolve and measure the compounds it is intended to quantitate. Because no preliminary sample purification, other than deproteinization, is needed, the possibilities for technical errors are reduced. Moreover, the high stability of the compounds measured in the homogenization buffer permits the use of an automatic sample injector at room temperature. Therefore, many samples can be reliably analyzed with very little operator attention.

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GRADIENT REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF NATURALLY OCCURRING RETINOIDS

HAROLD C. FURR

Department of Biochemistry and Biophysics, Iowa State University of Science and Technology, Ames, IA 50011 (U.S.A.)

OLIVIER AMÉDÉE-MANESME

Unité de Recherche d'Hépatologie Infantile, INSERM U56, et Département de Pédiatrie, Université Paris Sud, Hôpital de Bicêtre 94720, Bicêtre (France)

and

JAMES A. OLSON*

Department of Biochemistry and Biophysics, Iowa State University of Science and Technology, Ames, IA 50011 (U.S.A.)

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SUMMARY

A gradient reversed-phase high-performance liquid chromatographic technique is described for the facile separation and quantitation of the naturally occurring retinoids: retinoic acid, retinol, and retinyl esters. An octadecylsilane column (Waters μBondapak C_{18}) is used, with gradient elution from methanol—water (80:20) (solvent A) to 70% or 100% methanol—tetrahydrofuran (50:50) (solvent B) at 2.0 ml/min; detection is by absorbance at 325 nm. Analysis can be completed, with return to starting conditions, in 25—30 min. The method is inherently flexible: retinyl esters can be eluted as a group, with little resolution, by gradient to 100% solvent B, or mostly resolved by gradient to 70% solvent B; separation of retinoids more polar than retinoic acid can be achieved by use of greater proportions of water in solvent A. The separation of vitamin A compounds from extracts of human, rat, and pig liver and from rat kidney by this technique is described.

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INTRODUCTION

Vitamin A and its derivatives (retinoids) occur in nature as compounds having a wide range of polarities, from the very non-polar vitamin A esters to retinoic acid and its water-soluble derivatives (retinoyl β-glucuronide, retinotaurine, etc.). Because of this wide range of polarities, the analysis of retinoids in biological tissues provides an excellent example of the "general elution problem" in liquid chromatography, the separation in a single chromatographic run of compounds having a range of polarities. Previous workers [1—7] have used reversed-phase high-performance liquid chromatography (HPLC) with a variety of isocratic, discontinuous step, and gradient elution eluents to separate these retinoids, but these techniques seemed to us either to lack resolution, to lack flexibility, or to require long analysis times. We propose here a gradient reversed-phase HPLC system which provides good resolution among most of the classes of retinoids found in biological tissues, is readily adapted to suit the analysis of retinoids required, and is relatively rapid.

MATERIALS AND METHODS

Gradient reversed-phase HPLC

Retinoids were analyzed by gradient reversed-phase chromatography on a 10-μm octadecylsilane column (μBondapak C_{18}, 30 cm × 3.9 mm I.D.; Waters Assoc., Milford, MA, U.S.A.) preceded by a guard column (8 cm × 2 mm I.D.) packed with Co:Pell ODS (Whatman Chemical Separation, Clifton, NJ, U.S.A.). Isocratic elution with solvent A, methanol—water (80:20) for 5 min at 2.0 ml/min was followed by a linear gradient to an appropriate percentage (70% or 100%) of solvent B, methanol—tetrahydrofuran (50:50) over a 10-min period; isocratic elution with this final solvent composition continued for 5—10 min, in order to elute retinyl esters. A linear gradient over 5 min back to solvent A restored the system for the next sample. Reagent grade, filtered solvents (0.5-μm Fluoropore filters, Millipore, Bedford, MA, U.S.A.) were used. Waters Assoc. Model 6000A and M45 pumps were controlled by a Waters Model 660 solvent programmer; sample introduction was via a Waters Model U6K loop injector. Detection was by ultraviolet absorption at 325 nm (Model LC-75 detector, Perkin-Elmer, Norwalk, CT, U.S.A.) with quantitation by electronic integration (Model 3390A integrator, Hewlett-Packard, Palo Alto, CA, U.S.A.). A standard curve of integrator peak area versus microgram quantity of vitamin A (retinol and the retinyl component of retinyl palmitate) was prepared and confirmed by daily chromatography of standards. For additional confirmation of the identity and purity of retinoids eluted by this chromatographic system, a photodiode-array spectrophotometric detector (SPD-M1A, Shimadzu Scientific Instruments, Columbia, MD, U.S.A.) was used to determine absorption spectra of individual peaks. All separations were conducted at ambient temperature (20—25° C).

Retinoid standards

All-*trans* retinoic acid, retinol, retinal, retinyl acetate, and retinyl palmitate were purchased commercially (Sigma, St. Louis, MO, U.S.A.); when necessary

they were purified by reversed-phase HPLC essentially as described above. [11,12-^{3}H] All-*trans* retinyl acetate was graciously provided by Hoffmann-La Roche (Basel, Switzerland). Anhydroretinol was prepared by acid-catalyzed dehydration of retinol [8]. The long-chain fatty acyl esters of retinol (i.e., retinyl myristate, palmitate, palmitoleate, stearate, oleate, linoleate, linolenate, and arachidate) were prepared by reaction of retinol with the corresponding acyl chlorides [9]. Synthetic 4-oxo-retinoic acid and retinoyl β-glucuronide were generously provided by Dr. Arun Barua. Solutions of standards were prepared in acetonitrile (to avoid hydrolysis and transesterification) containing 0.1% butylated hydroxytoluene (BHT).

Extraction of biological tissues

Female Sprague-Dawley-derived rats (Holtzman, Madison, WI, U.S.A.) were maintained on a vitamin A-deficient diet (ICN Nutritional Biochemicals, Cleveland, OH, U.S.A.) supplemented with a daily oral dose of 100 μg retinyl acetate in corn oil. At six weeks of age each rat was given 0.57 μCi (26 μg) [11,12-^{3}H] all-*trans* retinyl acetate by intragastric infusion in corn oil. Seven days later the animals were sacrificed under diethyl ether anesthesia, and the liver and kidneys were removed, weighed, and frozen. For analysis, each tissue was first thawed and then ground thoroughly by mortar and pestle with 2—3 times its weight of anhydrous sodium sulfate, and then was extracted with dichloromethane [10]; the liver extract was diluted to 50 ml and the kidney extract diluted to 25 ml with dichloromethane. Aliquots of these extracts were added to 0.1 ml of 0.1% BHT in ethanol and evaporated under a gentle stream of argon, then dissolved in 0.1 ml of 2-propanol plus 0.05 ml dichloromethane for injection onto the chromatograph. Fractions (2 ml) of the HPLC effluent in 10 ml Biofluor liquid scintillation cocktail (New England Nuclear, Boston, MA, U.S.A.) were counted in a liquid scintillation counter (LS 7500, Beckman Instruments, Irvine, CA, U.S.A.); counts were corrected for efficiency of counting by use of an external standard.

A small portion (20 mg) of human liver obtained by needle biopsy (autopsy sample from a normal subject) was similarly extracted and the extract dissolved in 0.1 ml of 2-propanol [11]. Liver from a two-month-old pig was obtained at slaughter, and representative portions were analyzed similarly. All manipulations were carried out under yellow light (Westinghouse F40 Gold fluorescent lamps).

Saponification and acid-catalyzed dehydration of liver extracts

Small volumes (0.3 ml) of the dichloromethane extract of rat liver were evaporated just to dryness. For saponification of the sample, 1 ml of 10% methanolic sodium hydroxide was added and hydrolysis was allowed to proceed overnight at room temperature. After addition of 1 ml water, the non-saponifiable lipid was extracted with hexane; the hexane extracts were then evaporated and the residue was dissolved in 2-propanol for injection onto the chromatograph. For acid-catalyzed dehydration of the liver extract, the concentrated sample was dissolved in 1 ml of 10% ethanolic hydrochloric acid, overlaid with 1 ml hexane, and left overnight at room temperature [8]. After addition of 1 ml aqueous 1 *M* sodium hydroxide, the lipids were

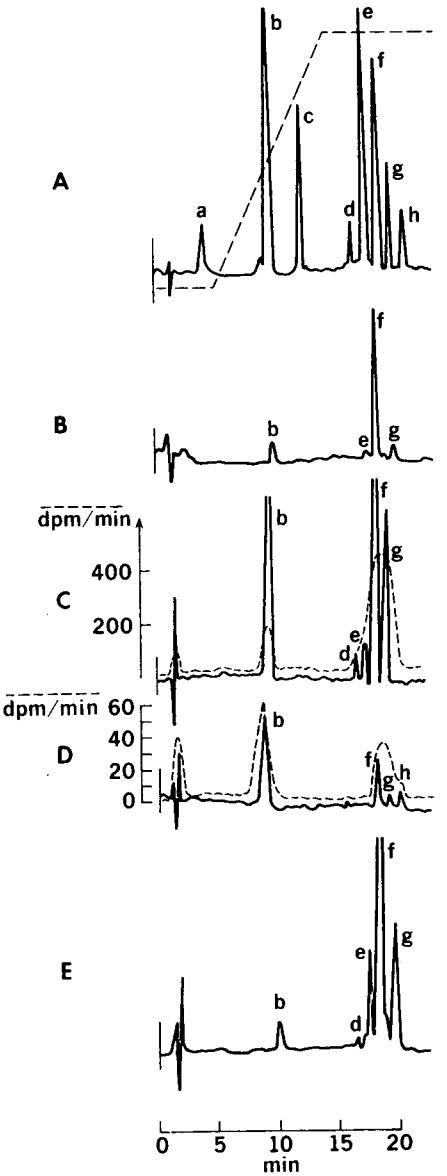

Fig. 1. Gradient reversed-phase HPLC of retinoids. (A) Analysis of retinoid standards. —, absorbance at 325 nm; - - - -, change of solvent composition from 0% solvent B to 70% solvent B. For other chromatographic conditions see Table I. (B) Analysis of vitamin A compounds in an extract of pig liver (20 mg sample). (C) Analysis of vitamin A compounds in an extract of liver from a rat given an oral dose of [^{3}H]retinyl acetate. A 2-ml aliquot of a total of 50 ml extract (equivalent to 0.4 g liver) was analyzed. —, Absorbance at 325 nm; - - - -, radioactivity. (D) Analysis of vitamin A compounds in an extract of kidneys from a rat given an oral dose of [^{3}H]retinyl acetate. A 10-ml aliquot of a total of 25 ml extract (equivalent to 0.96 g kidney) was analyzed. —, Absorbance at 325 nm; - - - -, radioactivity. (E) Analysis of vitamin A compounds in an extract of human liver (20 mg sample). Peaks: a = retinoic acid; b = retinol; c = retinyl acetate; d = retinyl linolenate; e = retinyl myristate plus retinyl palmitoleate plus retinyl linoleate; f = retinyl palmitate plus retinyl oleate; g = retinyl stearate; h = retinyl arachidate.

extracted with hexane, and the hexane extracts concentrated and dissolved in 2-propanol as described above.

RESULTS

Separation of retinoids

The separation of retinoid standards by this gradient reversed-phase HPLC system is shown in Fig. 1A. The major classes of naturally occurring retinoids are clearly separated: retinoic acid, retinol, and long-chain fatty acyl retinyl esters, thus allowing rapid quantitation of these classes of retinoids in biological tissue extracts. Gradient elution to 70% solvent B (i.e., to a final solvent composition of methanol—tetrahydrofuran—water of 59:35:6) allows resolution of most of the commonly occurring retinyl esters, with the following exceptions: retinyl myristate, palmitoleate, and linoleate were unresolved, as were retinyl palmitate plus oleate. Identity of these retinoids in biological tissue extracts was confirmed by their co-elution with authentic standards. Fig. 1 B—E shows the application of this chromatographic system to extracts of several biological tissues: porcine liver, rat liver and kidney, and human liver.

Quantitation of retinoids

Using the detector and integrator described above, the standard curve of peak area versus micrograms of vitamin A (retinol and the retinyl component of retinyl palmitate) was linear up to 5 μg, and curvilinear but usable up to 20 μg. Amounts less than 10 ng were not reproducibly detectable and quantifiable with this detector and integrator. Reproducibility was determined by repetitive injections of retinol and retinyl esters standards (Table I).

TABLE I

CHROMATOGRAPHIC CONDITIONS

Column	μBondapak C_{18} (octadecylsilane), 30 cm × 3.9 mm I.D.
Mobile phases	Solvent A: methanol—water (80:20) Solvent B: methanol—tetrahydrofuran (50:50)
Flow-rate	2.0 ml/min
Gradient conditions	5-min isocratic solvent A, followed by linear gradient over 10 min to 70% or 100% solvent B, held at this composition for 5—10 min; 5-min linear gradient back to solvent A
Detector wavelength	325 nm
Temperature	Ambient
Sensitivity	To 10 ng retinol or retinyl ester
Reproducibility (retinol and retinyl ester standards)	Within-day, standard error of mean = 2.7% (n = 5) Between-day, standard error of mean = 1.6% (n = 10)

Saponification and dehydration of liver extracts

After saponification of the liver extract, retinyl esters disappeared with a concomitant increase in the area of the retinol peak (Fig. 2B). Acid-catalyzed dehydration of liver extract, on the other hand, abolished the peak due to retinol, while giving rise to peaks having retention times similar to that of anhydroretinol, 14 min (Fig. 2C).

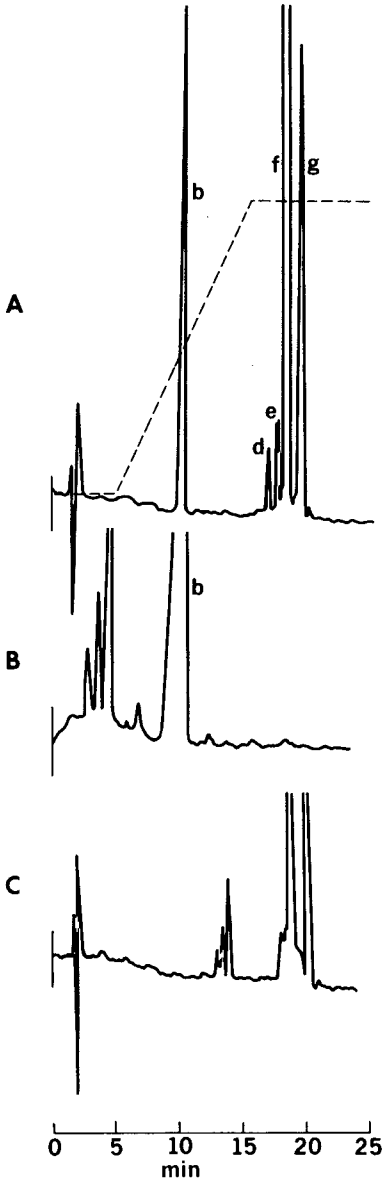

Fig. 2. Saponification and acid-catalyzed dehydration of liver extracts. (A) Gradient reversed-phase HPLC of rat liver extract (0.3 ml of 50 ml extract, equivalent to 0.06 g liver). (B) Analysis of saponified rat liver extract. (C) Analysis of acid-catalyzed dehydrated rat liver extract. Chromatographic conditions as in Fig. 1.

Three-dimensional recording of chromatogram

The three-dimensional recording of absorption—wavelength—time as given by the photodiode-array spectrophotometric detector (Fig. 3) shows the presence of other light-absorbing eluates, in addition to the retinoids. However, none of these (with the exception of carotenoids in the human liver extract, retention time 20.5 min) co-elutes with retinol or retinyl esters.

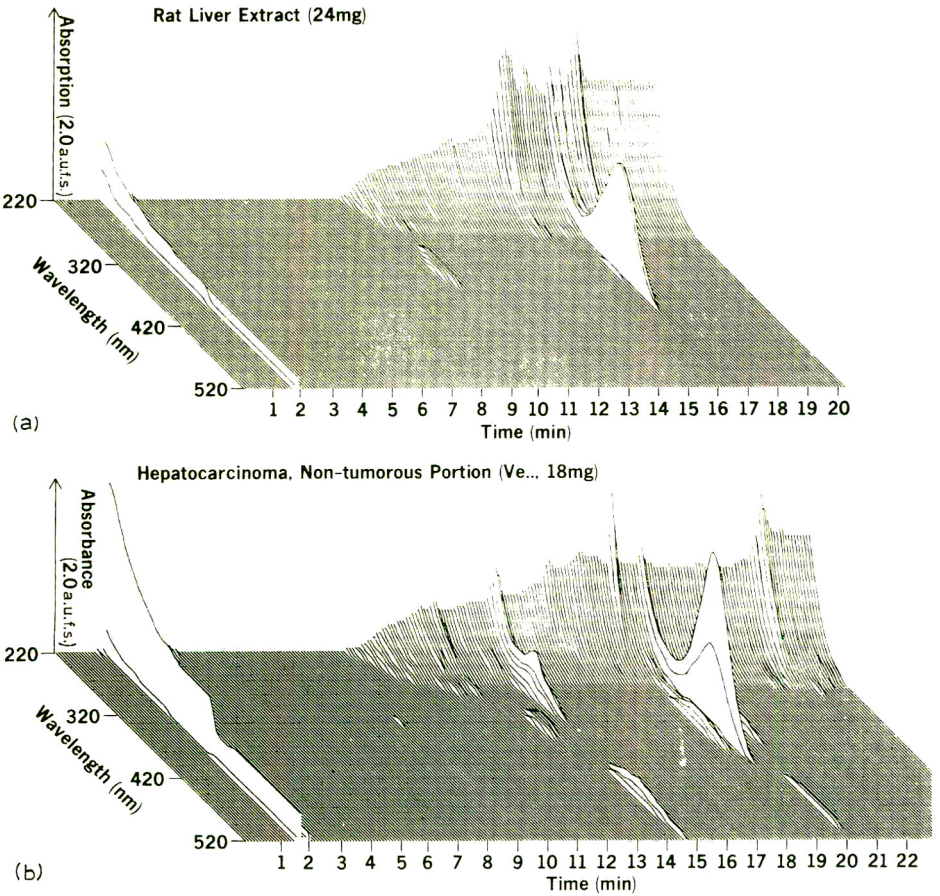

Fig. 3. Three-dimensional chromatograms of gradient HPLC separation of Vitamin A compounds from liver extracts. (A) Analysis of rat liver extract (equivalent to 24 mg liver). Isocratic elution for 5 min with 100% solvent A, followed by a linear gradient over 10 min to 100% solvent B, continued at this solvent composition for 5 min. Retention time for retinol, 10.7 min; retention times for long-chain retinyl esters, 16.4—17 min. (B) Analysis of human liver extract (18 mg liver). Isocratic elution for 5 min with 100% solvent A, followed by a linear gradient over 10 min to 70% solvent B, continued at this solvent composition for 10 min. Retention time for retinol, 10.9 min; retention times for long-chain acyl retinyl esters, 18.6—21.2 min; retention time for hydrocarbon carotenoids, 20.3 min.

Recovery of retinol and retinyl palmitate from HPLC

[^{3}H]Retinol and [^{3}H]retinyl palmitate from a rat liver extract were purified by gradient reversed-phase HPLC as described above. Aliquots of these fractions were re-chromatographed and the appropriate fractions collected and counted by liquid scintillation. The recoveries of radioactivity in retinol (two samples, 45 ng each) and retinyl palmitate (two samples, 230 ng each) averaged 96% (91--100%) and 102% (100—104%), respectively.

DISCUSSION

Tetrahydrofuran was chosen as the third component of this solvent system because it is completely miscible with water and with methanol, thus avoiding any problems of solvent compatibility. This provides a flexible chromatographic system: the solvent gradient can be readily run to 100% solvent B, methanol—tetrahydrofuran (50:50) for the rapid elution of retinyl esters, or to a less non-polar composition [e.g., to 70% solvent B, methanol—tetrahydrofuran—water (59:35:6)] for improved resolution of retinyl esters. If determination of retinol and retinyl esters, but not of retinoic acid, is desired, the initial isocratic portion of the elution solvent program may be omitted and the gradient begun with 30% solvent B, methanol—tetrahydrofuran—water (71:15:14). If, on the other hand, resolution of vitamin A metabolites more polar than retinoic acid is desired, a more polar initial solvent composition (with inclusion of a low concentration of salt to decrease peak tailing of ionizable retinoids [2, 3] and a longer gradient may be used. For example, using an immediate 15-min gradient from methanol—water (70:30), [containing 0.1% ammonium acetate], to methanol—tetrahydrofuran (50:50) the uncorrected retention times are: 4-oxo-retinoic acid, 4.1 min; retinoyl β-glucuronide, 7.9 min; retinoic acid, 9.0 min; retinol, 11.3 min; retinyl acetate, 12.7 min; retinyl palmitate, 16.5 min. Alternatively, the gradient elution system of Silva and DeLuca [12], which uses a solvent gradient from 10 m*M* aqueous ammonium acetate—methanol (98:2) to methanol, might be easily used as the initial gradient, with elution by methanol—tetrahydrofuran as the latter component of the solvent gradient.

Because *cis—trans* isomers of each of the retinoid classes are not well separated by this elution technique, all the geometric isomers of each class can be quantitated as a single peak. Retinal is not satisfactorially resolved from retinol by this column and solvent combination. However, quantitatively important levels of retinal have not been shown in biological tissues other than the eye [13]; thus the lack of resolution of retinal from retinol does not appear to be a serious handicap in most studies of vitamin A metabolism.

Although isocratic elution methods have been developed to give complete resolution of long-chain fatty acyl esters of retinol [14—16], the gradient elution method described here has the advantage of producing sharper peaks, thus facilitating quantitation of retinyl esters in small samples. Although several pairs of retinyl esters are not resolved by this technique (e.g., retinyl oleate co-elutes with retinyl palmitate, and linoleate and palmitoleate with myristate), the ester profile from any given tissue appears to be characteristic of that tissue: human liver retinyl esters appear to have a fatty acid composition similar to that of rat liver and pig liver, but decidedly different from that of rat kidney or rat mammary tissue (cf. Fig. 1; ref. 17).

This method owes its high specificity for vitamin A to the fact that few other compounds in biological tissues have appreciable light absorption at 325 nm, and to the fact that all of these interferences (except β-carotene) are well separated chromatographically from retinol and retinyl esters. This separation is dramatically demonstrated by the three-dimensional chromatograms (absorption—wavelength—time) of tissue extracts (Fig. 3). The saponification

procedure applied to a liver extract confirms that there were no other compounds present contributing to the absorbance of the retinyl esters, and the dehydration procedure demonstrates that no other compounds contributed to the absorbance peak attributed to retinol in this extract. Chromatographic analysis of liver extracts from a rat previously given [^{3}H]retinyl acetate showed that the radioactivity co-eluted with peaks identified on the basis of retention times as retinol and retinyl esters.

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DETERMINATION OF NICOTINIC ACID IN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

YASUTO TSURUTA and KAZUYA KOHASHI

Faculty of Pharmacy and Pharmaceutical Sciences, Fukuyama University, Sanzo, Higashimuramachi, Fukuyama 729-02 (Japan)

and

SATOSHI ISHIDA and YOSUKE OHKURA*

Faculty of Pharmaceutical Sciences, Kyushu University 62, Maidashi, Higashi-ku, Fukuoka 812 (Japan)

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SUMMARY

A sensitive method for the determination of nicotinic acid in serum is described which employs high-performance liquid chromatography with fluorescence detection. Nicotinic acid and 2-chloronicotinic acid as an internal standard in deproteinized serum are reacted with N,N′-dicyclohexyl-O-(7-methoxycoumarin-4-yl)methylisourea in acetone to give the corresponding fluorescent 4-hydroxymethyl-7-methoxycoumarin esters. The compounds are separated by reversed-phase chromatography on LiChrosorb RP-18 with isocratic elution using aqueous acetonitrile containing a small amount of sodium 1-hexanesulphonate as a mobile phase. The detection limit of nicotinic acid in serum was 0.2 nmol/ml. The method requires only 100 μl of serum.

INTRODUCTION

Nicotinic acid belongs to the vitamin B complex and it is a component of nicotinamide nucleotides. It shows pharmacological activities such as a vasodilator effect [1], and hypolipaemic action [2, 3], and it has a prophylactic effect in atherosclerosis [4]. Nicotinic acid has also been used for the treatment of pellagra [5]. These activities are dependent on the concentration of nicotinic acid in blood [6]. For the biomedical investigation of nicotinic acid

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and for the monitoring of the compound in blood during therapy, a sensitive and selective method is required.

Various methods have been reported for the determination of nicotinic acid in biological materials. Microbiological methods [7, 8] are sensitive but not selective; only the total amount of nicotinic acid and nicotinuric acid, a metabolite of nicotinic acid, can be determined in a narrow concentration range. The colorimetric method based on the König reaction [9] requires separation of nicotinic acid by thin-layer chromatography (TLC). The method is insensitive and requires 2—3 ml of serum. Liquid chromatography using an anion-exchange column [10] is time-consuming; reversed-phase high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection [11] is much more sensitive, but still requires rather a large amount of serum (500 μl).

Recently, N,N′-dicyclohexyl-O-(7-methoxycoumarin-4-yl)methylisourea (DCCI) and N,N′-diisopropyl-O-(7-methoxycoumarin-4-yl)methylisourea (DICI) have been reported as fluorescent derivatization reagents of aliphatic and aromatic carboxylic acids [12,13]. These reagents give 4-hydroxymethyl-7-methoxycoumarin esters of the corresponding acids when reacted in organic solvent such as tetrahydrofuran or benzene at room temperature, but no ester is formed from nicotinic acid under these reaction conditions.

We found that both DCCI and DICI react with nicotinic acid in acetone in tightly closed test-tubes at a higher temperature to yield highly fluorescent esters which could be separated from the components of the reagent blanks by TLC or HPLC. On this basis we developed a sensitive method for the determination of nicotinic acid in a small amount of serum using reversed-phase HPLC with fluorescence detection. The fluorescence intensity of the reaction product with DCCI was greater than that with DICI and therefore DCCI was used in this method. Human serum fortified with nicotinic acid was used as a model to establish suitable conditions.

MATERIALS AND METHODS

Materials and apparatus

Nicotinic acid, 2-chloronicotinic acid, and silica gel TLC plates (Wakogel B-5, 5 × 10 cm, 250 μm thick) were purchased from Wako (Osaka, Japan). DCCI and DICI were kindly supplied by Dr. S. Goya (Faculty of Pharmaceutical Sciences, Kumamoto University, Kumamoto, Japan) and were used without further purification. Sodium 1-hexanesulphonate was obtained from Tokyo-kasei (Tokyo, Japan). Other chemicals and solvents were of reagent grade. Normal sera were obtained from healthy volunteers (male, 21—52 years of age) of the Faculty of Pharmaceutical Sciences, Kyushu University, in the usual manner.

The HPLC system was a Yanaco L-200 liquid chromatograph (Yanagimoto, Kyoto, Japan) equipped with a Shimadzu FLD-1 fluorescence detector fitted with a coated mercury lamp and an EM-4 cut-off filter. A stainless-steel column (150 × 4.0 mm I.D.) was packed with LiChrosorb RP-18 (Merck Japan, Tokyo, Japan), particle size 5 μm, using a slurry technique [14]. Uncorrected fluorescence spectra were measured with a Hitachi MPF-4 spectrofluorometer in micro quartz cells (3 × 1.0 cm optical pathlength; 500 μl volume); spectral

bandwidths of 5 nm were used in both the excitation and emission sides of the monochromator.

Procedure

To a mixture of 100 μl of serum and 200 μl of aqueous 2-chloronicotinic acid (50 nmol/ml) as an internal standard placed in a 10-ml glass-stoppered centrifuge tube, 1.5 ml of acetone were added to precipitate the protein. The mixture was shaken for 10 min, followed by centrifugation (800 *g*, 5 min). The supernatant (1.3 ml) was transferred to a 10-ml glass-stoppered centrifuge tube and washed with 1.5 ml of chloroform. After centrifugation, 200 μl of the aqueous layer were transferred to a 10-ml glass-stoppered tube containing 100 μl of 0.05 *M* hydrochloric acid. The mixture was evaporated to dryness in vacuo at 25—30° C. To the residue, 100 μl of acetone were added and the resulting solution was shaken vigorously for 10 min, followed by centrifugation (800 *g*, 5 min). To 50 μl of the acetone solution (placed in a screw-capped 1.5-ml vial; Gasukuro Kogyo, Tokyo, Japan) 50 μl of 5m*M* DCCI solution in acetone were added. The tube was tightly closed and heated in a boiling water bath for 15 min. A 10-μl aliquot of the reaction mixture was injected into the chromatograph and eluted with acetonitrile—water (4:6, v/v) containing 5 m*M* sodium 1-hexanesulphonate. The flow-rate was 1.3 ml/min. The column temperature was ambient (approximately 25° C).

For the establishment of a calibration curve, a series of nicotinic acid standard solutions (5—200 nmol/ml) containing 2-chloronicotinic acid (50 nmol/ml) were prepared, and the mixtures of normal serum (100 μl) and standards (200 μl) were treated as described above. The peak height ratios of nicotinic acid and 2-chloronicotinic acid derivatives were plotted against the concentration of nicotinic acid.

TLC of the reaction mixture of nicotinic acid and 2-chloronicotinic acid with DCCI

A mixture (50 μl) of nicotinic acid and 2-chloronicotinic acid (each 100 nmol/ml) in acetone was treated with DCCI as described. The resulting mixture was applied on a silica gel thin-layer plate, and then developed with benzene—ethyl acetate (1:2, v/v) at approximately 25° C. The fluorescent bands corresponding to DCCI esters of nicotinic acid (R_F 0.41) and 2-chloronicotinic acid (R_F 0.68) were scraped off and the esters were extracted with acetone. The extraction solvent was removed in vacuo.

RESULTS AND DISCUSSION

HPLC conditions

Fig. 1 shows the chromatograms obtained with an acetone solution of nicotinic acid and 2-chloronicotinic acid, and with acetone alone as reagent blank. Although many peaks are observed in the reagent blank (Fig. 1b), which are probably due to decomposition products of DCCI under the conditions of the derivatization reaction, the peaks of the DCCI esters of nicotinic acid and 2-chloronicotinic acid are well separated (retention times 6.0 and 10.0 min, respectively) from the peaks of the reagent blank. The yields of both com-

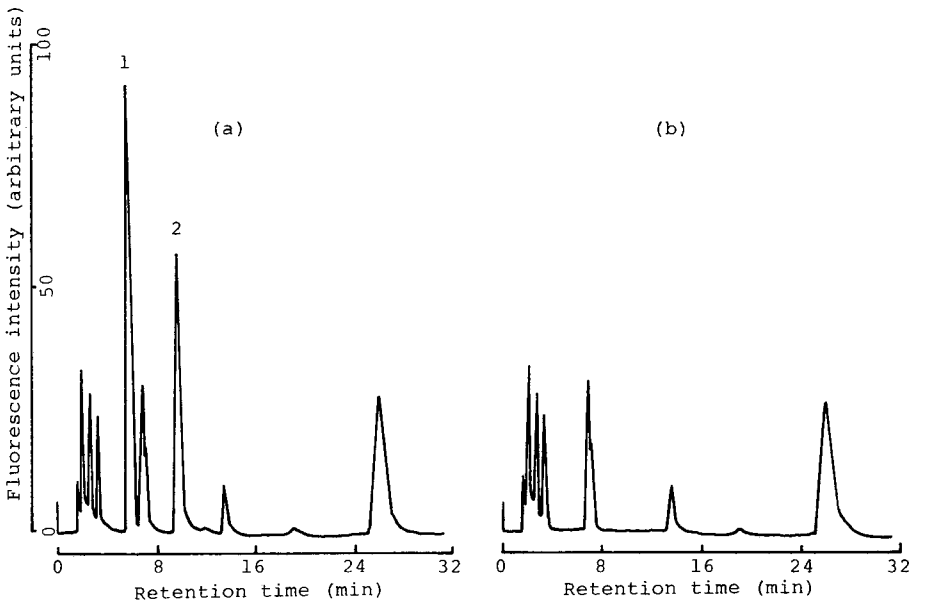

Fig. 1. Chromatograms of (a) DCCI esters of nicotinic acid and 2-chloronicotinic acid (internal standard), and (b) reagent blank. Aliquots (50 μl) of an acetone solution of nicotinic acid and 2-chloronicotinic acid (100 nmol/ml of each), and of acetone (blank) were subjected to the derivatization reaction. 1 = Nicotinic acid derivative; 2 = 2-chloronicotinic acid derivative.

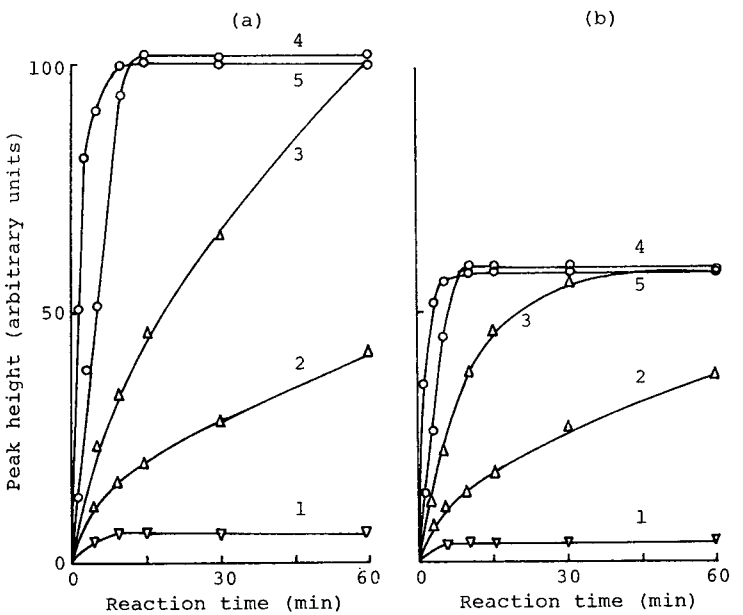

Fig. 2. Effect of reaction time and temperature on the peak heights of (a) nicotinic acid and (b) 2-chloronicotinic acid derivatives. Aliquots (50 μl) of an acetone solution of nicotinic acid and 2-chloronicotinic acid (100 nmol/ml of each) were treated with DCCI for various lengths of time at the following temperatures: 1, 20°C; 2, 40°C; 3, 60°C; 4, 80°C; 5, 100°C.

pounds are reproducible and there are linear relationships between peak heights and the amounts of nicotinic acid and 2-chloronicotinic acid esters. The eluates containing peak 1 and peak 2 (Fig. 1a) have fluorescence excitation maxima at 325 nm and 335 nm, respectively, and emission maxima at 395 and 390 nm, respectively.

The concentration of acetonitrile in the mobile phase affects the separation of the peaks. At concentrations above 50%, the peak for nicotinic acid overlaps with the nearest peak of the blank, while at a concentration of 35% or less there is a delay in the elution rate with broadening of the peaks. The presence of sodium 1-hexanesulphonate in the mobile phase does not affect the retention times of any of the peaks at concentrations of 0.5—10 m*M* but it sharpens the peaks at concentrations of 3 m*M* and above. In practice, 40% acetonitrile and 5 m*M* sodium 1-hexanesulphonate in the mobile pahse were used. When methanol was used in place of acetonitrile in the mobile phase, the peaks were broadened.

The formation of fluorescent derivatives of nicotinic acid and 2-chloronicotinic acid with DCCI is dependent on reaction time and temperature (Fig. 2). Maximum and constant peak heights for both compounds were obtained when the reaction was carried out at 80—100° C for approximately 10 min or longer. Heating at 100° C for 15 min was selected for convenience.

DCCI is soluble in polar and non-polar solvents, while nicotinic acid and 2-chloronicotinic acid are only soluble in polar solvents such as water, acetone and alcohols. Therefore, these solvents were examined for their usefulness in the derivatization reaction. Acetone gave the most intense and reproducible peaks with both acids; in contrast alcohols gave much smaller and unreproducible peaks (Table I). DCCI is decomposed to 4-hydroxymethyl-7-methoxycoumarin and other products in the presence of water under the conditions of the derivatization reaction. No derivative formation occurs in acetone if the reaction mixture contains water at 20% or higher.

The final reaction mixture in the procedure recommended in this work is stable for more than 2 h at room temperature with or without protection from light.

TABLE I

EFFECT OF SOLVENT ON THE DERIVATIZATION REACTION OF NICOTINIC ACID AND 2-CHLORONICOTINIC ACID WITH DCCI

Aliquots (50 μl) of a mixture of nicotinic acid and 2-chloronicotinic acid (100 nmol/ml of each) in the given solvent were subjected to the derivatization reaction.

Solvent	Relative peak height*	
	Nicotinic acid	2-Chloronicotinic acid
Acetone	100	60
Methylcellosolv	69	48
n-Propanol	72	54
Ethanol	56	32
Methanol	2	2

*Height of the peak for nicotinic acid obtained in the reaction in acetone was taken as 100.

Determination of nicotinic acid in serum

The deproteinization of serum can be achieved by adding acetone (final concentration approximately 83%) to serum diluted with an aqueous solution of the internal standard. Shaking for more than 5 min gives constant peak height ratios of nicotinic acid to 2-chloronicotinic acid, thus shaking for 10 min is recommended in the procedure. Washing of the deproteinized sample with chloroform serves to minimize the peaks ascribable to some substances occurring in serum.

Water should be removed from the deproteinized sample before derivatization for the reasons mentioned above. This can be done by adding diluted hydrochloric acid and then evaporating to dryness in vacuo. A very small amount of hydrochloric acid is required for the satisfactory extraction of nicotinic acid and 2-chloronicotinic acid with acetone from the resulting dried residue.

Fig. 3 shows typical chromatograms obtained from the serum of a healthy man who had not been administered nicotinic acid, and from the same serum fortified with nicotinic acid. Peaks for nicotinic acid and 2-chloronicotinic acid are separated by HPLC from all other peaks present in the serum sample and the reagent blank. Endogenous nicotinic acid in the serum produces only a small peak (Fig. 3a) because of its low concentration.

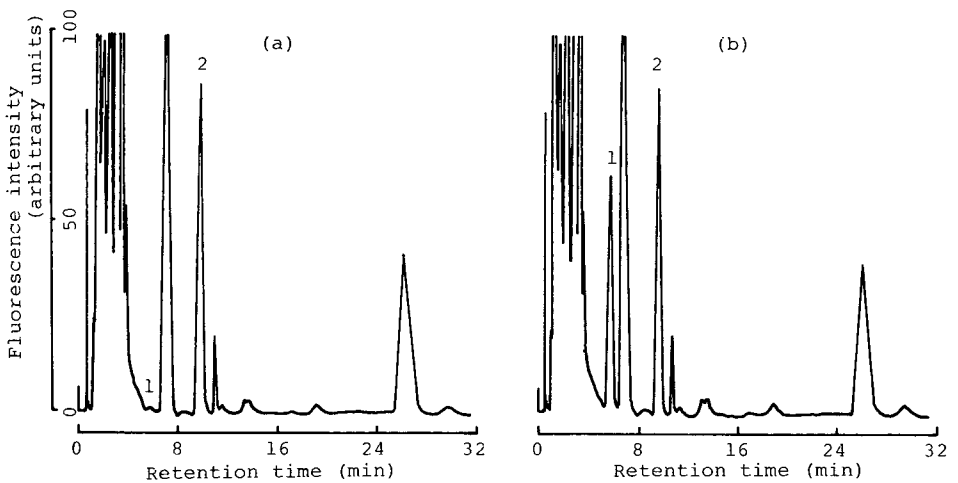

Fig. 3. Chromatograms from (a) normal serum and (b) serum fortified with nicotinic acid (50 nmol/ml). 1 = Nicotinic acid; 2 = 2-chloronicotinic acid.

The fluorescence excitation and emission spectra of peaks 1 and 2 in Fig. 3a or b, those of peaks 1 and 2 in Fig. 1a, and those of the DCCI esters of nicotinic acid and 2-chloronicotinic acid which were separated from other reaction products by TLC, were identical.

A linear relationship was observed between the peak height ratios of nicotinic acid to 2-chloronicotinic acid esters and the amounts of nicotinic acid added to serum in amounts between 100 and 400 nmol/ml. The recoveries of nicotinic acid and 2-chloronicotinic acid (100 nmol/ml of each) from serum were 81.5 ± 1.8% and 79.5 ± 1.5% (mean ± S.D., n = 10 for each), respectively.

The recoveries were calculated from the values obtained with the fortified serum samples and a nicotinic acid standard solution (50 nmol/ml) containing 2-chloronicotinic acid (50 nmol/ml).

The limit of detection for nicotinic acid in serum was 0.2 nmol/ml, at a signal-to-noise ratio of 2. The sensitivity is higher than that of the HPLC method with UV detection [11], but is not high enough to measure precisely endogenous levels of nicotinic acid in normal serum.

The precision was established by repeated determinations using normal serum to which 20 or 100 nmol/ml nicotinic acid was added. The coefficients of variation were 6.5 and 1.7% (n = 10 in each case), respectively. Nicotinamide and nicotinuric acid did not interfere with the nicotinic acid determinations.

This study provides the first HPLC method with fluorescence detection for the determination of nicotinic acid. The method may be applied in routine biomedical studies of nicotinic acid and for monitoring the drug during therapy.

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SIMULTANEOUS DETERMINATION OF METOPROLOL AND METABOLITES IN URINE BY CAPILLARY COLUMN GAS CHROMATOGRAPHY AS OXAZOLIDINEONE AND TRIMETHYLSILYL DERIVATIVES

OLLE GYLLENHAAL* and KURT-JURGEN HOFFMANN

Departments of Analytical Chemistry and Pharmacokinetics and Drug Metabolism, AB Hässle, S-431 83 Mölndal (Sweden)

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SUMMARY

A method for the determination of metoprolol and its main metabolites in urine is presented. The method comprises derivatization of the aminopropanol side-chain with phosgene at alkaline pH and isolation in an organic phase at acidic pH. After trimethylsilylation, separation and quantification are performed by capillary column gas chromatography with flame ionization detection.

The reaction is performed at pH 12 with 60 μl of 2 *M* phosgene in toluene added in three portions. Diethyl ether—dichloromethane is used as extraction medium and bis(trimethylsilyl)acetamide as silylating agent.

With spiked samples linear standard curves were obtained for metoprolol and three of its main metabolites with a detection limit varying between 4 and 20 μmol/l of urine. The method was applied to urine samples from a normal individual who had taken 292 μmol of metoprolol as tartrate.

INTRODUCTION

Metoprolol is mainly metabolized by oxidative deamination and O-dealkylation with subsequent oxidation and aliphatic hydroxylation [1]. These metabolites account for 85% of the dose in man [1]. A survey of the metabolism is given in Fig. 1.

The metabolic hydroxylation of drugs by individuals can in several instances be divided into poor and extensive hydroxylators [2]. The first and most well known example is debrisoquine [3]. This drug is commonly used to classify individuals into rapid or poor hydroxylators, the property being genetically controlled. Papers have appeared that indicate that this might also

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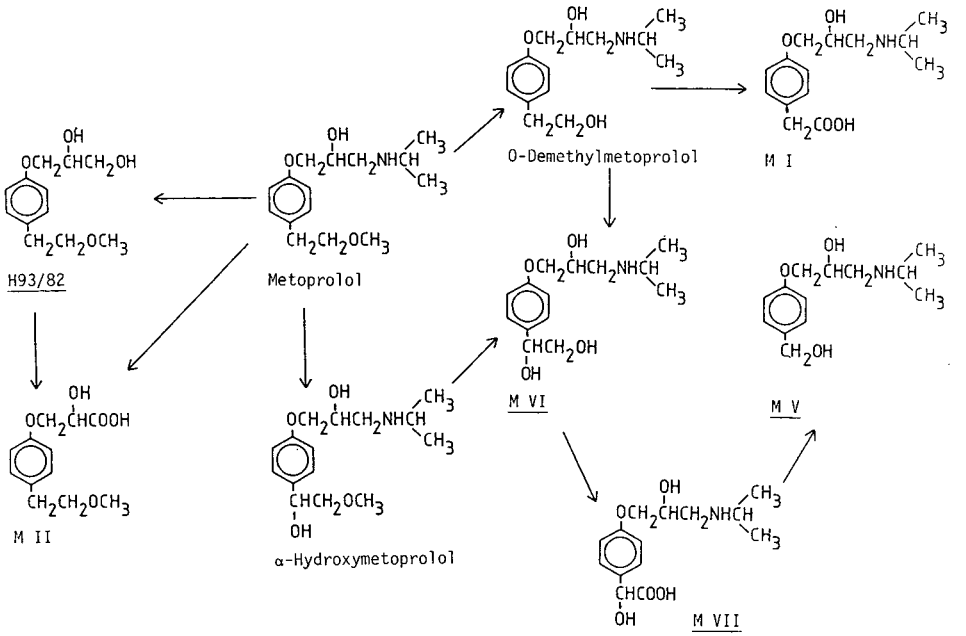


Fig. 1. Identified metabolites of metoprolol. Underlined metabolites have only been detected in the rat.

be the case for metoprolol [4, 5], but contradictory data have been published recently [6]. Benzylic hydroxylation only accounts for about 10% of the total metoprolol dose recovered in urine in man [1]. It has therefore been suggested that the formation of the acid metabolite M I (Fig. 1, H 117/04) by O-dealkylation and subsequent oxidation is under genetic control as well [4]. To enable a study of this hypothesis we have developed a method for the simultaneous determination of metoprolol and its main metabolites. Previous methods for their determination have been based on work with radioactively labelled metoprolol [1]. A mass fragmentographic method is only capable of measuring the basic compounds [7]. This is also true for some liquid chromatographic methods [8—10]. Different methods have been used in the same study [11, 12] for the quantification of basic and acidic metabolites. A simultaneous method would therefore be attractive.

As the main metabolite of interest (M I) is an amino acid (Fig. 1), liquid chromatography would be a possible separation system. However, even if the expected urinary concentrations of some metabolites might be in the higher μmol/l range, direct injection of the sample onto a reversed-phase column with ultraviolet (UV) detection lacks selectivity. To isolate the amino acid by extraction requires blocking the amino group or the carboxylic group.

Carbonic dichloride (phosgene) was recently shown to be a convenient reagent for the cyclization of metoprolol to an oxazolidineone derivative suitable for gas chromatography with nitrogen-selective detection [13]. A two-phase system was used to minimize plasma interference in the derivatization step.

The strategy in the present work was to block the functional groups in the

aminopropanol side-chain by oxazolidineone formation and to isolate the neutral or acidic derivatives by extraction into an organic phase at low pH prior to silylation.

EXPERIMENTAL

Apparatus

Gas chromatography. A Varian 3700 gas chromatograph equipped with flame ionization and thermionic detectors was used. The glass column (120 × 0.2 cm I.D.) was filled with 3% Hi-EFF-8BP (cyclohexanedimethanol succinate). The carrier gas was nitrogen at a flow-rate of 45 ml/min. The injector, column and the detector temperatures were maintained at 300°C, 240°C and 300°C, respectively.

The instrument was also adapted for capillary columns [14]. The capillary columns were of borosilicate glass (25 m × 0.37 mm I.D.) and fused silica (25 m × 0.31 mm I.D.). Both were siloxane-deactivated and coated with SE-54. Helium was used as carrier gas with an inlet pressure of 50 kPa giving a linear velocity of 30 cm/sec. The split flow-rate was approximately 50 ml/min, with make up gas at 30 ml/min. The temperature of the oven was 180°C for 1 min after injection and then increased to 240°C at 10°C/min. The injector and the detector temperatures were 280°C and 300°C, respectively.

The gas chromatograph was also equipped with an automatic injection unit, a Varian 8000 autosampler. The air inlet pressure was kept at 45 kPa and μ-vials were used to allow two injections of each sample. The system was occasionally flushed with ethyl acetate at higher pressure to prevent clogging of the waste exit by solid silylation reaction products.

The peak areas were evaluated by a 3390A Hewlett-Packard integrator.

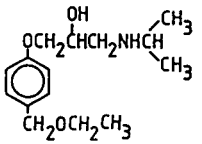
Liquid chromatography. The system consisted of an Altex 110 A pump, a Rheodyne injection valve fitted with a sample loop (100 μl) and a Cecil 212 variable-wavelength UV detector. The column (stainless steel 150 × 4.5 mm I.D.) was filled with 5-μm LiChrosorb RP-8 (E. Merck, Darmstadt, F.R.G.). The mobile phase was 0.01 *M* tetrabutylammonium, 9% acetonitrile in phosphate buffer pH 2 (I = 0.1). The flow-rate was 1 ml/min and the UV absorption of the eluate was measured at 272 nm. Prior to injection, samples were flushed with nitrogen to remove any trace of toluene which would otherwise interfere with the UV detection.

Mass spectrometry. Mass spectra of the derivatives were recorded on a Finnigan MAT 44 S gas chromatograph—mass spectrometer upon electron impact at an ionization energy of 70 eV using a packed OV-17 column. The mass spectra were acquired by a Finnigan MAT SS 200 data system followed by normalization and background subtraction.

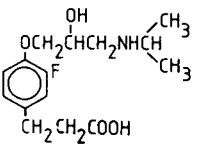
Reagents and chemicals

Metoprolol tartrate, internal standard 1 (IS 1, H 87/31), metabolite I (M I, H 117/04) and internal standard 2 (IS 2, H 177/56) as hydrochlorides, O-demethylmetoprolol (H 105/22), α-hydroxymetoprolol (H 119/66) and metabolite VI (M VI, H 119/72) as 4-hydroxybenzoates, metabolite V (M V, H 119/68) and metabolite VII (M VII, H 119/77) as neutral salts with sodium

acetate, and metabolite II (M II, H 104/83) and metoprolol oxazolidineone (H 151/38) were synthesized at the Department of Organic Chemistry, AB Hässle. The structures are given in Figs. 1 and 2. Tetrabutylammonium hydrogen sulphate was from the same source.



IS 1



IS 2

Fig. 2. Structures of internal standards.

Diethyl ether (Ph. Eur. grade) was from Standard Färg (Dörsjö, Sweden), dichloromethane, HPLC grade, from Rathburn (Walkerburn, U.K.) and acetonitrile from Reagenta (Uppsala, Sweden). Phosgene 2 *M* in toluene purum was from Fluka (Buchs, Switzerland), bis(trimethylsilyl)acetamide (BSA) from Macherey and Nagel (Düren, F.R.G.).

Buffers were prepared from sodium phosphates (Merck).

The oxazolidineone of M I was prepared by reacting 225 mg of the compound dissolved in 100 ml of water and 2 ml of buffer pH 12 with phosgene added in 100-μl portions with vigorous magnetic stirring. The pH of the solution was maintained by the aid of a pH meter and additions of 1 *M* sodium hydroxide. A total of 3 ml of phosgene in toluene was added. The reaction was followed by injecting 1-μl volumes into the liquid chromatographic system. The formed derivative was then isolated by extracting three times with 40 ml of diethyl ether at pH < 3. The collected ether fractions were dried with sodium sulphate and the solvent evaporated. White crystalline needles were obtained.

A similar approach was used for the synthesis of the oxazolidineones of α-hydroxymetoprolol and propranolol. Here the derivatives were extracted at neutral pH with dichloromethane, which was washed with dilute acid and then water prior to evaporation.

Stock solutions were prepared in 0.01 *M* hydrochloric acid: IS 1 550 μmol/l, M II 830 μmol/l, metoprolol and α-hydroxymetoprolol 750 μmol/l and M I 1.5 mmol/l.

The extraction solvent with marker was prepared by dissolving 8.8 μmol of propranolol oxazolidineone in 300 ml of diethyl ether and 200 ml of dichloromethane.

Buffer pH 12 was prepared and tested as follows: 0.5 *M* trisodium phosphate was adjusted with 5 *M* sodium hydroxide until the pH was 12 after mixing two parts of buffer with two parts of urine and one part of 0.01 *M* hydrochloric acid.

Determination of metoprolol and metabolites in urine

A 1-ml volume of urine sample was mixed with 1 ml of buffer pH 12, 0.5 ml of 0.01 *M* hydrochloric acid and 200 μl of internal standard solution IS 1 550 μmol/l. The mixture was agitated; a 20-μl portion of phosgene in toluene

was added and the agitation continued for at least 30 sec. The procedure was repeated twice. The solution was then made acid, pH < 3, by the addition of 0.5 ml of 1 *M* sulphuric acid and extracted with 5 ml of the extraction solvent (diethyl ether—dichloromethane, 3:2) containing the marker. After centrifugation, 4 ml of the organic upper phase were taken to dryness by a stream of nitrogen. The residue was dissolved in 30 μl of BSA. The reaction time was at least 30 min at room temperature.

Before injection, or loading the automatic sampling vials, 120—170 μl of dichloromethane were added. Two 1—2 μl injections of each sample were made in the split mode. By automatic injection each cycle took 25 min.

Standard samples were prepared by adding 0.5 ml of a solution of the compounds to be analysed to 1 ml of blank urine. Samples at four concentrations were analysed in duplicate. Standard curves were constructed by plotting the area ratios to the marker versus the concentration of the compound to be measured. In a typical experiment the standard concentrations of the standard samples were in the ranges 4—26 (M II), 5.5—37 (metoprolol), 18—71 (α-hydroxymetoprolol) and 56—300 (M I) μmol/l of urine.

RESULTS AND DISCUSSION

The cyclization reaction

The aminopropanol side-chain reacts with phosgene as outlined in Fig. 3 and the derivatives formed have no basic properties. The lactic acid M II does not react under the present conditions. The hydrochloric acid formed during the reaction causes a shift in the pH of the aqueous phase towards the acid side.

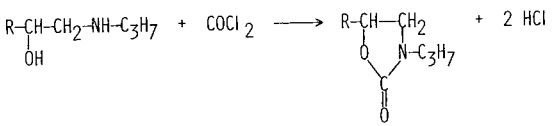


Fig. 3. Cyclization of the aminopropanol side-chain with phosgene.

Identification of formed oxazolidineone and trimethylsilyl derivatives

The structures of the formed derivatives were confirmed by mass spectrometry after gas chromatographic separation. The most important ions are listed in Table I. A complete spectrum of the metoprolol oxazolidineone has been published elsewhere [13]. In general, the oxazolidineones give weak molecular ions and have *m/z* 56 as base peak. This ion may be identical with the ion formed by fragmentation of boronate derivatives of alprenolol [15]. The trimethylsilyl derivatives have *m/z* 73 as base peak in three out of four instances. The molecular ions are weak except for IS 2 (22%). Consequently, the possibilities for mass fragmentographic quantitation using electron-impact ionization seem rather limited.

Optimum pH for the derivatization of M I

The derivatization conditions were studied by the aid of liquid chromatography. The aqueous phase could be injected directly and the formation of the derivative as well as the disappearance of the parent compound could be

TABLE I

SIGNIFICANT MASS SPECTRAL DATA OF METOPROLOL AND METABOLITES AS DERIVATIVES

Compound	Trimethylsilyl		Oxazolidineone	Relative intensity (%)					
	ether	ester		56	73 (TMS)	100	M − 45	M − 15	M⁺ (*m/z*)
M II	x	x			100		8	10	8 (384)
IS 1			x	100		38	18		15 (293)
Metoprolol			x	100		65	90		28 (293)
Propranolol			x	100		48		5	78 (285)
O-Demethyl-metoprolol	x		x	25	100	12	1	22	8 (351)
α-Hydroxy-metoprolol	x		x	20	75		100	3	— (381)
M I		x	x	18	100			5	4 (365)
IS 2		x	x	81	100	18	14	22	22 (397)

followed. To do this by gas chromatography would have required extraction and further derivatization.

In a previous paper it was shown that metoprolol in aqueous solution could be derivatized with 20 μl of 2 *M* phosgene in toluene [13]. However, with plasma present, the reaction was facilitated by using a two-phase system with a distinct organic phase present. It is probable that in both instances the reaction takes place in the organic phase. With M I being an amino acid with poor partition properties to organic solvents, the reaction might take place either in the aqueous phase, where water competes for phosgene, or in the minute organic toluene phase with poor solubility of the reactant.

Optimum pH of the reaction was found to be near pH 12 (Table II). As the reagent is consumed the pH falls. It is therefore necessary to use buffers with a high buffer capacity. This is also evident from the sample with 0.1 *M* sodium hydroxide (Table II).

TABLE II

INFLUENCE OF pH ON THE DERIVATIZATION OF M I WITH PHOSGENE AS MONITORED BY LIQUID CHROMATOGRAPHY

Method: 1.0 ml of buffer (ionic strength 1), 1.0 ml of M I (1 μmol in 0.01 *M* hydrochloric acid), 20 μl of 2 *M* phosgene in toluene, 20 μl analysed by liquid chromatography after evaporation of remaining toluene.

Initial pH of reaction solution	pH after phosgene reaction	Peak height (mm)	
		Derivative (n = 2)	Unreacted M I
7.0	n.m.**	6	275
8.1	7.3	12	293
9.4	7.4	29	273
11.0	10.4	77	n.m.
11.7	11.0	120	n.m.
12.1	11.4	104	n.m.
12.5	11.7	101	n.m.
12.6*	9.7	96	n.m.

*0.1 *M* sodium hydroxide.
**n.m. = not monitored.

Optimum amount of phosgene reagent for the reaction

The yields of derivative using varying amounts of 2 *M* phosgene in toluene are illustrated in Table III. It is apparent that at least one 20-μl portion is required and even more derivative can be formed by adding further reagent as the pH is still sufficiently high. Three successive additions of the reagent with agitation periods 30 sec long in between were selected (Table IV). The pH was readjusted to 12 after the third 20-μl portion and after the second 50-μl portion (Table IV).

TABLE III

OPTIMAL AMOUNT OF PHOSGENE REAGENT FOR THE DERIVATIZATION OF M I AS MONITORED BY LIQUID CHROMATOGRAPHY

Method: see Table II.

Added 2 *M* phosgene (μl)	Peak height (mm)	
	M I derivative (n = 2)	Unreacted M I (n = 2)
50	110	28
20	108	57
10	85	118
5	36	163
2	8	119
1	4	144
0.5	1	155
0	0	30
40 (2 × 20)	163	28

TABLE IV

EFFECT OF REPEATED ADDITIONS OF THE PHOSGENE REAGENT ON THE YIELD OF DERIVATIVE OF M I

Method: see Table II, 200 μg of H 151/38 (marker, metoprolol oxazolidineone [13]). The average of the two highest yields was arbitrarily set to 100%.

Addition number	Volume (μl)									
	20		50		100		200		500	
1	66	68	74	68	73	71	60	62	60	60
2	88	83	80	92						
3	91	101	75*	79*						
4	99*	87*								

*Denotes adjustment of the pH.

Isolation of metoprolol and metabolites from urine

Dichloromethane and diethyl ether alone were not capable of extracting all compounds quantitatively (> 95%) using a phase ratio of 4:1 at acidic pH (Table V). By combining ether and dichloromethane 3:2 and a 2:1 phase volume ratio, quantitative extraction was also obtained with M II being the

most polar metabolite according to the extraction data. Metoprolol as oxazolidineone has been shown to be extracted with ease by hexane—dichloromethane 4:1 and the same phase ratio [13].

TABLE V

ISOLATION OF METOPROLOL AND METABOLITES FROM THE AQUEOUS PHASE AFTER CYCLIZATION WITH PHOSGENE

Liquid chromatography of the aqueous phase, 0.5 *M* hydrochloric acid, before and after equilibration with the organic phase. Propranolol used as marker.

Organic phase	V_{org}/V_{aq}	Recovery in the organic phase (%)			
		M II	O-Demethyl-metoprolol	α-Hydroxy-metoprolol	M I
Dichloromethane	2:1	63	95	95	95
	4:1	76	>95	n.m.*	n.m.
Diethyl ether	2:1	89	78	63	79
	4:1	90	n.m.	n.m.	90
Dichloromethane—diethyl ether (3:2)	2:1	95	>95	>95	>95

*n.m. = not monitored.

Separation and quantification system

The selectivity and the sensitivity of the present liquid chromatographic system was not sufficient to allow quantification of metoprolol and metabolites after derivatization and extraction of urine samples. The packed column with 3% Hi-EFF-8BP was not capable of separating metoprolol from α-hydroxymetoprolol but was used briefly to check silylation conditions. Instead the possibility of using capillary column gas chromatography was investigated. Flame ionization detection was chosen in order to make quantification of the lactic acid, M II, derivative possible. This was not the case with the thermionic detector. Capillary columns 25 m long coated with SE-54 were used. The temperature of the column oven was programmed in order to elute the more volatile and the least volatile derivatives within a reasonable time as well as to retain peak height. The resolution of α-hydroxymetoprolol and M I was improved by using helium instead of nitrogen as carrier gas. A chromatogram with metoprolol and most of the metabolites is shown in Fig. 4.

Trimethylsilylation before gas chromatography

Carboxylic and remaining hydroxylic groups were protected by trimethylsilylation prior to the gas chromatographic separation step. BSA [bis(trimethylsilyl)acetamide] was preferred over BSTFA (corresponding trifluoroacetamide) since the latter reagent produced several interfering peaks in the region of interest. Hexamethyldisilazane—trimethylchlorosilane was less reactive than BSA with the carboxylic acids. At least 10 μl of the BSA reagent were required to derivatize the compounds of interest isolated from a high concentration urine sample. A 30-μl volume was selected as a safe over-capacity when samples were to be analysed at a later stage. The reaction itself appeared to be

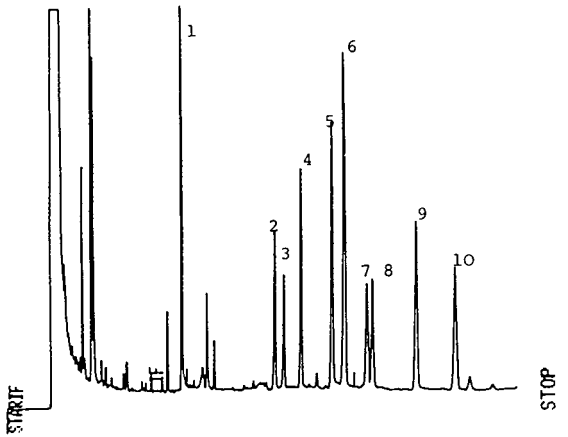


Fig. 4. Gas chromatogram of metoprolol and metabolites after phosgene derivatization, extraction and trimethylsilylation. 1 = M II, 2 = M V, 3 = metoprolol, 4 = M V, 5 = O-demethylmetoprolol, 6 = propranolol (marker), 7 = α-hydroxymetoprolol, 8 = M I, 9 = M VI, 10 = M VII. Retention time of M VII 16 min. Structures of the parent compounds are given in Fig. 1, and gas chromatographic conditions in the Experimental section.

virtually instantaneous. The reagent excess was not evaporated as the carboxylic acid trimethylsilyl esters tended to decompose in the absence of reagent. This instability was more pronounced with M II than with M I.

Selection of internal standard

Because of the variety of chemical structures of the compounds in this study, the selection of an internal standard was bound to be a compromise. At an early stage of method development, IS 2 (Fig. 2) was investigated as a potential internal standard for M I.

However, precision data were inferior to those obtained with the propranolol oxazolidineone as marker. Also the area ratios versus IS 1 spread more than versus propranolol oxazolidineone. IS 1 is an isomer of metoprolol (Fig. 2) and was used in the phosgene-based method for metoprolol mentioned [13] and is now also used in the electron-capture gas chromatographic method for metoprolol [16]. Thus, for quantitative purposes the propranolol oxazolidineone marker was preferred but IS 1 was added to check the phosgene reaction. Both were used in a fairly large amount compared with some of the compounds to be measured. Approximately 110 μmol of each were added per sample to minimize disturbances from endogenous components present.

As no related acid was available, M II, the lactic acid metabolite was also quantified by the aid of propranolol oxazolidineone despite the large difference in retention time (6 min). Stearic acid might be an alternative when M II only is studied. Its trimethylsilyl ester has only a 1 min longer retention in this gas chromatographic system. The acid can be added to the extraction medium and an excess is recommended as endogenous octadecanoic acids may be present.

Standard curves

Standard curves were constructed after analysing samples prepared by adding metoprolol and metabolites to urine to give the anticipated concentrations. A positive intercept of the slope of M II was observed and is due to chromatographic interference. In some samples this was a problem as the integrator recognized only one peak. A gas chromatogram with M II from urine is shown in Fig. 5.

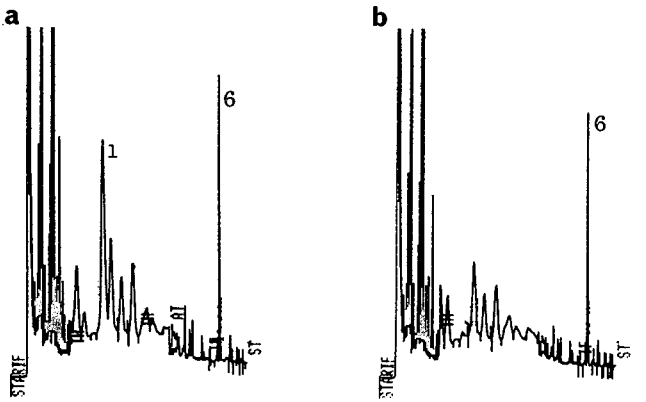


Fig. 5. Gas chromatograms of M II in urine: (a) 17 μmol/l added to urine, (b) blank urine. Peak identification as in Fig. 4, 6 = marker. Chart speed ten times higher in the region where M II elutes and the attenuation decreased × 2.

Linear-regression analysis gave correlation coefficients > 0.999 except for M I (0.987). Preliminary analysis of O-demethylmetoprolol in urine from several individuals who had taken metoprolol never gave concentrations above 4 μmol/l. Thus, standard curves were not constructed for this metabolite.

Precision and absolute yield

The precision of the method was studied by the analysis of spiked urine samples from six individuals. The results are listed in Table VI together with the absolute yield. This was obtained by comparison with solutions of synthesized derivatives and the pure compounds. From Table VI it is obvious that urine from different individuals has a negligible influence on the spread for

TABLE VI

PRECISION AND YIELD OF THE METHOD

Method: see Experimental, urines from six individuals were spiked.

	Precision (urine, R.S.D., %)	Concentration (μmol/l)	Yield (%)	
			Urine (n = 6)	Water (n = 3)
M II	11.5	42	93	86
Metoprolol	4.5	37	92	88
α-Hydroxymetoprolol	4.1	71	72	72
M I	6.4	75	56	86

TABLE VII

DETERMINATION OF METOPROLOL AND METABOLITES IN URINE FROM A HEALTHY VOLUNTEER AFTER INGESTION OF 292 μmol OF METOPROLOL TARTRATE

Fraction (h)	M II		Metoprolol		O-Demethylmetoprolol		α-Hydroxymetoprolol		M I	
	μmol/l	μmol excreted	μmol/l	μmol excreted	μmol/l	μmol excreted	μmol/l	μmol excreted	μmol/l	μmol excreted
0—6	66	16	22	5.2	<4	—	50	12	369	87
6—12	4	1	13	3.6	<4	—	30	9.3	133	38
12—24	<4	—	<4	—	<4	—	19	7.7	38	15

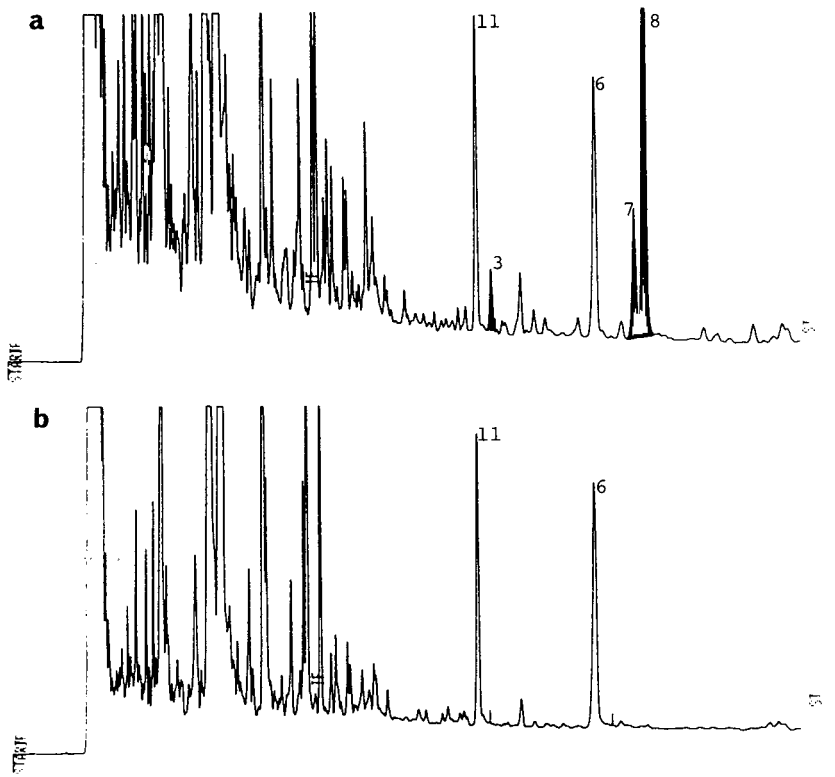


Fig. 6. Gas chromatograms of metoprolol and metabolites after the analysis of urine from an individual who had been given 292 μmol of metoprolol tartrate: (a) 0—6 h fraction, (b) blank urine from the same individual. Peak identification as in Fig. 4, 11 = IS 1. Retention time of M I 12.8 min. For gas chromatographic conditions see Experimental and for levels found see Table VII.

α-hydroxymetoprolol and M I at these levels. However, in the case of M II, some of the blank urines contained a peak near that of the compound of interest, thus affecting the precision in its determination. The absolute yield of M I in the organic phase was about two-thirds that obtained when a pure aqueous solution was derivatized in parallel (Table VI). In the other instances the recovery from aqueous and urine samples were the same. A further 10% of M I could be recovered if the aqueous phase was isolated and derivatized anew after readjustment of the pH. Further studies of the derivatization of M I in urine might be worthwhile. However, since the actual levels were adequate for

the present method this was not deemed necessary at the present time.

Blank urine from two individuals was spiked with M II, α-hydroxymetoprolol and M I to varying concentrations by an independent person. The eight samples were then analysed by the present method. The levels found correlate well (better than 0.997) and the slopes were between 0.90 and 1.10.

Determination of metoprolol and metabolites in urine samples

An individual known to be a normal hydroxylator was given 292 μmol of metoprolol as tartrate. Urine was collected in fractions during 24 h and was analysed according to this method. The concentrations found are presented in Table VII. A gas chromatogram of 0—6 h fraction together with a blank is shown in Fig. 6. In agreement with data reported earlier based on radioactivity measurements [1], the metabolite M I is the major urinary excretion product in man after an oral dose of metoprolol. Unchanged drug and α-hydroxymetoprolol are present in urine as well as the lactic acid metabolite M II. The method in this paper revealed that 67% of the given dose can be accounted for.

Samples with M VI and M VII (Fig. 1) were prepared to a concentration of 20 μmol/l of blank urine each. After analysis the peaks in the chromatogram were matched with peaks in the same region from the individual who had been given metoprolol. However, on the basis of retention times, corresponding peaks in the biological samples could not be detected.

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DEVELOPMENT OF A STANDARDIZED ANALYSIS STRATEGY FOR BASIC DRUGS, USING ION-PAIR EXTRACTION AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

VI. DRUG LEVEL DETERMINATION IN SALIVA

G. HOOGEWIJS and D.L. MASSART*

Farmaceutisch Instituut, Vrije Universiteit Brussel, Laarbeeklaan 103, B-1090 Brussels (Belgium)

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SUMMARY

Assay methods for measuring saliva levels of carbamazepine and its active metabolite, and of amidopyrine are developed using a standardized analysis strategy. Both assay methods include ion-pair extraction of the analytes with octylsulphate as the counter-ion and chromatography on a CN-bonded phase using a hexane—dichloromethane—acetonitrile—propylamine mixture as the mobile phase. Both methods are applied to patient samples.

INTRODUCTION

It has been suggested that saliva might be substituted for plasma in therapeutic drug monitoring and pharmacokinetic studies. Observations and considerations supporting this suggestion include the following. (1) It has been established that for a number of drugs the saliva concentration is proportional to the plasma concentration. (2) For some drugs it has been shown that the saliva concentration is equal to the free drug concentration in plasma, i.e. the pharmacologically active portion of the total drug concentration in plasma. (3) Saliva is readily available and can be collected by non-invasive techniques, which is of great practical importance particularly for paediatric, geriatric and out-patients.

The advantages and possibilities of drug level determinations in saliva have been reviewed by Danhof and Breimer [1] and by Horning et al. [2]. From these reviews it appears, however, that extensive research is still needed before the suggested substitution of saliva for plasma could be entirely

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justified. This implies, among others, drug level determinations in an extensive number of samples and for a wide variety of drugs. It could therefore be advantageous to use a standardized analytical procedure which is applicable to a large number of drugs and requires only minor modifications in order to make the assay method selective for the drug under investigation. In our laboratory such a standardized analysis strategy has been developed for basic drugs. Its philosophy and advantages [3, 4] as well as its application to the analysis of pharmaceutical dosage forms [5, 6] and cosmetics [7] have been reported previously. It is the aim of the present paper to demonstrate the usefulness of the strategy for drug level determinations in saliva. This will be done by constructing a method for the determination of carbamazepine and its active metabolite and for amidopyrine.

EXPERIMENTAL

Apparatus

A Varian 8500 liquid chromatograph was used, equipped with a Valco loop injector (sample loop volume 100 μl), a fixed-wavelength (254 nm) ultraviolet (UV) detector, a Varian 9176 recorder and a Varian Vista CDS 401 chromatographic data system. Chromatography was performed on a MicroPak CN-10 column (particle size 10 μm), 300 × 4 mm (Varian, Palo Alto, CA, U.S.A.). The characteristics of the briefly used LiChrosorb CN column were: particle size 10 μm, dimensions 250 × 4 mm (E. Merck, Darmstadt, F.R.G.).

Chemicals and reagents

Sodium *n*-octylsulphate (for tenside tests) was purchased from Merck. *n*-Hexane, dichloromethane and acetonitrile were HPLC grade and purchased from Merck or Fluka (Buchs, Switzerland). All other reagents were analytical reagent grade and obtained from Merck, except propylamine which was purchased from Fluka. Carbamazepine and carbamazepine-10,11-epoxide were kindly supplied by Ciba-Geigy, and amidopyrine was a kind gift from Hoechst.

Collection of saliva samples

Method construction was performed using saliva samples from different donors not undergoing drug therapy. Only whole saliva, obtained without stimulation of the salivary flow, was used. Samples of patients and volunteers were obtained in the same way. No special precautions concerning, for example, beverage and food intake of the donors were taken. The samples were briefly centrifuged before sample preparation in order to precipitate debris.

Extraction procedure

To 1 ml of saliva standard or sample, 100 μl of internal standard solution were added in glass centrifuge tubes equipped with PTFE-covered screw-caps. After vortexing, 10 ml of 0.05 *M* sodium *n*-octylsulphate solution in phosphate buffer pH 3.0 (I = 0.4) were added. The mixture was homogenized by vortexing, then 5 ml of chloroform were added. Partitioning was performed by gently shaking the tubes longitudinally in a shaking bath for 30 min (vortexing

for a few minutes might be equally efficient but was not investigated). After centrifugation, the aqueous layer was discarded and 4 ml of the organic solvent were transferred to a clean vial with conical bottom and evaporated to dryness under a gentle nitrogen stream at ± 40° C. Each extract was reconstituted just prior to chromatography with 200 µl of dichloromethane; 100 µl were injected.

The extraction of plasma was also carried out using the method described above. The plasma proteins, however, were precipitated with 2 ml of acetonitrile, the supernatant was transferred to a new centrifuge tube and the acetonitrile was evaporated in a water bath, before addition of the counter-ion solution [8].

Assay standards

Assay standards were prepared by spiking drug-free saliva or plasma with 100 µl of an aqueous solution of the analytes to give the desired concentrations. The stock solution of carbamazepine and carbamazepine-10,11-epoxide was prepared by dissolving the drugs in a few millilitres of acetonitrile and then bringing to volume with double-distilled water.

Internal standard solutions

The internal standard solution for the carbamazepine assay contained 1.0 µg of nitrazepam per 100 µl of water. A fresh solution was made each day. The internal standard solution for the amidopyrine assay contained 500 ng of promethazine · HCl per 100 µl of water.

Recovery

The overall recoveries were established by interpolation of the peak area of the extracted analytes on a calibration curve (peak area versus concentration) of standards prepared in dichloromethane.

Quantitation

The peak area ratio of analyte to internal standard was used as quantitation criterion. The peak area ratios were plotted against concentration to obtain standard calibration curves. A new calibration curve was made with each sample set.

RESULTS AND DISCUSSION

The standardized analysis strategy [3—8] consists of an ion-pair extraction step using chloroform as the solvent and either sodium *n*-octylsulphate (NaOS) at pH 3.0 or di-(2-ethylhexyl)phosphoric acid (HDEHP) at pH 5.5 as ion-pairing reagents, followed by direct HPLC analysis of the extract using a CN-bonded phase with either hexane—dichloromethane—acetonitrile—propylamine (50:50:25:0.1) or acetonitrile—water—propylamine (90:10:0.01) as standardized eluent. Selectivity of the method for a particular analysis problem is obtained by optimizing the volume ratio of the mobile phase components. The polarity of the analyte usually determines which of the counter-ions and which of the eluents should be used, although other factors such as the opportunity of detection at wavelengths in the lower UV region might

influence the choice of eluent. In the present study, a fixed-wavelength (254 nm) detector was used, ruling out this possibility, but even then sensitivity aspects (see, for example, the carbamazepine assay) might influence the eluent choice. As was demonstrated previously [3, 5], HDEHP is a more powerful ion-pairing reagent than NaOS and should therefore be preferred. However, in the present work all extractions are performed using NaOS extraction to demonstrate that this reagent also allows high extraction yields. Furthermore, it should be noted that due to the low pK_a (5.0) of aminopyrine, no ion-pair formation with HDEHP at pH 5.5 would be possible. The foregoing remarks demonstrate the need for flexibility in a standardized analysis scheme, and although both the stationary and the mobile phases, the extraction solvent, the pH, and ion-pairing reagents are standardized, care has been taken during the conception and development of the strategy to allow enough versatility to be able to meet certain special needs in a particular analysis problem.

Carbamazepine

Since at 254 nm normal-phase chromatography affords higher sensitivity than the reversed-phase mode [9], the carbamazepine assay method was developed by use of the hexane—dichloromethane—acetonitrile—propylamine eluent. The original (50:50:25:0.1) volume ratio was optimized by halving the dichloromethane content in order to separate carbamazepine and its active metabolite, carbamazepine-10,11-epoxide, and to resolve both compounds from caffeine and other sample components. At a flow-rate of 2 ml/min, caffeine, carbamazepine and carbamazepine-10,11-epoxide gave retention times of 4.4, 6.7 and 11.5 min, respectively. Several drugs were chromatographed to test for interference and for use as internal standard. Their retention times relative to carbamazepine are listed in Table I. Nitrazepam was selected as internal standard. None of the drugs investigated interfered with either

TABLE I

RETENTION TIMES, RELATIVE TO CARBAMAZEPINE, OF VARIOUS DRUGS

Drug	Relative retention time
Thioridazine	0.65
Nitrazepam	0.75
Amidopyrine	0.83
Procaine	0.85
Carbamazepine*	1.00
Phenylbutazone	1.28
Mesoridazine	1.33
Carbamazepine-10,11-epoxide	1.72
Antipyrine	2.15
Chlordiazepoxide	2.28
Primidone	3.40
Phenobarbital	3.40
Paracetamol	3.87
Diphenylhydantoin	4.45
Quinidine	>5
Procainamide	>5

*Retention time = 6.7 min.

carbamazepine, carbamazepine-10,11-epoxide or nitrazepam. Since large intra- and inter-individual differences in UV-absorbing saliva constituents have been observed [10] the method was applied to a large number of saliva samples from various donors, collected at different times of day. No interference from endogenous compounds was observed. The analytical recoveries of carbamazepine and its 10,11-epoxide from both saliva and plasma were assessed at the 1 μg/ml level using the octylsulphate extraction technique. The values obtained are presented in Table II. As would be expected the recoveries are comparable for both analytes and are higher from saliva than from plasma. It should be noted that storage of the evaporated extracts at 4° C during 24 and 48 h results in losses of both analytes of about 10% and 25%, respectively.

TABLE II

PERCENTAGE OVERALL RECOVERY FOR CARBAMAZEPINE AND CARBAMAZEPINE-10,11-EPOXIDE FROM SALIVA AND PLASMA

Samples of 1 μg/ml for each drug were used; $n = 6$.

Sample	Percentage recovery	
	Carbamazepine	10,11-Epoxide
Saliva	95.8 ± 2.0%	96.8 ± 2.9%
Plasma	87.0 ± 3.2%	89.3 ± 3.8%

The linearity of the calibration curves in both saliva and plasma was evaluated for carbamazepine in the 0.1—10.0 μg/ml concentration range and for the 10,11-epoxide in the 0.1—5.5 μg/ml range. The standard curves were rectilinear in the range tested for both analytes and both matrices (for carbamazepine mean $r^2 = 0.999$, for the 10,11-epoxide mean $r^2 = 0.998$). A chromatogram of a saliva standard is shown in Fig. 1. The within-day precision of the assay method was evaluated at the 0.5 μg/ml saliva and 3.0 μg/ml saliva levels by analysing replicate spiked samples ($n = 6$). Coefficients of variation of 4.2% and 2.9%, respectively, for carbamazepine and 4.9% and 3.7%, respectively, for the epoxide metabolite were found. The limits of detection at a signal-to-noise ratio of 3 were estimated to be 0.004 μg of carbamazepine per ml of saliva and 0.040 μg of carbamazepine-10,11-epoxide per ml of saliva.

In order to demonstrate the usefulness of the method it was applied to saliva samples of patients treated with Tegretol®. Although most of these patients received various other drugs as well, no interference was observed. The purity of the extracts is demonstrated in Fig. 2, which shows a chromatogram obtained from a patient treated with Tegretol (200 mg, four times a day) and Largactil® (25 mg, three times a day). The sample was collected 1 h after the last tablet intake and carbamazepine and 10,11-epoxide levels were found to be 4.17 and 0.20 μg/ml, respectively. The chromatogram also shows an unidentified peak with a retention time of 2.5 min. It is interesting to note that this peak was only present in saliva from patients on carbamazepine and that the area of this peak increased with increasing carbamazepine and 10,11-epoxide levels. It might therefore be a metabolite of carbamazepine. It can be concluded that the method is very useful for assaying carbamazepine and its

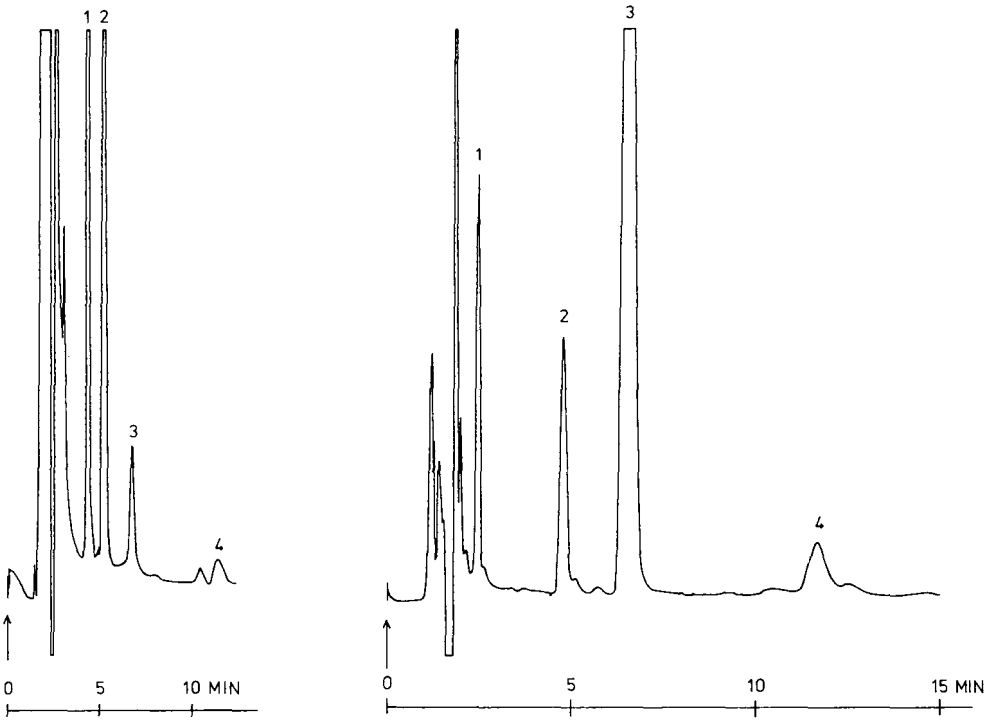


Fig. 1. Chromatogram of a spiked saliva extract. Peaks: 1 = caffeine, 2 = nitrazepam (internal standard), 3 = carbamazepine (106 ng/ml), 4 = carbamazepine-10,11-epoxide (102 ng/ml). Column: MicroPak CN-10 (10 μm, 300 × 4 mm). Mobile phase: *n*-hexane—dichloromethane—acetonitrile—propylamine (50:25:25:0.1). Flow-rate: 2 ml/min. Detection sensitivity: 0.01 a.u.f.s.

Fig. 2. Chromatogram of a saliva sample from a patient treated with Tegretol and Largactil. Peaks: 1 = unknown substance, probably a metabolite, 2 = caffeine, 3 = carbamazepine (4.17 μg/ml), 4 = carbamazepine-10,11-epoxide (0.20 μg/ml). The chromatographic conditions are the same as in Fig. 1, except detector sensitivity which was 0.02 a.u.f.s.

epoxide metabolite in saliva. It also should be valuable for assaying the drugs under study in paired samples of saliva and plasma in order to elucidate further the relationship between saliva and plasma levels of both carbamazepine and its epoxide metabolite.

We were able to find only one HPLC method designed for measuring carbamazepine levels in saliva. This method, reported by Westenberg et al. [11], has proved very valuable since it allowed the existence of a correlation to be established between carbamazepine levels in plasma and saliva. However, although the method has been applied to a large number of saliva samples from seven patients receiving carbamazepine as long-term medication, it did not allow the detection of the 10,11-epoxide metabolite in saliva. This is certainly due to the rather low sensitivity (0.4 μg/ml) for the metabolite. Our HPLC method, which is sensitive to 40 ng of 10,11-epoxide per ml, revealed the presence of the 10,11-epoxide in every saliva sample from patients receiving Tegretol, analysed so far.

Amidopyrine

The hexane—dichloromethane—acetonitrile—propylamine eluent was also used for the determination of amidopyrine in saliva and plasma. Optimization of the volume ratio of the mobile phase constituents was done in parallel on a LiChrosorb CN column and on a MicroPak CN column. Volume ratios of 70:25:5:0.1 and 25:50:25:0.1, respectively, giving capacity factors of 4.3 and 4.8, respectively, were found to be appropriate with respect to peak shape, retention, analysis time and interference from sample constituents and possible co-administered drugs. The substantial difference between both volume ratios once again shows that (1) considerable differences between CN columns of different manufacturers exist, although both columns are packed with the same packing material, and (2) brand-to-brand and lot-to-lot differences do not jeopardize the standardized strategy since the volume ratio of the mobile phase constituents can easily be adapted to the particular column and analysis problem.

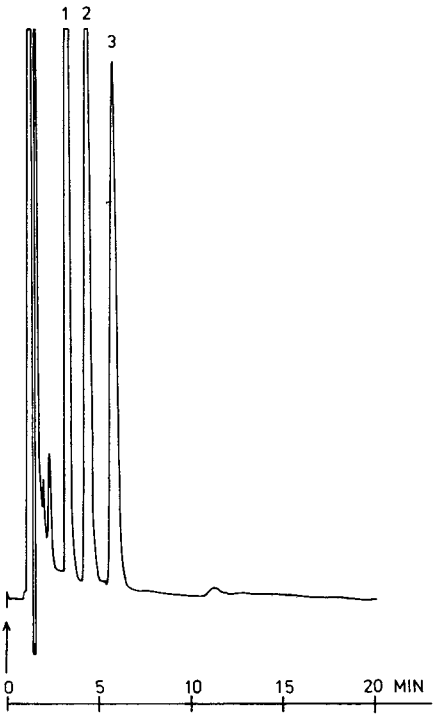
Although both columns combined with the appropriate mobile phase were equally efficient for the determination of amidopyrine in saliva and plasma, the MicroPak-CN column combined with hexane—dichloromethane—acetonitrile—propylamine (25:50:25:0.1) was chosen for subsequent use. This choice, however, is arbitrary. The retention times relative to amidopyrine of a number of drugs chromatographed to test for interference and for use as internal standard, are tabulated in Table III. Promethazine was chosen as internal standard.

TABLE III

RETENTION TIMES, RELATIVE TO AMIDOPYRINE, OF VARIOUS DRUGS

Drug	Relative retention time
Diazepam	0.38
Propyfenazone	0.40
Triflupromazine	0.50
Chlorimipramine	0.60
Amitriptyline	0.66
Procaine	0.73
Promethazine	0.74
Thioridazine	0.79
Imipramine	0.81
Amidopyrine*	1.00
Mesoridazine	1.55
Antipyrine	1.88
Diphenylhydantoin	2.17
Nortriptyline	2.24
Paracetamol	3.86
Primidone	3.97
Procainamide	>5
Phenobarbital	>5
Phenylbutazone	>5
Lidocaine	>5

*Retention time = 4.5 min.



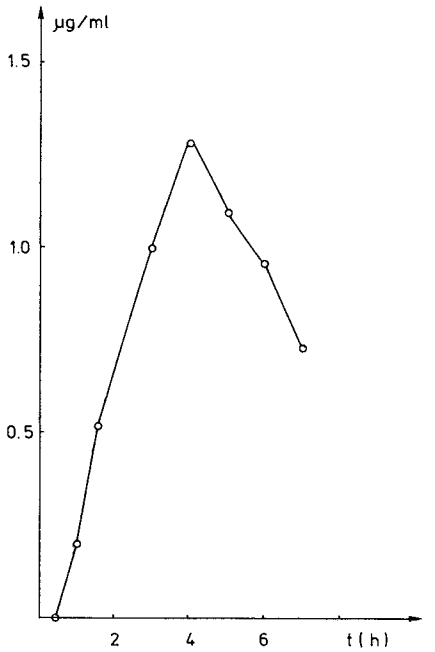


Fig. 3. Chromatogram of a spiked saliva extract. Peaks: 1 = caffeine, 2 = promethazine (internal standard), 3 = amidopyrine (1 µg/ml). Column: MicroPak CN-10 (10 µm, 300 × 4 mm). Mobile phase: *n*-hexane—dichloromethane—acetonitrile—propylamine (25:50:25:0.1). Flow-rate: 2 ml/min. Detector sensitivity: 0.02 a.u.f.s.

Fig. 4. Saliva concentration—time profile of a volunteer who ingested 300 mg of amidopyrine.

The analytical recoveries of amidopyrine from both saliva and plasma were assessed in the 1—3 µg/ml range. The mean values obtained were 94.7 ± 3.9% and 93.9 ± 4.2%, respectively (n = 7). Here also losses of about 10% occur when the evaporated extracts are stored at 4° C for 24 h. The calibration curves in both saliva and plasma were rectilinear up to at least 5 µg/ml (mean r^2 = 0.998). A chromatogram of a saliva standard is shown in Fig. 3. The within-day precision was evaluated at the 200 ng/ml and 3.0 µg/ml levels by analysing replicate spiked samples (n = 6). Coefficients of variation of 5.8% and 3.4%, respectively, were found, and the detection limit at a signal-to-noise ratio of 3 was estimated to be 20 ng/ml of saliva.

In order to demonstrate the usefulness of the method it was applied to a study of the pharmacokinetics of amidopyrine in one subject. The volunteer was given a single oral dose of 300 mg of amidopyrine; saliva samples were collected 0.5, 1, 1.5, 3, 4, 5, 6 and 7 h after ingestion of the cachet. The results are reported in Fig. 4 as a saliva concentration—time profile. It can be seen that the peak level is reached between 3 and 5 h after ingestion.

CONCLUSION

It can be concluded that the standardized analysis strategy which was previously shown to be applicable to pharmaceutical dosage forms and cosmetics, is also applicable to drug-level determinations in saliva. This was exemplified for carbamazepine and its active metabolite, on the one hand, and amidopyrine on the other.

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CHROMBIO. 2142

THE APPLICATION OF REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY TO IN VITRO DRUG METABOLISM STUDIES WITH N-ALKYLARYLAMINES

N.J. GOODERHAM* and J.W. GORROD*

Department of Pharmacy, Chelsea College, University of London, Manresa Road, London SW3 6LX (U.K.)

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SUMMARY

Analytical procedures have been investigated for the separation, detection, identification and quantitation of some metabolites of N-benzyl-4-substituted anilines. Techniques based on gas—liquid chromatography were investigated and found to be unsatisfactory. By the use of reversed-phase high-performance liquid chromatography with gradient and ion-pairing techniques, methods were devised for the simultaneous analyses of a variety of metabolites. The method involves minimum sample work-up (acetonitrile precipitation) and allows easy and prompt analysis in biological media avoiding undue decomposition of unstable metabolites.

INTRODUCTION

In the course of our studies on the in vitro metabolism of nitrogenous xenobiotics, we found N-benzyl-4-substituted anilines are converted to a variety of compounds. Our preliminary experiments with thin-layer chromatographic (TLC) techniques indicated that metabolism involved nitrogen-oxidation, N-dealkylation and ring-hydroxylation, the resulting products having diverse physiochemical characteristics, stability and quantities in which they were produced.

A review of the literature indicated numerous methods were available for the detection and quantitation of in vitro metabolic N-alkylaniline N-dealkylation including colorimetric assays based on the work of Brodie and Axelrod

*Present address: Department of Pharmacology, University of Minnesota, Minneapolis, MN 55455, U.S.A.

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[1] and Nash [2], radiometric [3] and gas—liquid chromatographic (GLC) techniques [4]. Similarly, ring-hydroxylated products of N-alkylanilines have been determined by spectrophotometry [5] and more recently by GLC [6—8]. In contrast, studies on N-alkylaniline N-oxidation have suffered from daunting analytical problems due to the inherent instability of these compounds. Previously, non-specific colorimetric analytical procedures, in which the primary N-oxidised product was inferred rather than demonstrated, were used [9], recently a colorimetric assay for primary and secondary arylalkyl hydroxylamino compounds in drug metabolism studies has been described [10]. Because of stability problems, GLC of this type of N-oxidised compounds has proved elusive, but not impossible, since GLC of the trimethylsilyl derivatives of the N-oxidised metabolite of N-methyl-4-aminoazobenzene has been reported [11]. High-performance liquid chromatography (HPLC) has been used in drug metabolism studies since it offers many advantages over more traditional analytical techniques, especially when dealing with particularly labile compounds, recently exemplified with N-hydroxynaphthylamines [12].

Since we wished to study in vitro N-dealkylation, ring-hydroxylation and N-oxidation of N-alkylanilines, we required a method of analysis whereby all these pathways could be followed simultaneously. Results with GLC partly met our criteria, however, analysis of N-oxidation products was unsatisfactory. Therefore, we turned our attention to HPLC and report here our findings for both analytical procedures.

EXPERIMENTAL

Materials

Aniline, 4-chloroaniline, 4-toluidine, N-benzylaniline · HCl, benzaldehyde and heptanesulphonic acid (sodium salt) were purchased from BDH (Poole, U.K.) as the purest grade available. Phenyl-N-(4-substituted)phenylnitrones and N-benzyl-N-(4-substituted)phenylhydroxylamines were synthesised as reported elsewhere [13]. The Schotten-Baumann reaction as described by Vogel [14] was used to prepare the N-benzoyl-4-substituted anilines, all melting points agreed with literature values. N-Benzyl-4-aminophenol was purchased from Eastman (Rochester, NY, U.S.A.). Acetonitrile (HPLC grade) was obtained from Rathburn Chemicals (Walkerburn, U.K.). All other solvents used were purchased from BDH and glass distilled prior to use.

Gas—liquid chromatography

A Perkin-Elmer F33 gas chromatograph equipped with a flame ionisation detector and a 0—2.5 mV Hitachi-Perkin-Elmer 159 chart recorder were used. Precoiled 1 m × 6.4 mm O.D. glass columns were packed as follows: Column A, 3% OV-17 on 80—100 mesh AW DMDCS treated Chromosorb G; Column B, 3% OV-1 on 80—100 mesh AW DMDCS treated Chromosorb G.

The columns were conditioned at a temperature 10°C higher than the operating temperature for 48 h. Prior to use, both columns were silanized in situ with 3 × 5 μl of hexamethyldisilazane. Gas pressures were nitrogen 140 kPa, hydrogen 119 kPa and air 175 kPa. Columns were connected to glass-lined inlet ports to minimise the risk of catalytic thermal breakdown. Solutions

of the test compounds were prepared in methanol (1 mg/ml) and 1—2 μl aliquots injected onto the column.

High-performance liquid chromatography

The liquid chromatograph comprised two pumps (Altex Model 110A), coupled to a mixing chamber and interfaced with a microprocessor solvent gradient programmer (Altex Model 420), a syringe loading sample injector valve (Rheodyne 7120) fitted with a 100-μl sample loop. Detection was a variable-wavelength UV spectrophotometer detector (Pye Unicam LC3) and chart recorder (Tekman TE200). The column was a 250 × 5 mm smooth bore seamless annealed 316 stainless-steel tube packed with Spherisorb 5 ODS (HPLC Technology, Wilmslow, U.K.), a microparticulate, 5-μm particle size, reversed-phase material consisting of a C_{18} stationary phase on a silica backbone. A 50 × 5 mm guard column packed with Partisil Co:Pell ODS (Whatman, Maidstone, U.K.), and placed immediately before the analytical column was routinely used. The columns were conditioned by passing appropriate mobile phase through the system for about 2 h prior to use.

Solvents used in the analyses (primarily water and acetonitrile) were filtered through glass fibre grade GF/F paper (Whatman), then degassed under vacuum for 10 min, followed by a 5-min purge with a fine stream of helium obtained using a sintered glass tube. When heptanesulphonic acid in acetic acid was incorporated in the mobile phase, it was added prior to helium purging.

Reference compounds were freshly prepared in methanol (1 mg/ml) and aliquots (10—20 μl) were injected onto the column.

Quantitative analysis of N-benzyl-4-substituted aniline metabolism by HPLC

Authentic samples of reference compounds were prepared in acetonitrile. Aliquots (100 μl) of these were used to spike typical microsomal incubates (3 ml) maintained on ice to give mixtures containing between 5 and 500 nmol of reference compound. The appropriate internal standard (100 nmol in 50 μl acetonitrile) was added and the contents of the flask were quantitatively transferred to a screw-capped tube (10 ml, Sovirel) using acetonitrile (2 ml) (HPLC grade). The tubes were capped, shaken and centrifuged at 10,000 *g* (Sorvall RB2 centrifuge) for 10 min to sediment the protein and particulate matter precipitated by the acetonitrile. The supernatant, an aqueous mixture of acetonitrile (60%), was transferred into fresh tubes and used directly for HPLC analysis. Aliquots (100 μl) were injected onto the column and peak height ratios of the reference compounds to internal standard were plotted against concentration.

RESULTS AND DISCUSSION

Gas—liquid chromatography

The retention times of the compounds are given in Table I. At the oven temperature required to obtain reasonable retention times, aniline and benzaldehyde eluted with the solvent front. Moreover, when aniline and benzaldehyde were injected simultaneously, they produced an additional peak with a retention time of 2.6 min on column A and 1 min on column B. Further

investigation indicated this peak was due to the formation of benzylideneaniline. Of the other compounds examined, N-benzylaniline, N-benzoylaniline and N-benzyl-4-aminophenol chromatographed satisfactorily.

Two peaks were produced when α,N-diphenynitrone was injected onto column A or B, although TLC indicated the reference sample was pure. The peak at retention time 12.6 min on column A and 4.4 min on column B, is probably due to the nitrone, whereas the peak at retention time 2.6 min on column A and 1.0 min on column B, is due to a breakdown product, probably benzylideneaniline. This was confirmed when the chromatographic characteristics of authentic benzylideneaniline were determined. N-Benzyl-N-phenylhydroxylamine was found to yield multiple products on both columns. Of these, the two major peaks had similar retention times to α,N-diphenylnitrone and benzylideneaniline, a third peak corresponded with that obtained for authentic N-benzylaniline.

TABLE I

GAS—LIQUID CHROMATOGRAPHIC SEPARATION OF N-BENZYLANILINE AND SOME POTENTIAL METABOLITES

Compound	Retention time (min)	
	Column A*	Column B**
Aniline	—	—
Benzaldehyde	—	—
N-Benzylaniline	3.0	1.3
Benzanilide	8.4	3.4
N-Benzyl-4-aminophenol	10.2	4.2
α,N-Diphenylnitrone***	12.6, 2.6	4.4, 1.0
N-Benzyl-N-phenylhydroxylamine***	12.6, 2.6, 3.0 + others	4.4, 1.0, 1.3 + others

*Column A: 3% OV-17 on 80—100 mesh AW DMDCS Chromosorb G; 1 m glass; nitrogen, 140 kPa; oven temperature 220° C; injection temperature 250° C.
**Column B: 3% OV-1 on 80—100 mesh AW DMDCS Chromosorb G; 1 m glass; nitrogen, 140 kPa; oven temperature 165° C; injection temperature 200° C.
***Indicates compounds decomposed during chromatography.

Re-examination of α,N-diphenylnitrone chromatographically demonstrated that the products obtained depended upon the amount of nitrone applied. When concentrations of the magnitude expected in metabolic experiments (100 nmol per 100 μl of extracted, concentrated sample) were injected, the peaks at retention time 12.6 min (column A), and 4.4 min (column B) disappeared leaving only the peaks due to benzylideneaniline. This degradation did not appear to be quantitatively reproducible. Therefore, GLC was rejected as a method suitable for the simultaneous determination of the major products of N-benzylaniline metabolism.

High-performance liquid chromatography

By employing mobile phase 1 and the gradient elution programme described in Table II, it was possible to resolve mixtures of N-benzyl-4-substituted

anilines and their potential metabolites. The various compounds eluted discretely, the peak shapes being symmetrical and reproducible, and could be analysed within a reasonable time. The metabolites of N-benzylaniline also chromatographed well under these conditions with the exception of α,N-diphenylnitrone and N-benzyl-4-aminophenol, which tended to co-chromatograph. A complication arose due to the appearance of a minor peak associated with the aminophenol. Aminophenols are easily oxidised in aqueous solution [15], N-benzyl-4-aminophenol is no exception, aqueous solutions of which quickly turn brown on standing, especially in bright light [6]. The oxidised intermediates produced in these reactions are probably of a quinoneimine structure. The minor peak associated with N-benzyl-4-aminophenol could be an ionised species present in equilibrium with the unionised compound, a phenomenon observed with other compounds during HPLC with aqueous mobile phases [16].

TABLE II

HPLC RETENTION TIMES FOR N-BENZYL-4-SUBSTITUTED ANILINES AND THEIR POTENTIAL METABOLITES

Mobile phase 1 (M1): pump A, water (distilled twice); pump B, acetonitrile.
Mobile phase 2 (M2): pump A, 0.005 *M* acetic acid containing 0.005 *M* heptanesulphonic acid (pH of mixture = 3.5 ± 0.1); pump B, acetonitrile containing 0.005 *M* acetic acid + 0.005 *M* heptanesulphonic acid
Programme: solvent gradient, flow-rate 1.5 ml/min; time zero, pump B = 39% of mobile phase, time 8 min, pump B = 39 to 60% of mobile phase in 5 min, time 17 min, pump B = 60 to 39% of mobile phase in 1 min.
Analytical column, 250 × 5 mm packed with Spherisorb 5 ODS; detection, UV 254 nm; temperature, ambient.

Compound	Retention time (min)	
	M1	M2
Aniline	4.5	4.7
Benzaldehyde	6.2	6.4
α,N-Diphenylnitrone	8.4	8.6
N-Benzoylaniline*	10.9	11.1
N-Benzyl-4-aminophenol	8.7	12.7
N-Benzyl-N-phenylhydroxylamine	14.2	14.8
N-Benzylaniline	17.2	18.0
4-Chloroaniline	7.2	ND**
α-Phenyl-N-(4-chloro)phenylnitrone	12.5	ND
N-Benzoyl-4-chloroaniline*	15.2	16.0
N-Benzyl-N-(4-chloro)phenylhydroxylamine	17.0	ND
N-Benzyl-4-chloroaniline	20.0	ND
4-Toluidine	5.7	ND
α-Phenyl-N-(4-tolyl)nitrone	11.2	ND
N-Benzoyl-4-toluidine	13.2	ND
N-Benzyl-N-(4-tolyl)hydroxylamine	14.8	ND
N-Benzyl-4-toluidine	18.8	ND

*Compound used as an internal standard.
**ND = not determined.

Further experiments were performed in which N-benzyl-4-aminophenol was chromatographed in the presence of heptanesulphonic acid (HSA), an ion-pairing agent. The results are presented in Table III. Heptane sulphonic acid did not alter chromatographic properties of α,N-diphenylnitrone or N-benzyl-4-aminophenol and just lowering the pH of the mobile phase with acetic acid appeared to cause breakdown of the aminophenol. However, lowering the pH of the medium with acetic acid enabled an ion-pair to be formed between HSA and N-benzyl-4-aminophenol. The resulting chromatography of the aminophenol is pH-dependent, being optimum at pH 3.5 with negligible change observed for the other potential metabolites (see Fig. 1a and b).

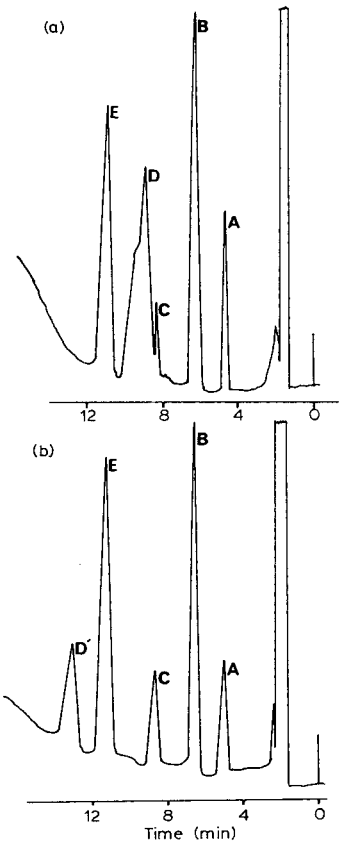

Fig. 1. The influence of heptanesulphonic acid and acetic acid on the HPLC separation of metabolites of N-benzylaniline. (a) Without HSA and acetic acid; mobile phase 1, water—acetonitrile. (b) With HSA and acetic acid; mobile phase 2, water—acetonitrile with 0.005 *M* HSA and 0.005 *M* acetic acid added to each component. Peaks: A = aniline; B = benzaldehyde; C = α,N-diphenylnitrone; D = N-benzyl-4-aminophenyl; D' = N-benzyl-4-aminophenol/HSA ion pair; E = benzanilide.

Quantitative analysis of metabolites of N-alkyl-4-substituted anilines using HPLC

Once optimum chromatographic conditions were established, calibration graphs were obtained for the potential metabolites with the exception of

TABLE III

EFFECT OF HEPTANE SULPHONIC ACID AND pH ON THE HPLC OF THE METABOLITES OF N-BENZYLANILINE

See Table II for details of programme and mobile phase 1.

Mobile phase	Retention time (min)				
	Aniline	Benz-aldehyde	α,N-Diphenyl-nitrone	N-Benzyl-4-aminophenol	Benz-anilide
Mobile phase 1	4.5	6.2	8.4	8.7	10.9
Mobile phase 1 + 0.005 *M* HSA + 0.001 *M* acetic acid (pump A, pH = 3.8)	4.6	6.3	8.6	10.2	11.1
Mobile phase 1 + 0.005 *M* HSA + 0.0025 *M* acetic acid (pump A, pH = 3.65)	4.6	6.3	8.6	12.2	11.1
Mobile phase 1 + 0.005 *M* HSA + 0.005 *M* acetic acid (pump A, pH = 3.5)*	4.7	6.4	8.6	12.7	11.1
Mobile phase 1 + 0.005 *M* HSA	4.5	6.2	8.4	8.7	10.9

*Equivalent to mobile phase 2 in Table II.

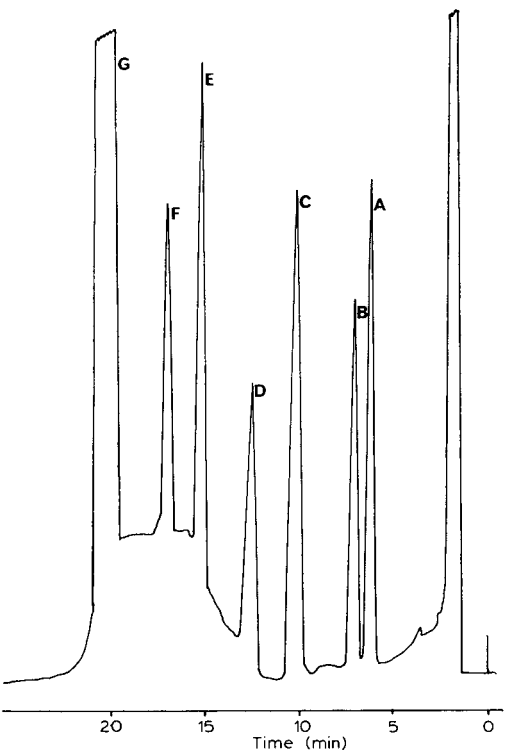

Fig. 2. HPLC separation of N-benzyl-4-chloroaniline and its metabolites. Peaks: A = benzaldehyde; B = 4-chloroaniline; C = benzanilide (internal standard); D = α-phenyl-N-(4-chloro)-phenylnitrone; E = N-benzoyl-4-chloroaniline; F = N-benzyl-N-(4-chloro)phenylhydroxylamine; G = N-benzyl-4-chloroaniline.

N-benzyl-N-(4-tolyl)hydroxylamine. This compound, which was the least stable disubstituted hydroxylamine synthesised, appeared to undergo almost total degradation in aqueous media at pH 7.4 to yield numerous products, predominantly the nitrone, together with benzaldehyde and the parent amine. Consequently, should N-benzyl-N-(4-tolyl)hydroxylamine be metabolically formed, it would almost certainly undergo spontaneous decomposition whilst in its incubation media. Therefore, it was not included in the spiked solutions.

Some of the compounds tested have low aqueous solubility and problems of co-precipitation (with protein) were envisaged during sample work-up. However, careful handling, including keeping samples cool, but avoiding temperatures of less than 10° C, ensuring minimum analytical delay and the storage of samples in the dark prior to analysis, avoided problems. Plots of amounts of compound present in the mixture against peak heights recorded after chromatography demonstrated linear correlations with coefficients greater than 0.997. A chromatogram showing the separation of N-benzyl-4-chloroaniline and its metabolites in a spiked microsomal incubate is given as an example in Fig. 2.

Complete separation of N-benzyl-4-substituted anilines and their potential metabolites in biological media has been achieved by reversed-phase gradient elution HPLC. In one instance, separation was facilitated by the use of an ion-pairing technique. Our procedures are relatively straightforward and allow diect analysis of products of in vitro metabolism. The methods described avoid the ambiguity associated with former analytical methods and have been used to investigate the enzymology involved in the in vitro metabolism of N-benzyl-4-substituted anilines [16].

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DETERMINATION OF OMEPRAZOLE AND METABOLITES IN PLASMA AND URINE BY LIQUID CHROMATOGRAPHY

PER-OLOF LAGERSTRÖM* and BENGT-ARNE PERSSON

Department of Analytical Chemistry, AB Hässle, S-431 83 Mölndal (Sweden)

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SUMMARY

Omeprazole, a substituted benzimidazole and a new gastric acid inhibitor, has been determined in plasma and urine, together with three of its metabolites — the sulphide, the sulphone and the hydroxy compound. The methods comprise extraction from the biological materials with methylene chloride, followed either by direct injection of the extract onto a normal-phase liquid chromatography column or evaporation, dissolution and injection onto a reversed-phase system. The compounds were detected using ultraviolet spectrometry. The absolute recoveries obtained were mostly above 95%. The minimum determinable concentration for omeprazole was 20 nmol/l in plasma (relative standard deviation 10—15%) and 50 nmol/l in urine. The metabolites could also be determined at the same levels.

INTRODUCTION

Studies both in vitro [1, 2] and in vivo [3, 4] have shown that omeprazole, 5-methoxy-2-{[(4-methoxy-3,5-dimethyl-2-pyridinyl)methyl]sulfinyl}-1H-benzimidazole (Fig. 1, I), is a potent inhibitor of gastric acid secretion. The mechanism of omeprazole is different from that of H_2-receptor antagonists and anticholinergic agents and it functions by direct interaction with the proposed gastric proton pump, the H^+, K^+-ATPase [5]. This may be a highly selective clinical means of suppressing the acid secretory process. The effect of omeprazole in dog and in man has been shown not to be correlated to the peak concentration in plasma but to the area under the plasma curve. Since a large number of blood plasma samples have continuously been generated in the documentation of this new drug, a simple method to determine omeprazole in plasma was required to monitor these samples.

The metabolism of omeprazole has been studied by Hoffmann et al. [6]. This present paper describes methods for the determination of omeprazole and three

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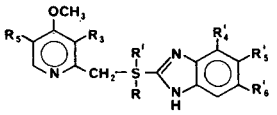

	R_3	R_5	R	R'	R_4'	R_5'	R_6'
Omeprazole(I)	CH_3	CH_3	O	-	H	OCH_3	H
Sulphone (II)	CH_3	CH_3	O	O	H	OCH_3	H
Sulphide (III)	CH_3	CH_3	-	-	H	OCH_3	H
Hydroxyomeprazole (IV)	CH_3	CH_2OH	O	-	H	OCH_3	H
H 168/24(V)	CH_3	CH_3	O	-	H	CH_3	H
H 153/52(VI)	H	H	O	-	CH_3	H	CH_3

Fig. 1. Chemical structures of omeprazole, metabolites and internal standards.

of its metabolites (Fig. 1) — the sulphone (II) and the sulphide (III) in plasma, and the hydroxy metabolite (IV) in plasma and urine. The methods comprise extraction from plasma into methylene chloride followed either by direct injection of part of the organic extract onto a normal-phase liquid chromatography (LC) column or, for the more polar hydroxy metabolite, evaporation of the organic extract, dissolution into an aqueous phase and injection onto a reversed-phase column. The compounds are detected by an ultraviolet (UV) monitor.

Using the method for omeprazole in plasma more than 25,000 analyses have been performed during the last five years. This large number of analyses also initiated the development of a totally automatic method based on Technicon's FAST®-LC system [7].

Recently, a paper on the reversed-phase LC determination of omeprazole has been published [8].

EXPERIMENTAL

Apparatus

The liquid chromatograph was composed of an Altex 110A LC-pump (Altex Scientific, Berkeley, CA, U.S.A.) and an LDC Spectromonitor III (Laboratory Data Control, Riviera Beach, FL, U.S.A.) UV detector. The automatic injector was either a Waters WISP 710B (Waters Assoc., Milford, MA, U.S.A.) or a Kontron MSI 660 (Kontron Electrolab, London, U.K.) using an injection volume of 150 μl. The separation columns were of precision-bore stainless steel (150 × 4.5 mm) with end fittings of modified Swagelok connections and were home-packed either with LiChrosorb Si 60, 5 μm (E. Merck, Darmstadt, F.R.G.) or with Polygosil C_{18}, 5 μm (Macherey-Nagel, Düren, F.R.G.). In the reversed-phase system a precolumn was used (Brownlee Labs, Spheri-5, RP-8, 30 × 4.6 mm).

Reagents

Methylene chloride, methanol and ammonium hydroxide solution (25%) (pro analysi grade, Merck) were used. Acetonitrile was of HPLC grade (Rathburn Chemicals, Scotland, U.K.). All reagent and buffer solutions were prepared with analytical reagent grade chemicals. Omeprazole (Hässle, Mölndal, Sweden) fulfilled the quality requirements of the Pharmacopoeia Nordica.

Omeprazole, the metabolites and internal standards were supplied by the Department of Organic Chemistry, Hässle. For chemical structures, see Fig. 1.

Standard solutions

A standard solution for plasma determination of omeprazole and metabolites (60 μmol/l) was prepared by dissolving 2 mg of each compound in 20 ml of methanol and diluting to 100 ml with carbonate buffer pH 9.3, $I = 0.1$. A 100-μl volume of the standard solution was added to a large number of 5-ml centrifuge tubes which were kept frozen at −18°C for no longer than three months. Plasma standards were prepared by adding 1 ml of blank plasma to the tubes containing standard solution, at the time of analysis.

For the urine analysis, the standard solution used had double the concentration of each compound (120 μmol/l). Again, 100 μl of standard solution were stored at −18°C in centrifuge tubes and 1 ml of urine was added to make a urine standard, at the time of analysis.

Stock solutions of internal standard for omeprazole in plasma (VI) and for hydroxyomeprazole in plasma and urine (V) contained 5—10 mg per 100 ml of methanol—carbonate buffer, and were kept in a refrigerator for not more than one month.

Sample preparation

Plasma. The plasma method was optimized for the determination of omeprazole and the sulphone. The concentration of the sulphide in human plasma is usually too low to be determined. The frozen plasma sample is allowed to thaw at room temperature and is mixed and centrifuged. A 1-ml aliquot of the sample is transferred to a centrifuge tube, mixed with 100 μl of sodium dihydrogen phosphate 1 mol/l (final pH 6.5—7.0) and 100 μl of the internal standard solution (VI) and is then extracted with 1 ml of methylene chloride by shaking for 10 min. After centrifugation twice for 10 min at 2500 *g*, the aqueous upper layer is aspirated and discarded. Part of the organic layer is transferred to sample vials for the automatic injector; 150 μl are injected onto the normal-phase LC column (Fig. 2).

In certain series of plasma samples it was also of interest to determine the content of the sulphide (III); for example, if the sulphide had been given as a drug, in which case V was used as internal standard instead of VI. Furthermore, a slight modification in mobile phase composition was made (Fig. 3).

For the determination of the more hydrophilic hydroxy metabolite (IV) in plasma, the following procedure was used. The frozen plasma sample is allowed to thaw at room temperature and is mixed and centrifuged. Then 1 ml of the sample is transferred to a centrifuge tube, mixed with 100 μl of sodium dihydrogen phosphate 1 mol/l (final pH 6.5—7.0) and 100 μl of the internal standard (V), and is then extracted into 10 ml of methylene chloride by shaking for 10 min. After centrifugation for 10 min (2500 *g*), the aqueous layer is aspirated and discarded. An 8-ml volume of the organic layer is transferred to a conical centrifuge tube and evaporated under nitrogen flow. The residue is dissolved in 500 μl of 20% acetonitrile + 80% phosphate buffer pH 7.5, $I = 0.05$, and 150 μl are injected by means of the automatic injector onto the reversed-phase column (Fig. 4).

Urine (omeprazole, its sulphone and hydroxyomeprazole). The procedure is the same as for the determination of hydroxyomeprazole in plasma (Fig. 5).

Chromatography

The chromatographic separation for the plasma method (omeprazole and the sulphone) is made on a silica column with a mobile phase of methylene chloride containing 3.5% of a solution of 5% of concentrated ammonium hydroxide in methanol. For the determination of omeprazole sulphide (III) the methanol content was decreased to 2.0%. The flow-rate was 1.5 ml/min and the eluent was monitored by UV detection at 302 nm.

In the method for hydroxyomeprazole in plasma and for omeprazole and metabolites in urine, a reversed-phase system is used with a mobile phase containing acetonitrile and phosphate buffer pH 7.5 (I = 0.05) (30:70, v/v). The flow-rate was 1 ml/min and the detector wavelength the same as in the normal-phase method.

Quantification is based on peak height measurements and internal standardization.

Determination of distribution ratios

The distribution ratios for omeprazole, the sulphone (II), the sulphide (III), the hydroxy metabolite (IV) and the internal standards (V and VI) between methylene chloride and water at pH 6.5—7.0 were determined by equilibration in centrifuge tubes. As aqueous phase, phosphate buffer solutions (I = 0.10) were used. After phase separation by centrifugation, the concentration of the compounds in the organic phase was determined by LC. In the aqueous phase the concentration was determined after repeated extraction of an aliquot by methylene cloride, and measurement in the organic phase.

Stability

Standard solutions of omeprazole were kept at pH 9 to ensure good stability during storage. The stability of omeprazole at −18° C in plasma at pH 7.5 and pH 9 was studied. Authentic plasma samples were divided in two parts, and carbonate buffer was added to one of the samples to give a final pH 9. The two samples were then divided into several samples to provide a sufficient number of samples for a long-term study. Samples were then analysed over a period of one year.

Another study was performed in which authentic plasma samples stored at room temperature for 0, 1, 2, 3 and 4 days were analysed for omeprazole.

RESULTS AND DISCUSSION

Extraction

Omeprazole, the metabolites discussed here and the internal standards V and VI are ampholytes and have two dissociation constants: 2—5 for the pyridine nitrogen and 8—11 for the imidazole nitrogen (Table I). This means that a pH between 6 and 7 is appropriate for extraction of the compounds into an organic phase. Omeprazole is easily extracted into methylene chloride. The distribution ratios for the compounds studied are shown in Table I. Using equal

TABLE I

DISTRIBUTION RATIOS (D) OF OMEPRAZOLE, METABOLITES AND INTERNAL STANDARDS BETWEEN METHYLENE CHLORIDE AND PHOSPHATE BUFFER SOLUTIONS

pH 6.5—7.0, I = 0.10. pK^* is the mixed dissociation constant.

Compound	D	p$K^{*\,*}_{(1)}$	p$K^{*\,**}_{(2)}$
Omeprazole (I)	180	4.2	9.0
Sulphone (II)	300	3.5	7.8
Sulphide (III)	3100	5.2	11.5
Hydroxyomeprazole (IV)	2.4	2.1	9.0
V	450	4.2	9.2
VI	160	4.3	9.4

*p$K^*_{(1)}$ refers to the pyridine nitrogen [9].

**p$K^*_{(2)}$ refers to the benzimidazole nitrogen [9].

phase volumes, the theoretical absolute recovery is ⩾ 99% for all of the compounds except for the hydroxy metabolite. For the extraction from plasma, equal phase volumes were used, while an eight times larger volume of organic phase was needed to obtain a high recovery for the more polar hydroxy metabolite.

Chromatography

Normal-phase systems. In the determination of omeprazole in plasma a normal-phase separation system was chosen since the sample work-up could be limited to extraction into methylene chloride and injection of an aliquot of the extract onto the LC column. Moreover, the chromatograms are relatively free from interfering peaks. The mobile phase contains methylene chloride as main component as in the extraction solvent, and with dilute ammonia solution in methanol as modifier. The concentration of ammonia is sufficiently low not to be deleterious for the stationary phase and the columns show good long-term stability. The normal-phase method is optimized for the determination of omeprazole and its sulphone (II), the latter being subject to slight interference by an adjacent peak (Fig. 2). In a limited number of studies the sulphide had to be determined and the methanol content was then lowered from 3.5% to 2.0% to give this compound a suitable retention. Using this phase, omeprazole and the sulphone could be determined as well, but with a 2—3 times lower sensitivity because of the larger retention volume (Fig. 3).

Reversed-phase methods. In urine, the compound of most interest seems to be the hydroxy metabolite (IV) [6], and the analytical method was thus focused on the determination of this compound. A reversed-phase system was chosen in which the hydroxy metabolite (IV) elutes ahead of omeprazole and the sulphone. The pH of the mobile phase was 7.5 to ensure stability of both the compounds and the stationary phase. After extraction into methylene chloride and evaporation of the solvent, the residue is dissolved in a solvent containing a lower content of acetonitrile than in the mobile phase in order to obtain a concentration in the starting zone of the column. For the determination of hydroxyomeprazole in plasma the same sample preparation and chromatographic system were used (Fig. 4).

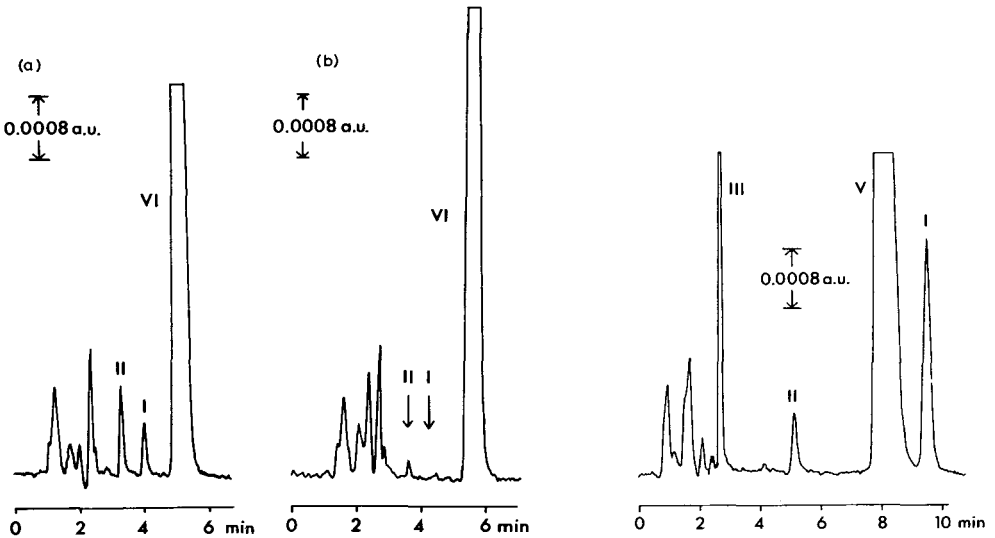

Fig. 2. Omeprazole (I) and the sulphone (II) in a plasma sample from a patient administered omeprazole. Packing material: LiChrosorb Si 60, 5 μm. Mobile phase: methanol (containing 5% of 25% ammonium hydroxide)—methylene chloride (3.5:96.5, v/v). (a) Sample: 150 μl of an extract from 1 ml of plasma containing omeprazole (I) 70 nmol/l and the sulphone (II) 110 nmol/l. (b) Sample: blank plasma.

Fig. 3. Omeprazole (I), the sulphone (II) and the sulphide (III) in a plasma sample from a patient administered omeprazole. Packing material: LiChrosorb Si 60, 5 μm. Mobile phase: methanol (containing 5% of 25% ammonium hydroxide)—methylene chloride (2.0:98.0, v/v). Sample: 150 μl of an extract from 1 ml of plasma containing the sulphide (III) 910 nmol/l, the sulphone (II) 170 nmol/l and omeprazole (I) 1300 nmol/l.

Selectivity

In both the normal-phase and the reversed-phase systems the separation between omeprazole and its main metabolites — the sulphone, sulphide and hydroxy compound — and the internal standards used was quite sufficient (Figs. 2—5). There is no interference in the chromatograms from the H_2-receptor antagonists, cimetidine and ranitidine, if they by chance should be present in the sample.

Stability

The results of the stability studies of omeprazole and its sulphone in authentic plasma samples (Table II) show clearly that plasma samples of omeprazole can be stored without any significant degradation at −18°C for one year. No significant difference was seen between storage at pH 9 and pH 7.5. Neither did four days at room temperature produce any degradation.

Urine samples for analysis were collected in bottles containing 2.5 ml of 1 mol/l sodium carbonate per hour of collection period, to buffer the urine to at least pH 8.

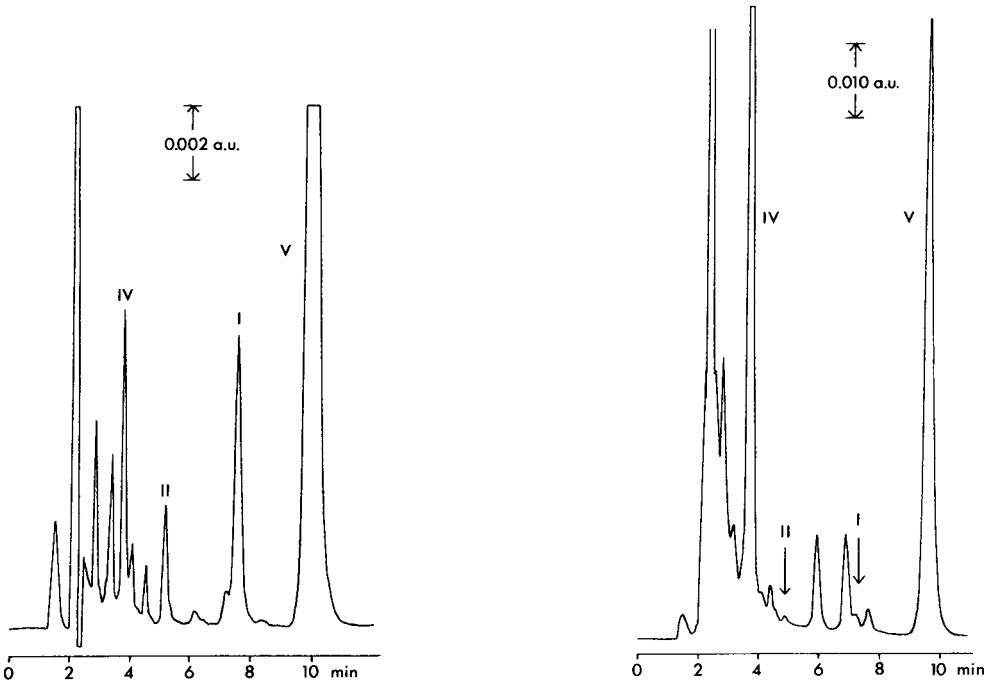

Fig. 4. Omeprazole (I), the sulphone (II) and the hydroxy metabolite (IV) in a plasma sample from a patient administered omeprazole. Packing material: Polygosil C_{18}, 5 μm. Mobile phase: 30% of acetonitrile in phosphate buffer pH 7.5, I = 0.05. Sample: 150 μl of an extract from 1.0 ml of plasma containing omeprazole (I) 650 nmol/l, the sulphone (II) 170 nmol/l and hydroxyomeprazole (IV) 350 nmol/l.

Fig. 5. Omeprazole (I), the sulphone (II) and the hydroxy metabolite (IV) in a urine sample from a patient administered omeprazole. Packing material: Polygosil C_{18}, 5 μm. Mobile phase: 30% of acetonitrile in phosphate buffer pH 7.5, I = 0.05. Sample: 150 μl of an extract from 1.0 ml of urine containing hydroxyomeprazole (IV) 5.5 μmol/l and < 100 nmol/l of omeprazole (I) and the sulphone (II).

Recovery and repeatability

Ten identical plasma samples containing either omeprazole, the sulphone and VI or the sulphide and V were analysed and the results were compared to direct injections of the corresponding compounds dissolved in methylene chloride. The results for the absolute recovery and the repeatability are given in Table III. As can be seen, the recovery for all of the compounds is more than 95%. The repeatability for omeprazole, the sulphone and sulphide is excellent. The minimum determinable concentration, defined as the level at which the relative standard deviation is 10—15%, is about 20 nmol/l for omeprazole and the sulphone, and about 50 nmol/l for the sulphide.

The recovery and repeatability for hydroxyomeprazole in plasma after reversed-phase chromatography are also shown in Table III.

The linearity ranges from 25—50 nmol/l to 50—100 μmol/l of plasma or urine.

In urine, the recovery and repeatability for omeprazole, the sulphone, the

TABLE II

STABILITY OF OMEPRAZOLE (I) AND THE SULPHONE (II) IN TWO PLASMA SAMPLES FROM PATIENTS ADMINISTERED OMEPRAZOLE

A: stored at −18°C at pH 7.5 and 9. B: stored in the dark at room temperature (+23°C). Initial concentration of omeprazole 2 μmol/l and of the sulphone 0.5 μmol/l.

Storage time		pH	Percentage of initial concentration	
			Omeprazole	Sulphone
A	1 (weeks)	7.5	100	100
		9	100	100
	5	7.5	104	97
		9	107	101
	15	7.5	102	101
		9	103	104
	52	7.5	102	99
		9	104	103
B	0 (days)	7.5	100	100
	1	7.5	103	97
	2	7.5	109	114
	3	7.5	110	104
	4	7.5	101	109

TABLE III

REPEATABILITY AND RECOVERY IN THE DETERMINATION OF OMEPRAZOLE, METABOLITES AND INTERNAL STANDARDS IN PLASMA AND URINE

The recovery given is the absolute recovery (n = 10).

Compound	Concentration (μmol/l)	Repeatability (S.D., %)	Recovery (%)	
			Calculated	Obtained
Plasma				
Omeprazole (I)	3.00	1.1	99	100
	0.30	3.5	99	98
	0.03	9.7	99	95
Sulphone (II)	2.60	1.5	99	98
	0.10	5.5	99	94
Sulphide (III)	6.30	1.0	99	102
	0.16	2.6	99	96
Hydroxyomeprazole (IV)	3.00	1.5	92	90
	0.10	5.7	92	87
V	3.00	1.5	99	98
VI	3.00	1.5	99	95
Urine				
Omeprazole (I)	100	1.5	99	93
	10	3.2	99	95
Sulphone (II)	10	1.5	99	95
Hydroxyomeprazole (IV)	10	1.5	92	90
	0.2	4.8	92	87
V	10	1.2	99	95

hydroxy metabolite and the internal standard V were determined in the same manner as in plasma. The results are shown in Table III. All recoveries are better than 95%, except for hydroxyomeprazole which has a recovery of only 85%. The actual concentrations of omeprazole and the sulphone in authentic samples are very low. The amounts excreted are much less than 1% of the given dose. The concentration of the main metabolite in urine, hydroxyomeprazole, is more than ten times higher. The minimum determinable concentration is 30 nmol/l for the hydroxy compound and 50 nmol/l for omeprazole and the sulphone, using 1 ml of urine.

Method reproducibility

The long-term reproducibility of the main plasma method was studied by analysis of identical samples. A large number of drug-free plasma samples was spiked with the same concentration of omeprazole and kept frozen for up to five months. During this time two samples were analysed each day of analysis. The results from five studies are given in Table IV and show that the mean value of each study lies between 99% and 103% of the nominal value, with a relative standard deviation of 3—4.5%.

TABLE IV

REPRODUCIBILITY OF THE NORMAL-PHASE PLASMA METHOD FOR OMEPRAZOLE

Identical plasma samples (spiked samples) were analysed, two samples per day of analysis, over a longer period of time. $\bar{m}$ = mean value of the results from each study expressed as a percentage of the nominal value. Concentration of omeprazole = 5 μmol/l.

Study No.	*n*	$\bar{m}$ (%)	S.D. (%)
A 106	94	99	4.0
A 109	92	104	3.3
A 203	42	101	4.5
A 207	64	99	4.3
A 211	67	99	3.5

Method comparison

Comparisons were made between two separate LC methods for omeprazole assay in plasma, the normal-phase method and a method based on Technicon's

TABLE V

COMPARISON BETWEEN A FAST-LC METHOD [7] AND THE NORMAL-PHASE METHOD FOR DETERMINATION OF OMEPRAZOLE IN PLASMA

Plasma samples from patients administered omeprazole were analysed.

Study No.	Mean ratio (FAST-LC/ normal phase)	S.D. (%)	*n*
1	0.97	7.0	61
2	1.01	8.7	100
3	0.95	7.0	45

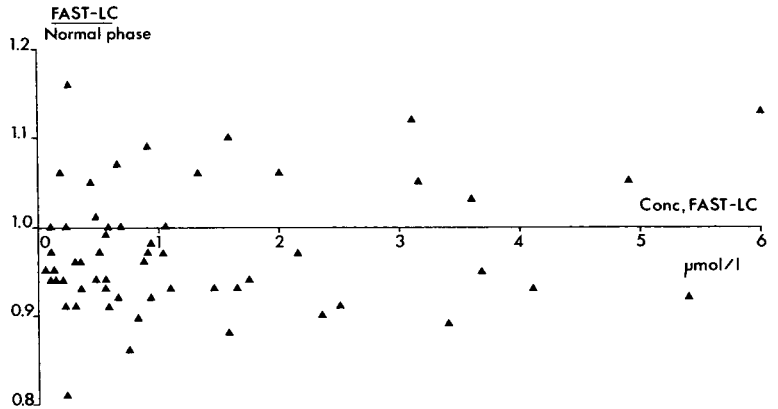

Fig. 6. Comparison between a FAST-LC method [7] and the normal-phase method for the determination of omeprazole in plasma. Plasma samples are from patients administered omeprazole. $n = 61$, $\overline{m} = 0.97$, S.D. = 7.0%.

Fully Automated Sample Treatment LC, FAST-LC [7]. The main differences between the methods are that the FAST-LC method comprises a fully automated sample work-up step including extraction in coils into isopropanol—chloroform, evaporation, dissolution in a dilute mobile aqueous phase and injection onto a reversed-phase LC column. The results from three studies are presented in Table V. As can be seen, the mean quotients of the results of the two methods are around 1.00 and the standard deviation of the quotients 7—9%. The concentration of the samples varied from 0.1 to 10 μmol/l of plasma. One comparative study is featured in Fig. 6. A detailed presentation of the FAST-LC method will be published shortly [7].

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AUTOMATED ANALYSIS OF ANTIEPILEPTIC DRUGS IN SERUM BY COLUMN-SWITCHING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

ALBERT NAZARETH*, LUIS JARAMILLO**, BARRY L. KARGER and ROGER W. GIESE*

Barnett Institute of Chemical Analysis and Materials Science, and College of Pharmacy and Allied Health Professions, Northeastern University, 360 Huntington Avenue, Boston, MA 02115 (U.S.A.)

and

LLOYD R. SNYDER

L.R. Snyder Inc., 2281 William Court, Yorktown Heights, NY 10598 (U.S.A.)

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SUMMARY

An automated high-performance liquid chromatographic column-switching system is presented for the analysis of antiepileptic drugs in serum. Initial results show that a reversed-phase extraction column works best overall when fitted with screens versus frits, and when packed with porous 30-μm particles as opposed to a pellicular packing of similar size or with smaller porous particles. The continuous analysis of primidone for over 2000 serum samples is achieved at a rate of twelve samples per hour with a single analytical column. An analogous boxcar high-performance liquid chromatographic system is also assembled and used to analyze two of four injected antiepileptic drugs at a rate of 40 samples per hour. For 1000 of these analyses, the coefficient of variation is 1% without an internal standard.

INTRODUCTION

The use of column-switching techniques for automated sample cleanup in high-performance liquid chromatography (HPLC) has been recently reviewed [1] with articles continuing to appear on this general method [2—8]. The

*Present address: Carter Wallace, Inc., Cranbury, NJ, U.S.A.
**Present address: Gillette Co., Boston, MA, U.S.A.

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major significance of this approach is that sample pretreatment has often been the rate-limiting step in analysis by HPLC.

In this article we extend column-switching HPLC to the analysis of antiepileptic drugs in serum. After first investigating the characteristics of particle size and type, screen versus frit end-fittings, and washing conditions in a reversed-phase extraction precolumn, we then illustrate a fully automated system that maintains high performance throughout the continuous analysis of over 2000 serum injections. We also demonstrate the capability and enhanced throughput of a boxcar configuration for our automated system.

EXPERIMENTAL

Apparatus

The fully automated HPLC system shown in Fig. 1 was constructed from a dual-piston Model 100 pump (Altex, Berkeley, CA, U.S.A.); a Model M 6000 HPLC pump (Waters Assoc., Milford, MA, U.S.A.); an autosampler and a peristaltic pump (Technicon Instruments, Tarrytown, NY, U.S.A.); two Model CV-6-UHPa-N60 pneumatically driven (nitrogen) six-port switching valves (Valco Instruments, Houston, TX, U.S.A.); and a Model 7010 sample injection valve (Rheodyne, Cotati, CA, U.S.A.), pneumatically driven with helium as described in the text. The injection valves and the autosampler were controlled by a Micromaster Model WP 6001 digital programmable timer (Minarik Electric, Los Angeles, CA, U.S.A.). Detection was carried out at 214 nm with a UV III spectrometer equipped with zinc source supply (Laboratory Data Control, Riviera Beach, FL, U.S.A.), using a dual-pen recorder (Linear Instruments, Reno, NV, U.S.A.).

The chromatographic system with boxcar capability involved two Model 110 A high-pressure pumps (Altex), a Series E-120 low-pressure pump (Eldex Labs., Menlo Park, CA, U.S.A.); a FAST-LC variable-wavelength photometric detector, equipped with a 12-μl flow cell (Technicon Instruments); and a SP-4100 computing integrator (Spectra-Physics, San Jose, CA, U.S.A.). The remaining components were the same as for the above system.

Stationary phases and columns

The analytical column was a FAST LC-8 column (150 × 4.6 mm) packed with 5-μm particles of porous silica bonded with dimethyl octylchlorosilane (Technicon Instruments); the short column was a 50 × 4.6 mm column packed with 5-μm particles of C_8 bonded phase packing material (Supelco, Bellefonte, PA, U.S.A.); and the extraction column, unless noted otherwise, was a 25 × 3.9 mm guard column (Waters Assoc.) dry-packed with 30-μm reversed-phase particles, LiChrosorb Si 60 (Supelco) bonded with C_{18} in our laboratory.

Other stationary phases employed for dry packing the extraction column included C_{18} Corasil®, 37—50 μm particle size (Waters Assoc.); LiChrosorb RP-18, 10 μm particle size (Alltech Assoc., Deerfield, IL, U.S.A.); and Supelcosil C_8, 5-μm particles (Supelco).

Reagents

Primidone and phenytoin were obtained from Applied Science Labs.

(Deerfield, IL, U.S.A.). Phenobarbital and carbamazepine were supplied by Technicon Instruments. Stock solutions of the antiepileptic drugs were made in methanol. Dilutions of the stock standards were made with pooled serum as required. Methanol was of chromatographic grade (J.T. Baker, Phillipsburg, NJ, U.S.A.). Sequanal grade triethylamine was purchased from Pierce Chemical (Rockford, IL, U.S.A.). Glass-distilled water was used throughout.

Analytical mobile phase

This reagent was 2.5 m*M* sodium dihydrogen phosphate in 45% methanol and contained 65 μl/l triethylamine. It was passed through a 0.45-μm filter (Millipore, Bedford, MA, U.S.A.) and degassed in vacuo prior to use.

Washing solution

Glass-distilled water degassed in vacuo was used throughout. Buffer was 0.1 *M* sodium monophosphate adjusted to pH 3.5 with phosphoric acid, and filtered as above.

Defibrination of plasma

Outdated, pooled blood bank plasma (1 l) was defibrinated by the addition of 2.22 g of calcium chloride followed by stirring for 1 h with a glass rod. After standing at room temperature for 2 h, the jelly-like mass was broken up with a glass rod and the clear supernatant was filtered through a Whatman filter paper, centrifuged, and frozen overnight. The supernatant was then thawed out at room temperature and filtered before use, giving a clear filtrate. The unused portion was frozen and re-filtered before use.

RESULTS AND DISCUSSION

Basic configuration

The basic configuration of our fully automated HPLC system with column-switching is shown in Fig. 1. Included is a six-port Valco loading valve (V_1) with a sample loop (L) and a six-port Valco injection valve (V_2), with a small,

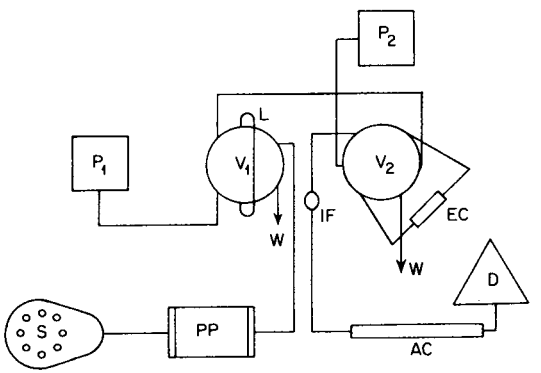

Fig. 1. Configuration of the fully automated HPLC system with on-line, solid-phase sample cleanup. S, auto sampler; PP, peristaltic pump; L, sample loop; V_1 and V_2, six-port switching valves; W, waste; P_2, mobile-phase pump; P_1, wash pump; EC, extraction column; AC, analytical column; IF, in-line filter; and D, detector.

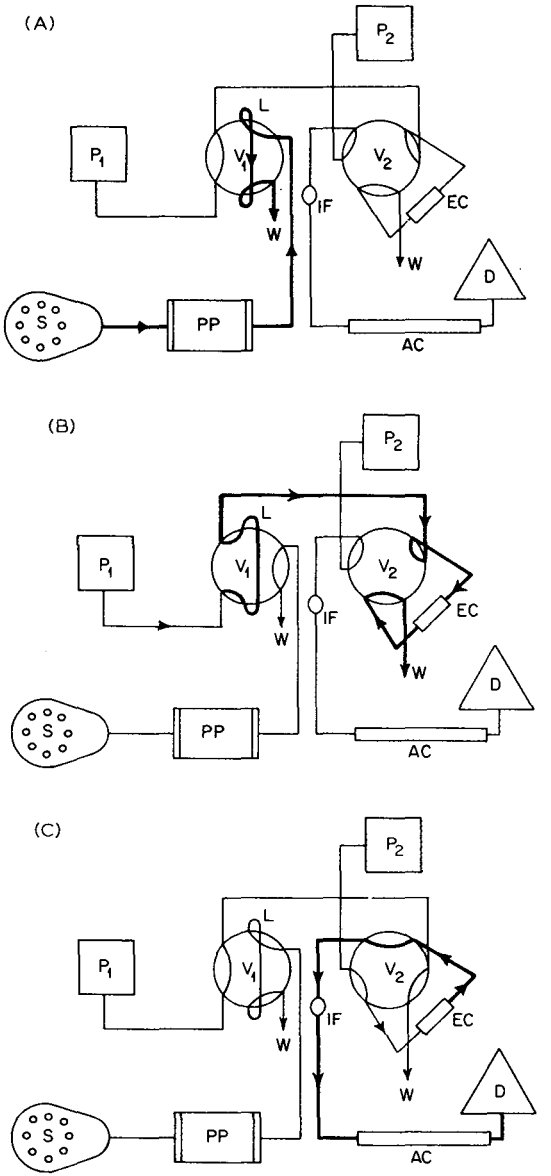

Fig. 2. Working cycle of the automated HPLC system involving solid-phase extraction and column-switching with the sample flow paths as heavy lines. (A) Activiation. The autosampler (S) is activated by the programmable timer to pick up the sample and fill the sample loop (L) by means of the peristaltic pump (PP) with excess going to waste (W). Simultaneously, the extraction column (EC) is equilibrated with flushing solvent (distilled water or buffer, pH 3.5) delivered by wash pump (P_1). (B) Loading. The loading valve (V_1) is rotated and the sample is washed onto the extraction column (EC). Since the extraction column is packed with reversed-phase material and conditioned with aqueous wash, the analyte(s) are adsorbed and enriched at the top of this column while the hydrophilic substances are washed through the extraction column to waste (W). (C) Injection. The injection valve (V_2) is next reversed and the sample components are back-flushed onto the analytical column (AC) for separation and analysis.

dry-packed extraction column (EC) for solid-phase extraction. The analytical column (AC) and the mobile-phase pump (P_2) are connected to V_2 whereas the wash pump (P_1) is connected to the V_1 loading valve, as is the autosampler (S) in series with the peristaltic pump (PP). The autosampler and the two valves are actuated automatically by a programmable timer based on a fixed time schedule. The whole working cycle in the analysis of a typical serum sample consists of an activation step, a loading step and an injection step, as illustrated and described in Fig. 2A—C. The heavy lines represent the flow-paths of the sample.

Extraction column

At the outset of our investigation, we were particularly encouraged by the results of Roth et al. [9] in the use of column-switching as a cleanup tool. These workers had reported on a system that successfully analyzed drugs in directly injected plasma, urine, or saliva samples. The system was based on the use of an extraction precolumn comprised of either reversed-phase or ion-exchange material, having a particle size of about 20—50 μm. After the drug was adsorbed from the sample, the precolumn was washed with water prior to elution of the drug with a backflush of mobile phase onto the analytical column. For a reversed-phase extraction precolumn, at least 1000 analyses could be conducted with the use of two such precolumns operated in parallel without a need for replacing the columns.

We began with a system similar to that reported by Roth et al. [9], having the components and configurations shown in Fig. 1, except for the sample delivery components (autosampler, peristaltic pump), programmable timer, and use of a single precolumn. The sample loop was filled by means of a syringe and the switching valves were actuated manually. The analysis of primidone spiked into serum was selected as an initial application. We considered that success with this highly polar drug, having weak adsorption characteristics for trapping onto a reversed-phase extraction column, would provide conditions also applicable to the other antiepileptic drugs having less polar structure.

We began our studies with an extraction column that was enclosed with 2-μm porous frits, and contained a porous 5-μm bonded-phase silica packing as opposed to much larger particles (Waters C_{18} Corasil) used by Roth et al. [9]. Our results with this approach were quite discouraging. After less than 100 consecutive serum injections of 100 μl each, we observed a severe increase in the pressure drop across both the extraction column and analytical column, and also a major drop in the overall plate number for the primidone peak.

We next packed our extraction column with the same type of material used by Roth et al. [9] and obtained significantly better results. After 75 serum injections of 100 μl each, there was no increase in back pressure across the extraction column, a moderate increase (17.7 bars) across the analytical column, and a decrease of 38% in the overall plate number. These results, however, still seemed to fall short of those reported by Roth et al. [9].

Our next step was to use an extraction column that we conjectured to be closer to that used by Roth et al. [9], comprising a standard, Waters guard column fitted with Waters screens (a retainer screen with 0.2-mm porosity against the particles, backed by a filter screen having 2-μm porosity), and we

repeated the 100 serum injections. This time our results agreeed with those of Roth et al. [9] in that there was no change in back pressure across either the extraction or the analytical column and the overall plate number remained constant. The importance on the use of screens instead of frits to prevent sample blockage has also been recently emphasized by Roth [10].

Based on these results, we decided to re-examine in more detail the role of the particle size and type in the extraction column, employing the Waters screens instead of porous frits as endfittings. The four reversed-phase silica particles that we tested and their performances are summarized in Table I.

TABLE I

PARAMETERS AFFECTED BY VARIATION OF PARTICLE SIZE AND TYPE IN THE EXTRACTION COLUMN

Particle size and type in the extraction column*	Changes in parameter after 100 injections of 100 μl of serum spiked with primidone			
	Increase in pressure across the extraction column (bars)	Increase in pressure across the analytical column (bars)	Initial and final plate numer of the analytical column**	Absolute recovery of primidone (%)
5-μm C_8	13.6	27.2	7080, 2600	100
10-μm C_{18}	3.4	13.6	6305, 3560	100
Corasil (37—50 μm, C_{18})	0	1.36	7400, 7450	5
30-μm C_{18}	6.8	0	8325, 7942	>90

*The extraction column was a 25 × 3.9 mm column fitted with a retainer screen and 2-μm filter screen (Waters Assoc.).

**Plate number was calculated using toluene and the mobile phase of the analytical column.

Although the recovery of primidone with the 5-μm, C_8 porous particles was quantitative throughout 100 injections of 100 μl each of serum spiked to a concentration of 20 μg/ml with this drug, the plate number of the analytical column dropped significantly. Also, a gradual increase in pressure developed across both the extraction and analytical columns. With a 10-μm C_{18} porous packing in the extraction column, the quantitative recovery of primidone was maintained, but the reduction in plate number was still significant. Once again an increased pressure developed in the analytical column, although less than before. As already reported above, we observed no significant change in the pressure drop across either the extraction or analytical columns, nor in the plate number of the analytical column, when a Corasil C_{18} pellicular packing was used. However, now we observed that the recovery of primidone was only about 5% with this packing.

The best overall results were obtained with a porous 30-μm C_{18} packing, as shown in Table I. This column gave a greater than 90% recovery of primidone, essentially no change in either the pressure drop or plate number of the analytical column, and underwent an intermediate increase in pressure across the extraction column. The higher recovery of drug with this packing relative

to the pellicular packing is readily explained by the higher surface area of the porous particle, as opposed to that of the pellicular Corasil packing. (For a less polar drug such as carbamazepine, or the drugs analyzed by Roth et al. [9], the reversed-phase Corasil pellicular packing does give complete recovery in our system.)

The overall poor performance of the 5- and 10-μm packings, including the previously observed similar shortcomings of porous frits versus screens as retainer fittings, is not completely clear. Perhaps the causes relate to plugging from microparticles or other species present in the serum. Nevertheless, we chose to continue using an extraction column fitted with the Waters screens and packed with porous 30-μm C_{18} particles for our subsequent work.

Recovery of other drugs, and water versus buffer wash

The recovery of three other commonly used antiepileptic drugs was next determined on the fully automated system shown in Figs. 1 and 2. Serum samples spiked with known amounts of drugs were injected onto the extraction column via a 20-μl sample loop followed by forward wash with either distilled water or a low-pH buffer at a flow-rate of 2 ml/min. After washing for 4 min, the system was switched to backflush mode. When distilled water was used as a flushing solvent, high recoveries were obtained in all cases, as shown in Table II, except for phenobarbital. Apparently this latter drug, having a pK_a of 7.3 [11], was ionized and lost during the wash cycle with water. This ionization was suppressed by the acidic wash buffer, yielding a good recovery for phenobarbital and the other drugs as well. A representative chromatogram is shown in Fig. 3.

TABLE II

DRUG RECOVERY FROM SERUM BY AUTOMATED SOLID-PHASE EXTRACTION AS A FUNCTION OF WATER VERSUS BUFFER WASH

The drugs were dissolved together in serum at a concentration of 10—20 μg/ml, based on weight, and this solution was analyzed by the system shown in Fig. 1 using a 20-μl sample injection.

Drug	Percentage recovery*	
	Water wash	Buffer wash**
Primidone	89	91
Phenobarbital	0	91
Phenytoin	81	95
Carbamazepine	98	95

*Average, absolute recoveries were determined based on ten injections.
**0.1 *M* Sodium phosphate buffer, pH 3.5.

Although the data are not shown, we also examined the influence of protein binding on drug recovery. This study was done by comparing the recovery of these same drugs spiked to the same concentration in water versus serum. Identical recoveries were obtained, demonstrating that protein binding (ranging from 0% to 10% for primidone, to 65—85% for carbamazepine [12]) had no overall effect on drug capture by our extraction column.

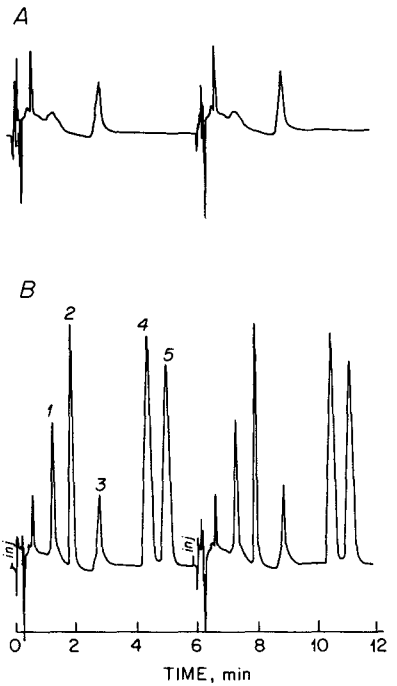

Fig. 3. Representative HPLC chromatograms of pooled serum samples using the systems in Figs. 1 and 2; (A) 20 μl of blank serum pool; (B) 20 μl of serum spiked with primidone (10 μg/ml), phenobarbital (20 μg/ml), phenytoin (15 μg/ml) and carbamazepine (10 μg/ml). A buffer wash (pH 3.5) was delivered by pump P_1. Peaks: 1 = primidone, 2 = phenobarbital, 3 = impurity, 4 = phenytoin, 5 = carbamazepine.

Valve and timing optimization

Encouraged by these results, as summarized in Tables I and II, we introduced further optimization into the system of Figs. 1 and 2 in two respects. First, we reduced the switching time of the injection valve, V_2, to minimize pressure surges on the analytical column, by replacing a Valco Model CV-6-UHPa-N60 with a Rheodyne valve (Model 7010) fitted with a Model 7001 solenoid (Rheodyne). We operated the solenoid with helium in conjunction with a Humphrey TAC^2 41PP valve. Secondly, the sequential steps of activation, loading and injection were synchronized so that the system handled twelve serum samples per hour. This was accomplished primarily by simultaneously conducting sample pickup into the injection loop (40 sec) and water wash of the extraction column (40 sec), while having these events begin 50 sec after the previous sample had been injected onto the analytical column.

Over 2000 injections

With the optimized version for the system shown in Figs. 1 and 2, as summarized in Table III, we achieved 2112 continuous 20-μl serum injections spiked with primidone with practically no change in the analytical column back pressure. The chromatographic column lost less than 25% of its starting plate number. The extraction column was repacked and its filter replaced whenever its back pressure increased more than 6.8 bars, which typically occurred every

TABLE III

PRESSURE DROP AND EFFICIENCY OF THE ANALYTICAL COLUMN DURING OVER 2000 SERUM INJECTIONS USING THE SYSTEM SHOWN IN FIG. 1

Number of injections	Pressure drop across the analytical column (bars)	Plate number of the analytical column*
0	123.8	8400
146	125.8	8150
249	129.2	8580
462	127.2	8680
760	125.8	7360
1090	125.8	7940**
1261	122.4	6740
1393	129.2	7120
1637	129.2	7390
1877	141.4	6240
2112	144.2	6500

*Evaluated as described in Table I.
**The analytical column was reversed.

Fig. 4. Typical chromatograms using the automated HPLC system shown in Figs. 1 and 2: (A) 20 μl of serum blank; (B) 20 μl of serum spiked with 10 μg/ml of primidone. A water wash was delivered by pump P_1.

200—300 serum injections. The analytical column was periodically washed with tetrahydrofuran to remove any strongly retained components, and was reversed once during the overall experiment. The recoveries and precision obtained day-to-day with different extraction columns were consistent, as was the absence throughout of any baseline disturbance or additional peaks. The within-day

precision was quite high, ranging from 0.8% to 2.5%, coefficient of variation (C.V.). A representative chromatogram is shown in Fig. 4.

Boxcar configuration

Boxcar chromatography is an advanced column-switching technique placing multiple samples simultaneously into an analytical column, for enhanced sample throughput [13]. To demonstrate this increased throughput capability for our system, we converted it to a boxcar configuration, as shown in Fig. 5. This latter configuration differs from that shown in Fig. 1 by the addition of another pump (P_3), a six-port valve (V_3) and a short column (SC). These new components provide an initial separation and heart-cutting of the sample.

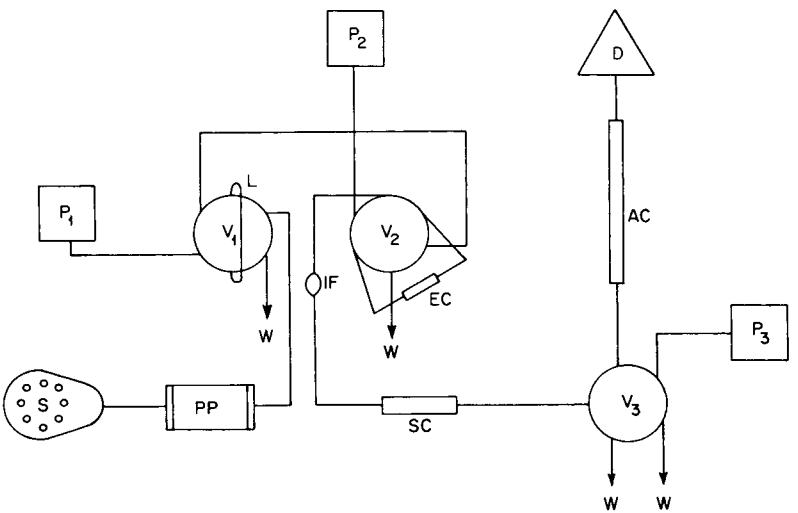

Fig. 5. Configuration of the boxcar HPLC system. S, autosampler; PP peristaltic pump; L, sample loop; V_1, V_2 and V_3, six-port switching valves; W, waste; P_2 and P_3, mobile-phase pumps; P_1, wash pump; EC, extraction column; SC, short column; AC, analytical column; IF, in-line filter; D, detector. The sequential steps for this system, controlled by a digital programmable timer, are as follows: (1) activate the auto sampler, S, to fill up the sample loop, L, while equilibrating the extraction column (EC) with aqueous wash; (2) switch valve V_1 to solid-phase extract the drug in EC; (3) switch valve V_2 to back-flush the drug onto the short column (SC); (4) switch valve V_3 to heart-cut the drug(s) into the analytical column, AC, for further separation prior to detection, D.

With this boxcar system we successfully analyzed primidone and phenobarbital in serum samples containing a mixture of primidone, phenobarbital, phenytoin and carbamazepine at an increased rate of 40 samples per hour. Over 1000 serum samples were analyzed with a C.V. of 1%, without the use of an internal standard (data not shown). A representative chromatogram is shown in Fig. 6.

The timing for this analysis was as follows: (1) sample loading, 20 sec; (2) sample extraction, 48 sec; (3) back elution, 20 sec; (4) pre-separation on short column, 32 sec; and (5) heart cutting onto the analytical column, 30 sec. This timing achieved complete filling of the sample loop; no memory effects from a previous sample; and 100% transfer of the analyte drugs from the short column to the analytical column.

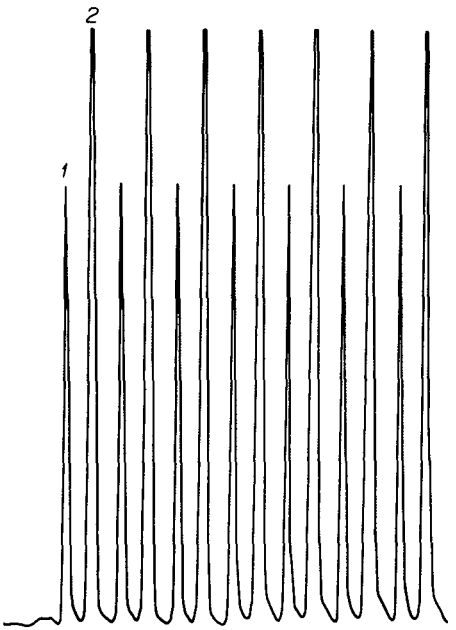

Fig. 6. Chromatogram showing the analysis of primidone (1) and phenobarbital (2) in serum at a rate of 40 samples per hour using the boxcar system shown in Fig. 5.

Aside from the use of parallel columns, further increases in sample throughput utilizing boxcar chromatography potentially may be achieved by additional optimization of the extraction column, since the use of this column currently is the slowest step.

CONCLUSION

Clearly, automated column-switching HPLC is a viable technique for therapeutic drug monitoring. At least for the repetitive analysis of a pooled serum spiked with antiepileptic drugs, we have achieved high throughput, excellent precision, and long-term system stability.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF BUPIVACAINE IN HUMAN SERUM

R.L.P. LINDBERG* and K.K. PIHLAJAMÄKI

Department of Pharmacology, Institute of Biomedicine, University of Turku, Kiinamyllynkatu 10, SF-20520 Turku (Finland)

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SUMMARY

A selective high-performance liquid chromatographic method for the determination of bupivacaine in human serum is described. The technique is based on a single extraction of the drug from alkalinized serum with a mixture of hexane—isopropanol—chloroform. Desmethyldoxepin is used as internal standard. The chromatographic system consists of a home-packed Nucleosil C_8 (10 μm) column; the mobile phase is acetonitrile—0.05 *M* potassium phosphate buffer (pH 3.3) (28:72, v/v).

The method can accurately measure serum bupivacaine concentrations down to 20 μg/l using 500 μl of sample. The coefficient of variation for intra-assay variability of bupivacaine is 2.1% (n = 13) and for inter-assay variability of bupivacaine 5.7% (n = 11) at 1.00 mg/l. The calibration graph is linear over the range 0.02—5.00 mg/l and the extraction efficiency is 91.8 ± 3.8% (± S.D., n = 7).

The method is accurate and sensitive for both clinical and pharmacokinetic studies on bupivacaine in man. The method is applied to the analysis of serum samples obtained from orthopaedic patients during both spinal and epidural analgesia.

INTRODUCTION

Local anaesthetic agents are an important part of modern anaesthesiology. Most of the systemic toxic reactions from the local anaesthetics are correlated with high concentrations of these agents in the circulation [1]. Determination of serum concentrations of local anaesthetics can therefore make their use safer for the patients undergoing various anaesthetic procedures.

Bupivacaine hydrochloride (Marcain®) is a new amide-type local anaesthetic agent of long duration of action [2] which is gaining more and more popularity, especially in obstetrics, orthopaedics and post-operative analgesia [3—5].

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Several gas chromatographic methods for the determination of bupivacaine have been reported [6—12]. However, these methods involve various extraction steps and are rather laborious and time-consuming. Gas chromatography—mass spectrometry has also been used in the analysis of local anaesthetic agents [13].

No high-performance liquid chromatographic (HPLC) methods for the determination of bupivacaine in human serum have been published. We describe here an accurate, rapid and sensitive procedure for the detection of bupivacaine using HPLC. The method allows a more rapid measurement of therapeutic and toxic concentrations of bupivacaine in serum. Because of the high sensitivity this method is also very suitable for pharmacokinetic studies on bupivacaine.

We applied the procedure described herein for monitoring bupivacaine serum concentrations during spinal and epidural analgesia in various orthopaedic operations.

EXPERIMENTAL

Reagents and chemicals

The following reagents and chemicals were used: bupivacaine hydrochloride (Marcain, A.B. Bofors Nobel Pharma, Bofors, Sweden) and desmethyldoxepin hydrochloride (Pharmaceutical Div., Pennwalt, Rochester, NY, U.S.A.); *n*-hexane, isopropanol, chloroform and acetonitrile were of analytical reagent grade (E. Merck, Darmstadt, F.R.G.).

Internal standard. A solution of desmethyl doxepin (100 mg/l) was prepared by dissolving 11.4 mg of desmethyldoxepin hydrochloride in 100 ml of distilled water. A second solution containing desmethyldoxepin (5 mg/l) was prepared by dilution with distilled water.

Drug standards. Working drug solution contained 50 mg of bupivacaine in distilled water. Drug serum standards were prepared by spiking blank control serum with appropriate microlitre volumes of working drug solution to obtain seven serum standards with the following concentrations of bupivacaine: 0.1, 0.25, 0.5, 0.75, 1.0, 2.5 and 5.0 mg/l.

Extraction procedure

To a 0.5-ml serum sample, 0.1 ml of desmethyldoxepin solution (5 mg/l) was added. The serum was made alkaline by adding 0.5 ml of sodium hydroxide solution (0.1 mol/l). Bupivacaine and the internal standard were extracted with 5.0 ml of a mixture of hexane—isopropanol—chloroform (30:60:10, v/v/v) by shaking for 20 min. The organic phase was separated by centrifugation and transferred to a clean test-tube. The organic mixture was evaporated at 50°C under a gentle stream of air. The residue was reconstituted with 100 µl of phosphoric acid (0.05 mol/l) and 50 µl were injected into the chromatograph. The concentration of bupivacaine in the serum sample was determined from a calibration curve of peak height ratio (drug/internal standard) versus drug concentration in serum standards carried through this procedure.

High-performance liquid chromatography

The analysis was carried out on a Model SP 740 HPLC system (Spectra-

Physics, Santa Clara, CA, U.S.A.) using an HP 1030B ultraviolet detector (Hewlett-Packard, Waldbronn, F.R.G.) set at 210 nm. An SP 4100 computing integrator (Spectra-Physics) was used to calculate peak heights. The mobile phase consisted of acetonitrile—0.05 *M* potassium phosphate buffer (28:72, v/v) (pH 3.3) and the flow-rate was 2.0 ml/min. The reversed-phase column was home-packed with 10-μm Nucleosil C_8 (Macherey-Nagel, Düren, F.R.G.) and measured 30 cm × 3.9 mm I.D.

Application of the method

To five patients suffering from rheumatic arthritis (four women and one man, age 26—61 years, height 158—177 cm, weight 72—87.5 kg) 150 mg of bupivacaine hydrochloride were given for epidural analgesia before various orthopaedic operations. For spinal analgesia 22.5 mg of bupivacaine hydrochloride were given to three women (age 65—71 years, height 153—175 cm, weight 52—71.5 kg). All the patients abstained from eating and drinking for at least 6 h before and 4 h after the operations. They were also without their usual antirheumatic and other drugs.

Nine blood samples were drawn from cubital venous cannulas until 3 h after administration of the bupivacaine. The blood samples were kept in a refrigerator at 4° C and centrifuged 3—9 h after drawing. After centrifugation the sera were deep-frozen and kept at —20° C until analysed.

RESULTS AND DISCUSSION

Chromatograms of extracts from blank serum, blank serum spiked with 1.0 mg/l bupivacaine and the serum sample of a patient during spinal analgesia are illustrated in Figs. 1A—C, respectively. Bupivacaine and the internal standard, desmethyldoxepin, were well separated and no interference was noted. The retention times of bupivacaine and the internal standard were 4.15 and 5.70 min, respectively, which means that the total chromatographic run time was only about 7 min. Thus it is possible to determine 30 serum samples in duplicate during an 8-h working day.

The calibration graph was linear for samples over the concentration range studied here, 0.02—5.00 mg/l. The least-squares linear regression line which represents the best fit of the bupivacaine data had an equation of $Y = 0.55X + 0.10$ (Y = peak height ratio, drug/internal standard, and X = bupivacaine concentration). The correlation coefficient was > 0.999.

The precision was assessed by multiple analyses of seven standard serum pools in the concentration range 0.10—5.00 mg/l. Inter-assay variability was determined over a period of three months. The coefficients of variation for intra-assay and inter-assay variability of bupivacaine are given in Table I. The values for intra-assay variability vary from 1.0% to 5.1% and for inter-assay variability from 5.7% to 10.5% in the range studied. The results demonstrate the high accuracy and reproducibility of the method.

From a comparison of bupivacaine peak heights obtained from direct injection of aqueous solutions and from samples carried through the assay procedure, the extraction efficiency was estimated as 91.8 ± 3.8% (± S.D., n = 7) (Table II). The coefficient of variation for recovery was 4.2% (n = 7) over the concentration range 0.10—5.00 mg/l.

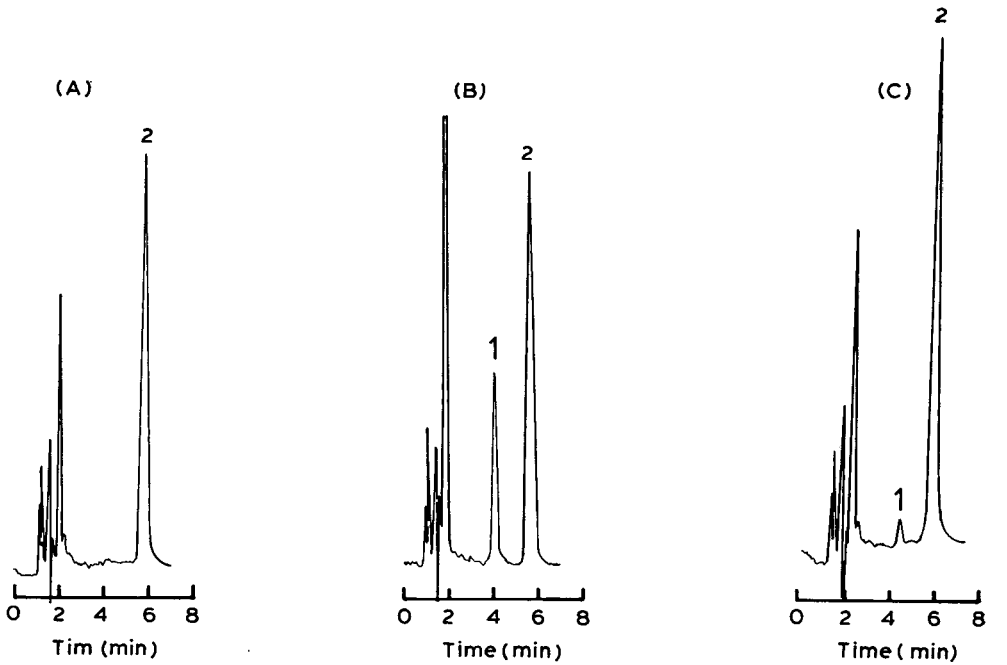

Fig. 1. Liquid chromatograms of the extracts from blank serum (A), blank serum spiked with 1.0 mg/l bupivacaine (B), and from a serum sample of a patient during spinal analgesia 30 min after the beginning of the spinal block (C). The retention time of bupivacaine (1) is 4.15 min and that of internal standard, desmethyldoxepin (2), 5.70 min. The concentration of bupivacaine in the patient's serum (C) is 0.087 mg/l. For chromatographic conditions, see text.

TABLE I

INTRA- AND INTER-ASSAY REPRODUCIBILITY OF HPLC FOR THE DETERMINATION OF BUPIVACAINE IN HUMAN SERUM

Concentration (mg/l)	Coefficient of variation (%)	
	Intra-assay (n = 13)	Inter-assay (n = 11)
0.10	2.4	8.1
0.25	5.1	10.5
0.50	4.0	9.5
0.75	4.1	6.6
1.00	2.1	5.7
2.50	2.5	7.3
5.00	1.0	6.2

If the limit of sensitivity is defined as that signal which is three times higher than the background signal, this method can be used to determine serum containing 20 μg of bupivacaine per litre. Greater sensitivity may be possible by increasing the volume of serum used. Berlin et al. [8] reported the accuracy of the gas chromatographic method as 15 μg/l and Reynolds and Beckett [6] as 40 μg/l. A detection limit of 1 μg/l using gas chromatography has been

TABLE II

BUPIVACAINE DETERMINATION IN HUMAN SERUM

Bupivacaine concentration (mg/l)	No. of parallel determinations	Recovery (mean) (%)	Standard deviation (%)
0.10	13	89.7	3.5
0.25	13	91.4	4.0
0.50	13	98.4	4.1
0.75	13	94.2	4.0
1.00	13	86.1	5.8
2.50	13	92.2	3.4
5.00	13	90.5	4.4
Range 0.10—5.00	7	91.8	3.8

published [12], but the coefficient of variation for that measurement was 15%, so the value is very dubious.

Because our orthopaedic patients were suffering from rheumatic arthritis and using many drugs, we tested a lot of different drugs for possible interference by injecting stock solutions of these compounds into the chromatograph. Diclofenac, indomethacin, cortisol, fentanyl, salicylate, furosemide, amiloride and hydrochlorthiazide did not interfere.

Fig. 2 shows the serum concentrations of bupivacaine in our rheumatic patients. The peak plasma concentration after the extradural administration of 150 mg of bupivacaine hydrochloride has been shown to be at the level 1.0—1.2 mg/l [14], which is identical with our results.

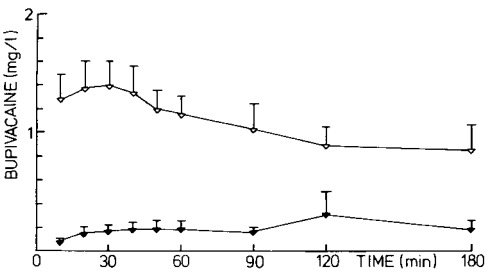

Fig. 2. Mean (+ S.E.M.) serum concentrations of bupivacaine during spinal (▼) (n = 3) and epidural (▽) (n = 5) analgesia.

After spinal analgesia with 15 mg of bupivacaine hydrochloride, Dennhardt and Konder [15] have shown the blood peak concentration of bupivacaine to be 0.30 ± 0.13 mg/l. Tucker and Mather [16] have reported the blood/plasma concentration coefficient for bupivacaine to be 0.73 ± 0.08 in normal adult males with normal blood pH values. Our patients were not suffering from chronic pulmonary or renal diseases, so we can suppose they had quite normal blood pH values. Because they did not have abnormally low serum protein values either, we can use the concentration coefficient of 0.73 when comparing our serum concentrations with whole blood concentrations of bupivacaine. Our results are at the same level as the values obtained by Dennhardt and Konder [15].

A small increase in our mean values at 120 min following spinal analgesia could be explained by the occurrence that one of our patients was transferred from the operating table to his bed some minutes before drawing the 120-min sample. This move might have caused an increase in the pressure of the intradural space thus augmenting the absorption of bupivacaine from the cerebrospinal fluid.

In conclusion, the HPLC method for bupivacaine in human serum that has been developed has been demonstrated to be accurate, selective, simple and rapid for the analysis of bupivacaine. The method has also been successfully applied in studies of spinal and epidural analgesia of orthopaedic patients.

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Note

The effect of the presence of glucose on the determination of amino acids by gas chromatography

D. LABADARIOS*, G.S. SHEPHARD, I.M. MOODIE and E. BOTHA

Metabolic Unit Tygerberg Hospital, Department Internal Medicine, University of Stellenbosch and the M.R.C. National Research Institute for Nutritional Diseases, P.O. Box 63, Tygerberg 7505 (Republic of South Africa)

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The speed, sensitivity and low initial capital outlay makes gas chromatography (GC) an ideal tool in the analysis of amino acids in biological fluids and tissues. Despite the advantages GC has to offer, its acceptance as a routine tool in amino acid analysis is significantly hampered by the laborious and time-consuming steps necessary in sample purification prior to derivatisation. The main approach in plasma sample purification over the past ten years has been one of deproteinisation followed by ion-exchange clean up of the protein-free extract [1—5]. Although this approach yields a product pure enough for derivatisation, the recovery and reproducibility of results for certain amino acids are variable and often unacceptable. The ion-exchange resins that have been used seem to irreversibly [4, 6, 7] bind certain amino acids or reduce [8] their elution by on-column reactions.

MATERIALS AND METHODS

Crystalline amino acids and heptafluorobutyric anhydride were obtained from Sigma (St. Louis, MO, U.S.A.). All other chemicals were obtained from E. Merck (Darmstadt, F.R.G.). Venous blood was obtained from laboratory personnel in the fasting state following their expressed consent. Plasma (0.5 ml) was deproteinised using methanol—concentrated hydrochloric acid (4:1; 2 ml), centrifuged at 2000 *g* at 4°C for 15 min and the supernatant filtered through a Millex 0.22-μm filter (Millipore). N-Heptafluorobutyryl isobutyl ester (HBB) derivatives of the amino acids were prepared as previously described [9], but modified for derivatisation of 25-μg quantities of each amino acid [10]. GC was performed using conditions described elsewhere [10].

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Glucose when derivatised was present in approximately the concentration found in the plasma of normal subjects.

RESULTS AND DISCUSSION

GC analysis of derivatised crystalline amino acid standards is shown in Fig. 1. Glucose when taken through the same derivatisation procedure, yielded the products whose chromatogram is shown in Fig. 2. The interference caused by glucose in the analysis of amino acid standards is shown in Fig. 3 and the identification, by retention times, of glucose as the interfering compound in the analysis of plasma amino acids in Fig. 4.

There are two possible mechanisms which may explain the interference caused by glucose, a reducing sugar, during amino acid derivatisation. Firstly, the hydroxyl groups of glucose may react with heptafluorobutyric anhydride forming derivatives [11] which co-elute with hydroxyproline, proline, methionine and aspartic acid, thus making the quantitation of these amino acids unattainable. Secondly, and in view of the high temperatures employed during the derivatisation, glucose may react with free amino acids forming the so-called Maillard compounds [12]. These latter compounds may themselves contribute directly to the spurious peaks observed or indirectly following their esterification and acylation during derivatisation. Under mild conditions of temperature (37°C) lysine will be the main contributor to the Maillard reactions [13]. However, with increasing severity of temperature conditions (> 90°C), as employed in the derivatisation of amino acids, glycine,

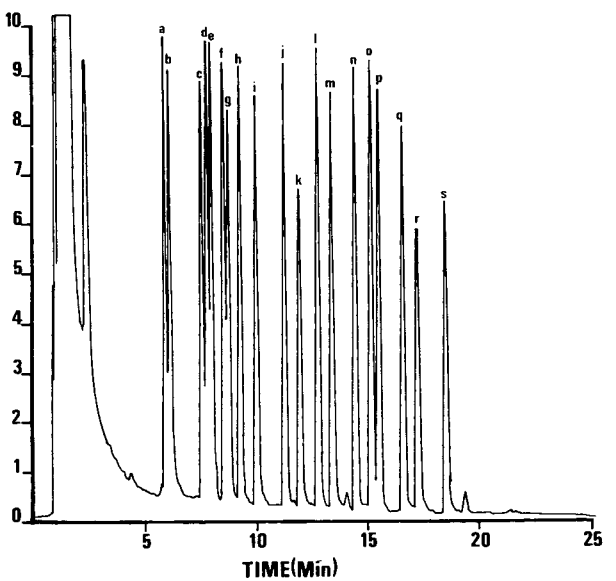

Fig. 1. GC separation of HBB derivatives of amino acid standards. Peaks: a = alanine; b = glycine; c = valine; d = threonine; e = serine; f = leucine; g = isoleucine; h = norleucine (internal standard); i = proline; j = hydroxyproline; k = methionine; l = aspartic acid; m = phenylalanine; n = glutamic acid; o = lysine; p = tyrosine; q = arginine; r = histidine; s = tryptophan.

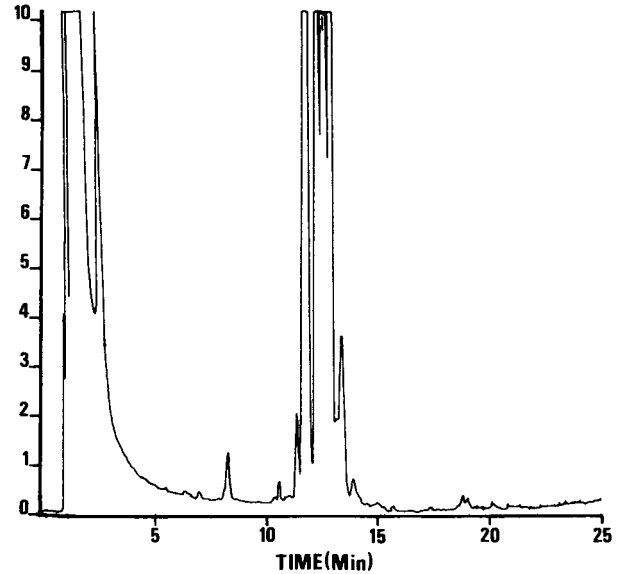

Fig. 2. Chromatogram of the product from glucose subjected to the derivatisation procedure.

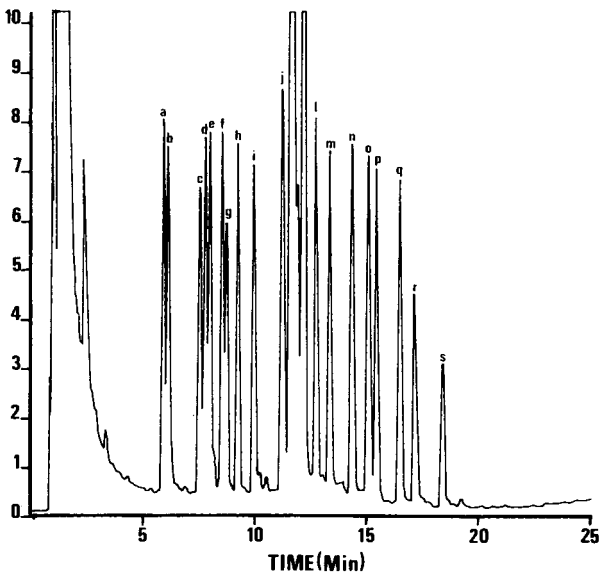

Fig. 3. GC separation of HBB derivatives of amino acid standards in the presence of derivatised glucose. Peak identification as in Fig. 1.

arginine, aspartic acid and glutamic acid will also participate in the Maillard reactions [14].

It would therefore appear that elimination of the glucose induced interference, by means such as enzymic degradation, will improve the qualitative and quantitative analysis of plasma amino acids. This approach to plasma purification is currently under investigation.

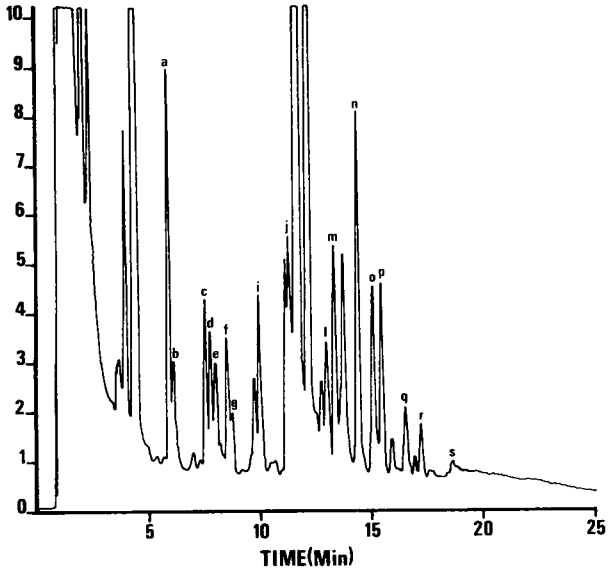

Fig. 4. GC separation of HBB derivatives of plasma amino acids showing the glucose-induced interference. Peak identification as in Fig. 1.

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We thank SABAX SA for the donation of the gas chromatograph.

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Note

Measurement of homovanillic acid in human plasma by high-performance liquid chromatography with electrochemical detection

PETER Q. HARRIS*

**Department of Psychiatry, Veterans Administration Hospital, White River Junction, VT 05001 (U.S.A.) and Dartmouth Medical School, Hanover, NH 03756 (U.S.A.)*

NICHOLAS G. BACOPOULOS

Departments of Pharmacology and Psychiatry, Dartmouth Medical School, Hanover, NH 03756 (U.S.A.)

and

SUSAN J. BROWN

Department of Psychiatry, Veterans Administration Hospital, White River Junction, VT 05001 (U.S.A.) and Dartmouth Medical School, Hanover, NH 03756 (U.S.A.)

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Several recent reports have discussed the possible use of plasma homovanillic acid (HVA) as an index of brain dopaminergic function [1—6]. Except for one study [6], determination of plasma HVA has been by means of gas chromatography with mass spectrometry (GC—MS). High-performance liquid chromatographic (HPLC) methods found to be suitable for measuring HVA in cerebrospinal fluid (CSF), have not been considered suitable for plasma or serum because compounds having long retention times have made repeated injections at short intervals impossible [7]. Because GC—MS methods were not readily available to us, we developed an HPLC method to measure HVA in human plasma.

EXPERIMENTAL

Samples were obtained from normal controls and psychiatric inpatients before and after administration of neuroleptic medication [6]. Informed consent was obtained from all subjects. Morning fasting blood was drawn into

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heparinized Vacutainer tubes. Plasma was separated within 1 h of collection and 2—4 replicate aliquots of 0.5—2.5 ml were placed in glass scintillation vials. For each 0.5 ml of plasma, 0.25 ml of 1 mol/l hydrochloric acid, 20 μl of 10% EDTA and 20 μl of 10% sodium metabisulfite were added. Samples were stored at —70°C until the time of assay, which was generally within one month after collection. Samples could be stored for six months without apparent loss of HVA.

A modification of the HVA extraction method described by Bacopoulos et al. [1] was used. To determine percent recovery, 50 μl of 0.001 mol/l hydrochloric acid containing 10 ng HVA was added to replicate patient samples and/or to replicate control samples run simultaneously. To determine linearity of extraction from plasma, recoveries of 1, 5, 10, 15, 20, and 25 ng of HVA added to a pooled plasma sample were determined. Volumes of plasma of 0.5, 1.0, 1.5, 2.0, and 2.5 ml were extracted using each amount of HVA standard. Recovery was measured as the difference between HVA in the sample containing standard and the same sample without added HVA.

Lipids were extracted at room temperature using two hexane washes of 5 ml (for 0.5- and 1.0-ml plasma samples) or 10 ml (for 1.5—2.5 ml plasma samples). Following extraction of lipids, samples were transferred to centrifuge tubes and proteins were precipitated by adding perchloric acid to achieve a final concentration of 0.3 mol/l. The precipitate was removed by centrifugation at 22,000 *g* for 15 min. A measured aliquot of supernatant (0.5—2.7 ml depending on original sample size) was added to glass scintillation vials containing potassium chloride to saturate. HVA was extracted into 6 ml of ethyl acetate added to each vial by shaking the samples for 15 min at high speed using a mechanical shaker. After a 15-min centrifugation at low speed to separate the organic and aqueous phases, a 4.5-ml aliquot of the ethyl acetate extract was evaporated to dryness at 28°C under nitrogen. Dried samples were capped and stored frozen until the time of injection into the chromatograph.

A Bioanalytical Systems liquid chromatography system consisting of an M-45 solvent delivery system, an LC-4A amperometric detector connected to a glassy carbon (TL-5) working electrode, and a RYT recorder were used. The Biophase ODS, 5-μm column, 25 cm × 5 mm I.D., was protected by a guard column of the same material. An oxidation potential of 0.72 V with respect to an Ag/AgCl reference electrode was applied. The mobile phase consisted of 50 m*M* sodium acetate containing 100 mg/l EDTA and 12% methanol, with an apparent pH of 4.4. Flow-rate was 1.1 ml/min at ambient temperature. Elution was isocratic.

Samples were reconstituted with 300 μl of mobile phase and filtered through a 0.45-μm RC 45 filter (Bioanalytical Systems) before injection into a 100-μl sample loop. A sensitivity of 2 nA full scale was used for 0.5- and 1.0-ml samples; for larger samples a 5-nA sensitivity was used.

RESULTS AND DISCUSSION

Using HPLC with electrochemical detection, we have measured HVA levels in 342 plasma samples (Fig. 1). Excluding the extreme high values (> 45 ng/ml), the mean (± standard error) plasma HVA levels were: controls, 9.8 ± 0.5 ng/ml

(n = 22), unmedicated subjects, 9.2 ± 1.8 ng/ml (n = 25), subjects receiving neuroleptics, 17.3 ± 1.1 ng/ml (n = 234). These values compare favorably with previously reported values for plasma HVA determined using GC—MS [5].

Peak identity was based on retention characteristics (Fig. 2) and a comparison of hydrodynamic voltammograms for reference HVA and the HVA peak in plasma (Fig. 3). The closeness of the voltammograms demonstrates that no interfering substances were co-eluting with plasma HVA [8] and allows quantitation of the plasma peak. The standard curve for HVA was

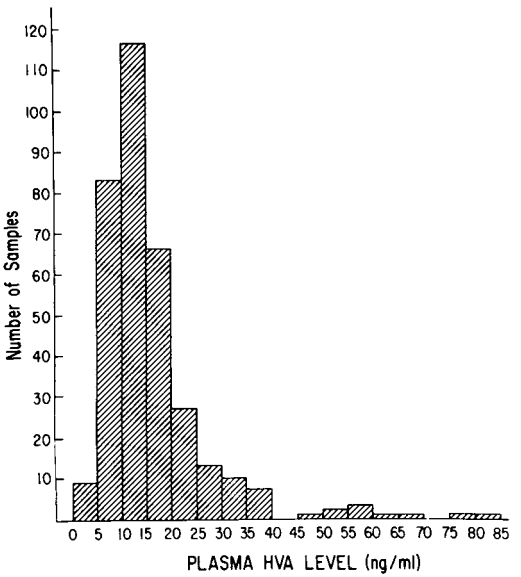

Fig. 1. Levels of homovanillic acid (HVA) measured in 342 plasma samples using HPLC with electrochemical detection.

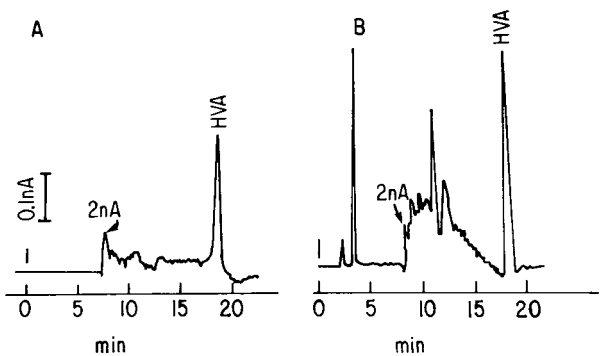

Fig. 2. Comparison of chromatograms for a 500-pg HVA standard (A) and a 0.5-ml plasma sample (B). Retention time for the plasma peak was identical with that for reference HVA and changed in an identical manner when the pH or methanol concentration of the mobile phase was changed. Reference HVA added to plasma produced an increase in the size of the plasma HVA peak with no evidence of a shoulder or split-peak. Mobile phase: 50 m*M* sodium acetate containing 100 mg/l EDTA and 12% methanol, pH 4.4; retention time for HVA: 17 min 50 sec.

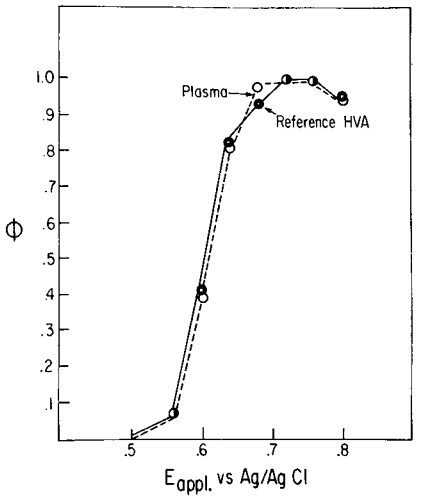

Fig. 3. Hydrodynamic voltammograms for the HVA reference compound and the peak in human plasma. Abscissa: oxidation potential vs. Ag/AgCl reference electrode. Ordinate: ratio of response at a particular potential to the maximal response. The close identity of the two curves demonstrates the purity of the plasma HVA peak.

linear to 100 ng, the highest concentration tested, and passed through zero. Therefore, no HVA was being retained by the column and there was no baseline interference with the peak of interest. As little as 100 pg HVA could be measured.

Precision of the analytical procedure was determined by chromatographing aliquots of plasma samples. The day-to-day precision measurement produced a coefficient of variation of 3.9% (n = 4).

Extraction procedures described were highly reproducible with consistent results for a large number of extractions performed during a two-year period. Recovery of HVA, determined in over 300 different plasma samples, was generally 28—30%. It was the same for 1—25 ng of HVA added to a given volume of plasma and for a given amount of HVA added to different volumes (0.5—2.5 ml) of plasma. It was not altered by different amounts of endogenous HVA in different plasma samples or by neuroleptic medication. If samples are carefully prepared and stored, the standard deviation (S.D.) for four replicate samples can be as low as 0.1 to 0.5 ng/ml (Table I).

Some precautions should be taken in order to achieve consistent extraction of HVA from plasma. During the hexane washes, samples should be shaken only very gently because vigorous shaking will turn the sample into a gel [1]. In addition, incomplete separation of the ethyl acetate and aqueous layers may be a problem with 2.5-ml plasma samples and can be overcome by centrifuging the samples at low speed for 5 min, shaking the vials to break up the gel-layer that forms between the organic and aqueous phases, and re-centrifuging.

Variation between replicate plasma samples depends on a number of factors other than reliability and reproducibility of extraction and chromatography procedures. Particular care should be taken that samples are not thawed and re-frozen prior to extraction. In addition, long-term storage of small sample

TABLE I

PLASMA HVA LEVELS

Plasma HVA levels are measured in four replicate samples obtained from ten subjects and demonstrate the reliability of the extraction, separation and quantitation methodes described in the Experimental section.

Sample		Plasma HVA (ng/ml, mean ± S.D.)
Control	A	10.2 ± 0.3
	B	8.5 ± 0.5
	C	12.8 ± 0.2
	D	11.0 ± 0.1
	E	10.4 ± 0.2
Subject	A	15.0 ± 0.5
	B	4.3 ± 0.4
	C	13.5 ± 0.3
	D	14.0 ± 0.2
	E	29.8 ± 0.5

volumes in scintillation vials is inadvisable because these samples have a tendency to dry down on the bottom of the vial.

One objection to the use of HPLC methods to measure HVA in plasma or serum has been that compounds in the blood are retained on the column for long periods of time, making repeated injections at reasonable intervals of time impossible [7]. Using the methods we have described, and an initial plasma volume of 0.5 ml, injections can be made every 19 min. There are peaks which elute after the HVA peak, but these are generally not a problem for small plasma samples. For samples larger than 0.5 ml plasma, we have found it necessary to stop injections and wash the column with mobile phase for 1—1.5 h after every six to eight injections. Samples larger than 0.5 ml may be desirable when plasma HVA levels are expected to be less than 3 ng/ml. However, an easier alternative is to reconstitute the ethyl acetate extract with less mobile phase and inject the entire sample.

In summary, we have described a reliable method for extraction of HVA from plasma, separation of HVA from other plasma constituents present in these extracts, and quantitation of amount of HVA in the isolated peak. Injections can be made at 19-min intervals without interference from compounds having longer retention times than HVA. Values obtained are consistent with previously reported values for plasma HVA obtained by GC—MS. HPLC is simpler, less expensive and more widely available than GC—MS. Therefore, measurement of plasma HVA using HPLC instead of GC—MS may facilitate further investigation of the relationship between plasma HVA and central dopaminergic function.

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Note

Determination of methylprednisolone and methylprednisolone acetate in synovial fluid using high-performance liquid chromatography

M. ALVINERIE* and P.L. TOUTAIN

Station de Pharmacologie-Toxicologie, INRA, 180 chemin de Tournefeuille, 31300 Toulouse (France)

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Methylprednisolone acetate (MPA) administered intraarticularly is widely used in both human and veterinary medecine for the relief of various acute and chronic joint diseases [1]. After its administration, MPA must be hydrolysed

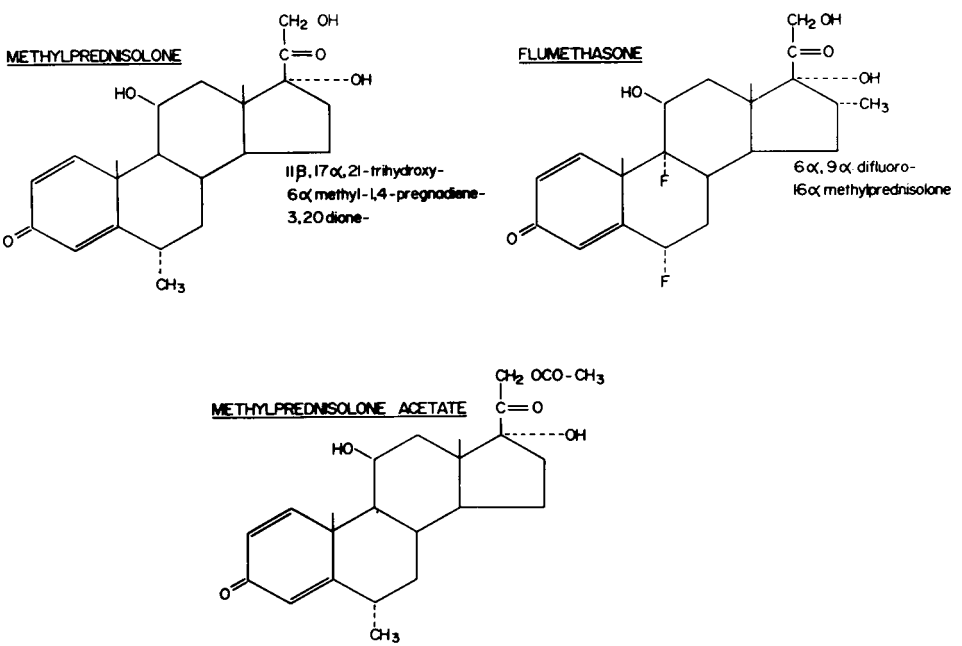

Fig. 1. Chemical structures of the investigated glucocorticoids.

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to the pharmacologically active parent drug, methylprednisolone (MP) (Fig. 1). In whole blood, a rapid rate constant for MPA hydrolysis has been reported [2]. In contrast, no information is available concerning the hydrolysis of MPA to MP in synovial fluid.

Several mehods [3, 4] have been described to measure selectively the concentration of MP in plasma by use of high-performance liquid chromatography (HPLC); however, no method has been developed for the simultaneous assay of MP and MPA in various biological fluids.

Using the advantages of a common normal-phase chromatographic method that has been successfully applied to a large number of steroids [5, 6], the present report describes a sensitive and specific procedure for the simultaneous determination of MP and MPA in synovial fluid.

EXPERIMENTAL

Apparatus

A constant-volume high-performance liquid chromatograph (Waters Assoc., Milford, MA, U.S.A.), consisting of a Model M45 pump, a U6K injector and a Model M440 detector, was used. The column (10 × 0.8 cm I.D.) was packed with 10-μm silica gel (Radial Pak B, Waters Assoc.) and was included in a radial compression system (Module RCM 100, Waters Assoc.).

Reagents

MP, MPA and flumethasone reference standard were purchased from Sigma (St. Louis, MO, U.S.A.). Dichloromethane was obtained from Farmitalia Carlo-Erba (Milan, Italy).

Standards

Each steroid was dissolved in methanol at a concentration of 1 mg/ml. The working standard mixture was prepared by diluting the stock solution 1:100 in the elution solvent. Flumethasone was used as internal standard at a concentration of 1 mg/ml in methanol; a working solution was prepared by diluting the stock solution 1:10 in methanol.

Operating conditions

The mobile phase was prepared by mixing exact volumes of dichloromethane, methanol and glacial acetic acid (96.8:2.4:0.8, v/v). The solution was stirred and degassed. A constant flow-rate of 1.4 ml/min was maintained. The radial compression pressure was 100 bars and inlet pressure was 15 bars.

The ultraviolet detector was set at 254 nm; the sensitivity of the detector was 0.001 a.u.f.s. (absorbance units full scale). The system was operated at ambient temperature (18—20° C).

Retention time and selectivity

Normal-phase liquid chromatography showed a high selectivity in the separation of glucocorticoids; a good separation was obtained in 8 min (Table I).

TABLE I

RETENTION TIMES AND CAPACITY FACTORS OF SELECTED GLUCOCORTICOIDS

Component	Capacity factor (k')	Retention time (min)
Methylprednisolone acetate	1.2	4
Flumethasone	2.3	6
Methylprednisolone	3.4	8

Extraction procedure

In a 30-ml tube (Corex, U.S.A.) were placed 100 μl of sample, 10 μl of the working solution of internal standard (equivalent to 0.1 μg of flumethasone), 1 ml of 0.1 *M* sodium hydroxide and 10 ml of dichloromethane. The tubes were shaken for 10 min and centrifuged at 8400 *g* for 10 min. The dichloromethane layer was aspirated off and evaporated at 40°C under a stream of nitrogen gas to prevent oxidation. The sample extract was reconstituted with 100 μl of eluent, mixed using a vortex mixer, and the total was then injected on to the column.

Calibration

Pooled bovine synovial fluids were spiked with MP and MPA to give concentrations in the range 0.02—1 μg/ml. A constant amount (10 μl) of working solution of the internal standard was added to each sample. Pooled synovial fluid samples were run through the procedure and calibrations curves constructed by plotting the peak height ratio for each compound with respect to the internal standard against the amount of compound added to each synovial sample. Least-squares regression analysis was used to determine the slope, intercept and correlation coefficient for each compound in the concentration range tested (Table II). The response of the HPLC system was linear from 0.02 to 1 μg/ml for each of the two compounds.

The analytical recovery of the steroids was measued by comparing the chromatographic peak heights from the analysis of biological samples which were spiked with 100 ng of MP and MPA to the peak height resulting from a direct injection. The recovery of all steroids from synovial fluid was 80—85% when approximately 85—90% of the dichloromethane layer was available for evaporation.

TABLE II

LEAST-SQUARES REGRESSION STATISTICS FOR HPLC CALIBRATION DATA OF METHYLPREDNISOLONE AND METHYLPREDNISOLONE ACETATE IN SPIKED SAMPLES

Component	Slope	Intercept	Correlation coefficient (n = 6)
Methylprednisolone	0.0105	+0.045	0.997
Methylprednisolone acetate	0.0405	+0.015	0.998

Precision

The intraassay variation was determined by analysing synovial fluid samples spiked with 100 ng of MP and MPA. Coefficients of variation were 5.8% (n = 8) and 4.9% (n = 8), respectively.

The interday variability of the assay over a period of a week (n = 5) was determined by analysing samples spiked with 100 ng of MP and MPA. Coefficients of variation were 8.7% and 7.5%, respectively.

Drug disposition study

To test ability of this method to detect MP and MPA in synovial fluid of treated animals, 200 mg of methylprednisolone acetate (Depomedrol; Upjohn, Paris, France) were injected into the tibiotarsal joint of a cow. Synovial fluid samples were collected every day during the first week, and every three days for two months after administration. In order to prevent hydrolysis of the parent compound, samples were immediately stored at −21°C until analysis.

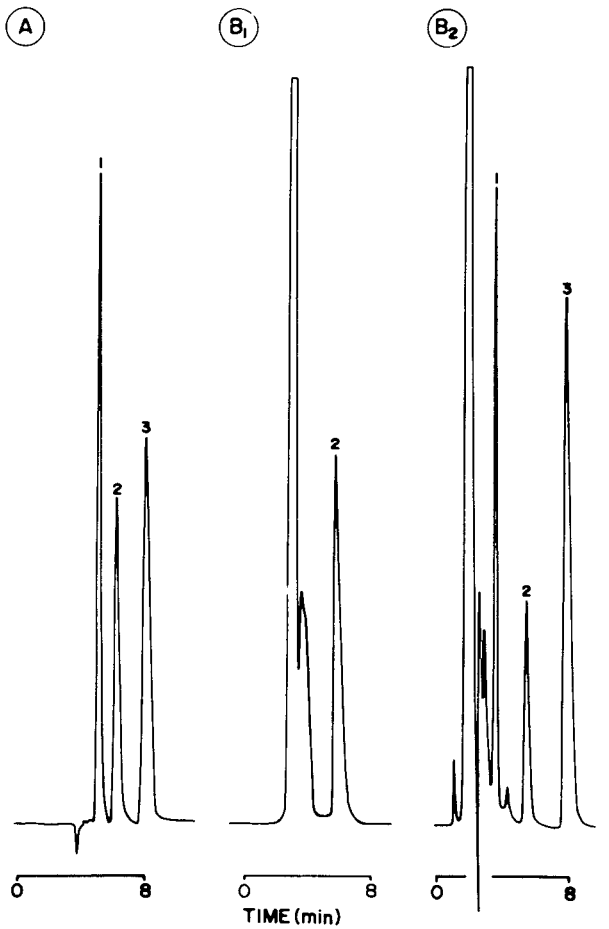

Fig. 2. (A) Chromatogram of a mixture of 100 ng each of MPA, flumethasone and MP. (B_1) Chromatogram of extracted blank synovial fluid containing only internal standard. (B_2) Chromatogram of extracted synovial fluid after administration of MPA with serum levels of 230 ng/ml for MP and 100 ng/ml for MPA. Peaks: 1 = methylprednisolone acetate, 2 = flumethasone, 3 = methylprednisolone.

RESULTS AND DISCUSSION

The purpose of this study was to develop a precise and sensitive technique which would allow the simultaneous determination of MP and its ester (MPA) in synovial fluid. In addition, a suitable technique for this purpose must include the choice of a suitable internal standard with good separation from the glucocorticoids to be measured. In this respect flumethasone was used and the method described in this report meets these criteria.

Fig. 2A shows a chromatogram of a mixture of 100 ng each of MPA, flumethasone and MP, demonstrating the clear separation of the glucocorticoids using the column and the mobile phase described above. The longest retention was approximately 8 min for MP, which is the least polar of the three assayed compounds.

Fig. $2B_1$ shows a typical chromatogram of a synovial fluid sample, spiked with internal standard, from an untreated cow. No major endogenous peak that would interfere with the resolution of the glucocorticoids was encountered.

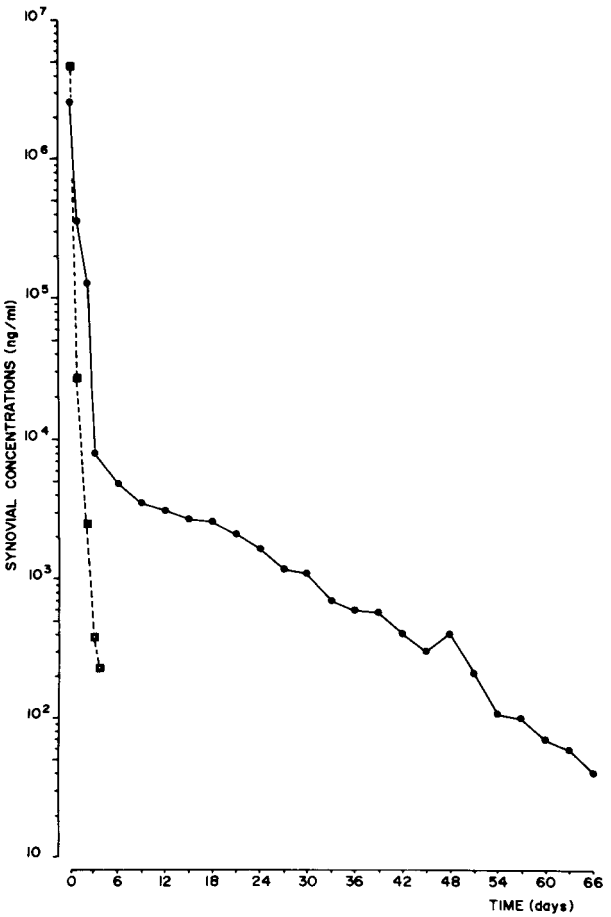

Fig. 3. Synovial concentration—time curve in the cow given a 200-mg intraarticular dose of MPA. ■, MPA; ●, MP.

Fig. $2B_2$ displays a chromatogram of a synovial fluid sample from an animal injected intraarticularly with MPA.

Fig. 3 presents a typical synovial fluid concentration—time profile of MP and MPA after intraarticular administration. MPA concentration declines very rapidly until 24 h and then more slowly. For MP the highest concentration was observed at the first sampling; thereafter, MP concentrations decreased very rapidly for the first week and more slowly between one and nine weeks after administration.

The fact that MP remains for so long in synovial fluid must be considered significant from a pharmacological point of view; other more extensive studies about the disposition of MPA in synovial fluid after intraarticular administration will be reported elsewhere.

In conclusion, a selective and sensitive method has been developed for MP and MPA assay in synovial fluid. The detection limit of the proposed method using the equipment described was 10 ng for MPA and 20 ng for MP based on a 100-μl synovial fluid sample; this could be enhanced by increasing the amount of sample, thus permitting the accurate measurement of concentrations as low as 50 ng/ml.

ACKNOWLEDGEMENTS

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Note

Determination of mequitazin in human plasma and urine by capillary column gas—liquid chromatography—mass spectrometry

J.-B. FOURTILLAN*, J. GIRAULT, S. BOUQUET and M.-A. LEFÈBVRE

Laboratoire de Chimie Thérapeutique, U.E.R. de Médecine et Pharmacie, 34 Rue du Jardin des Plantes, 86034 Poitiers Cédex (France)

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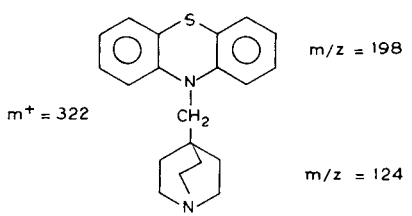
Mequitazin, 10-(3-quinuclidinylmethyl)phenothiazine (LM-209), is a new phenothiazine derivative which has been described as a potent H_1 antagonist with few or no sedative side-effects [1]. Related to the intensity of its pharmacological effects, mequitazin is administered orally at low dosages, varying between 5 and 10 mg per tablet. At these doses, the circulating plasma levels are such that a very sensitive and specific assay is required. Although the determination of radioactivity in rat and dog after oral or intravenous administration of ^{35}S-labelled mequitazin has been described [2], no chromatographic method has been published previously.



This work describes an assay using combined gas—liquid chromatography and mass spectrometry, the sensitivity of which (0.5 ng of mequitazin per ml of plasma) enables plasma concentrations to be monitored for over 72 h after the oral administration of a 10-mg single dose of mequitazin to healthy volunteers [3]. This assay was employed for a chronopharmacological study of mequitazin [4].

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EXPERIMENTAL

Reagents

LM-209 (mequitazin) and IBF-28145 (internal standard) were from Pharmaka Labs., Gennevilliers, France. Sodium hydroxide (Prolabo, Paris, France), hydrochloric acid and twice-distilled water were used in the preparation of 0.2 *M* hydrochloric acid and 2.0 *M* sodium hydroxide solutions. Diethyl ether (Merck, Interchim, Montluçon, France) and ethyl acetate (Fluka, Interchim, Montluçon, France) nanograde quality were used without further purification. Methanol and sodium chloride were from Prolabo.

Apparatus

Samples were analysed by mass fragmentography using a Hewlett Packard 5985 mass spectrometer equipped with a Hewlett Packard 5840 gas—liquid chromatograph, fitted with a solid injector (Ros model). The fused-silica capillary column (25 m × 0.23 mm I.D.) was wall-coated with the liquid phase CP Sil 5. The injection port temperature was set at 320°C and samples were injected at an initial oven temperature of 210°C. The temperature was programmed at a rate of 10°C/min up to 310°C. Helium was used as carrier gas at an inlet pressure of 0.9 kg/cm^2, which gave a constant flow-rate of 1.2 ml/min through the capillary column. The falling needle of the solid injector was cleaned every day to prevent drug adsorption. The operating conditions of the mass spectrometer were: separator temperature 280°C, source temperature 200°C, ionization energy 70 eV (electron-impact mode) and trap current 300 μA.

Extraction procedure

In a 20-ml screw-capped tube, 2 ml of plasma or 1 ml of urine were supplemented with 1 ml of 2.0 *M* sodium hydroxide saturated with sodium chloride, 5 ng of internal standard (50 μl of 100 ng/ml IBF 28145 in methanol solution) and 7 ml of diethyl ether. The mixture was shaken mechanically for 10 min and centrifuged for 10 min at 3000 *g* and 0°C. In a 10-ml screw-capped tube, 6 ml of the organic phase were added to 1 ml of 0.2 *M* hydrochloric acid saturated with sodium chloride. After shaking for 10 min and centrifuging for 5 min, the organic phase was carefully discarded using a Pasteur pipette. The aqueous phase was made alkaline with 0.8 ml of 2.0 *M* sodium hydroxide and extracted with 6 ml of diethyl ether. The mixture was shaken and centrifuged. The upper organic layer, transferred to a new 10-ml screw-capped tube, was evaporated to dryness under a gentle stream of nitrogen at 30°C. The residue was dissolved in 20 μl of methanol and an aliquot (2 μl) was injected into the chromatograph.

The retention times of internal standard (IBF 28145) and mequitazin were 5.0 and 5.75 min, respectively.

Mass spectrometric analysis

Figs. 1 and 2 show the electron-impact mass spectra of underivatized mequitazin and IBF 28145, respectively. A base peak appeared at *m/z* 124 and *m/z* 110, respectively, which resulted from fragmentation by cleavage of the side-

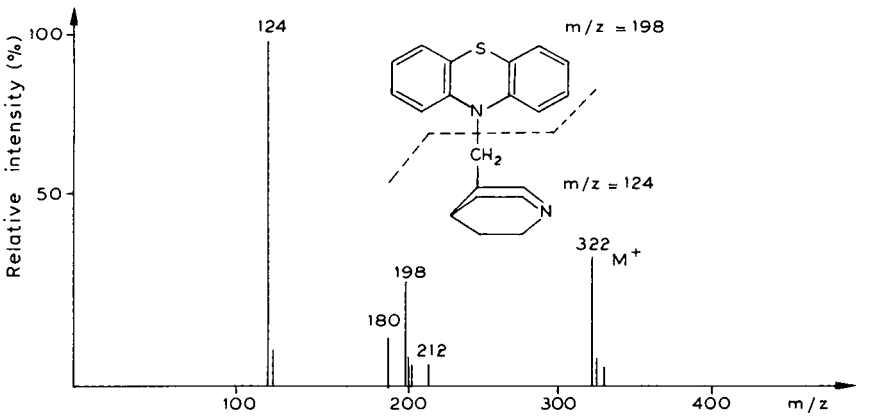

Fig. 1. Typical electron-impact spectrum and chemical structure of underivatized mequitazin.

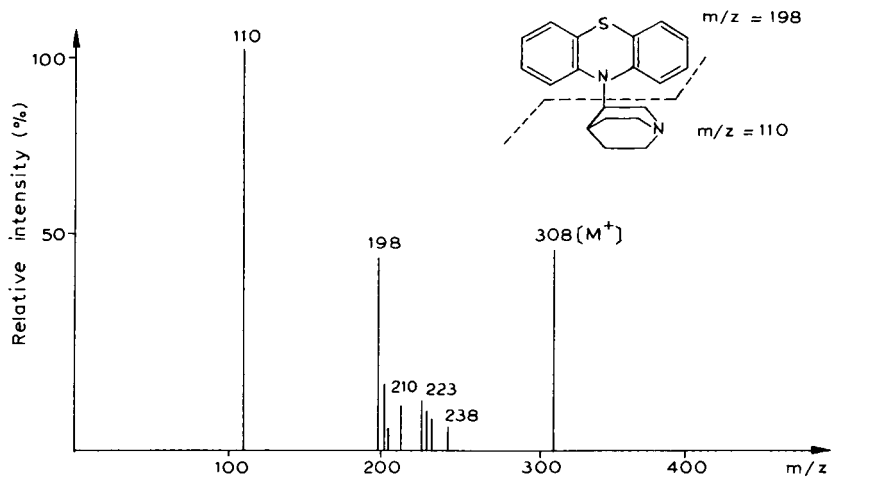

Fig. 2. Typical electron-impact spectrum and chemical structure of underivatized IBF 28145 (internal standard).

chain methylquinuclidinyl or quinuclidinyl. Because of possible interference from endogenous material, these ions were not chosen for quantification in biological samples; the molecular ions (*m/z* 322 and *m/z* 308) were used.

A standard curve (0, 0.5, 1, 2, 5, 10, 20, 40, 60, 80 and 100 ng of mequitazin per ml of plasma) was constructed from the simple linear relationship between the ion intensity ratios and the concentrations of mequitazin and internal standard.

The regression line of the data corresponding to the experimental points was drawn through the origin.

Mequitazin in human plasma

Eight healthy human subjects (six men and two women) were each given a single 5-mg oral dose of mequitazin (one tablet of 5.0 mg). Blood was collected in heparinized Vacutainer tubes at intervals during the 12 h following the dose. Plasma was separated by centrifugation and frozen at −20°C until assayed.

RESULTS AND DISCUSSION

Fig. 3 shows a chromatogram obtained with plasma containing 5 ng/ml internal standard and 15 ng/ml mequitazin using total ion current detection. Blank plasma or urine samples gave no interfering peaks on the chromatogram. A calibration curve prepared from a range of plasma levels (0, 5, 10, 20, 40, 60, 80 and 100 ng of mequitazin per ml of pooled plasma) indicated that intensity ratios of ions *m/z* 322 and 308 were linear when plotted against the concentrations of mequitazin. The lower limit of sensitivity was 0.5 ng of mequitazin per ml of plasma or urine.

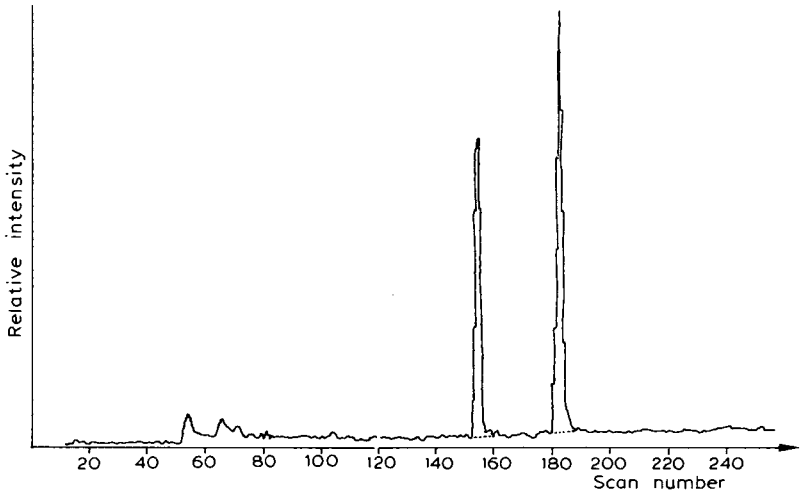

Fig. 3. Total ion current of a sample spiked with IBF 28145 (internal standard) and mequitazin.

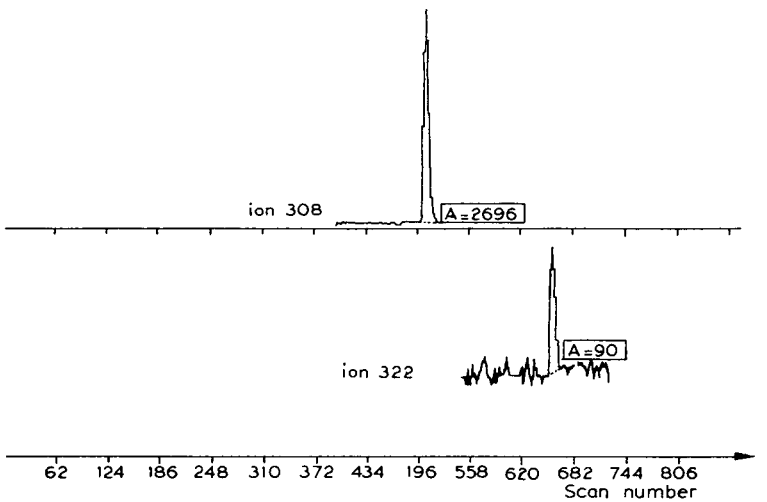

Fig. 4. Peaks obtained by mass fragmentography with plasma containing 5.0 ng/ml IBF 28145 (amplification 2800, *m/z* 308) and 0.5 ng/ml mequitazin (amplification 2800, *m/z* 322).

Fig. 4 shows a run obtained with a human plasma sample when 0.5 ng of mequitazin was added to 1.0 ml of plasma. This concentration gave for *m/z* 322 a peak area of 90 with an amplification value equal to 2800. The upper limit of linearity was at least 100 ng/ml.

The coefficient of correlation of the linear calibration was 0.9991 and the equation is of the type $Y = aX + b$, namely $Y = 0.1039X + 0.0124$.

Repeatability intra-day assays were performed on two pools of human plasma samples containing 1.0 and 10.0 ng/ml mequitazin (Tables I and II). The accuracy of the technique is good: the mean ratios of the peak areas (mequitazin/internal standard) were 0.034 ± 0.003 (± S.E.M.) and 0.693 ± 0.027 for the low- and high-concentration plasma samples, respectively. This mass fragmentographic method is presumed to be specific for the intact compound in urine and plasma.

The procedure was applied to numerous plasma and urine samples in pharmacokinetic studies. Fig. 5 shows the plasma concentration versus time run of mequitazin over a 252-h period, following multiple doses of 5 mg administered at 12-h intervals to healthy volunteers.

Fig. 6 represents a mass fragmentogram from a pharmacokinetic study corresponding to the plasma concentrations shown in Fig. 5.

TABLE I

REPEATABILITY ASSAY WITH MEQUITAZIN PLASMA LEVEL OF 1.0 ng/ml

Sample No.	Peak area ratio mequitazin/internal standard
1	0.038
2	0.032
3	0.027
4	0.033
5	0.046
6	0.039
7	0.024
8	0.030
Mean ± S.E.M.	0.034 ± 0.003

TABLE II

REPEATABILITY ASSAY WITH MEQUITAZIN PLASMA LEVEL OF 10.0 ng/ml

Sample No.	Peak area ratio mequitazin/internal standard
1	0.99
2	0.82
3	0.92
4	1.09
5	0.98
6	0.97
7	0.94
8	0.99
Mean ± S.E.M.	0.963 ± 0.027

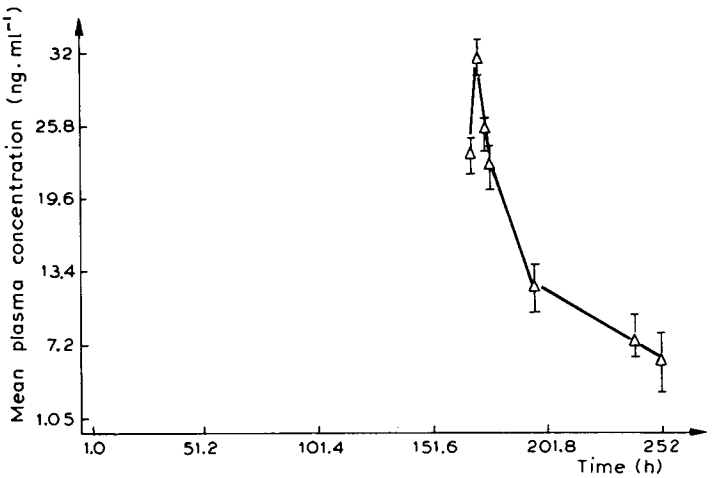

Fig. 5. Plasma concentrations of mequitazin, after multiple doses of 5 mg, every 12 h, over 252 h, to healthy volunteers. Each point represents the mean ± S.E.M. from six subjects.

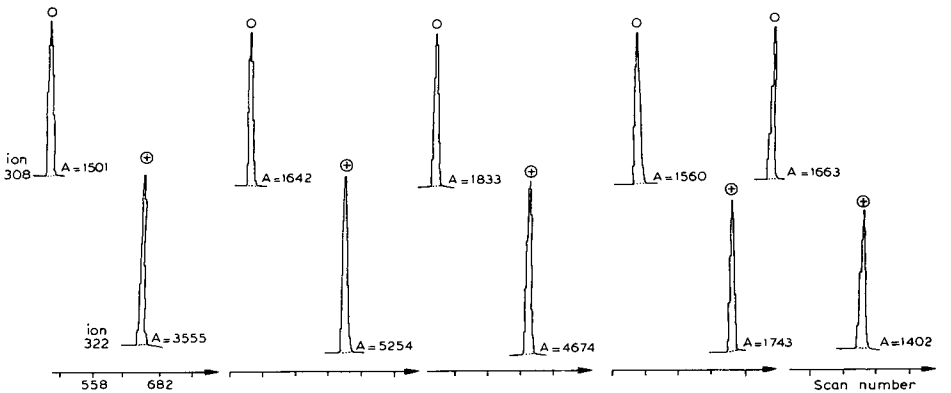

Fig. 6. Chromatograms obtained by mass fragmentography with plasma containing 5 ng/ml IBF 28145 (○) (amplification 2600, m/z = 308) and 23.5, 32, 26, 11.5 or 8.7 ng/ml mequitazin (⊕) (amplification 2600, m/z = 322).

Plasma mequitazin levels were determined in six healthy human volunteers over 32 h following a single 5-mg oral dose, and over 252 h after multiple oral doses of mequitazin. The terminal half-life was 48.4 ± 11.8 h (mean ± S.E.M.).

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Note

Rapid analysis of valproic acid by gas chromatography

ARVIND B. REGE

Department of Pharmacology, Tulane University School of Medicine, New Orleans, LA 70112 (U.S.A.)

JUAN J.L. LERTORA

Departments of Medicine and Pharmacology, Tulane University School of Medicine, New Orleans, LA 70112 (U.S.A.)

and

LUANN E. WHITE and WILLIAM J. GEORGE*

Department of Pharmacology, Tulane University School of Medicine, New Orleans, LA 70112 (U.S.A.)

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Several methods are described in the literature for the analysis of plasma valproic acid. These include gas chromatography (GC) [1—13], high-performance liquid chromatography (HPLC) [14—16], enzyme immunoassay (EMIT) [17], and fluorescence immunoassay [18]. Although a number of methods are available, certain problems exist, such as the assays being either time-consuming, expensive, non-specific, insensitive and/or difficult to conduct. Two major characteristics of valproic acid which have restricted methods development are its lack of ultraviolet (UV) absorbance at higher wavelengths (> 235 nm) and its high volatility as the free acid. Because of this lack of UV absorption, HPLC methods which were developed, required derivatization to a phenacyl ester before being detected at 246 or 247 nm [14, 15]. A sensitive HPLC method based on UV detection at 210 nm was developed but this method requires a controlled evaporation step during specimen preparation [16]. While many of the methods are effective, they require more time to conduct than is desirable.

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Other methods employ GC or enzyme immunoassay (EMIT) techniques which are not as time-consuming, but which are not as reproducible. A critical comparison of GC and EMIT methods has been reported recently showing that although the two methods produced equivalent results for control samples, there was a highly significant proportional error of 26% when the same patient sample was split and analyzed by both methods [19]. The consistently higher values which were obtained by EMIT were probably due to cross reactivity of some unsaturated metabolites of valproic acid which reportedly interfere with the enzyme immunoassay. Among the GC methods, ethyl acetate [10] and isoamyl acetate [20] have been added to the extracted free acid prior to concentration by evaporation to minimize volatilization of valproic acid. To circumvent concentration by evaporation, several methods [2, 3, 5, 7, 9, 11, 12] use a small volume of extraction solvent such as chloroform, dichloromethane, ethyl acetate, or carbon disulfide. In other methods, the efficiency of extraction is increased by adding saturated potassium dihydrogen phosphate to the plasma prior to extraction [8, 12]. Even in another method, extraction has been eliminated [13], but this method employs electron capture which does not require the concentrations needed for flame ionization detection. Reproducibility has consistently been a problem in the analysis of valproic acid in plasma using methods which employed small volumes of organic solvents for the extraction of free acid. The use of chloroform or dichloromethane for extraction has been previously evaluated [9] and causes undesirable complications such as formation of emulsions, high background and residue deposition on the flame ionization detector.

Because a rapid, sensitive, yet easy, method is required for the day-to-day analysis of valproic acid, the present phenacylbromide derivatization method was developed.

MATERIALS AND METHODS

Pure valproic acid was obtained from Saber Labs. A stock valproic acid standard (9.05 mg/ml) was prepared by diluting 100 μl of pure valproic acid to 10 ml with 0.2 *M* ammonium hydroxide. Working valproic acid standards were then prepared by diluting 55.3 μl of the stock valproic acid to 10 ml with drug-free plasma. Aliquots (0.3 ml) of this working solution were stored at -20°C and used as required. Such solutions are stable for at least two months. Standard regression lines were obtained by the analysis of standards containing 200, 100, 50, 25, 10 and 5 μg/ml valproic acid in drug-free plasma.

Valproic acid controls

Control serum specimens of valproic acid were obtained from UTAK Labs. (valproic acid control, 55 μg/ml); Hyland Diagnostic serum anticonvulsant control Level I (70 μg/ml); and serum anticonvulsant control Level II (125 μg/ml).

Stock *n*-caproic acid was obtained from Chem Service Labs. and diluted to 1 mg/ml in 0.2 *M* ammonium hydroxide. A working solution of 50 μg/ml was prepared in 0.5 *M* ammonium hydroxide.

Phenacylbromide was diluted to 10 mg/ml in acetonitrile and may be stored for up to four weeks.

All reagents and solvents were of analytical grade.

Extraction from plasma and derivatization

The standards, controls or specimens (0.25 ml) are placed into 10-ml glass tubes, with PTFE lines and screw tops, and 0.25 ml of the working internal standard and 0.25 ml of 1 *M* sulfuric acid are added. The tubes are vortexed gently for 15 sec and 4 ml of pentane are added to each tube and the tubes capped and vortexed for 2 min. The tubes are centrifuged at 3000 *g* for 5 min and 3 ml of the upper pentane layer are transferred to a conical tube. Triethylamine (20 μl) and phenacylbromide (20 μl) are added to each tube and the tubes vortexed for 15 sec. The sample is evaporated under nitrogen to dryness by placing the tubes in a water bath at 50°C for 5 min. The residue remaining in the tube is dissolved in 50 μl of methanol and 3—5 μl are injected onto the GC column.

The instrument employed was a Shimadzu GC 6 AM gas chromatograph equipped with a flame ionization detector and a glass column (1.83 m × 5 mm O.D., 3 mm I.D.) packed with 3% OV-17 on 100—120 mesh Gas-Chrom Q (Applied Science). The analysis was carried out isothermally with a column temperature of 205°C and a detector and injector temperature of 250°C. Nitrogen was used as the carrier gas at a flow-rate of 80 ml/min. Sensitivity and range were 1 mΩ and 80 mV, respectively. The recorder output was 10 mV.

RESULTS

A standard regression line for known concentrations of valproic acid (5—200 μg/ml) added to drug-free plasma was determined and the assay is linear for these concentrations of valproic acid in plasma.

The estimation of the precision and accuracy in the analysis of valproic acid is shown in Table I. The accuracy of the method was tested by repeated analysis (n = 10) conducted with three commercial controls. The obtained range for the UTAK control (55 μg/ml) was found to be 52.35—58.04 μg/ml; that for the Hyland anticonvulsant Level I (70 μg/ml) was 67.34—73.04 μg/ml; and that for the Hyland anticonvulsant Level II (125 μg/ml) was 121.60—128.53 μg/ml. Intra-assay coefficient of variation ranged from 1.66 to 1.80%. Inter-assay coefficient of variation ranged from 2.39 to 3.31%.

Chromatograms obtained from the analysis of valproic acid in samples containing other anticonvulsants and theophylline and employing *n*-caproic acid as an internal standard are shown in Fig. 1. Chromatogram A (Fig. 1A) was obtained using Hyland TDM anticonvulsants Level II containing carbamazepine, ethosuximide, phenobarbital, phenytoin, primidone, theophylline and valproic acid. Except for valproic acid, none of these agents were detectable on the chromatogram and did not interfere with the assay. Chromatogram B (Fig. 1B) was obtained using UTAK valproic acid control containing 55 μg/ml. The peaks of the chromatogram are sharp; the internal standard elutes prior to the valproic acid. Chromatogram C (Fig. 1C) was

TABLE I

PRECISION AND ACCURACY (n = 10) IN THE ANALYSIS OF VALPROIC ACID

Valproic acid controls	Target value (μg/ml)	Range (μg/ml)	Mean value (μg/ml)	Standard deviation (μg/ml)	Coefficient of variation (%)
Intra-assay					
UTAK Hyland anticonvulsant Level I	55	53.81—56.44	54.76	0.97	1.77
Hyland anticonvulsant Level II	70	68.05—72.03	70.33	1.27	1.80
	125	122.50—127.60	124.40	2.07	1.66
Inter-assay					
UTAK Hyland anticonvulsant Level I	55	52.35—58.04	55.15	1.83	3.31
Hyland anticonvulsant Level II	70	67.34—73.04	70.39	2.22	3.15
	125	121.60—128.53	124.69	2.99	2.39

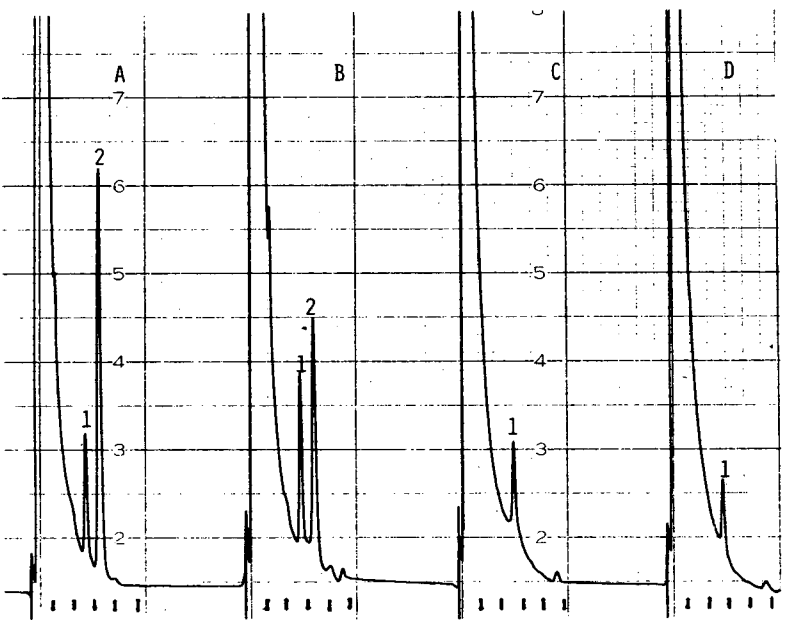

Fig. 1. Chromatograms of plasma extracts. (A) Hyland TDM anticonvulsant Level II control, containing carbamazepine (16 μg/ml), ethosuximide (125 μg/ml), phenobarbital (60 μg/ml), phenytoin (24 μg/ml), primidone (15 μg/ml), theophylline (30 μg/ml), and valproic acid (125 μg/ml); (B) UTAK valproic acid control (55 μg/ml); (C) drug-free plasma to which was added carbamazepine, ethosuximide, phenobarbital, phenytoin, primidone and theophylline (20 μg/ml of each); (D) drug-free plasma. Peaks: 1 = phenacyl derivative of n-caproic acid (internal standard); 2 = phenacyl ester of valproic acid.

obtained emploing drug-free plasma to which carbamazepine, ethosuximide, phenobarbital, phenytoin, primidone and theophylline had been added. Again, as in the Hyland anticonvulsant Level II, there was no interference from these agents; the only peak discernable is the internal standard. Chromatogram D (Fig. 1D) was obtained with drug-free plasma. The only peak is the internal standard and this indicated that plasma constituents do not interfere with the assay.

DISCUSSION

The objective of this study was to develop an analytical method for the determination of valproic acid that was suitable for a clinical laboratory. It was not only important that the assay be rapid and simple, but also that it be accurate and reproducible. A GC method was chosen over HPLC or EMIT because of lack of UV absorbance of valproic acid with the HPLC and interference by other substances with the EMIT method. Ideally, the method would not include a derivatization or evaporation step; however, this was essential to obtain the sensitivity and reproducibility that was required. Many of the GC methods in the literature are either very difficult [10], time-consuming [6] or lack reproducibility [10]. This method employs several steps described by Gupta et al. [6] with a number of modifications to simplify the method and decrease the assay time without compromising the sensitivity or reproducibility.

A single extraction of the valproic acid with pentane under acidic conditions was found to be sufficient to recover most (> 90%) of the valproic acid from the plasma. Further extraction with pentane under basic or neutral conditions was not necessary as no interfering substances were observed in the chromatograms. Derivatization of valproic acid was found to be necessary in order to obtain the desired sensitivity and to reduce the volatility of the compound during evaporation. A derivative was formed with phenacylbromide to form the phenacyl ester of valproic acid and *n*-caproic acid. Derivatization with phenacylbromide was selected for the following reasons: (1) the derivatization is extremely simple and rapid; (2) the phenacyl ester is less volatile than the free acid; and (3) the sensitivity with flame ionization detection is increased because of a greater number of carbon atoms (i.e. derivatization). The derivatization of valproic acid and *n*-caproic acid is very simple and quick requiring only the addition of triethylamine and phenacylbromide and occurs in a few seconds. The sample is immediately ready for subsequent steps.

Evaporation of the pentane was found to be necessary because of the volume of pentane required to extract the valproic acid. The pentane could be very quickly evaporated by placing the sample in a water bath at 50°C under a stream of nitrogen. By employing the stream of nitrogen, evaporation time was decreased from 1 h to 3 or 4 min. Not only does this save time, but there is less chance of decomposition of the sample. The phenacyl ester of valproic acid and of *n*-caproic acid is less volatile than the free acid and no loss of the sample is observed during this step.

n-Caproic acid was employed as the internal standard because it has similar properties to valproic acid with respect to extraction from the plasma by pentane and elution time during chromatography. The phenacyl ester derivative

of *n*-caproic acid elutes from the column during chromatography prior to the phenacyl ester derivative of valproic acid. The peaks of the internal standard are sharp and separate well from the solvent peak and the valproic acid peak. Other methods employ cyclohexane carboxylic acid which elutes after the valproic acid. The use of *n*-caproic acid as an internal standard decreases the assay time of each sample because it elutes before the valproic acid.

The standard curve for valproic acid was linear at the concentrations employed in the assay (5—200 μg/ml). A correlation coefficient of 0.9999 was obtained with the regression line passing through the origin. The detection limit of the assay was 5 μg/ml, and the sensitivity of the assay could certainly be increased by the use of larger volumes of plasma, but would serve no practical purpose since the therapeutic range is 55—100 μg/ml.

This method for the analysis of valproic acid is both accurate and precise. The accuracy was measured by multiple analysis of control samples. The value obtained for the controls was very close to the actual value in each case with a low coefficient of variation, as shown in Table I. This is a major criterion for an analytical method.

Another important asset of this method is the lack of interference from plasma constituents or from other anticonvulsants that might be used in combination with valproic acid.

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Note

Determination of 3-deazaguanine in mice plasma by high-performance liquid chromatography

BANGARU CHANDRASEKARAN* and BACH ARDALAN

Norris Cancer Hospital and Research Institute, University of Southern California Comprehensive Cancer Center, 1441 East Lake Avenue, Los Angeles, CA 90033-0804 (U.S.A.)

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3-Deazaguanine (3-DG), 6-amino-1,5-dihydro-4H-imidazo[4,5-C]pyridin-4-one is a synthetic analogue of guanine in which the nitrogen in the 3-position has been replaced with a carbon and hydrogen atom [1]. 3-DG has been shown to be a potential antitumor and antiviral agent [2]. The growth inhibitory activity of 3-DG in bacterial as well as in mammalian systems, has been shown to be the result of its conversion to 3-DG-containing nucleotides [3,4]. 3-DG inhibited several cell types including L1210 [5,6], HeLa [7], Ehrlich ascites [4], EMT-6 [8], primary Chinese hamster embryo cells [9], hamster ovary cells [10] and KB cells [4]. 3-DG has been presumably reported to inhibit the two enzymes involved in the purine biosynthetic pathway namely, inosine monophosphate dehydrogenase (IMPDH) and hypoxanthine—guanine phosphoribosyl transferase (HGPRT) which are probably the sites of cellular action.

To understand the fate of 3-DG in the biological system and also to study the mechanism of action of the drug in detail, we developed a sensitive high-performance liquid chromatographic (HPLC) method of determination of 3-DG and the efficacy of the method is analyzed in plasma and urine of mice and the results of the observation are discussed in this communication.

EXPERIMENTAL

Apparatus

Separation was achieved on a Model 6000 A solvent delivery system, Model U6K universal injector (Waters Assoc., Milford, MA, U.S.A.) and Model SF-720 spectroflow monitor (Schoeffel Instruments, Westwood, NJ, U.S.A.). Peak areas, retention time and concentrations based on standards were calculated with a Model 720 system controller and a Model 730 data module (Waters Assoc.).

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Column

The column used for reversed-phase HPLC was 300 × 4 mm μBondapak C_{18} (Waters Assoc.). The column was prepacked with 10-μm (average diameter) porous silica particles to which octadecyl groups were covalently bonded through a Si—O—Si bond.

Reagents

Ammonium formate (Sigma, St. Louis, MO, U.S.A.), trichloroacetic acid (Baker, Phillipsburg, NJ, U.S.A.), methanol (HPLC grade, Fisher, Fairlawn, NJ, U.S.A.) and glass-double-distilled water were used in preparing buffers and all other aqueous solutions. All solutions used in the HPLC system were filtered through a membrane filter (average pore size 0.2 μm; Millipore, Bedford, MA, U.S.A.) and degassed under vacuum immediately prior to use in the HPLC system.

Drug

3-Deazaguanine was obtained from Warner-Lambert (Ann Arbor, MI, U.S.A.). A stock solution of 10 mg/ml of the drug was prepared in 0.05 *M* hydrochloric acid and further dilutions were made with glass-distilled water and used as standards.

Internal standard

Fluorouridine (FUR) was used as the internal standard for 3-DG. A stock solution of 10 m*M* FUR was prepared in glass-distilled water and stored at −20°C and further dilutions were made immediately prior to use.

Buffer

A solution of 5 m*M* ammonium formate was freshly prepared in glass-double-distilled water and filtered through a membrane filter and degassed under vacuum immediately prior to use in the HPLC system.

Sample collection and storage

Female B6DZF_1 mice (20—23 g) were used in all the experiments. The animals were housed under natural lighting and fed a standard laboratory chow (Wayne Lab. Animal Diets, Chicago, IL, U.S.A.) ad libitum. Each mouse was administered intraperitoneally with 0.2 ml of 3-DG in normal saline (5 mg/ml). About 0.5 ml of blood was collected at different time intervals (0, 2, 5, 15, 30, 60, 90, 120, 240, 360, 720, 1440 min) from retroorbital sinus of mice using heparinized microhematocrit capillary tubes and immediately centrifuged at 12,000 *g* for 2 min. Plasma was separated and stored in ice until the collection was completed and then kept frozen at −20° C.

Reversed-phase HPLC determination of 3-DG

A 25-μl aliquot of plasma was placed in Eppendorff microcentrifuge tubes and 75 μl of 6% trichloroacetic acid were added to deproteinize plasma and vortexed with 100 μl of water. The solutions were centrifuged for 5 min and an aliquot of 20 μl was injected and chromatographed. The drug was monitored by its absorbance at 254 nm. The areas under the peaks were integrated with

the data module and system controller and the amount of drug in the plasma was calculated as follows: The amount of drug per ml of sample:

$$\left[\frac{\text{Area}_{\text{test}}}{\text{Area}_{\text{standard}}}\right]_{\text{sample}} \times \text{amount of standard} \times \frac{\text{ml sample}}{\text{volume of standard}}$$

Animal experiment

3-DG was injected into mice, three in each group. Plasma levels of drug were analyzed in a total number of eighteen mice, which were divided into six groups of three mice in each group. Blood was drawn twice from each mouse, i.e. a total number of 36 times was drawn from eighteen mice at various time intervals as described earlier.

HPLC conditions

Initially, the column was in 100% methanol phase and the column was washed with glass-distilled water for 30 min and regenerated for drug analysis by pumping 5 m*M* ammonium formate for a further 30 min. Flow-rate was maintained at 1 ml/min, the temperature was 24°C at 0.01 a.u.f.s.

RESULTS AND DISCUSSION

A chromatogram demonstrating the resolution of 3-DG is shown in Fig. 1. It can be seen from Fig. 1 that 3-DG has a retention time of 11 min at a flow-rate of 1 ml/min and it is sufficiently resolved to allow accurate quantitation. Identification of the peaks found in the chromatograms of plasma or urine were observed by matching the retention times of peaks from plasma or urine with those of standard compounds. Further confirmation was achieved by addition of a known quantity of each standard to a duplicate sample of plasma or urine containing drug, followed by chromatography of both samples. It is evident that the characteristics of the identified peaks remained unchanged, although they were appropriately taller with the added standard, and no new peaks emerged.

Separation of 3-DG and FUR is shown in Fig. 2. FUR has the retention time of 16.5 min at the flow-rate of 1 ml/min. There is no non-specific peak corresponding to FUR in control plasma or urine.

Quantitation of 3-DG at different concentrations ranging from 100 ng/ml to 1 mg/ml showed that the linearity of the drug was observed in the concentrations ranging from 1.0 to 100 μg/ml. The peak areas corresponding to 3-DG concentrations of 1, 5, 10, 50 and 100 μg/ml were $20.63 \cdot 10^4$, $109 \cdot 10^4$, $219 \cdot 10^4$, $1024 \cdot 10^4$ and $2072 \cdot 10^4$, respectively. The efficiency of the HPLC separation allows a low detection of 100 ng/ml or 2 ng per injection.

The imprecision of the determination is a correlation of the relative standard deviation of the yield and this amounts to less than 1% for the blood plasma determination of 3-DG (Table I). The yield of procedure was determined by adding 40, 20 and 10 μg of 3-DG to each milliliter of three normal plasma samples and then processing the samples as described in the Experimental

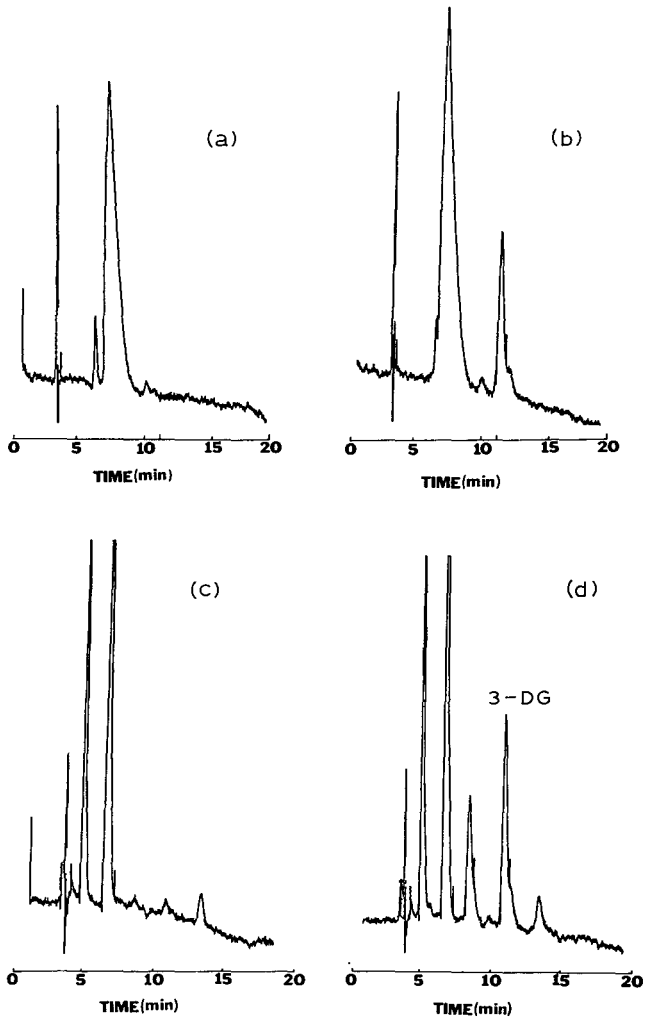

Fig. 1. Chromatograms obtained from control plasma (a); plasma containing drug (b); control urine (c); and urine containing drug (d). Plasma and urine were spiked with 3-DG to obtain a concentration of 2.5 μg/ml and 5.0 μg/ml, respectively and the deproteinized supernatants were chromatographed. Column: μBondapak C_{18} (300 × 4 mm); buffer: 5 m*M* ammonium formate (native pH); flow-rate: 1 ml/min; detector: 254 nm; 0.01 a.u.f.s.; temperature: 24°C.

section. The mean overall yield was found to be 91.7 ± 3.7%. The correction factor for the yield is therefore 1.09 in plasma.

When plasma was spiked with 40, 20 and 10 μg/ml 3-DG and aliquots were processed as described in Experimental and quantitated at daily intervals for a week, it was shown that the drug is stable in plasma under the experimental conditions. When the above experiment was repeated by spiking urine with 3-DG and immediately filtering through a membrane filter and chromatographing an aliquot, there was about 40—50% loss of parent drug in urine. This loss could be accounted for by the presence of a new peak which has a retention time of about 8 min (Fig. 1c and d).

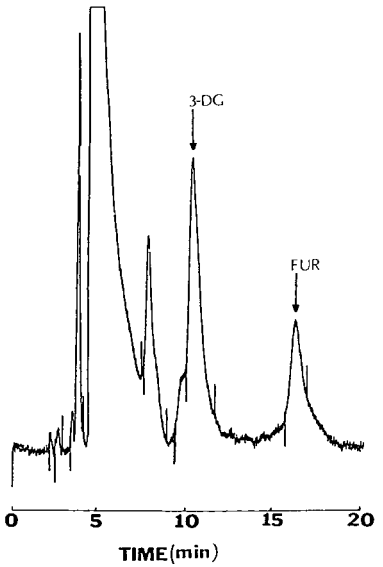

Fig. 2. Chromatogram representing the separation of 3-DG and the internal standard FUR in plasma. Conditions are the same as in Fig. 1.

TABLE I

PRECISION OF THE HPLC ASSAY OF 3-DEAZAGUANINE

$n = 3$.

Compound	Amount injected (μg/ml)	Peak area*			Mean ± S.D. (%)
3-Deazaguanine	10	219	219	218	218.66 ± 0.21
	5	110	111	110	110.33 ± 0.21
	1	21.4	21.5	21.5	21.466 ± 0.21

*Actual peak area = peak area $\times 10^4$.

We made an attempt to quantitate 3-DG in 24-h urine specimens of mice after administration of the drug but we could not detect the presence of the drug in the first and second 24-h urine specimens.

When an aliquot of 20 μl filtrate of control plasma was chromatographed, there was no non-specific peak corresponding to 3-DG. Retention times and peak areas in the chromatograms of authentic drug and plasma containing drug were found to be the same at isocratic conditions and so no attempt was made to purify plasma further for drug assay. Analysis of drug in plasma showed the presence of drug (5.0 ± 0.52 μg/ml) within 2 min after administration, reaching a maximum at 5 min (6.1 ± 0.68 μg/ml) and steadily decreasing thereafter. Drug could be quantitated in up to 2-h specimens (0.37 ± 0.02 μg/ml) and it disappeared completely from blood by 3 h (Fig. 3).

FUR was selected to serve as internal standard in the determination of 3-DG since the two compounds have the same absorption maxima, extraction and precision properties. It is also a readily available compound and its retention time under the experimental conditions is quite convenient to use it as an internal standard for 3-DG.

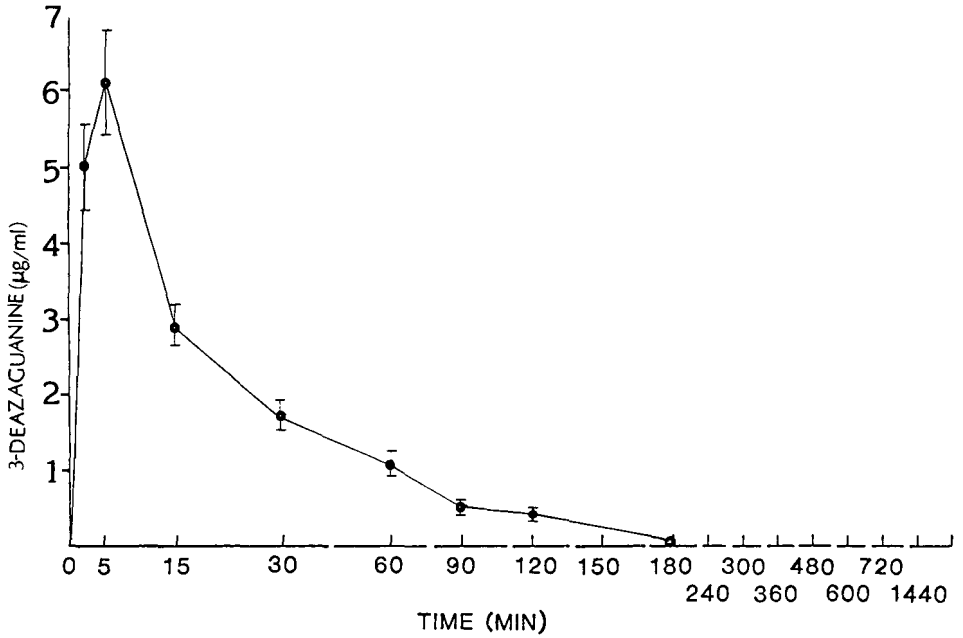

Fig. 3. Analysis of 3-DG in mice plasma. A 1-mg amount of 3-DG was injected into each mouse and a minimum number of three blood withdrawals of mice were quantitated at every time point.

The reversed-phase HPLC method for the separation and quantitation of 3-DG from plasma with UV absorption detection is a rapid, efficient, selective, sensitive and quantitative method. There is no method available in the literature for the estimation of the drug. 3-DG is soon to enter Phase I study. The elucidation of the method will throw some light on the pharmacology of the drug.

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Note

A novel method for the quantitation of 6-mercaptopurine in human plasma using high-performance liquid chromatography with fluorescence detection

N.K. BURTON*, G.W. AHERNE and V. MARKS

Division of Clinical Biochemistry, University of Surrey, Guildford, Surrey, 5XH GU2 (U.K.)

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6-Mercaptopurine (6-MP) has been used since 1953 to induce and maintain remissions in both acute and chronic leukaemias [1, 2]. Most regimens utilise 6-MP in combination with other cytotoxic drugs such as methotrexate and vincristine (e.g. POMP regimen) [3]. Dosages in these regimens have remained fairly constant, usually 75 mg/m^2/day, although individuals may have their doses reduced to avoid toxic effects. Herber et al. [4] have shown that there is no correlation between drug dose and biological effects although the same laboratory has reported a relationship between the red cell 6-thioguanine nucleotide concentration and response in childhood leukaemia [5]. To evaluate whether there is a relationship between 6-MP plasma levels and response pharmacokinetic studies are needed. Confirmation of a relationship would allow "tailoring" of the drug dose to the individual patient to produce optimum response to treatment.

In the past, various methods have been used to quantitate 6-MP levels in plasma including colorimetric and fluorometric assays [6, 7], gas chromatography (GC) [8, 9] and high-performance liquid chromatography (HPLC) [10—12]. The colorimetric and fluorometric assays lacked the sensitivity needed for accurate pharmacokinetic studies and therefore GC and HPLC methods are preferred as they can combine both sensitivity and selectivity, although some methods have a long sample turnover time which produces problems when manual injection is the only method available [13, 14]. Jonkers et al. [15] have developed a fluorescent method but this method needs post-column derivatisation which requires specialised equipment. This laboratory has used various published methods [10—12] but we have found that plasma samples tend to produce interfering peaks which could not be removed by changing the extraction process and a different technique was sought.

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In 1981, Newton et al. [16] reported a method for selectively measuring biological thiols by their selective reaction with monobromobimane (mBBr) and subsequent separation on reversed-phase HPLC. On reaction with thiols mBBr produced highly fluorescent products.

This paper describes how the selective reaction of mBBr with thiols can be utilised to selectively and sensitively measure 6-MP levels in plasma using reversed-phase HPLC with fluorescence detection.

EXPERIMENTAL

Materials

6-MP, 2-mercaptopurine (2-MP), 6-mercaptopurine riboside (MPR), 6-mercaptopurine riboside-5^1-phosphate (MPRP), 6-methylmercaptopurine (6MMP), adenine (Ad), xanthine (Xan) and uric acid (UA) were all obtained from Sigma (Poole, U.K.). Cysteine (Cys), glutathione (GSH) and guanine (Gua) were purchased from BDH (Eastleigh, U.K.). Hypoxanthine (Hx) was procured from Nutritional Biochemicals (Cleveland, OH, U.S.A.). Methotrexate (MTX) was kindly supplied by Lederle (Gosport, U.K.) and mBBr was obtained from C.P. Labs. (Bishops Stortford, U.K.). Methanol was HPLC grade from Fahrenheit Laboratory Supplies (Rotherham, U.K.). Chloroform, acetone, glacial acetic acid and sodium hydroxide were all analytical grade obtained from May and Baker (Dagenham, U.K.)

Stock solutions of 1 mg/ml of MPR and MPRP were made up in methanol and stored at 4°C. 6-MP and possible cross-reactants were stored at 4°C as 1 mg/ml stock solutions in 0.02 *M* sodium hydroxide, mBBr was stored at −20°C as 100 μg/ml stock solution in acetonitrile.

All stock solutions were protected from light during storage.

Methods

Plasma (1 ml) and 2 ml of methanol were placed in a 5-ml screw-top vial, vortex-mixed for 30 sec and centrifuged for 10 min at 2750 *g* on a Beckman Model J-6 centrifuge (Beckman-RIIC, High Wycombe, U.K.). The supernatant was decanted into a 10-ml screw-top test tube and 6 ml of chloroform were added and mixed on a rotating mixer. The phases were then separated by centrifuging at 2750 *g* for 10 min. The chloroform layer was discarded and the aqueous layer was again washed with 6 ml of chloroform as above. After the second wash 500 μl of the aqueous phase was transferred to a 5-ml screw-top glass vial which had been precooled on ice for 10 min. A 25-μl aliquot of a 100-μg/ml solution of monobromobimane was added and the mixture incubated in the dark at 4°C overnight. After incubation the reaction mixture was put on ice and 25 μl of glacial acetic acid was added and vortex-mixed. The mixture was then centrifuged at 9950 *g* for 2 min in an Eppendorf 5412 bench centrifuge (Anderman & Co., East Molesey, U.K.) to remove any precipitate. A 50-μl aliquot of the mixture was injected into the HPLC system.

The chromatographic procedure was performed using a Kipp Analytica 9208 pump (MSE Scientific Instruments, Crawley, U.K.) connected to a Waters 420 AC fluorescence detector (Waters Assoc., Harrow, U.K.) equipped with a 395-nm band-pass excitation filter and a 455-nm long-pass emission filter.

A 150 × 5 mm, 5-μm Hypersil-ODS column (HETP, Macclesfield, U.K.) was used for the separation. The mobile phase consisted of acetone—water (20:80) with 1% glacial acetic acid added and buffered to pH 4.0 with 1 *M* sodium hydroxide. The flow-rate was 1 ml/min with a back pressure of 15 MPa. 6-MP standards in plasma underwent the extraction and analytical procedure in parallel with the quality controls and samples. The standard curve was obtained by plotting peak height of the 6-MP—mBBr adduct against concentration of 6-MP in each standard.

RESULTS AND DISCUSSION

Typical chromatograms obtained from blank plasma and plasma spiked with 6-MP are shown in Fig. 1. The 6-MP adduct has a retention time of 7.2 min which allows a good separation from other peaks. The sample turnover time of approximately 12 min allows rapid quantitation of samples.

Previously published methods for the quantitation of 6-MP in plasma using reversed-phase HPLC and UV detection tend to suffer from a long sample turnover time or a high background interference close to the peak of interest.

The procedure uses a similar sample preparation to Narang et al. [12] utilising the low solubility of 6-MP in organic solvents. Methanol is used to precipitate protein in the sample and is later selectively removed by washes in chloroform.

Plasma standards give a linear response over the range 20—500 ng/ml (equation of line $y = 0.221x + 3.73$, correlation coefficient = 0.999) with coefficients of variation being given in Table I. A quadratic function may also be

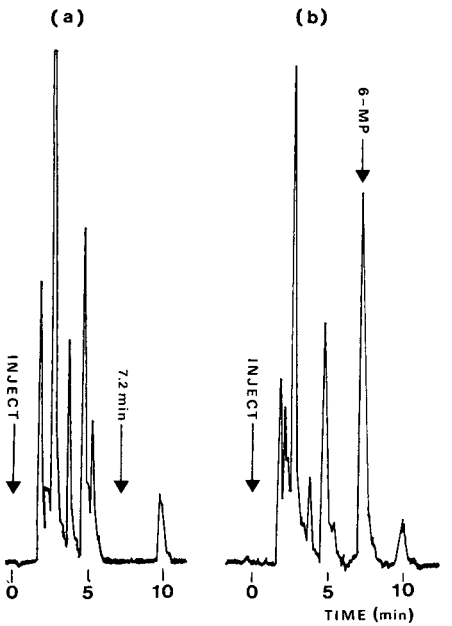

Fig. 1. Typical HPLC tracings of (a) blank plasma showing retention time of 6-MP peak; (b) plasma spiked with 500 ng/ml 6-MP.

TABLE I

MEAN OF FIVE STANDARD CURVES SHOWING STANDARD DEVIATIONS (S.D.) AND COEFFICIENTS OF VARIATION (C.V. %)

Concentrations of standards (ng/ml)	Mean peak height (mm)	S.D.	C.V. (%)
500	118.0	5.15	4.36
200	53.4	3.02	5.66
100	25.7	1.64	6.40
50	14.6	0.79	5.39
20	7.6	0.42	5.47

fitted to the data shown (equation: $y = 1.373 + 0.269x - 7.057 \cdot 10^{-5} x^2$). There is no significant difference between the standard curve fitted to this quadratic function and that fitted by linear regression as shown by a paired *t*-test. Also a paired *t*-test comparison of plasma samples calculated by both methods showed no significant difference. Within the limits of the standard curve specified, a linear response may be assumed, although beyond these bounds this correlation breaks down. We have found that the standard range is satisfactory for the measurement of therapeutic levels of 6-MP in patients and suggest that any samples above this range should be diluted until within the standard curve. Dilutions should be made with normal human plasma. The sensitivity of the assay, defined as the concentration of 6-MP giving a signal-to-noise ratio of 2:1, was approximately 20 ng/ml for a 50-μl injection. Adapting a theoretical definition [17], i.e. sensitivity equals 2 S.D. of the intercept at zero divided by the slope of the standard curve, the sensitivity was 18 ng/ml. Although a number of possible internal standards were considered no suitable compound was found. However, an internal standard, though desirable, is not essential in the method as described, since the standards employed are prepared in a matrix, i.e. plasma, identical to that of the sample, and are subsequently treated in parallel with them. Inter-assay and intra-assay coefficients of variation are shown in Table II for two quality controls.

No interference was found with the following compounds: 2-MP, MPR, MPRP, 6MMP, Ad, Xan, UA, Cys, GSH, Gua, Hx or MTX. Thiols in plasma, such as cysteine, do react with bimanes producing peaks near the solvent front (i.e. 1—4 min after injection). With the standard range quoted above, endogenous thiols do not affect the reaction with 6-MP since addition of more bimane after incubation does not increase the 6-MP peak suggesting that this reaction has gone to completion.

TABLE II

INTER-ASSAY AND INTRA-ASSAY VARIATION OF HIGH AND LOW QUALITY CONTROLS (HQC AND LQC) MADE FROM POOLS OF PATIENTS PLASMA

	Inter-assay (n = 4)			Intra-assay (n = 5)		
	Mean concentration (ng/ml)	S.D.	C.V. (%)	Mean concentration (ng/ml)	S.D.	C.V. (%)
HQC	160.0	6.63	4.14	162.1	6.20	3.82
LQC	34.6	3.29	9.48	33.1	3.14	9.46

We have found that the reaction of bimane with 6-MP will proceed to completion in 2 h at room temperature. However, a problem arose with this incubation method since a small interfering peak was seen in blank plasma which effectively decreased the sensitivity of measurement to 50 ng/ml. This interfering peak is not seen in the 4°C incubation and is probably a breakdown product of the bimane. This method was used to measure the pharmacokinetics of 6-MP in two acute lymphoblastic leukaemia (ALL) patients on maintenance therapy. The results are shown in Fig. 2. The patients were given their normal oral dose of 6-MP after an overnight fast and on a separate occasion after a standard breakfast. It can be seen from the results that there is a substantial decrease in absorption and bioavailability of the drug, as measured by AUC, when the dose was administered after a meal. The peak plasma levels obtained in the two subjects after an overnight fast correlate with levels published by Zimm et al. [18]. In their paper Zimm et al. suggest that the presence of food in the stomach during oral administration of 6-MP may cause variations in plasma levels. Our preliminary results confirm this suggestion and further studies are now underway to investigate this observation more closely.

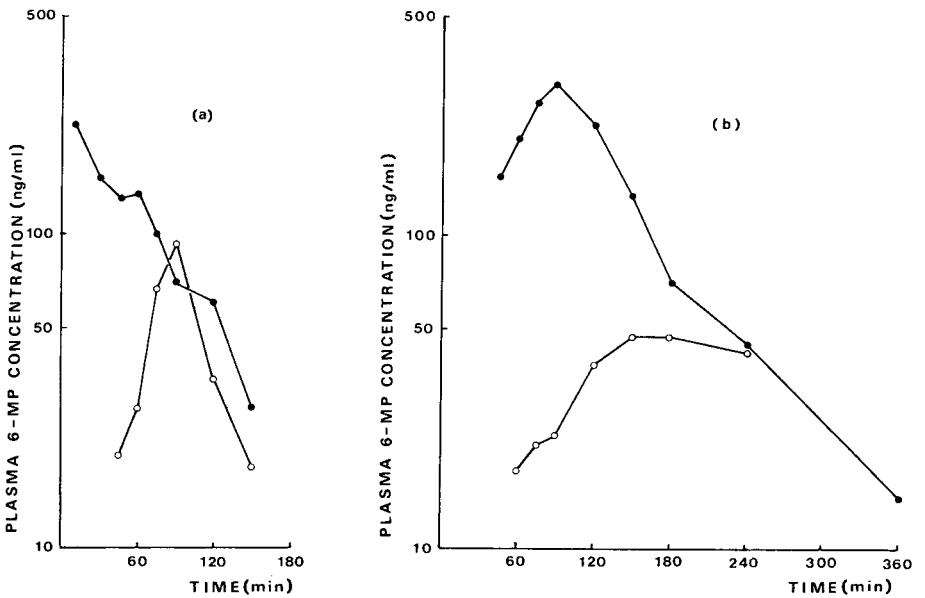

Fig. 2. Pharmacokinetics following oral administration of 6-MP in two patients with ALL. (a) Patient JR: age 10 years, dose 60 mg; (b) patient MB: age 63 years, dose 125 mg. •, Dose administered after overnight fast; ○, dose administered 15 min after standard breakfast. Area under curve (AUC) (ng/ml min) patient JR: post fast: 13255, post breakfast: 4693; patient MB: post fast: 33772, post breakfast: 6952.

Although we have only used the procedure for the measurement of pharmacokinetic samples, by decreasing incubation time to 1 or 2 h it could be used with decreased sensitivity for therapeutic drug monitoring.

The method described allows a specific and sensitive assay for the measurement of 6-MP in human plasma, which because of the rapid separation of fluorescent adduct by HPLC will facilitate further pharmacokinetic studies on this important anti-leukaemic drug.

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Note

Quantitative determination of premazepam in human plasma by high-performance liquid chromatography

ALBERTO BERNAREGGI*, BRUNO RATTI and ARMANDO TOSELLI

Pharmacokinetics and Metabolism Department, Research Laboratories Gruppo Lepetit S.p.A., Via Durando 38, 20158 Milan (Italy)

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Premazepam*, 1,7-dihydro-6,7-dimethyl-5-phenylpyrrolo-[3,4-*e*][1,4]-diazepin-2(3H)one (Fig. 1), is a pyrrolodiazepine which originates from the search for drugs with a clear-cut dissociation between the desired pure anti-anxiety activity and undesired sedative effects.

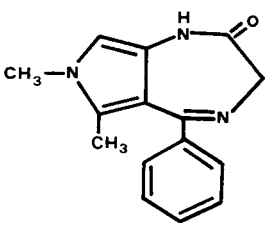

Fig. 1. Chemical structure of premazepam.

Premazepam shows benzodiazepine-like activity in the pharmacological tests considered to be predictive of anti-anxiety activity in man [1, 2]. Unlike benzodiazepines, premazepam does not induce ataxia or sedation at comparable or much higher doses than those effective in the above-mentioned tests.

Premazepam binds selectively to brain benzodiazepine receptors in vitro and in vivo [1, 2], acting also as a benzodiazepine antagonist at these receptors, i.e. it reduces or abolishes the effects of diazepam on motor coordination or muscular tone [1].

In a pilot study on tolerance and efficacy of premazepam, the drug was

*International non-proprietary name (World Health Organization).

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well tolerated by fifteen anxious patients up to 75 mg twice daily, and as judged by many rating scales for the evaluation of anxiety caused an improvement in clinical symptoms [3].

The aim of this work was to develop a sensitive, selective and reproducible method for assaying premazepam in human plasma.

Several analytical methods have been published for the determination of benzodiazepines in biological fluids by thin-layer chromatography (TLC), gas chromatography (GC) with sensitive and specific detection, and high-performance liquid chromatography (HPLC) [4, 5].

GC analysis is often unsuitable for the assay of some benzodiazepines because of their thermal instability or the need for derivatization. Some trials conducted on premazepam using this technique were unsuccessful.

A TLC method, validated for the concentration range 0.3—3.0 μg/ml, was previously developed [6] for assaying premazepam in the plasma of laboratory animals.

HPLC followed by UV detection was also found to be suitable for the determination of premazepam [7] and it was used in developing the present method for assaying the drug in human plasma. This method proved to have a higher sensitivity than the TLC method [6] since the application range tested was 20—200 ng/ml. Moreover, the HPLC method was particularly suitable for routine analysis using an automatic sampler.

Polarography was also used for the determination of some benzodiazepines in biological fluids [8], because of the presence of the reducible groups >C=N—, =N<O, and —NO_2 in the molecules [9]. The polarogram of premazepam displays a reduction wave with $E_{1/2}$ = —0.9 V under the conditions reported elsewhere [7]. This property of premazepam suggested the application of an electrochemical detector, a system more sensitive than ultraviolet (UV) detection, for the quantitative determination of premazepam in plasma after HPLC. Trials are in progress.

EXPERIMENTAL

Materials

Premazepam (Fig. 1) was Lepetit analytical standard with a chemical purity of 99.9%. Betamethasone (Fig. 2), crystalline, from Sigma (St. Louis, MO, U.S.A.), was used as the internal standard. Solvent and reagents were high purity grade from Merck (Darmstadt, F.R.G.). Distilled water was filtered through a Milli-Q system (Millipore, Bedford, MA, U.S.A.).

Plasma was from healthy volunteers.

The Bransonic 12 ultrasonic equipment was supplied by Smithkline (Soest,

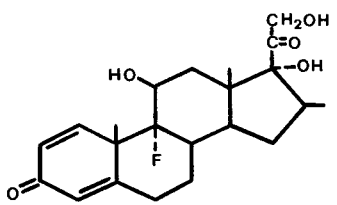

Fig. 2. Chemical structure of betamethasone, internal standard.

The Netherlands). The Continental Alter 2864 shaker was from Passoni (Milan, Italy).

A Supelcosil LC-18 DB column, 15 cm × 4.6 mm, particle size 5 μm, from Supelco (Bellefonte, PA, U.S.A.), was used as the stationary phase.

Instrumentation

A Waters Assoc. liquid chromatograph equipped with a Waters Model 620 solvent programmer, a Waters Model 440 absorbance detector, a WISP Model 710A automatic sampler, and a Tarkan 600 W+W recorder connected to an HP 3357 data system, or a Hewlett-Packard Model 3380A integrator, was used for the quantitative determination of premazepam with reference to the internal standard.

Standard solutions

For premazepam, 10 mg of premazepam were dissolved in 100 ml of acetonitrile—2-propanol (1:1). For internal standard, 10 mg of betamethasone were dissolved in 10 ml of acetonitrile—2-propanol (1:1).

Extraction procedure

A 1-ml volume of human plasma was pipetted into a screw-cap tube containing 1 ml of 0.2 *M* carbonate/bicarbonate pH 10 buffer solution and 0.1 ml of 0.05% ascorbic acid solution. The internal standard, betamethasone (500 ng), was added and then solid sodium chloride was dissolved until saturation by vortex mixing. The pH was adjusted to 10—10.5 with 0.5 *M* sodium hydroxide and the sample was extracted with 15 ml of chloroform by shaking for 10 min at 300 inversions per min. After centrifuging at 3000 *g* for 10 min (ambient temperature), the organic phase was transferred to another screw-cap tube containing 1 ml of ammonium hydroxide.

The tube was shaken for 5 min at 300 inversions per min and was centrifuged at 3000 *g* for 10 min (ambient temperature). The aqueous phase was aspirated; 12 ml of the organic phase were poured into a conical tube and evaporated to dryness in a water bath (37°C) under a stream of nitrogen.

The residue was concentrated at the bottom of the tube by washing the walls with chloroform saturated with ammonia and by evaporating the solvent in a water bath (37°C) under nitrogen.

Chromatography

The residue was redissolved in 20—25 μl of acetonitrile—2-propanol (1:1) and transferred to the microvial of the HPLC automatic sampler.

A 10-μl aliquot of the extracted sample was injected onto a Supelcosil LC-18 DB column (stationary phase). Isocratic elution was made using a mixture of 22% B in A as mobile phase, where A is 0.1 *M* ammonium hydrogen phosphate—ammonium dihydrogen phosphate solution pH 7.5, and B is acetonitrile. The flow-rate was 2 ml/min, and UV detection was at 254 nm. The retention times of premazepam and betamethasone were 6.5 and 20 min, respectively.

RESULTS

Plasma samples from untreated subjects, processed by the method described, gave tracings without any peaks that could interfere with the determination of premazepam or the internal standard (Figs. 3 and 4).

Plasma samples of untreated healthy subjects were spiked with premazepam and betamethasone (internal standard) in order to determine the recovery, precision (repeatability), accuracy and linearity of the method. Five samples were prepared for each of the following concentrations of premazepam: 20, 50, 100, 200 ng/ml of plasma. The samples were extracted and analysed as described.

The results of the recovery trials are given in Table I. The mean recovery of premazepam relative to betamethasone internal standardization from human plasma was 63—82% in the concentration range tested, indicating that premazepam is extracted less than the internal standard used. The lowest recovery (63%) was for the lowest concentration (20 ng/ml). The true value of the

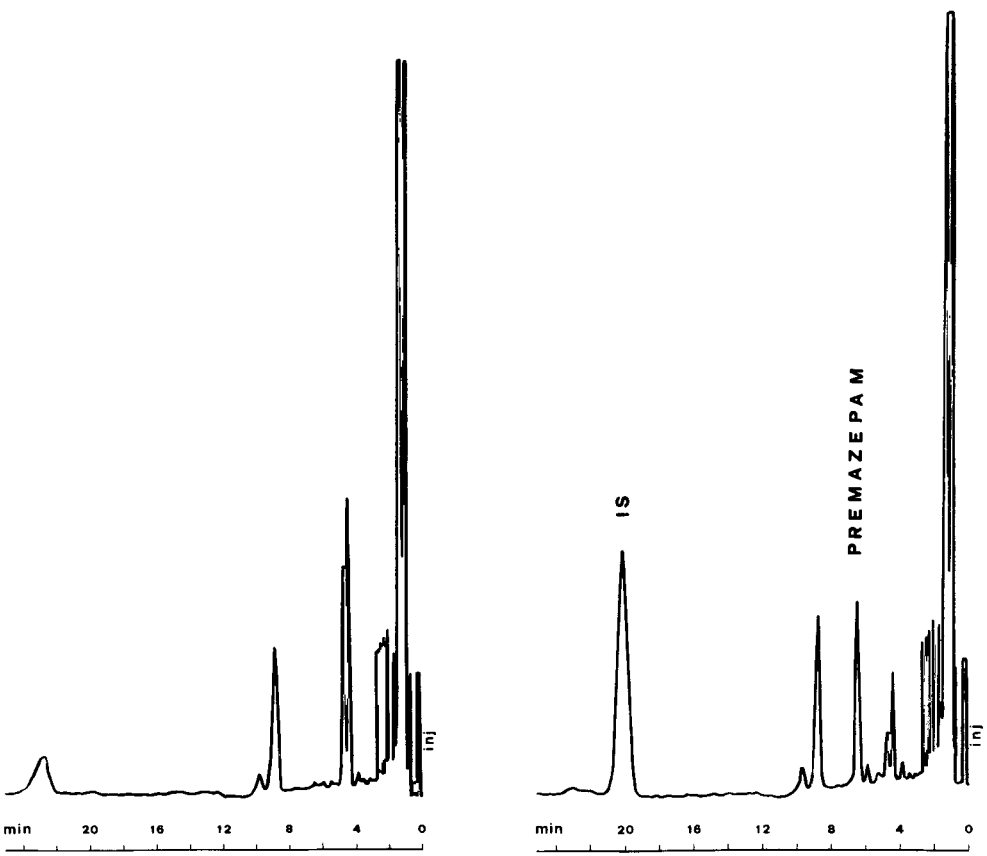

Fig. 3. Chromatogram of a plasma sample of an untreated subject. The retention times of premazepam and betamethasone (internal standard) are 6.5 and 20 min, respectively.

Fig. 4. Chromatogram of a plasma sample of an untreated subject, spiked with premazepam (100 ng/ml) and the internal standard (IS, 500 ng/ml).

TABLE I

RECOVERY OF PREMAZEPAM FROM HUMAN PLASMA SAMPLES (RELATIVE TO BETAMETHASONE)

Added (ng/ml)	Found (ng/ml)					Mean ± S.D. (ng/ml)	R.S.D.	Mean recovery (%)
20.5	11.9	12.6	14.5	13.8	12.0	13.0 ± 1.1	8.5	63.2
50.5	38.1	36.4	40.9	34.7	39.8	38.0 ± 2.5	6.6	75.2
100.8	74.4	76.8	76.5	75.0	77.2	76.0 ± 1.2	1.6	75.4
205.9	165.5	164.7	171.0	174.2	164.4	168.0 ± 4.4	2.6	81.6

plasma concentration in a plasma sample can be found by the regression analysis equation given below.

The precision of the method was established by the value of the relative standard deviation (R.S.D.) and ranged from 1.6% to 8.5% (Table I) in the concentration range analysed.

The accuracy of the method was calculated using the twenty determinations reported in Table I, with a mean recovery of 73.8% and an R.S.D. of 10.4% over the concentration range analysed.

The linear-regression analysis made with twenty values of X (premazepam added, ng/ml) and twenty values of Y (premazepam found, ng/ml) (Table I) provided the equation $Y = 0.8348X - 5.1102$ (correlation coefficient, $r = 0.9987$).

DISCUSSION

In setting up the extraction procedure, a simple extraction with chloroform of premazepam and the internal standard, followed by evaporation under nitrogen, gave varying recoveries of premazepam from spiked plasma samples particularly at the lowest concentrations.

Initial trials indicated that the critical step was the evaporation of the organic phase under nitrogen, after which the recovery of premazepam was found to be non-reproducible, probably due to the adsorption of premazepam on the glass. In our efforts to improve the precision of the method, several trials were conducted and the use of ammonia gave the best results. The organic phase has to be saturated with ammonia before evaporating it under nitrogen and chloroform saturated with ammonia has to be used to collect the residue at the bottom of the conical tube.

It is well known that the pyrrole ring is sensitive to oxidation [10]. Thus, ascorbic acid was added in the tube as anti-oxidizing agent before extracting the sample.

The validation range was established on the basis of the plasma concentration of premazepam expected in humans after the administration of the drug. The method was applied to observe the time course (Fig. 5) of premazepam in the plasma of subjects ($n = 4$) treated with a single 30-mg oral dose [3]. Premazepam was rapidly absorbed by the gastrointestinal tract, reaching plateau plasma concentrations of 330—370 ng/ml 1.5—6 h after administration. The concentration of premazepam decreased thereafter with a mono-

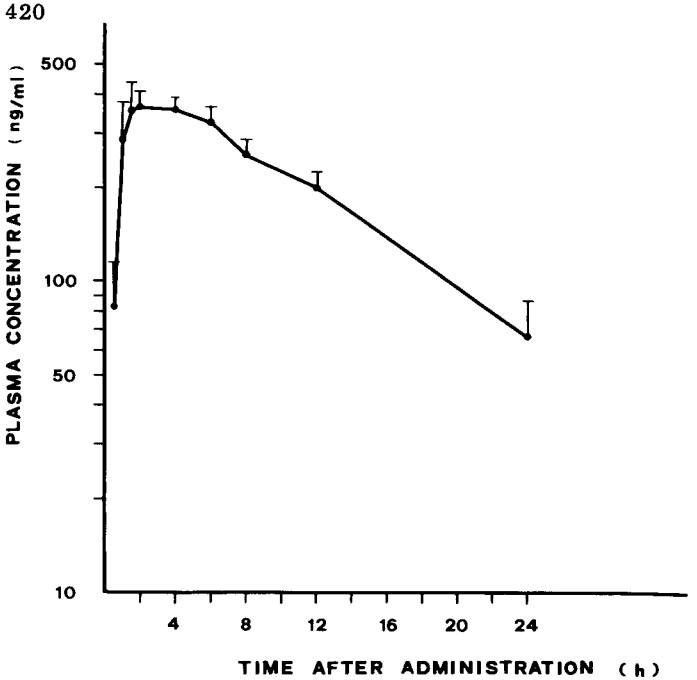

Fig. 5. Mean plasma levels ± S.E. of premazepam in man (n = 4) after a 30-mg oral dose.

exponential slope characterized by an elimination half-life of about 8 h. Twenty-four h after administration, the plasma concentration declined to about 70 ng/ml.

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Note

Ion-exchange high-performance liquid chromatography in drug assay in biological fluids

IV. Nadolol diastereomers: demonstration of pharmacokinetic and binding equivalence

V.K. PIOTROVSKII*, Yu.A. ZHIRKOV and V.I. METELITSA

The Institute of Preventive Cardiology of the U.S.S.R. Cardiology Research Center, Cherepkovskaya, 15-a, 121500, Moscow (U.S.S.R.)

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Nadolol, *cis*-5-{3-[(1,1-dimethylethyl)]-2-hydroxypropoxy}-1,2,3,4-tetrahydro-2,3-naphthalenediol, is a new β-adrenoceptor blocking agent used in the treatment of angina pectoris and hypertension [1]. Its molecule contains three chiral centres, but due to the *cis*-configuration of the ring hydroxyls, only four enantiomers (two pairs of optical antipodes) are possible. Nadolol stereoisomers were separated for the first time in 1974 [2]. It was demonstrated that there are two racemic mixtures called racemate A and racemate B which can be comparatively easily separated by fractional crystallization. Racemate A consists of (*d*)-side-chain-(*l*)-cyclohexene ring and (*l*)-side-chain-(*d*)-cyclohexene ring nadolol, and racemate B consists of (*d*)-side-chain-(*d*)-cyclohexene ring and (*l*)-side-chain-(*l*)-cyclohexene ring nadolol. It was also shown that racemate B provides three times more β-blocking and antiarrhythmic activity than racemate A. Thus the separate determination of nadolol racemates in patients' biofluids is of interest since they also can exert a different pharmacokinetic behaviour.

Recently a paper was published [3] in which the high-performance liquid chromatographic (HPLC) separation of racemates on a reversed-phase column was described. Earlier we developed an assay for nadolol (as a racemic mixture) in serum and urine using HPLC on a cation-exchange column [4]. The aim of the present work was to widen the possibilities of our method to determine racemates A and B separately in human blood and urine. We used a column packed with Nucleosil 5-SA, of particle size 5 μm, which provided a higher efficiency than the Partisil 10 SCX used previously [4].

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EXPERIMENTAL

Apparatus and columns

The chromatographic system consisted of a Model 100A high-pressure pump (Altex, Berkeley, CA, U.S.A.), a Model 7125 injection valve (Rheodyne, Catabi, CA, U.S.A.) and a fluorescence detector FluoroMonitor III (Model 1311; LDC/Milton Roy, Riviera Beach, FL, U.S.A.). The chromatograms were recorded and processed on a Model 3390A reporting integrator (Hewlett-Packard, Avondale, PA, U.S.A.). The column, 250 × 4.6 mm, was packed with Nucleosil 5-SA (5 μm particle size; Alltech, Deerfield, IL, U.S.A.). The precolumn, 40 × 3.2 mm, was packed by us with Partisil 10-SCX (10 μm particle size).

Reagents and standards

Nadolol standard substance was kindly given by Squibb, Hounslow, U.K. Procainamide hydrochloride used as internal standard was obtained from Serva (F.R.G.). Acetonitrile (LiChrosolv®), orthophosphoric acid, diethylamine and 0.05 mol/l sulphuric acid were analytical grade (E. Merck, Darmstadt, F.R.G.). Pentane was "Resi-Analyzed" grade (J.T. Baker, U.S.A.). Water for the mobile phase and preparation of standard solutions was purified using a Milli RO/Milli Q system (Millipore, Bedford, MA, U.S.A.). Other reagents were chemically pure (Reachim, U.S.S.R.).

Extraction procedure

Serum or urine (1 ml) was placed in a PTFE-lined screw-cap tube and 0.1 ml of internal standard solution (1—10 μg/ml) was added. Then 0.1 ml of 1 mol/l potassium hydroxide and 7 ml of pentane—amyl alcohol mixture (4:1) were added; the tube was then vortexed for 1 min. After centrifugation for 2 min the upper layer was transferred into a conical tube, 0.1 ml of 0.05 mol/l sulphuric acid was added, and the tube was vortexed for 1 min. After brief centrifugation at 750 *g*, the organic layer was discarded and the acidic extract was neutralized with 1 mol/l potassium hydroxide to pH 6—7. Then an aliquot of the extract was injected onto the column.

Chromatographic conditions

To prepare the mobile phase, acetonitrile and water were mixed (15:85) and 0.2% (v/v) of orthophosphoric acid (1.71 g/cm^3) was added. The pH was then adjusted to 4.1 by the addition of diethylamine. The flow-rate was 1 ml/min. In the detector a Zn lamp was used as the light source for excitation (214 nm). An emission filter of 230—400 nm was used.

Quantitation

The reporting integrator calculated the peak area ratios of nadolol to internal standard (procainamide). Calibration lines were plotted in the range 20—1000 ng/ml nadolol for both serum and urine.

Pharmacokinetic study

A patient with stable angina pectoris received 80 mg of nadolol orally

(Corgard®, Squibb). Blood samples were taken before and 15, 30 and 45 min, and 1, 1.5, 2, 3, 4, 6, 8 and 24 h after drug administration. Serum was separated and frozen at −18° C until analysis.

Serum binding study

Protein binding of nadolol in serum was studied using equilibrium dialysis at 37°C in a PTFE cell with cellophane membrane. Fresh drug-free serum was spiked with nadolol (300 ng/ml) and after incubation at 37°C for 1 h was dialysed against 0.067 mol/l potassium phosphate buffer pH 7.4. Equilibrium was reached by 18 h. The concentrations of nadolol diastereomers in post-dialysis serum and buffer were determined as described and the fraction of bound drug was calculated as the drug level in serum minus the drug level in buffer divided by the drug level in serum.

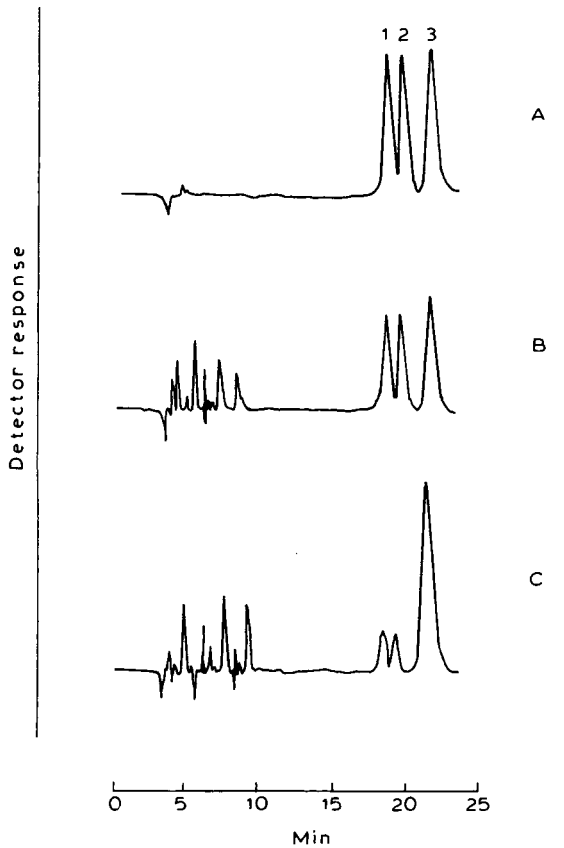

Fig. 1. (A) Chromatogram of nadolol and procainamide standard substances on the cation-exchange column Nucleosil 5-SA. Peaks: 1 = racemate B, 2 = racemate A, 3 = procainamide. (B) Chromatogram of an extract of blank serum spiked with 500 ng/ml nadolol and procainamide. (C) Chromatogram of an extract of patient's serum obtained 6 h after oral administration of 80 mg of nadolol.

RESULTS

The conditions described provide good separation of nadolol diastereomers, as demonstrated in Fig. 1A. Peaks 1 and 2 correspond to racemates A and B. The peak with a retention time of 22.55 min corresponds to procainamide. The areas of peaks 1 and 2 are equal, thus the ratio of the racemates in the nadolol standard substance used in the experiments is 1:1. The same ratio was found by Matsutera et al. [3]. For peak identification the substance was enriched by racemate A, which is more soluble in acetonitrile [2]. In the enriched sample the second peak became higher than the first. Thus on the cation-exchange column racemate A has a greater retention time (19.6 min) than racemate B (18.7 min) in contrast with the result obtained with the reversed-phase column [3].

Chromatogram B (Fig. 1) was obtained from an extract of blank serum spiked with 500 ng/ml of nadolol and procainamide. As can be seen, the racemate peak areas are equal on the chromatogram of the extracts as well as of the nadolol standard substance. This means that the mixture is not enriched by one of the racemates during the extraction. The drug-free serum extract contained no peaks that could interfere with nadolol or the internal standard.

The calibration graphs were linear in the nadolol concentration range of 20—1000 ng/ml. The coefficient of variation at 20 ng/ml was 7.8% for racemate A and 8.4% for racemate B (n = 5). Almost the same results were obtained with urine.

Chromatogram C (Fig. 1) is from an extract of patient's serum obtained 6 h after nadolol administration. The peaks of the diastereomers were equal again. In Fig. 2 the serum concentrations of the nadolol diastereomers 0—24 h after drug administration are plotted. The upper curve represents the total

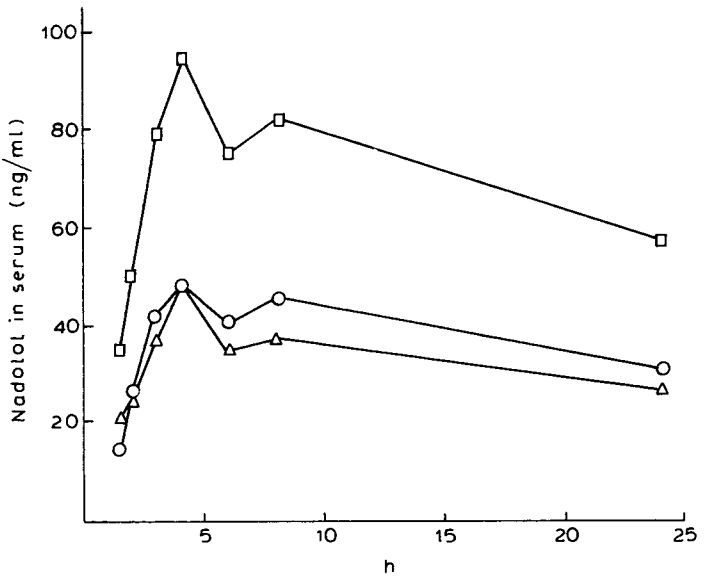

Fig. 2. Nadolol diastereomer concentrations in patient's serum after nadolol administration (80 mg orally). △, Racemate A; ○, racemate B; □, total nadolol (sum of diastereomer levels).

nadolol concentration (the sum of the racemate concentrations). It is evident that the difference in racemate levels is small and does not exceed the determination error at all the time points. The half-lives of the nadolol diastereomers were estimated from the last two points and were 30 h for racemate A and 33 h for racemate B. Areas under the concentration—time curves from 0 to 24 h assessed by the trapezoidal rule were 883 and 767 ng h/ml for racemates A and B, respectively. The serum binding study showed that the concentrations of the nadolol diastereomers in post-dialysis serum and buffer were equal. The bound fraction was 49.6 ± 5.0% (n = 3).

DISCUSSION

The modification of the assay method for nadolol described here allows the nadolol diastereomers (racemates A and B) in human serum and urine to be determined separately. It is interesting to note that for the separation of nadolol diasteromers on the reversed-phase column it is necessary to use alcohols or ethers only in the mobile phase; acetonitrile allows no separation [3]. In contrast, good separation was achieved on the cation-exchange column with a mobile phase containing acetonitrile.

Data obtained with one patient clearly demonstrate that there is no difference in the kinetics of the nadolol diastereomers. Their concentrations were close at all the sampling points. Half-lives and areas under the concentration—time curves were also close. We have demonstrated also that protein binding of the two nadolol diastereomers in serum does not differ.

There are data in the literature that the elimination of propranolol is stereoselective in dogs [5] and man [6]. In this case, however, the pharmacokinetics of the two enantiomers, which have an opposite configuration at the chiral carbon atom in the side-chain, are different. In nadolol racemates A and B two enantiomers are present so the racemates are equivalent in relation to the configuration at the side-chain chiral centre. This is probably why we have not found any difference in their pharmacokinetics. Also, the mechanisms of elimination of nadolol and propranolol differ substantially. While the latter is metabolized mainly in the liver [7], and this process is stereoselective, nadolol is excreted in the urine intact [8]. The renal excretion seems not to be stereoselective.

Thus it may be concluded that, in contrast with the pharmacological observations, which showed different β-blocking activity of the nadolol diastereomers, their pharmacokinetic behaviour is analogous.

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Note

Separation and identification of a plasma and urinary mono-acetylated conjugate of chloroquine in man by ion-pair high-performance liquid chromatography

NESBITT D. BROWN* and MARGARET G. STERMER-COX

Division of Biochemistry, Walter Reed Army Institute of Research, Washington, DC 20307 (U.S.A.)

BING T. POON

Division of Experimental Therapeutics, Walter Reed Army Institute of Research, Washington, DC 20307 (U.S.A.)

and

JEFFREY D. CHULAY

U.S. Army Medical Research Unit-Kenya, Box 401, Army Post Office, New York, NY 09675 (U.S.A.)

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Acetylation in vivo in man of certain therapeutic drugs, especially those containing primary and secondary amino groups, is used as a biological marker for assessing the genetic polymorphism of an individual [1—3]. Human subjects are phenotyped as either rapid or slow acetylators. While it is not clear whether the recognizable phenotyped acetylator can be determined by the acetylation of chloroquine to its N-acetyl metabolite, results from this study showed that the process of acetylation does occur during the metabolism of chloroquine in man.

In this report, we describe an ion-pair high-performance liquid chromatographic (HPLC) method for detecting and quantifying an acetylated conjugate of chloroquine in plasma and urine specimens. Fig. 1 represents a metabolic scheme showing the formation of N-acetyl(mono)desethylchloroquine. A pretreated organic extract of plasma or urine is required prior to analysis. Amounts as low as 10 ng on-column are quantifiable, and the analysis requires 12 min per sample. The use of an isocratic system minimizes the analysis time between runs.

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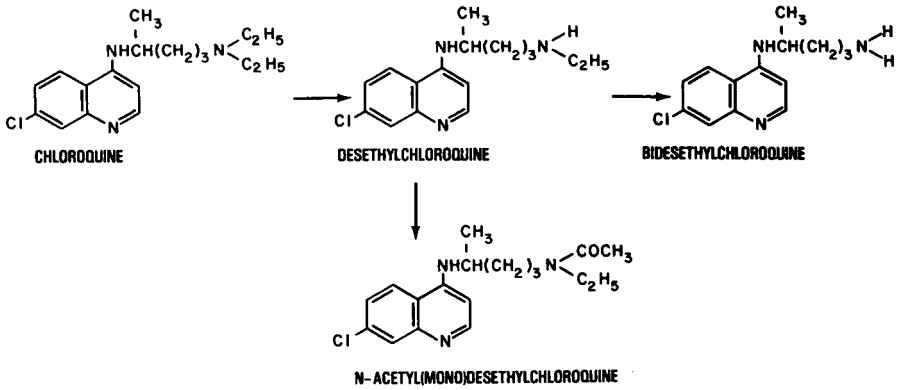

Fig. 1. Metabolic pathway for chloroquine in human subjects, showing the formation of N-acetyl(mono)desethylchloroquine.

We are currently employing this method in our laboratory for separating and identifying various 7-chloro-4-aminoquinoline metabolites associated with the metabolism of chloroquine in humans.

EXPERIMENTAL*

Apparatus

A Waters Assoc. (Milford, MA, U.S.A.) Model ALC/GPC 204 liquid chromatograph, equipped with two Model 6000A high-pressure pumps, a 660 solvent programmer, a U6K loop injector, a Model 440 absorbance detector, set at 340 nm and a Model 730 data module was used to conduct this study.

Reagents

All solvents and chemicals used in this investigation were either of spectroquality or of analytical grade. Acetonitrile was obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). PIC-B7 reagent (1-heptanesulfonic acid) was purchased from Waters Assoc. N-Acetyl(mono)desethylchloroquine was obtained as a gift from Dr. Frederick Churchill, III (C.D.C., Atlanta, GA, U.S.A.). Chloroquine disphosphate (Sigma, St. Louis, MO, U.S.A.), desethylchloroquine sesquioxalate, and bidesethylchloroquine hydrobromide (Inventory, Walter Reed Army Institute of Research, Washington, DC, U.S.A.) were used to prepare all working standards. Stock solutions containing 100 ng/ml of each compound were prepared in glass-distilled water. Working standards were prepared from the stock standards.

Procedure

A 300 mm × 3.9 mm I.D., 10-μm μBondapak C_{18} column (Waters Assoc.) was used to chromatograph chloroquine and its metabolites in standards and experimental samples. A description of the method has been published previously by our group [4].

*The manufacturers' names and products are given as scientific information only and do not constitute an endorsement by the United States Government.

Samples

Urine and plasma specimens were collected from two normal subjects. Each volunteer received a single 300-mg dose of chloroquine diphosphate. Biological specimens were taken before dosing (control) and 12 and 24 h after dosage. The extraction method used for our samples is described in an earlier report [4].

RESULTS AND DISCUSSION

The metabolism of chloroquine in humans has been widely discussed and debated by various research groups for many years, yet many questions on the pharmacokinetics of this drug remain unanswered. Recently, research by several groups [5, 6] has shown that HPLC can be utilized to separate and identify both major and minor metabolites present in physiological fluids. Work by our group has also shown that the mono- and di-ethylated metabolites of chloroquine were present in urine and plasma specimens of human subjects. At the same time, other 7-chloro-4-aminoquinoline analogues were suspected to be formed during this metabolic process. We now report the separation and identification of an N-acetyl conjugate of the primary metabolite of chloroquine in urine and plasma samples of man. N-Acetyl(mono)desethylchloroquine, the acetylated product of desethylchloroquine, was separated and quantified by HPLC and identified by chemical ionization mass spectrometry.

In utilizing this ion-pair reversed-phase HPLC method, various chloroquine analogues and experimental samples were chromatographed to determine the specificity and sensitivity of the procedure. Because of the similarities of chemical structures, separation of these 7-chloro-4-aminoquinolines is difficult. Results showed that the resolution for the series of compounds separated were excellent. At the same time, linearity was obtained for the various concentrations of each compound analyzed (5—200 ng).

Samples from two human subjects were chromatographed. Chromatograms shown in Fig. 2A and B depict two time frames for subject A. N-Acetyl(mono)-desethylchloroquine was observed in both the 12- and 24-h urine specimen. In calculating the total urinary output of chloroquine and its metabolites for subject A over the 24-h period, only 17% of the administered dose was recovered. Of this, 1.4% was N-acetyl(mono)desethylchloroquine. Bidesethylchloroquine (4.66%), desethylchloroquine (22.83%) and chloroquine (69.03%) constituted the remaining 7-chloro-4-aminoquinoline compounds present in the 24-h urine collection. Fig. 3 represents the 12-h plasma sample of subject A. N-Acetyl(mono)desethylchloroquine was also observed in this specimen. Chromatographic data of the 24-h plasma sample of subject A failed to show the presence of the N-acetylated metabolite.

The chromatograms shown in Fig. 2C and D show the separation of the urine specimen of subject B. Even though the excreted urinary volume for subject B was 50% greater than that of subject A, quantifiable amounts of N-acetyl(mono)desethylchloroquine were measurable in the 12-h urine sample. It represented 1.1% of the administered dose of chloroquine diphosphate. The 24-h urine specimen did not show a corresponding peak for the N-ace-

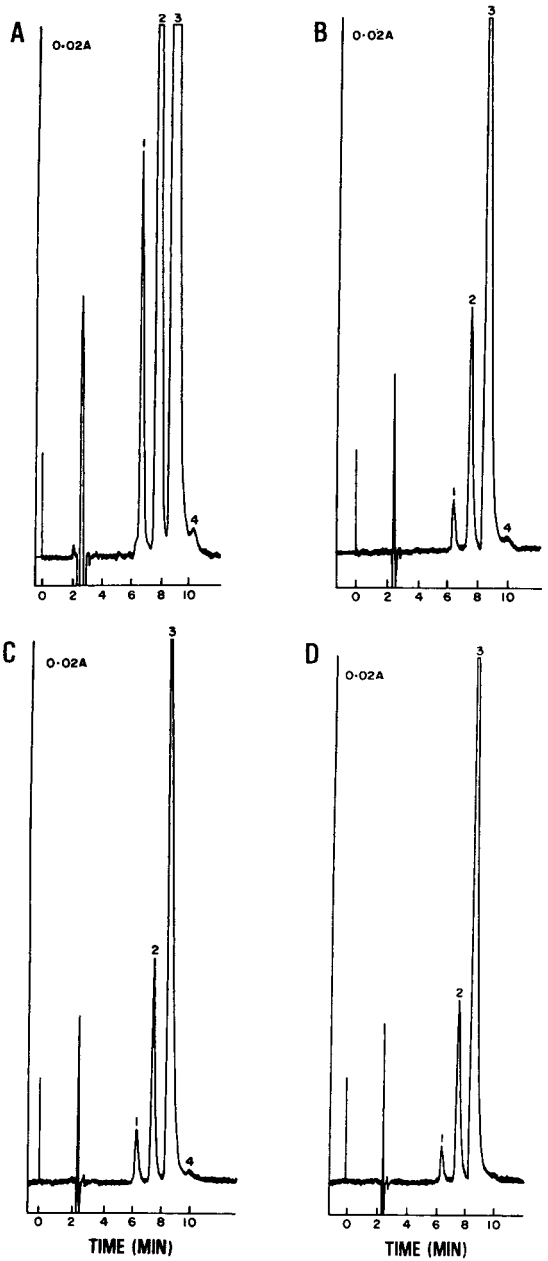

Fig. 2. Chromatograms of (A) the 12-h urine sample from human subject A; (B) the 24-h urine sample from human subject A; (C) the 12-h urine specimen from human subject B; (D) the 24-h urine specimen from subject B. Conditions: column: 300 mm × 3.9 mm I.D., μBondapak C_{18}; mobile phase: 0.02 *M* PIC-B7 reagent—acetonitrile (66:34); flow-rate: 1.0 ml/min; column temperature: ambient; sample volume: 10 μl; detection wavelength: 340 nm. Peaks: 1 = bidesethylchloroquine; 2 = desethylchloroquine; 3 = chloroquine, and 4 = N-acetyl(mono)desethylchloroquine.

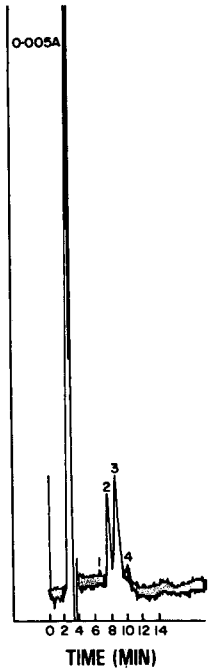

Fig. 3. Chromatogram of a 12-h plasma extract from subject A. Sample volume: 10 μl. Peaks: 1 = bidesethylchloroquine; 2 = desethylchloroquine; 3 = chloroquine; and 4 = N-acetyl(mono)desethylchloroquine.

tylated metabolite. However, the recoverable amounts of chloroquine and its two de-ethylated metabolites were measured and their amounts were similar to the total 24-h urinary values of subject A. Recovered percentages for these compounds were as follows: 5.8% for bidesethylchloroquine, 22.48% for desethylchloroquine, and 68.88% for chloroquine. The 12- and 24-h plasma sample of subject B did not contain any detectable amounts of N-acetyl(mono)desethylchloroquine.

While the experiment we describe in this study should have obvious importance in probing the metabolic fate of chloroquine in humans, many of the aspects involving the metabolism of this drug are far from being completely understood.

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CHROMBIO. 2154

Note

High-performance liquid chromatographic assay for aminoglutethimide and its acetylated metabolite in urine

M.O. KAMBLAWI, R.G. STEVENS and P.J. NICHOLLS*

Welsh School of Pharmacy, UWIST, P.O. Box 13, Redwood Building, Cathays Park, Cardiff CF1 3XF (U.K.)

(First received January 3rd, 1984; revised manuscript received March 7th, 1984)

Aminoglutethimide (AG), 3-(4-aminophenyl)-3-ethyl piperidine-2,6-dione, is an effective agent for the treatment of advanced breast cancer in post-menopausal women [1, 2].

Metabolism studies in man have shown that, after single doses of AG, large amounts are excreted unchanged in the urine together with smaller quantities of its acetylated metabolite, N-acetyl amino-glutethimide (AcAG) [3, 4]. Some minor metabolites of the drug have also been identified as N-formyl AG and nitroglutethimide [5]. N-Hydroxyl AG has been recently described as an auto-induced metabolite that appears in the urine on chronic dosing with AG [6].

In the rat the complete metabolic fate of AG has been determined using the ^{14}C-labelled drug [7] and only small quantities of AG are excreted in urine together with larger amounts of AcAG [8].

In order to study the inter-individual variation in the excretion of AG and its acetylation profile in man and other species, we developed a simple method for the quantitative determination of AG and AcAG in urine by high-performance liquid chromatography (HPLC) using aniline as internal standard. Recently an HPLC procedure for the assay of AG in plasma has been described [9]. However, the method does not determine AcAG.

EXPERIMENTAL

Materials

Aminoglutethimide was a gift from Ciba-Geigy (Horsham, U.K.).

N-Acetyl aminoglutethimide was synthesized according to the procedure described by Douglas [10].

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Aniline hydrochloride, 99% (Aldrich, Gillingham, U.K.), was used as the internal standard.

Dichloromethane (AnalaR, BDH, Poole, U.K.) was distilled and washed with 0.1 *M* phosphate buffer pH 7.0 prior to use.

The phosphate buffers used were prepared from the AnalaR grade mono- and di-sodium phosphates using mixtures of equimolar solutions.

HPLC grade acetonitrile (Rathburn, Walkerburn, U.K.) and AnalaR grade water (BDH) were used for chromatography.

Chromatography

Chromatographic analyses were performed on a component system consisting of a Constametric II G pump, equipped with a Rheodyne 7125 syringe-loading sample injector, a Spectromonitor III ultraviolet (UV) detector and a Tekman TE 200 flatbed recorder.

Samples were chromatographed at room temperature on a Spherisorb ODS column (30 cm × 4 mm I.D., 5 μm particle size) from Jones Chromatography (Llanbradach, U.K.).

The separations were obtained using acetonitrile—0.01 *M* phosphate buffer, pH 6.8 (22:68) as the mobile phase, with a flow-rate of 1.5 ml/min. Peaks were detected at 234 nm and peak areas were measured by triangulation.

Calibration curves

From stock solutions in methanol standard solutions were prepared containing mixtures of AG (in the range 0.05—5 mg), AcAG (in the range 0.025—2.5 mg) and aniline hydrochloride (0.2 mg) per ml of methanol. Replicate injections of 10 μl were made for each sample.

Assay method

Phosphate buffer, 0.1 *M*, pH 7 (5 ml) and aqueous aniline hydrochloride solution (1 ml containing 0.1 mg) were mixed with urine (5 ml). After addition of dichloromethane (10 ml) the mixture was vortexed for 2 min. The phases were then separated by centrifuging and the aqueous layer was re-extracted with dichloromethane (10 ml). The combined organic phase was dried with anhydrous sodium sulphate, filtered and evaporated under reduced pressure at 60°C. The residue was dissolved in methanol (0.5 ml). Replicate aliquots (10 μl) were injected into the column. Earlier investigations [3, 4] have shown this extraction procedure to produce reliably high recoveries of AG and AcAG.

RESULTS AND DISCUSSION

When AG, AcAG and aniline, dissolved in methanol, were applied together to the column a good separation was obtained, the retention times (and capacity factors) being 12.6 min (4.7), 14.8 min (5.7) and 8.6 min (2.9), respectively. Solutions of the compounds in methanol over the concentration ranges 0.05—5 mg/ml AG and 0.025—2.5 mg/ml AcAG with 0.2 mg/ml aniline hydrochloride were chromatographed in this system. Linear relationships between the ratio of peak areas (AG and AcAG to internal standard) and concentration

(mg/ml) of AG and AcAG were found that could be expressed by the following equations: for AG: $Y = 2.2192\,X + 0.2168$ ($r = 0.98$); for AcAG: $Y = 1.7155\,X - 0.0730$ ($r = 0.98$) where Y represents the peak area ratio and X the concentration (mg/ml).

Fig. 1 shows chromatograms obtained from extracts of a representative sample (Fig. 1A) from a human volunteer following oral ingestion of a 250-mg tablet of AG (Orimeten, Ciba-Geigy) and of blank urine (Fig. 1B). These chromatograms demonstrate the lack of interference and the specificity of the assay procedure for the measurement of AG and AcAG in human urine.

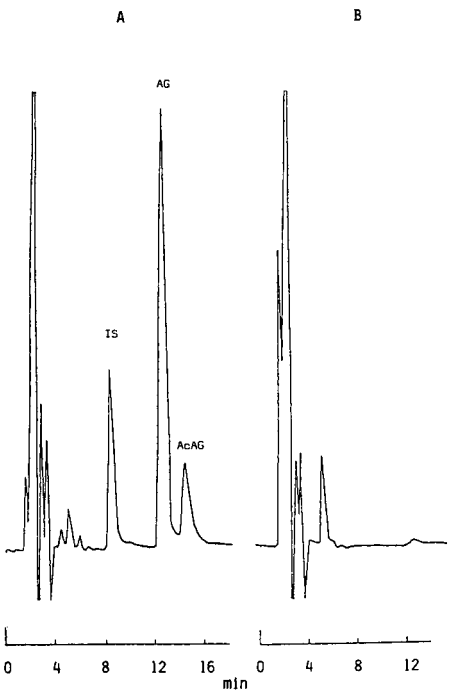

Fig. 1. Chromatograms of (A) extract of urine collected 0—24 h after the ingestion of a 250-mg tablet of aminoglutethimide; this urine contained 24.8% and 5.5% of the administered dose as AG and AcAG, respectively; (B) extract of blank human urine. UV detection: 234 nm. Peaks: IS = aniline; AG = aminoglutethimide; AcAG = N-acetyl aminoglutethimide.

Authentic samples of N-formyl AG (from AG) and nitroglutethimide (from glutethimide) were synthesized by standard methods and chromatographed in the above system. Nitroglutethimide had an elution time of 30 min, while N-formyl AG eluted between the AG and AcAG peaks. The formyl peak was never observed on the chromatograms from urine extracts of human subjects taking AG. Thus, as N-formyl AG is a minor metabolite, it was considered not to interfere with the assay of AG and AcAG. In a recent study in this laboratory, where urine extracts from patients receiving AG in various combinations with dextropropoxyphene, hydrocortisone, ibuprofen and paracetamol were chromatographed, no interfering peaks were detected.

Spiked blank human urine, containing mixtures of equal concentrations of AG and AcAG (at 10 and 20 μg/ml of urine) was carried through the ex-

traction and assay procedure. Recovery was consistent (90%) and the assay-variation (coefficient of variation, C.V., %) determined on six aliquots was 3.5% and 2.4% for AG and AcAG, respectively.

The lowest limit of detection was found to be approximately 0.2 μg in the methanolic solution injected onto the column. This is a more than adequate sensitivity for human urine in view of the relatively large doses employed in therapy and the extensive urinary excretion of the drug and its metabolites.

In a group of five male human volunteers with normal renal function receiving AG (250 mg) orally, the mean 24-h urinary excretion of unchanged drug and AcAG (as a percentage of the dose), determined by the above method, was 31.2 ± 10.1% S.D. and 10.3 ± 2.9% S.D., respectively.

The assay was also applied to the determination of AG and AcAG in urine of various animal species receiving AG (60 mg/kg) orally. Initially, representative samples of blank urine from each species were carried through the procedure. When chromatographed, the extracts presented similar elution profiles to those of blank human urine indicating the absence of interfering endogenous compounds. Following a dose of AG, peaks for AG and AcAG were detected in extracts of urine from rats, guinea pigs and rabbits. However, the extracts from these species contained additional materials (presumably other metabolites of AG) that were observed as peaks on the chromatograms. These did not interfere with the assay of AG and AcAG as they eluted prior to the internal standard. For the rat and guinea pig a further metabolite was eluted with a retention time of 17.9 min.

TABLE I

24-h URINARY EXCRETION OF AMINOGLUTETHIMIDE (AG) AND N-ACETYL AMINOGLUTETHIMIDE (AcAG) IN SEVERAL ANIMAL SPECIES AFTER AN ORAL DOSE OF AG (60 mg/kg)

Results are the means ± S.D. from four animals.

Species	Excretion (percentage of dose)	
	Ag	AcAG
Rat	1.8 ± 0.5	24.3 ± 6.2
Guinea pig	8.3 ± 1.1	4.2 ± 2.0
Rabbit	9.9 ± 4.3	8.0 ± 5.2

From the results presented in Table I it may be observed that unchanged AG was excreted in the urine of these animals to a small extent only. This was true for the excretion of AcAG in the guinea pig and rabbit. However, in the rat, AcAG appeared to be an important urinary metabolite.

In conclusion, this HPLC method is a simple, reproducible and sensitive procedure.

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Note

Rapid determination of metronidazole in human serum and urine using a normal-phase high-performance liquid chromatographic column with aqueous solvents

JOHN ADAMOVICS

Squibb Institute for Medical Research, New Brunswick, NJ 08903 (U.S.A.)

(First received January 12th, 1984; revised manuscript received March 26th, 1984)

Metronidazole [1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole] is effective against various infectious diseases caused by both aerobic and anaerobic microorganisms and sensitizes hypoxic cells to radiation damage. A large number of reversed-phase high-performance liquid chromatographic (HPLC) methods have been described to analyze metronidazole in body fluids [1—12]. The procedure reported is a normal-phase system with 2 min retention for metronidazole with a simple sample preparation.

MATERIALS AND METHODS

Reagents and materials

Metronidazole was supplied by the United States Pharmacopeia (U.S.P.). Ammonium phosphate and acetonitrile were HPLC grade, and the water used was doubly distilled. Human serum and urine samples were obtained from human volunteers and frozen at −20°C. Within a week of storage, each sample was thawed at room temperature and analyzed.

Standard solutions were prepared by accurately weighing approximately 50 mg of U.S.P. reference standard metronidazole and dissolving in 500 ml of mobile phase. This stock solution was accurately diluted with mobile phase 1:1, 1:10, 1:20, 1:50, 1:100 and 1:1000 for a wide range of concentrations used both for obtaining standard curves and needed for validation studies. These solutions were stored in glass at 0°C until needed.

Apparatus

Analyses were performed on a modular instrument with in-line solvent

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and precolumn filters of approximately 0.45 μm porosity. A saturator column containing 37-μm silica was located between the pump (Beckman-Altex 110A) and the autoinjector, which was either a Perkin-Elmer Model 420B or 600. Either a Kratos Model 773, a Perkin-Elmer Model 85 or Beckman 165 detector set to 320 nm was used. A laboratory automation system (Hewlett-Packard Model 3356) was used to process the data. A Kipp and Zonen two-pen recorder was also used to plot chromatograms as back-up to the computer.

Chromatographic conditions

Analytical columns successfully used were Chromegabond Diol, 5 and 10 μm particle size, 30 cm × 4.6 mm (E.S. Industries, Marlton, NJ, U.S.A.) and LiChrosorb Diol, 10 μm particle size, 25 cm × 4.6 mm (E. Merck, Darmstadt, F.R.G.). Mobile phase consisted of 0.005 *M* ammonium phosphate, adjusted to pH 6.5 with potassium hydroxide solution—acetonitrile (40:60) flow-rate 1 ml/min. Absorbance range adjustments are unnecessary with the computer system.

Sample preparation

Serum samples were thawed at room temperature. Afterwards, 1.0 ml from each sample tube was diluted with 1.0 ml of pH 6.0 buffer (1.65 g of dipotassium hydrogen phosphate and 98.35 g potassium dihydrogen phosphate dissolved in 1 l of distilled water) and transferred into a properly treated (cones soaked in distilled water for at least 1 h) Centriflo® membrane cone (Type CF50A).

The serum and phosphate buffer were centrifuged at 900 *g* for 15 min. An aliquot from the supernatant was mixed with an equal portion of mobile phase and injected. Urine samples were handled in a similar manner with 2.0 ml of urine mixed with 2.0 ml of phosphate buffer in the above mentioned membrane cone. Standards were dissolved in phosphate buffer, added to serum at 10 μg/ml or to urine at 100 μg/ml and were prepared with the appropriate clinical samples.

Procedure

Standard and sample solutions are injected in the sequence: $standard_1$, $standard_1$, $standard_2$, $sample_1$, $sample_n$, $standard_1$ or $_2$ ....., where *n* is number of samples and depends on the stability of the system. The first injection was rejected since it is the most susceptible to column anomalies. Responses of the two standards should be within 1.5%. Failure to achieve reasonable agreement requires reinjection, reweighing and preparation of the standard, reequilibration, or repair of the HPLC system. Purity of one standard should be calculated (as if the injection was an unknown) using integrators or computers. Obtaining the designated purity value verifies the system and arithmetic program. Quantitation of purity can be based on area or height.

Drug administration

Healthy male volunteers were administered metronidazole by 30-min intravenous infusions in a single dose, 500 mg. Metronidazole was purchased as a 1 mg/ml solution in isotonic citrate and phosphate-buffered saline. Administra-

tion was by intravenous infusion using a syringe pump (Harvard Apparatus Compact Infusion Pump).

RESULTS AND DISCUSSION

A rapid assay for metronidazole in human serum and urine has been developed using a normal-phase diol HPLC column with a reversed-phase type of mobile phase. Such a system is chromatographically stable with no deterioration even after 500 injections.

The sample preparation of serum in previously described methods required solvent pretreatment, centrifugation and addition of an internal standard for quantification [3,5,7,9,11]. The sample pretreatment in this method simply requires ultrafiltration and mixing of the supernatant with mobile phase. Internal standards were not required since recoveries obtained were consistent and near theory.

Chromatograms of human serum prior to dosing, at the end of infusion and 15 min after the end of infusion clearly show absence of any interferences (see Fig. 1). Fig. 2 shows a computer-enhanced chromatogram of control urine showing the absence of interference with metronidazole and Fig. 3 a typical chromatogram of drug in urine collected 0 to 2 h after dosing. Peak widths are exaggerated due to the time scale.

After 24 h, urinary excretion of metronidazole reached 22% of administered

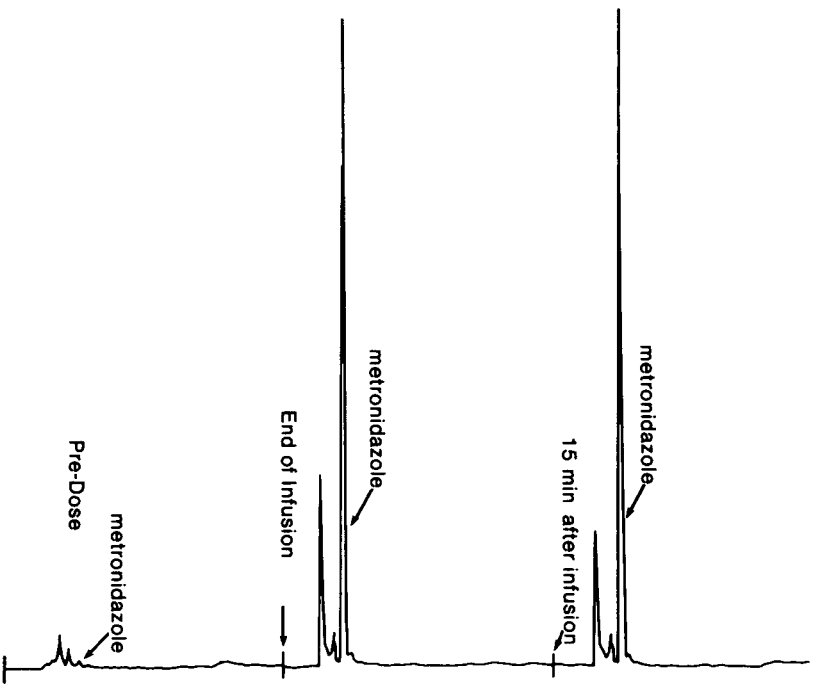


Fig. 1. Typical chromatogram of pre-dose serum, end of infusion (metronidazole serum concentration 4.2 μg/ml), 15 min after infusion (4.3 μg/ml) as recorded on a two-pen strip chart recorder. Metronidazole elutes in ca. 2 min.

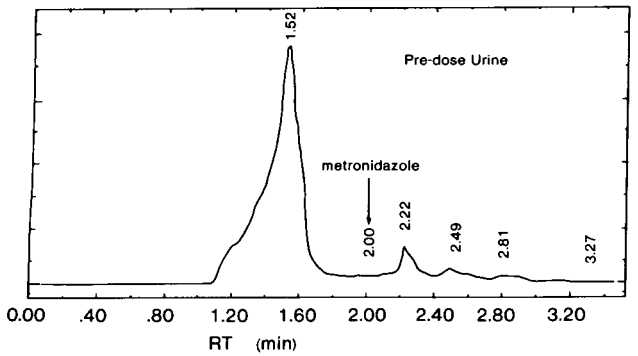


Fig. 2. Typical chromatogram of pre-dose urine sample.

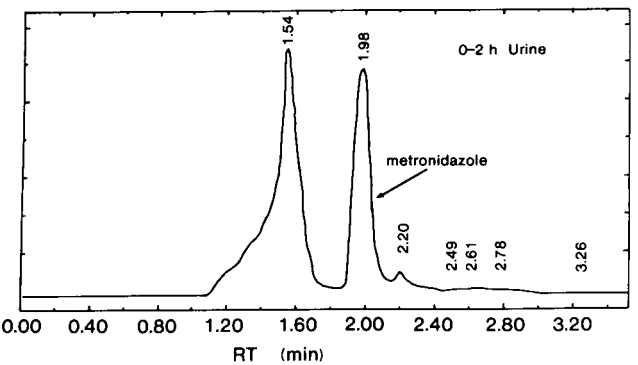
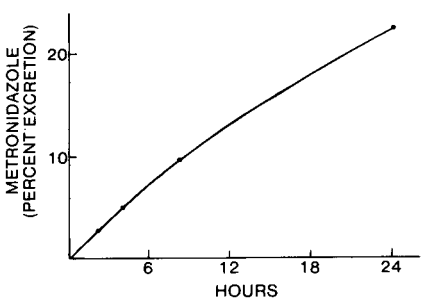


Fig. 3. Typical chromatogram of 0—2-h urine sample, 90 μg/ml.



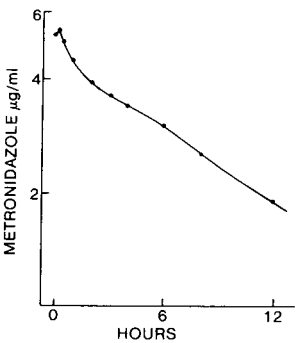


Fig. 4. Cumulative urinary excretion of metronidazole.

Fig. 5. Serum concentrations of metronidazole.

dose (see Fig. 4). The half-life of metronidazole in serum was 10.9 h (see Fig. 5) which agrees with previously reported data [2].

Reproducibility of six injections of 10 μg/ml metronidazole gave a relative standard deviation (R.S.D.) of 2.8% (x = $1.03 \cdot 10^5$ area units) and for 100 μg/ml, the R.S.D. was 1.1% (x = $1.02 \cdot 10^6$ area units). For a series of concentrations from 1 to 100 μg/ml, the correlation coefficient was >0.999. A plot

TABLE I

RECOVERY OF METRONIDAZOLE IN SERUM AND URINE

Serum (μg/ml)	Percent recovered	Urine (μg/ml)	Percent recovered
0.1	90.5	1	103
	95.0		100
1.0	95.0	10	94
	97.5		98
5.0	98.0	50	97
	99.0		96
10	101.0	100	99.5
	97.5		96.0
	X = 96.7		X = 97.9

passed through the origin. (The R.S.D. of the response normalized for concentration was 2.6%.)

Recovery studies in serum and urine are summarized in Table I.

The limit of quantitation for metronidazole in urine was determined to be 0.1 μg/ml, and in serum, 0.05 μg/ml.

In summary, a HPLC assay for metronidazole in body fluids using a simple sample treatment procedure has been described.

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Note

Simple and rapid high-performance liquid chromatographic analysis of cyclosporine in human blood and serum

ROBERT E. KATES* and ROBERTO LATINI*

Division of Cardiology, Stanford University Medical Center, Stanford, CA 94305 (U.S.A.)

(First received December 22nd, 1983; revised manuscript received March 28th, 1984)

Cyclosporine is a cyclic peptide (Fig. 1) of fungal origin which has proven to be a potent immunosuppressive agent in man [1]. Cyclosporine is effective in preventing rejection of transplanted organs including heart—lung, kidney, liver, pancreas and bone marrow [2].

Cyclosporine is a toxic drug with a narrow therapeutic index. Toxicity associated with cyclosporine includes non-specific immunosuppression, hepato

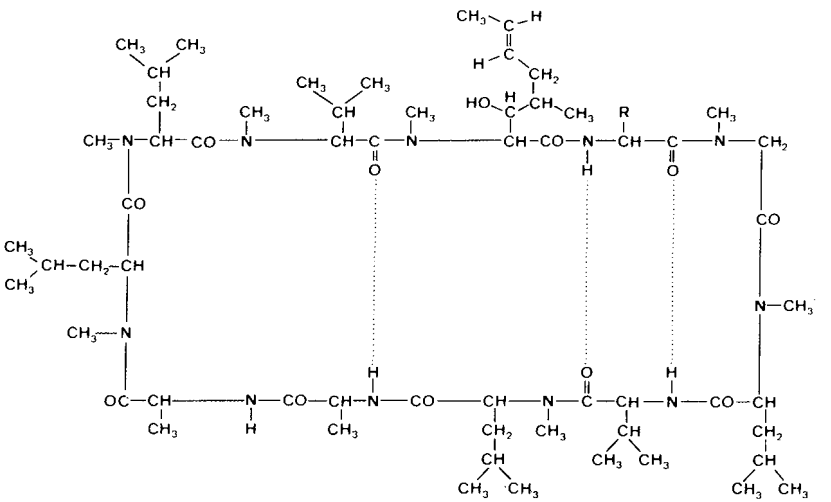

Fig. 1. Chemical structure of cyclosporine where R = $-CH_2CH_3$ and the internal standard, cyclosporin D where R = $-CH(CH_3)_2$.

*Present address: Istituto Mario Negri, Via Eritrea 62, Milan, Italy.

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and nephro toxicity and lymphoma [3,4]. It has been suggested that optimal use of cyclosporine requires careful titration of dosage and that serum or blood concentration monitoring of cyclosporine is advisable [5]. While a definitive therapeutic range of blood or serum concentrations has not been established, it has been reported that levels between 100 and 250 ng/ml are associated with adequate immunosuppression without a significant incidence of toxicity [6].

While several methods have been suggested for measuring cyclosporine blood or serum levels, most of these are not sufficiently sensitive to measure levels below 100 ng/ml and several are highly complex requiring special attention and equipment [7–9].

We have developed a rapid high-performance liquid chromatographic (HPLC) procedure for measuring cyclosporine in either whole blood or serum. This procedure is relatively simple and rapid, and has a lower limit of sensitivity below 50 ng/ml.

EXPERIMENTAL

Chemicals and reagents

Cyclosporine and the internal standard, cyclosporin D (Fig. 1), are obtained from Sandoz (Basle, Switzerland). Glass-distilled diethyl ether, acetonitrile, *n*-hexane, and methanol are obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Tris(hydroxymethyl)aminomethane is purchased from Aldrich (Milwaukee, WI, U.S.A.).

Instrumentation and chromatographic conditions

A Waters Instrument (Milford, MA, U.S.A.) Model 6000A HPLC pump, Model 480 variable-wavelength UV detector, Model 710B sample injector and Model 730 data module are employed. The column used is a Brown-Lee Labs. (Santa Clara, CA, U.S.A.) RP-8 MLPC analytical cartridge (10 cm × 4.6 mm I.D.; particle size 10 μm). A 3-cm RP-8 guard cartridge is positioned at the head of the column. The column is maintained at 70°C with an Eldex (Menlo Park, CA, U.S.A.) column heater. The heater is left on at all times and a constant flow is maintained through the column. The flow-rate of the mobile phase is 0.6 ml/min which produces a precolumn pressure of about 34 bars (500 p.s.i.). The effluent from the column is monitored at a wavelength of 215 nm.

Mobile phase

The mobile phase consists of a simple mixture of acetonitrile–water (72 : 28). This mixture is filtered and degassed by vacuum and sonication. The mobile phase is continuously recycled and replaced about every two weeks.

Preparation of extraction columns

The extraction of cyclosporine involves the use of a Baker-10 SPE extraction system (J.T. Baker, Phillipsburg, NJ, U.S.A.). The columns used are the 3-ml cyano disposable extraction columns. These columns are prepared by washing with 6 ml of methanol and then 6 ml of water under vacuum. They are left approximately one half to three quarters full of water until the sample is added.

Extraction procedure

One ml of whole blood or serum is added to PTFE-lined, screw-capped tubes. To the blood or serum is added 450 ng of internal standard, 3 ml of 0.1 *M* Tris buffer (pH 9.8) and 10 ml of diethyl ether. They are rocked for 20 min on a labquake rocker (Lab. Industries, Berkeley, CA, U.S.A.) and centrifuged for 20 min to separate the ether and aqueous layers. The diethyl ether is pipetted into a clean tube and the blood or serum residue is discarded. To the diethyl ether is added 200 μl of 75% methanol in water. The diethyl ether is then evaporated at room temperature on an N-Evap (Organomation, Northburough, MA, U.S.A.) with a gentle stream of nitrogen. Only the diethyl ether is evaporated, leaving 150—200 μl of the methanol—water remaining. To this are added another 100 μl of methanol. The methanol—water is then transferred to a 3-ml Baker extraction column. The residue is eluted onto the column with water. The column is then washed with 3 ml of 25% acetonitrile in water and 6 ml of *n*-hexane. The columns are then dried by drawing through air for 4 min. Cyclosporine and cyclosporin D are then eluted off the column with three washings of 200 μl of methanol. The methanol is collected into disposable tubes and evaporated to dryness with a stream of nitrogen at 50°C. The resulting residue is dissolved in 200 μl of the mobile phase. An aliquot of 165 μl is then injected onto the column.

Preparation of calibration standards

Stock solutions of cyclosporine and cyclosporin D are prepared in methanol and stored in amber bottles at room temperature. A stock solution of cyclosporin D is prepared in a concentration of 30 ng/μl. The stock solution of cyclosporine is prepared in a concentration of 10 ng/μl. This solutions is used to prepare standards for the calibration curve. The calibration curve samples are prepared by adding amounts of cyclosporine (50—800 ng) to whole blood or serum from a normal volunteer. These are then treated as described in the extraction procedure.

RESULTS AND DISCUSSION

As shown in Fig. 2B the retention times of cyclosporine and the internal standard are 9.2 and 11.8 min, respectively.

An unidentified peak elutes with a retention time of about 19 min, but does not interfere with the quantification of the cyclosporine or internal standard. As shown in Fig. 2A, blood from normal subjects, not taking cyclosporine has no interfering peaks eluting at times which would interfere with the analysis. Fig. 2B is a chromatogram of an analyzed whole blood sample to which had been added 200 ng of cyclosporine and the internal standard. Fig. 2C shows a chromatogram of a blood sample from a patient who was taking cyclosporine. The concentration in this sample was 304 ng/ml.

The chromatography is sensitive to slight changes in conditions. As reported by others [10] a column temperature of 70—75°C is essential for optimizing sensitivity and resolution. We also found that the mobile phase must be at least 68% acetonitrile; 72% is optimal in our system for a new column. We observed that resolution is greatly decreased at mobile phase flow-rates above 1 ml/min.

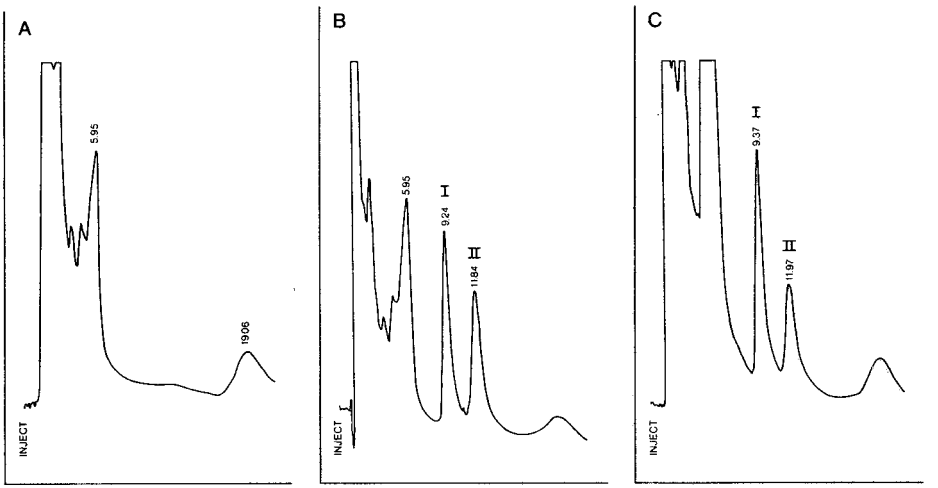

Fig. 2. Chromatograms of extracted whole blood samples. (A) Blood from normal healthy volunteer, not taking cyclosporine; (B) blood to which has been added 200 ng/ml of cyclosporine and the internal standard; (C) blood from a patient who was taking cyclosporine (the concentration in this patient sample is 304 ng/ml). Peaks: I = cyclosporine; II = cyclosporin D, internal standard.

For our conditions the optimal flow-rate is 0.6 ml/min. The flow-rate and column temperature are maintained constant at all times. Under these conditions, columns are stable and functional for at least six months.

Standard curves are prepared by plotting peak height ratios (cyclosporine/internal standard) against the amount added initially. These curves are linear over the range of interest, 50—800 ng. During the developmental phases of this procedure, curves were prepared daily. The variability in slope from seven daily standard curves was 21% (coefficient of variation, C.V.). The intercepts were not significantly different from zero.

The lower limit of sensitivity of the reported procedure is less than 50 ng/ml of blood. While our lowest calibration standard was 50 ng, our analytical range of interest was greater than 100 ng/ml. The peak height of a 50-ng standard was more than ten times the noise level, and samples with levels as low as 25 ng could easily be analyzed without modification. Analysis of samples with low concentrations (<50 ng/ml) of cyclosporine are best analyzed by using less internal standard and increasing the detector sensitivity. Fairly low sensitivity (0.05 a.u.f.s.) is sufficient for the range in which we have primary interest. To analyze samples with high concentrations (>800 ng/ml) we generally take smaller aliquots of blood (200 or 500 ml) and extract as described above.

The reproducibility of the procedure was evaluated by extracting and analyzing replicate blood samples containing 50, 200, 400 and 800 ng of cyclosporine. These data are summarized in Table I. The coefficients of variation, expressed as percent, for the 50, 200, 400 and 800 ng replicates are 3.3%, 7.6%, 7.2%, and 4.7%, respectively.

The extraction efficiency was determined by comparing peak heights of extracted standards with directly injected standards. This was carried out for both whole blood and serum. The recovery of cyclosporine was 34.7% and

TABLE I

REPRODUCIBILITY AT GIVEN PLASMA CONCENTRATIONS

Concentrations (ng/ml)	*n*	C.V. (%)
50	4	3.3
200	6	7.6
400	5	7.2
800	6	4.7

34.5% from whole blood and serum, respectively. Despite this low recovery, sensitivity was not a problem.

Several aspects of this analytical procedure deserve further amplification. The disposable CN columns which are used for the extraction of cyclosporine can be reused if washed with methanol (ca. 10 ml) between uses. We reuse these columns three times before discarding them, since we have noted that some columns tend to loose efficiency after three uses. Another consideration regarding the use of the CN columns is the eluting of the sample onto the column. The columns are at least half full of water when the sample, in ca. 300 μl methanol, is added. The columns are then filled with water to dilute the methanol and to facilitate mixing. If the methanol layer is drawn through the column without mixing or dilution, the cyclosporine will not be retained on the column.

A rather important step in the extraction is the addition of 200 μl of 75% methanol in water to the diethyl ether before drying. If the diethyl ether is dried completely, there is difficulty in redissolving the residue containing the cyclosporine and the internal standard. While the diethyl ether is evaporated the sample is not taken to dryness; the methanol—water mixture containing the drug and internal standard remain. This simple step helps overcome difficulties of low recovery which are encountered in the extraction.

In order to increase our capability, we are employing an automatic injector so that samples can be injected overnight. We have found that the use of plastic sample holders leads to several interfering peaks on the chromatogram. We therefore are using all glass sample holders to avoid this problem.

The majority of the literature concerning blood or plasma levels of cyclosporine has been based on a radioimmunoassay (RIA) procedure. While it has been suggested that this procedure is not highly specific for cyclosporine, the suggested plasma level guidelines for dosage adjustment are based on this method. We, therefore, analyzed several samples, both whole blood and serum, which had been analyzed by the RIA procedure. These samples were drawn from patients who had been taking cyclosporine for periods ranging from two weeks to several years. The results of this comparison are shown in Fig. 3. In general, the results of our HPLC analysis were considerably lower than those of the RIA analysis. This is consistent with similar HPLC—RIA comparisons reported by Carruthers et al. [9] and by Donatsch et al. [11]. In some patient samples, we noted a peak which eluted with a retention time similar to that of the internal standard. Since this interfering peak precluded our use of the cyclosporine D as an internal standard for those few samples in which it was

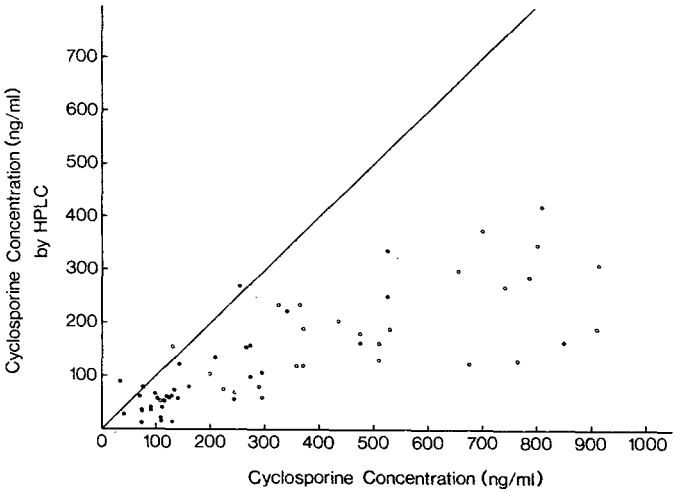

Fig. 3. Results of simultaneous analysis of whole blood (○) and plasma (●) samples by RIA and HPLC.

present, they were quantitated by means of a standard curve based on peak height of cyclosporine alone. These curves were linear over the range studied. It is interesting to speculate that this interfering peak may be due to a metabolite of cyclosporine. Since this peak eluted more slowly than the parent drug, it is probably less polar than cyclosporine. Its identity is currently being pursued.

While other methods have been developed for measuring cyclosporine in plasma or serum samples, they generally tend to be time-consuming and are not directly adaptable to analyzing whole blood. We developed the method presented here to facilitate the rapid and simple analysis of large numbers of whole blood samples from patients receiving cyclosporine following organ transplantation. The method reported here is sensitive, selective and equally adaptable for whole blood, plasma or serum samples. The advantage of this method over other procedures is that it is simple to perform and both the extraction and chromatography are rapid.

ACKNOWLEDGEMENTS

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Book Review

Quantitative analysis of steroids (Studies in Analytical Chemistry Series, Vol. 5), by S. Görög, Elsevier, Amsterdam, Oxford, New York, Tokyo, 1983, 440 pp., price Dfl. 225.00, US$ 95.75, ISBN 0-444-99698-2.

A number of books have been published on steroids, but this is the first devoted exclusively to their quantitative analysis under the scope of both pharmaceutical and biological—clinical analysis. The author's experience with his previous monograph (S. Görög and Gy. Szász: Analysis of Steroid Hormone Drugs, Akadémiai kiadó, Budapest, 1978) led him to combine the problems of drug analysis and those of steroid determination in biological fluids and tissues in one monograph in the right direction.

Some 400 communications on analysis of steroids appear annually in biochemical, pharmaceutical and endocrinological journals and as many papers not of a primary analytical character contain important information on analytical methodology and results. The book is a valiant attempt to cover the modern techniques of steroid determination with special emphasis on the developments in the past ten years.

The book is divided into eight chapters dealing with individual classes of steroids such as sex hormones and related materials, corticosteroids, cholesterol and related sterols, vitamin D and related materials, bile acids, cardiac glycosides, diosgenin and related sapogenins, and miscellaneous steroids such as ecdysones, steroid alkaloids and steroid quaternary ammonium compounds. As regards the analytical methods, all classical, spectroscopic, chromatographic and radioanalytical methods are discussed that are of general importance, biological assays and some of the very specialized biochemical techniques being omitted. The elucidation of steroid structures is not included and the spectroscopic methods such as infrared and NMR spectroscopy together with mass spectrometry are discussed only from the point of view of their quantitative analytical applications. Similarly, chromatography is also treated as a quantitative analytical tool, those techniques being emphasized which permit direct quantitative results: gas chromatography, high-performance liquid chromatography and thin-layer chromatography—densitometry. The theoretical and practical aspects of chromatographic separations (structure—chromatographic mobility relationships, solvent composition—mobility relationships, detection methods) are discussed only very briefly. Column and thin-layer chromatography are regarded as sample preparation steps for the subsequent quantitative analysis.

The book presents an excellent outline of applications of the various ana-

lytical techniques for steroids and by no means can be regarded as a laboratory handbook. Many citations, tables and figures enable the reader to obtain not only a general picture of the various methods and analytical problems, but the monograph also serves as a guide for reading original papers to those who intend to use the methods referred to.

Prague (Czechoslovakia) L. STÁRKA

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Volume 292

Volume 294

Author Index

Subject Index

Erratum

J. Chromatogr., 307 (1984) 351—359
Page 358, line 10, "so" should read "no".

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PUBLICATION SCHEDULE FOR 1984

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ELECTROPHORESIS

A Survey of Techniques and Applications

edited by Z. DEYL, Czechoslovak Academy of Sciences, Prague

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PART A: TECHNIQUES

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