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BIOMEDICAL APPLICATIONS

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Journal of Chromatography (incorporating *Biomedical Applications* and *Chromatographic Reviews*)
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MONTH	J	F	M	A	M	J	J	A	S	O	N	D
Journal of Chromatography	130 131	132/1 132/2 132/3	133/1 133/2	134/1 134/2	135/1 135/2	136/1 136/2 136/3	137/1 137/2	138/1 138/2	139/1 139/2	140/1 140/2 140/3	142 144/1	144/2 144/3
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Scope. The *Journal of Chromatography* publishes papers on all aspects of chromatography, electrophoresis and related methods. Contributions consist mainly of research papers dealing with chromatographic theory, instrumental development and their applications. The section *Biomedical Applications*, which is under separate editorship, deals with the following aspects: developments in and applications of chromatographic and electrophoretic techniques related to clinical diagnosis (including the publication of normal values); screening and profiling procedures with special reference to metabolic disorders; results from basic medical research with direct consequences in clinical practice; combinations of chromatographic and electrophoretic methods with other physicochemical techniques such as mass spectrometry. In *Chromatographic Reviews*, reviews on all aspects of chromatography, electrophoresis and related methods are published.

Submission of Papers. Papers in English, French and German may be submitted, if possible in three copies. Manuscripts should be submitted to:
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For further information, see page 3 of cover.

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HPLC is beautiful!

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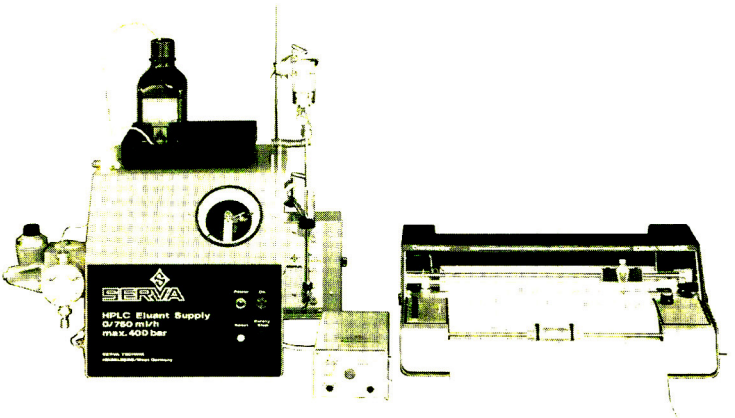

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REF. NO. 278

Isotachophoresis

Theory, Instrumentation and Applications

by F.M. EVERAERTS, J.L. BECKERS *and* TH. P.E.M. VERHEGGEN.

JOURNAL OF CHROMATOGRAPHY LIBRARY, Vol. 6

1976 xiv + 418 pages
US $61.50/Dfl. 160.00
ISBN 0-444-41430-4

This book is the only text currently available providing full information on the new separation technique known as Isotachophoresis, which competes with liquid and gas chromatography. All kinds of ionic materials can be separated and several classes of components can be analysed in quick succession as a proper rinsing of the equipment is all that is needed between separations. The various chapters of the book can be referred to more or less independently by scientists interested in fundamental aspects, by researchers intending to construct an instrument and by workers mainly concerned with analytical aspects.

CONTENTS: 1. Historical review. THEORY: 2. Principles of electrophoretic techniques. 3. Concept of mobility. 4. Mathematical model for isotachophoresis. 5. Choice of electrolyte systems. INSTRUMENTATION: 6. Detection systems. 7. Instrumentation. APPLICATIONS: 8. Introduction. 9. Practical aspects. 10. Quantitative aspects. 11. Separation of cationic species in aqueous solutions. 12. Separation of anionic species in aqueous solutions. 13. Amino acids, peptides and proteins. 14. Separation of nucleotides in aqueous systems. 15. Enzymatic reactions. 16. Separations in non-aqueous systems. 17. Counter flow of electrolyte. APPENDICES: A. Simplified model of moving-boundary electrophoresis for the measurement of effective mobilities. B. Diameter of the narrow-bore tube, applied for separation. C. Literature. Symbols and abbreviations. Subject index.

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CHROMATOGRAPHY '77

INTERNATIONAL SYMPOSIUM ON ADVANCES IN CHROMATOGRAPHY

The 12th International Symposium on Advances in Chromatography will be held in November 7-10, 1977 at the International Congress Centre RAI Amsterdam, The Netherlands.

The scope of the meeting will cover papers and informal discussion groups by outstanding researchers from throughout the world in all fields of chromatography. In particular, new developments in gas, liquid and high performance thin-layer chromatography will be included. There will also be a commercial exhibition of the latest instrumentation and books.

Participation in the symposium will be on the basis of invited papers as well as unsolicited contributions. Authors desiring to present papers must submit 500-word abstracts by April 1, 1977. The deadline for receipt of manuscripts of accepted papers is June 1, 1977. A group flight for U.S. delegates is being arranged departing New York on November 4 and returning November 12.

All correspondence concerning the symposium should be directed to:

Professor A. Zlatkis,
Chemistry Department,
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Houston, Texas 77004, U.S.A.

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ISOTOPES IN ORGANIC CHEMISTRY

edited by E. BUNCEL, Queen's University, Kingston, Ontario, Canada, and C.C. LEE, University of Saskatchewan, Saskatoon, Saskatchewan, Canada.

with a foreword by LARS MELANDER

The purpose of this series is to highlight the use and value of isotopes in diverse areas of organic chemistry. Each volume is devoted to one major area of current interest. The comprehensive surveys contained provide research workers with an invaluable reference source and, it is hoped, with a stimulus for further research.

Volume 2: Isotopes in Hydrogen Transfer Processes

1976 viii + 312 pages US $57.50/Dfl. 150.00 ISBN 0-444-41352-9

This, the second volume in the series, presents contributions by eight active research workers in the field of hydrogen transfer processes. The topics discussed include structural effects on proton transfer isotope effects; elimination reactions; biochemical processes; hydrogen atom transfer processes; oxidation processes and isotope exchange in carbonyl and in nitro compounds.

CONTENTS: Chapters 1. The effect of structure on the isotope effects in proton transfer reactions *(M. Kreevoy)*. 2. Deuterium exchange in carbonyl compounds *(G. Lamaty)*. 3. Proton transfers in nitro compounds *(K.T. Leffek)*. 4. Isotope effects in hydrogen atom transfer reactions *(E.S. Lewis)*. 5. Hydrogen isotope transfer in Biological processes *(H. Simon and A. Kraus)*. 6. Isotope effects in elimination reactions *(P.J. Smith)*. 7. Isotopes in Oxidation reactions *(R. Stewart)*. Indexes.

Volume 1: Isotopes in Molecular Rearrangements

1975 xvi + 320 pages US $38.50/Dfl. 100.00 ISBN 0-444-41223-9

Five active research workers in the field contributed to this book, covering the broad area of isotopes in molecular rearrangements. Topics dealt with include carbonium ion reactions; carbanion reactions, photochemical studies, mass spectral fragmentations and pericyclic reactions.

CONTENTS: Chapters 1. Deuterium labeling in carbonium ion rearrangements *(N.C. Deno)*. 2. Isotope effects in pericyclic reactions *(W.R. Dolbier, Jr.)*. 3. The elucidation of mass spectral fragmentation mechanisms by isotopic labelling *(J.L. Holmes)*. 4. Isotopes in carbanion rearrangements *(D.H.Hunter)*. 5. Utilization of deuterium labeling in organic photochemical rearrangements *(J.S. Swenton)*. Index.

"I strongly recommend the text as a valuable addition to any chemical library".

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Journal of Chromatography, 143 (1977) 117—123
Biomedical Applications

CHROMBIO. 020

DETERMINATION OF THE CONFIGURATIONS OF LACTIC AND GLYCERIC ACIDS FROM HUMAN SERUM AND URINE BY CAPILLARY GAS—LIQUID CHROMATOGRAPHY

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(Received May 20th, 1976)

SUMMARY

The separation of the enantiomers of lactic and glyceric acids can be achieved by capillary gas chromatography on SP-1000 using the corresponding O-acetylated menthyl esters. The structures of the derivatives were proved by proton magnetic resonance spectroscopy and mass spectrometry. The method has been used for the determination of the absolute configurations of lactic and glyceric acids isolated from serum and urine from different patients.

INTRODUCTION

Nowadays, inborn errors of metabolism are intensively studied and there is a growing interest in the determination of the absolute configurations of metabolic products in human blood and urine. Different optical isomers may originate from separate metabolic pathways and may reflect different enzyme defects. For example, excretion of *S*-glyceric acid is the result of *R*-glycerate dehydrogenase deficiency in hyperoxaluria type II [1]. , while *R*-glyceric acid has been found in a patient with the clinical symptoms of non-ketotic hyperglycinaemia [2]. Other examples recently investigated are *R*-methylsuccinic acid [3] and *S*-3,4-dihydroxybutyric acid [4]. The occurrence of *R*- and *S*-β-aminoisobutyric acid in man is well known [5], but the origin of the *S*-enantiomer has not yet been explained.

In our hospital, we were confronted with a child suffering from glyceric aciduria and another with an unexplained form of lactic aciduria. These patients prompted us to develop a gas chromatographic (GC) method for the determination of the absolute configurations of simple organic acids of this type.

In the literature, the resolution of racemic mixtures by GC has been achieved in two ways: (1) separation of the enantiomers with the use of a chiral stationary phase and (2) conversion of the enantiomers into diastereomers by a chiral reagent and the use of a non-chiral phase. Recently, Gil-Av and Nurok [6] published a comprehensive review on this subject. Pollock and Jermany [7] have achieved the separation of the enantiomers of some O-acetylated 2-hydroxy acids by GC of the 2-butyl, 3-methyl-2-butyl and 3,3-dimethyl-2-butyl esters. *S*-Lactic acid has been applied as an esterifying agent by several investigators for the separation of chiral alcohols, including menthol [6].

In this paper, we describe the separation of the diastereomers of the O-acetylated menthyl esters of lactic and glyceric acids by capillary GC on SP-1000, together with some recent clinical applications.

EXPERIMENTAL

Chemicals

R(+)-Glyceraldehyde, *S*(−)-glyceraldehyde and *R,S*-sodium lactate were purchased from Fluka (Buchs, Switzerland), *R,S*-calcium glycerate and (−)-menthol [(1*R*, 3*R*, 4*S*)-*p*-menthan-3-ol] from Aldrich Europe (Beerse, Belgium) and *R*-lithium lactate and *S*-lactic acid from Sigma (St. Louis, Mo., U.S.A.). *R*- and *S*-barium glycerate were synthesized by oxidation with HgO of *R*(+)- and *S*(−)-glyceraldehyde, respectively [8]; the salts were obtained as waxy masses with $[\alpha]_D^{20}$ values of +6.1° (*c* 1.8; H_2O) and −4.3° (*c* 4.0; H_2O), respectively. The purities of both compounds were tested by GC of the pertrimethylsilyl (TMS) derivatives on 5% GESE-52 [9]; they had the same retention time as the TMS derivative of *R,S*-glyceric acid. Their identity was further proved by gas chromatography—mass spectrometry (GC—MS) of the TMS derivatives. Capillary GC of the O-acetylated menthyl esters showed that during the oxidation reaction some racemization had occurred (*R*-barium glycerate, 88% *R* and 12% *S*; *S*-barium glycerate, 83% *S* and 17% *R*) (*cf.*, ref. 8). In general, free acids were obtained by treatment of the corresponding salts with Dowex 50-X8 (H^+) in water.

Synthesis of O-acetylated menthyl esters of lactic and glyceric acids

(−)-Menthol (300 mg) was added to 1—5 mg of dry hydroxy acid and esterified at 110° for 2 h by bubbling dry HCl gas through the solution [10]. Subsequently, the excess of HCl and menthol was removed by a gentle stream of nitrogen at 110°. To avoid possible losses of the volatile menthyl ester of lactic acid, it is advisable not to remove menthol completely. The residue was acetylated in 1 ml of pyridine—acetic anhydride (1:1) at 100° for 30 min. Finally, the solvent was evaporated in the presence of absolute ethanol and the residue dissolved in chloroform.

Capillary gas—liquid chromatography

A Varian Aerograph 2740-30-01 gas chromatograph equipped with a flame-ionization detector and a glass capillary column (25 m × 0.3 mm I.D.) coated with SP-1000 (LKB, Stockholm, Sweden) as stationary phase was used. For direct on-column injection (0.1—0.2 μl) without a stream splitter, a Pasteur pipette (length 140 mm, I.D. 1 mm) was used as the inlet, connected to the column with shrinkable PTFE. As the outlet to the detector, a second pipette (length 110 mm, I.D. 0.5 mm) was used. The carrier gas (nitrogen) flow-rate was 1 ml/min and the make-up gas (nitrogen) flow-rate was 30 ml/min. The injection port temperature was 200° and the detector temperature 220°; the oven temperature was 150° for lactic acid and 200° for glyceric acid.

Proton magnetic resonance spectroscopy

The proton magnetic resonance (PMR) spectra of the menthyl esters of *R,S*-lactic acid and *R,S*-glyceric acid as well as those of (—)-menthol, *R,S*-lactic acid and *R,S*-glyceric acid were recorded at 60 MHz with a Varian EM-360 spectrometer in CD_3OD as solvent and tetramethylsilane as internal standard at room temperature.

Gas chromatography—mass spectrometry

The 75-eV mass spectra of the O-acetylated menthyl esters of *R,S*-lactic acid and *R,S*-glyceric acid were recorded on a Jeol JGC-20 KP/JMS-D100/W-JMA combination at an ion-source temperature of 150°, an accelerating voltage of 3 kV and an ionizing current of 300 μA. As the column material, 5% GESE-52 on Chromosorb W AW DMCS, 100—120 mesh (HP), was used. The oven temperature was dependent on the sample.

Isolation of lactic and glyceric acids from urine or serum

Lactic and glyceric acids were extracted from urine with ethyl acetate as follows [11]. A 5-ml volume of saturated NaCl solution was added to 5 ml of urine, the mixture was acidified with concentrated HCl to pH 1—2 and subsequently extracted twice with 20 ml of ethyl acetate. The total organic phase was dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure. Glyceric acid was isolated from serum as follows. A 1-ml volume of serum was deproteinized with 9 ml of ethanol and the supernatant was evaporated in vacuo. The residue, dissolved in 1 ml of water, was applied to a DEAE-Sephadex A-25 anion-exchange column (6 × 0.7 cm) equilibrated with 20 ml of 0.5 *M* pyridinium acetate buffer. After washing with 10 ml of water, the organic acids were eluted with 15 ml of 1.5 *M* pyridinium acetate buffer [12]. Finally, the eluate was lyophilized.

RESULTS

To confirm the derivatization procedure, PMR spectroscopy was carried out after menthylation of the free acids and GC—MS after O-acetylation of the various esters. The following PMR data were obtained:

(a) Menthyl ester of *R,S*-lactic acid:

δ = 1.36 ppm (4.6 Hz), doublet, 3 H
δ = 4.22 ppm (4.6 Hz), quartet, 1 H } lactic acid unit

δ = 0.6—2.2 ppm, 18 H
δ = 4.7 ppm, multiplet, 1 H } menthyl unit

(b) Menthyl ester of *R,S*-glyceric acid:

δ = 3.76 ppm (4 Hz), doublet, 2 H
δ = 4.20 ppm (4 Hz), triplet, 1 H } glyceric acid unit

δ = 0.6—2.2 ppm, 18 H
δ = 4.7 ppm, multiplet, 1 H } menthyl unit

The mass spectra of the O-acetylated menthyl esters of *R,S*-lactic acid (M = 270) and *R,S*-glyceric acid (M = 328) are presented in Figs. 1 and 2, respectively. In both instances the original unnormalized spectra showed very small peaks at *m/e* [M + 1]. The most intense peaks in the spectra are related to the fragmentation pattern of the menthyl unit.

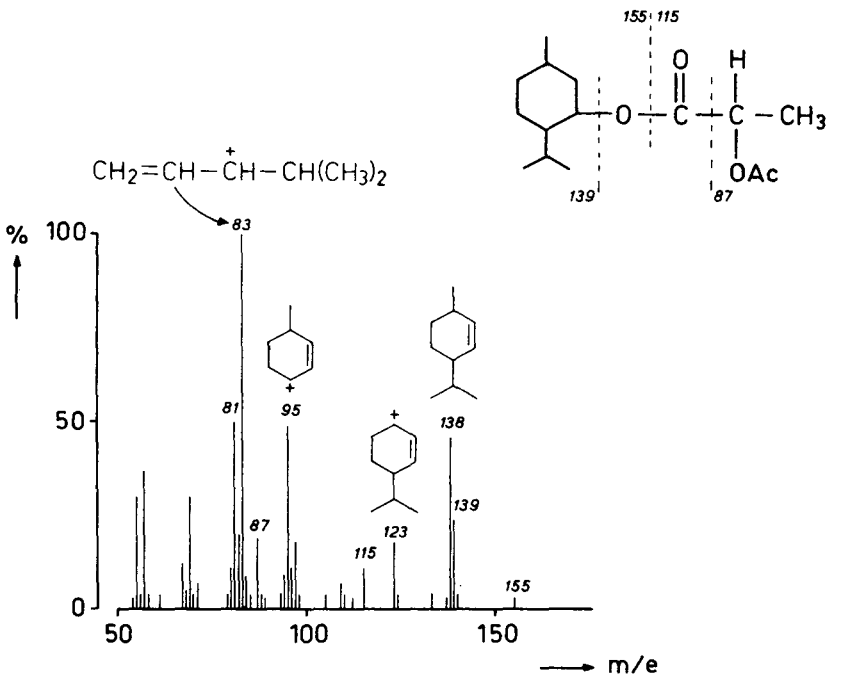

Fig. 1. Mass spectrum of the O-acetylated menthyl ester of *R,S*-lactic acid.

The gas chromatogram of the O-acetylated menthyl ester of *R,S*-lactic acid on SP-1000 is shown in Fig. 3a. The two peaks were identified by co-chromatography with the derivatives of the *R*- and *S*-enantiomers. Analysis of the commercially available *R*-lactic acid yielded 98.4% *R* and 1.6% *S*, while the *S*-enantiomer gave 99.4% *S* and 0.6% *R*. The origin of the contaminating optical antipodes is unknown; they might be intrinsic or be formed during the derivatization. For lactic acid, the resolution factor (the ratio of the retention times of configurational isomers) R_R/R_S was found to be 1.08.

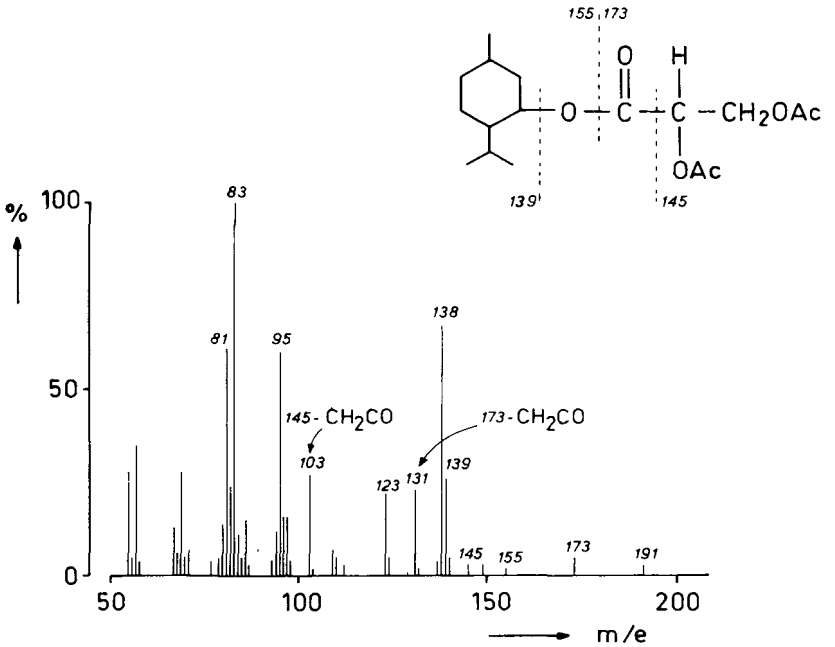

Fig. 2. Mass spectrum of the O-acetylated menthyl ester of *R,S*-glyceric acid.

The gas chromatogram of the O-acetylated menthyl ester of *R,S*-glyceric acid is shown in Fig. 3b. These peaks were identified in the same way as described for lactic acid. In this instance a resolution factor R_R/R_S = 1.04 was found.

The method has been applied to the determination of the absolute configurations of lactic and glyceric acids, present in large amounts in the urines and sera from some patients with unexplained metabolic disorders. Two applications are given below.

(1) A patient with lactic aciduria, but without concomitant hyperlactataemia

The lactic acid was isolated from urine by extraction with ethyl acetate. The extract was not further purified. After derivatization, only one main peak was found in the gas chromatogram, which had the same retention time as the derivative of *R*-lactic acid (Fig. 3c) (98.6% *R* and 1.4% *S*). Normally, only *S*-lactic acid occurs in human urine. *R*-Lactic acid was permanently present in this patient.

(2) A patient with glyceric acidaemia

The glyceric acid was isolated from urine by extraction with ethyl acetate and further purified by two-dimensional preparative paper chromatography. After derivatization, only one main peak was found in the gas chromatogram (Fig. 3d), having the same retention time as the authentic *R*-glyceric acid derivative (97.0% *R* and 3.0% *S*). The glyceric acid isolated from the serum was also the *R*-enantiomer (98.0% *R* and 2.0% *S*).

The clinical and biochemical aspects of both patients will be published elsewhere.

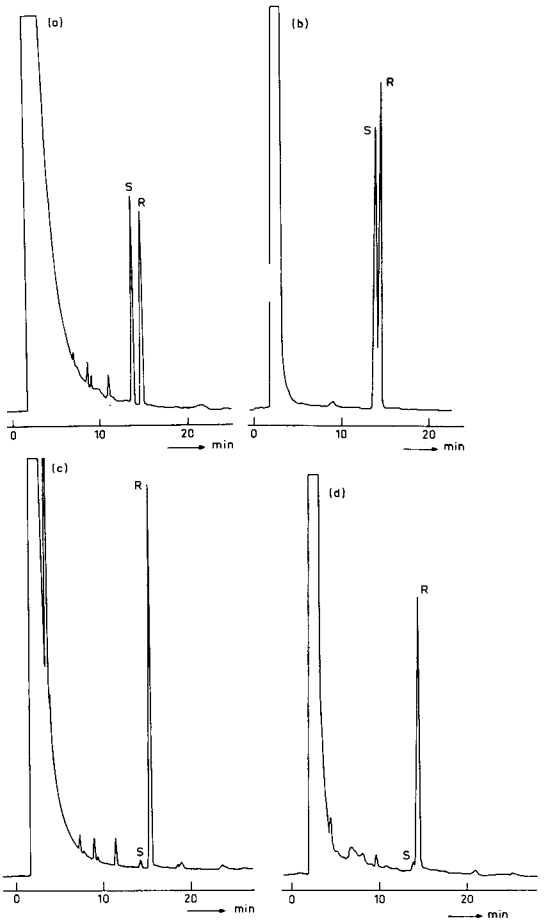

Fig. 3. Gas chromatograms of the O-acetylated menthyl esters of (a) synthetic *R,S*-lactic acid (150°), (b) synthetic *R,S*-glyceric acid (200°), (c) *R*-lactic acid isolated from urine (150°) and (d) *R*-glyceric acid isolated from urine (200°) on a glass capillary column (25 m × 0.3 mm I.D.) coated with SP-1000 as stationary phase (WCOT). The nitrogen flow-rate was 1 ml/min.

DISCUSSION

For the determination of the absolute configurations of lactic and glyceric acids, several methods have been applied in the literature. Firstly, it is possible to measure the optical rotation of the free acids or of suitable derivatives such as the salts or esters. The specific rotation values, $[\alpha]_D$, of the free acids are as low as 2—3° in water and are therefore not suitable for analytical purposes. The specific rotation values of the salts and alkyl esters are higher. Another disadvantage of the polarimetric method is the necessity to purify the isolated material highly. For these reasons, when only small amounts of material are available, the measurement of the optical rotation can give misleading results.

Secondly, in view of the high stereospecificity, the enzymes *R*- and *S*-lactate

dehydrogenase and *R*-glycerate dehydrogenase can be applied in order to establish the configurations of the acids. These methods can be used for small amounts of material. However, *R*- and *S*-lactate dehydrogenases do not only react with the natural substrate lactic acid, but also to some extent with other 2-hydroxy acids such as glyceric acid [13]. Ambiguous results may be obtained when mixtures of these acids are present in the materials to be analyzed. Therefore, it is necessary to remove any contaminating material which might also react with the enzymes. It should be noted that *R*-glycerate dehydrogenase does not react with *R*-lactic acid [14].

The technique presented here has been shown to be suitable for the configurational analysis of lactic and glyceric acids from physiological fluids, even when relatively small amounts of material are available. It is unnecessary to purify the starting material highly; simple isolation procedures are sufficient. Moreover, lactic and glyceric acids can be determined simultaneously.

ACKNOWLEDGEMENT

This investigation was supported in part with financial aid from Het Praeventiefonds.

REFERENCES

1 H.E. Williams and L.H. Smith, N. Engl. J. Med., 278 (1968) 233.
2 N.J. Brandt, K. Rasmussen, S. Brandt, S. Kølvraa and F. Schønheyder, Acta Paediat. Scand., 65 (1976) 17.
3 B. Zeitman and J.G. Lawless, Biochem. Med., 13 (1975) 111.
4 V. Fell, C.R. Lee and R.J. Pollitt, Biochem. Med., 13 (1975) 40.
5 E. Solem, E. Jellum and L. Eldjarn, Clin. Chim. Acta, 50 (1974) 393.
6 E. Gil-Av and D. Nurok, Advan. Chromatogr., 10 (1974) 99.
7 G.E. Pollock and D.A. Jermany, J. Gas Chromatogr., 6 (1968) 412.
8 A. Wohl and R. Schellenberg, Chem. Ber., 55 (1922) 1404.
9 H. Przyrembel, D. Bachmann, I. Lombeck, K. Becker, U. Wendel, S.K. Wadman and H.J. Bremer, Clin. Chim. Acta, 58 (1975) 257.
10 M. Hasegawa and I. Matsubara, Anal. Biochem., 63 (1975) 308.
11 S.K. Wadman, C. van der Heiden, D. Ketting and F.J. van Sprang, Clin. Chim. Acta., 34 (1971) 277.
12 R.A. Chalmers and R.W.E. Watts, Analyst (London), 97 (1972) 958.
13 L. Lundholm, E. Mohme-Lundholm and N. Svedmyr, Scand. J. Clin. Lab. Invest., 15 (1963) 311.
14 P.D. Dawkins and F. Dickens, Biochem. J., 94 (1965) 353.

Journal of Chromatography, 143 (1977) 125—136
Biomedical Applications

CHROMBIO. 024

RAPID CHROMATOGRAPHIC METHOD TO DETERMINE POLYAMINES IN URINE AND WHOLE BLOOD

HARVEY ADLER, MARVIN MARGOSHES* LLOYD R. SNYDER and CAROL SPITZER**

Technicon Instruments Corporation, Tarrytown, N.Y. 10591 (U.S.A.)

(Received August 17th, 1976)

SUMMARY

A procedure is described for the rapid determination of putrescine, spermine and spermidine in urine and whole blood. The samples are hydrolyzed with barium hydroxide and are neutralized with sulfuric acid. The polyamines are concentrated and separated from amino acids on a small bed of ion-exchange resin that then serves to load the samples on a two-channel, automated ion-exchange chromatography apparatus. As many as 100 samples can be analyzed in a 24-h period. The method has been shown to be applicable to the analysis of urine and whole blood samples, but further development is needed for application to serum samples.

INTRODUCTION

Since the initial report by Russell and colleagues [1, 2] the evidence has become well known for elevated urinary excretion of polyamines (putrescine, spermine, and spermidine) by some cancer patients. Evidence is accumulating also that some cancer patients have larger than normal amounts of spermidine in their serum [3—6]. Much of what is known of the relation between polyamine levels and cancer was summarized at a 1973 symposium [7] and has been briefly reviewed more recently by Savory and Shipe [8].

A number of methods have been devised to determine the polyamines in biological samples. The analytical state-of-the-art is well summarized in the symposium volume [7] and the review by Savory and Shipe includes several references to analytical methods. Most of the recently described methods have been based on separation by gas chromatography or by ion-exchange liquid chromatography. The separation times by gas chromatography have been reasonably short (15—20 min), but the sample preparation steps have been tedious

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[9,10]. Many of the ion-exchange chromatography methods have also required tedious sample preparation, and column time per analysis has typically been from 90 to 120 min [3, 11, 12].

The limitations of the analytical methods have undoubtedly slowed the progress of clinical studies on the significance of polyamine levels in the diagnosis and therapy of cancer. Savory and Shipe [8] concluded their review by saying: "The relationship between polyamines and cancer appears to have been well established, and there are sufficient data to indicate that measurements of polyamines in biological materials could provide a valuable test in the diagnosis of cancer and for monitoring therapy. *At the present time, there is a need for the development of precise and accurate methods with ultimate emphasis being placed on simplicity in order that large scale studies can be carried out.* Normal values of polyamines in serum and urine need to be established and further extensive clinical correlations are required." (Italics ours.)

It was our purpose in the work reported here to develop a rapid method of analysis to facilitate the clinical studies. The method measures free or total (free plus conjugated) polyamines in urine or whole blood, and with some additional development it should be useful in the analysis of serum. It employs commercially available equipment entirely, except for one module assembled from commercial parts. The equipment makes it possible to analyze 100 or more samples per day.

SUMMARY OF THE METHOD

The method has several features that distinguish it from earlier chromatographic methods for polyamine determination. These include novel procedures for sample hydrolysis and for separating and concentrating polyamines from the sample in addition to a rapid ion-exchange column separation. The details of the procedure are given below, in the Experimental section. In this section, the general features of the method are described and discussed briefly. The sequence of steps in an analysis are: (1) sample hydrolysis; (2) pre-concentration and group separation; and (3) chromatography. The description here is in the inverse of that order, so as to make clearer the relations between the steps.

Rapid chromatography

The primary objective of the method development program was to speed up the chromatographic analysis substantially from the state-of-the-art column time of 90—120 min per sample. (A newer method [13] requires only 35 min column time for the separation plus an unstated length of time to prepare the column for the next sample after each run.) In the method described here, the column time is 22 min including washing and regenerating the column for the next sample. The chromatographic separation is carried out on a dual-column analyzer [14] that is equipped for automated, unattended operation. It is possible to analyze six samples in little more than 1 h.

The column time was reduced by a combination of improvements over earlier methods:

(1) In the earlier methods [3, 11, 12] half or more of the column time was

needed to elute the amino acids in the sample. In the new method, the amino acids are separated from the sample before the chromatography.

(2) A combination of factors were optimized to speed up the chromatography generally. These included the use of small, uniform resin particles [15], a short column, moderately fast pumping rates (possible without excessive pressure because of the short column and the properties of the resin [15]), increased column temperature, and high ionic strength buffers.

(3) The elution times of the polyamines were so spaced as to minimize "dead" time between peaks. This was done with buffer gradient elution, using an ionic strength gradient.

(4) By gas-segmenting the column effluent stream, band spreading is considerably limited as the stream passes through the hydraulic system where it is reacted with ninhydrin reagent. In unsegmented systems that allow some spreading of the peaks, the separation achieved in the column must be greater than what is needed at the colorimeter (cf. discussion in ref. 19).

The amino acid analyzer [14] incorporates features that permit reliable unattended operation, and it can be left running with up to 80 samples in position for automatic loading and analysis. It can chromatograph 100 samples, plus a standard after each tenth sample, in only 20 h, leaving up to 4 h a day for routine maintenance. (In practice, only a few minutes should be needed for daily maintenance.) The instrument will stop itself after the last sample or if certain malfunctions occur.

Pre-concentration and group separation

The functions of this part of the procedure are to save time in the chromatography by avoiding the introduction of amino acids to the ion-exchange column, to enhance sensitivity by concentrating the polyamines before the sample is loaded on the column, and to protect the column from degradation by extraneous compounds and particulates in the samples. In doing this, advantage is taken of the cartridge that is the means of automated sample loading in the amino acid analyzer [14].

The cartridge holds a small amount of an ion-exchange resin similar to that in the column. A 2-ml portion of the sample prepared for analysis is pumped through the resin in the cartridge under conditions of pH and ionic strength selected for quantitative retention of the polyamines on the resin. Some of the amino acids in the sample will also remain on the resin, but they are removed by rinsing the resin with a flow of buffer.

Sample hydrolysis

In most of the prior analytical methods, the samples were prepared for chromatographic analysis by hydrolysis at high temperature in 6 *N* hydrochloric acid followed by neutralization with sodium hydroxide, or by hydrolysis with potassium hydroxide and neutralization with hydrochloric acid. Samples prepared by either procedure would not be satisfactory for the new method, because the high ionic strength of the hydrolysate would adversely affect the retention of the polyamines on the resin in the pre-concentration and group separation step. Hydrolysis of samples containing protein also produces amino acid contents large enough to prevent quantitative concentration of the polyamines in the cartridge.

It is a common procedure to follow hydrolysis by extraction of the polyamines into *n*-butanol, followed by evaporation of the butanol and dissolution of the residue in aqueous buffer. This procedure is effective in controlling the ionic strength of the sample and in concentrating the polyamines. However, it is time consuming, and it does not remove the amino acids from the sample.

In the present work, the samples were first deproteinized with sulfosalicylic acid and then hydrolyzed in the presence of barium hydroxide. Neutralizing the hydrolysate with sulfuric acid formed insoluble barium sulfate, so that the final solution did not have a high ionic strength. Freshly precipitated barium sulfate is well known to co-precipitate other compounds, and we found that as much as half of the polyamines were being lost in this way. We circumvented this potential problem by the conventional expedient of allowing the precipitate to stand overnight before taking off the clear supernatant.

Basically the same sample preparation method has been used to determine total polyamines in urine, serum or plasma, and whole blood samples, except that the whole blood samples are hemolyzed before being deproteinized. Data given below suggest that the procedure must be modified for the analysis of serum samples. When the free polyamines are to be determined, the only modification in the method is the elimination of the hydrolysis and neutralization steps.

EXPERIMENTAL

Equipment

The chromatography was conducted on a Technicon TSM Amino Acid Analyzer [14, 15] under the conditions listed in Table I. A buffer gradient was established as shown diagrammatically in Fig. 1. The chromatograms were integrated with an Autolab System AA Amino Acid Data Analyzer with two Analyzer Modules.

The apparatus shown in Figs. 2 and 3 was employed in loading the cartridges in the pre-concentration and group separation step. It was built up from the turntable assembly, positive displacement pump and pressure gauge of the

TABLE I

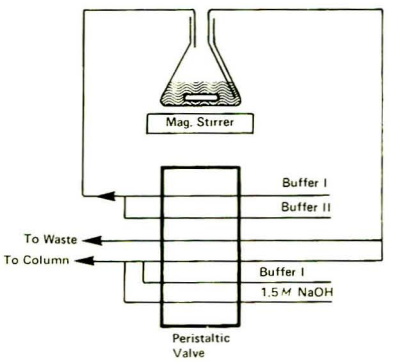
CHROMATOGRAPHY CONDITIONS

Resin: Technicon C-2, 8% cross-linked, sulfonic acid type, 8—12 μm diameter (Technicon Part No. T15-0356-42).

Column: 45 x 4 mm I.D.

Temperature: 80°.

Time (min)	Solution pumped	Flow (ml/min)	Volume in gradient flask (ml, start/end)
0—2	Buffer 1 to column	0.9	5.6/5.6
2—13.5	Buffer 2 to gradient	0.8	5.6/4.4
	gradient to column	0.9	
13.5—14	1.5 *M* NaOH to column	0.9	4.4/0
	gradient to waste	10	
14—22	Buffer 1 to gradient	1.6	0/5.6
	gradient to column	0.9	



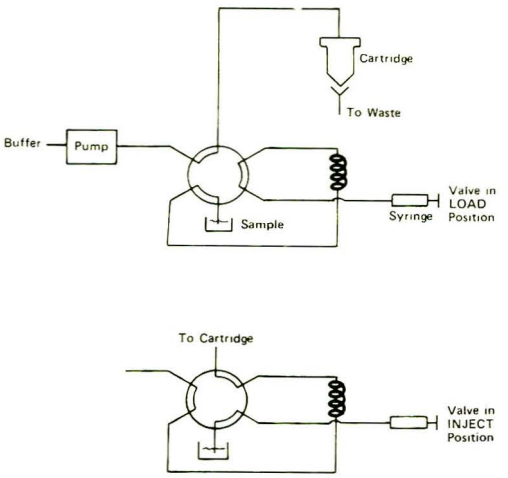


Fig. 1. Hydraulic diagram of the gradient buffer system. The peristaltic valve is a standard part of the amino acid analyzer.

Fig. 2. Hydraulic diagram of the cartridge loader, showing the two valve positions.

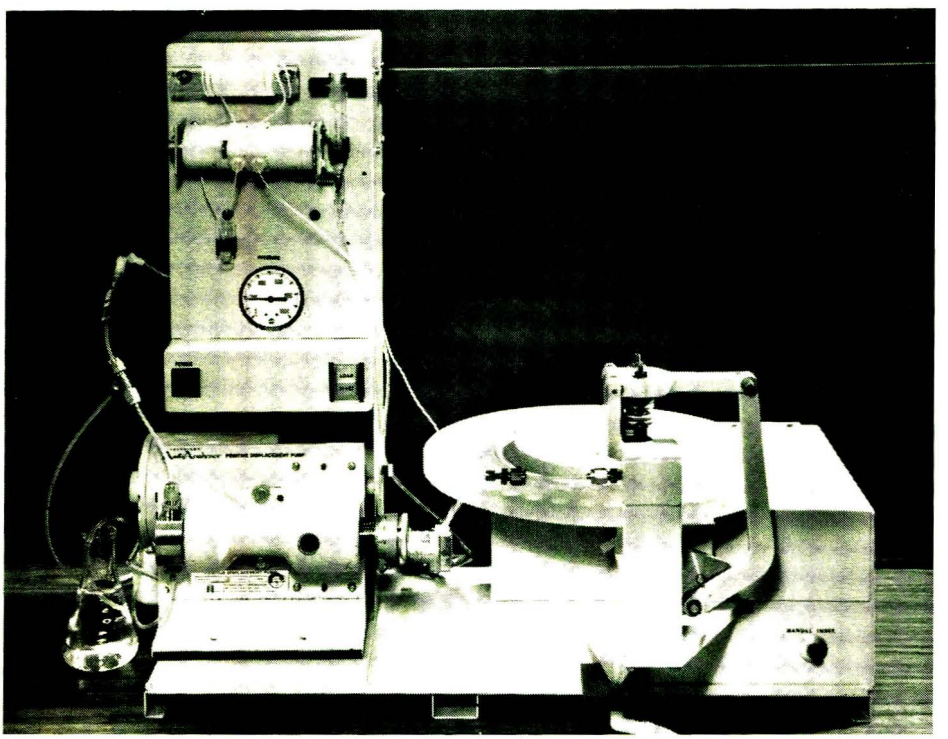


Fig. 3. The cartridge loader. The turntable for the cartridges is on the right, the pump at lower left, and the valve and sample loop at upper left.

Technicon Amino Acid Analyzer, a Chromatronix pneumatically actuated sampling valve, and a mechanical timer to control the sequence of operations. In using the cartridge loader, the operator sets the valve in the LOAD position and fills the sample loop by means of the syringe. The sample loop's length and diameter are chosen so that when the system is filled and the valve moved to the INJECT position it will contain 2.0 ml of sample. After the sample loop is filled, the operator presses the START button, with the valve still in the LOAD position. The pump starts, and buffer passes through the cartridge at 2 ml/min for 30 sec. The timer then causes the valve to switch to the INJECT position, and the sample is pumped through the cartridge in 1 min. The pump operates for 4 min more, passing buffer through the cartridge to remove the amino acids from the resin. The timer then causes the pump to turn off, the turntable assembly to move the next cartridge into position, and the system to stop in the STANDBY mode until the operator is ready to load the next sample.

Reagents

Stock citrate. Dissolve 420 g citric acid monohydrate and 165 g sodium hydroxide in approx. 1.5 l water, cool, and make up to 2.0 l with water.

25% Phenol. Mix 25 g phenol with sufficient water to give a total volume of 100 ml. This forms a two-phase liquid, that must be shaken well before use.

Buffer 1 (pH 5.25, 2.0 M Na^+). Dissolve 210.4 g sodium chloride in 600 ml water, add 200 ml stock citrate and 2 ml 25% phenol, and adjust to pH 5.25. Add 2 ml Brij-35 wetting agent (Technicon Part No. T21-0110-15) and make up to 2.0 l with water.

Buffer 2 (pH 5.25, 3.5 M Na^+). Dissolve 362.2 g sodium chloride in 600 ml water, add 200 ml stock citrate and 2 ml 25% phenol, and adjust to pH 5.25. Add 2 ml Brij-35 and make up to 2.0 l with water.

Buffer for the cartridge loader (pH 5.25, 0.35 M Na^+). Dissolve 17.5 g sodium chloride in 600 ml water, add 200 ml stock citrate and 2 ml 25% phenol and adjust to pH 5.25. Add 2 ml Brij-35 and make up to 2.0 l with water.

Sulfosalicylic acid. 3% (w/w) in water.

Triton X-100. Technicon Part No. T21-0188-06.

Ninhydrin reagent. Dissolve 10 g ninhydrin (Technicon Part No. T11-0102-25) in 500 ml methyl cellosolve and 250 ml buffered sodium acetate (Technicon Part No. T21-0105-16, 4 *M*, pH 5.5), make up to 1000 ml with water.

Hydrazine sulfate reagent. Dissolve 0.2623 g hydrazine sulfate in 600 ml water, add 5 ml Brij-35, make up to 1.0 l with water, and add one drop conc. sulfuric acid.

Standards

Putrescine, cadaverine, spermidine and spermine were used as received from Aldrich, Milwaukee, Wisc., U.S.A. To 750 ml water were added 3.0 mg putrescine, 10.0 mg cadaverine, 1.5 mg spermidine and 2.0 mg spermine. The pH of the solution was adjusted to between 3 and 4 with hydrochloric acid, and the solution was made up to 1.0 l with water.

Samples

Whole blood, serum or plasma, and 24-h urine collections were obtained from Technicon employees. Patient samples were provided by Drs. M. K. Schwartz and Y. Hirshaut of the Memorial Hospital and Sloan-Kettering Institute, New York, N.Y., U.S.A.

Procedure

Urine. Test 1 ml of clear urine for protein with a few crystals of sulfosalicylic acid. If a precipitate forms, deproteinize 10 ml urine with 100 mg sulfosalicylic acid, mix, and centrifuge. For free polyamines, add 0.3 ml stock citrate to 3.0 ml urine or the deproteinized supernatant, and adjust to pH 3—4 with 0.1 *M* sodium hydroxide or hydrochloric acid solution. For total polyamines, hydrolyze the sample as follows: To 5.0 ml of clear urine or deproteinized supernatant in a 15 ml glass centrifuges tube add 1.0 g barium hydroxide, mix, and heat 4 h at 100° in an oil bath. Cool, add 2 or 3 drops of phenolphthalein solution, and neutralize with concentrated sulfuric acid (caution: add acid dropwise with cooling) to disappearance of pink color. Leave at room temperature at least overnight (24 h is better) and centrifuge. To 3.0 ml of the clear supernatant add 0.3 ml stock citrate and adjust to pH 3—4.

Whole blood. Add 1 drop Triton X-100 to 1.0 ml whole blood and agitate ultrasonically to effect hemolysis. Add 9.0 ml sulfosalicylic acid solution, mix, and centrifuge. Treat the clear supernatant according to the procedures described above for urine samples.

Serum or plasma. The procedure for serum or plasma is like that for whole blood, except that the hemolysis with Triton X-100 is omitted. If 1 ml of serum is avaiable, deproteinize and follow the rest of the steps exactly as for whole blood. If 2 ml of serum is available, deproteinize with 8 ml sulfosalicylic acid solution and proceed as for whole blood.

Cartridge loading. The cartridges contain a sulfonic acid-type ion exchange resin (Technicon Type C-4, Part No. T15-0361-42) similar to the one used in the chromatography column, but with a larger particle size. A description of the cartridge loader and its method of use are given above in the Equipment section. The cartridges may be re-used, but growth of algae in the resin bed may limit their useful life. We routinely add a small amount of phenol to the buffers to inhibit growth of algae, and store the cartridges in a phenol solution.

Chromatography. The conditions for chromatography are listed in Table I. The chromatography conditions are the same for all types of samples, and blood and urine samples can be intermixed. Because of the smaller polyamine concentrations in serum and plasma, the colorimeter controls are adjusted for a higher sensitivity, and serum or plasma samples cannot be intermixed with blood or urine samples.

RESULTS

Fig. 4 is a chromatogram of a synthetic solution approximating the polyamine composition of hydrolyzed normal urine. The time scale on the abscissa is for the time of emergence of the eluate from the column, and the times above the peaks indicate when each arrives at the colorimeter; the time differ-

ence, about 10 min, is the residence time of the column effluent in the flow stream where it is mixed with reagents and incubated for color development. This residence time is nearly half the total analysis cycle, and it is not possible to change sample cartridges until the last peak has passed through the colorimeter. However, the interval is used to wash and regenerate the column. There would be little practical benefit if the chromatography conditions were changed to reduce the elution times. Cutting the elution time in half would only reduce the complete cycle from 22 to 16 min.

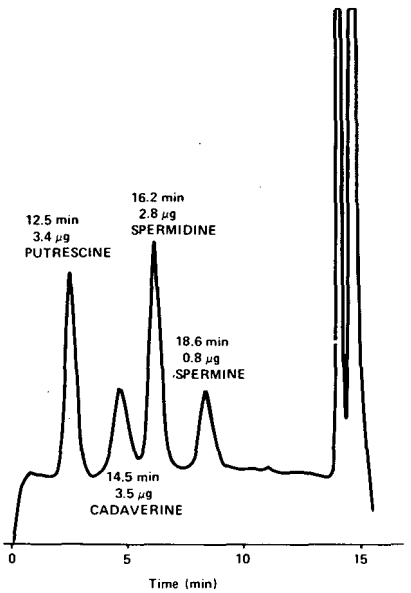


Fig. 4. Chromatogram of a standard solution.

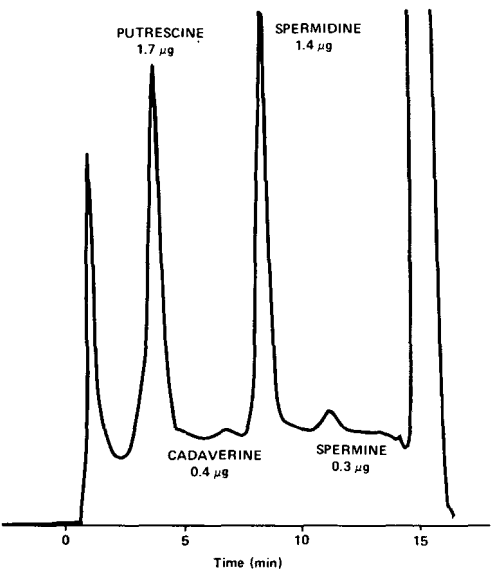
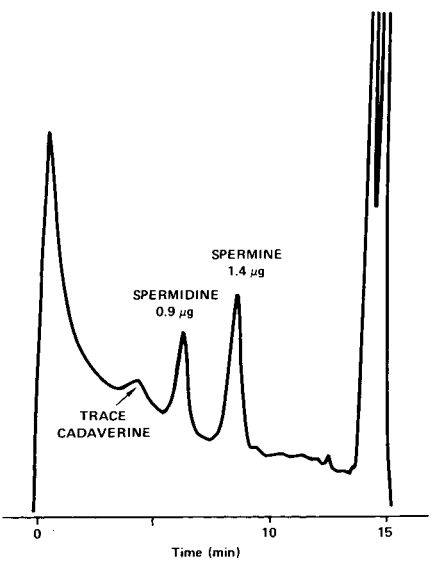


Fig. 5. Chromatogram of a hydrolyzed urine sample.

Figs. 5 and 6 show examples of chromatograms of samples of urine and whole blood after hydrolysis. The only significant difference from the chromatogram in Fig. 4 is that residual amino acids that remain on the cartridge appear at the beginning of the trace. These represent only trace amounts of the amino acids; it is advisable to handle the cartridges carefully after loading or the amino acid peak may increase due to contamination by these compounds from the operator's fingers.

The chromatogram of Fig. 7 illustrates the performance of the instrument under high-sensitivity conditions. Normal serum levels of spermidine are reported [4, 5] to be near 1/3 nmole/ml, or about 50 ng/ml. The figure shows that this amount of spermidine would be detectable in the chromatogram. However, we have not found spermidine in normal plasma samples or samples from cancer patients. Some possible reasons for this are discussed later in this publication.

Occasional chromatograms have shown peaks other than those shown in Figs. 5—7. A peak is sometimes observed just before the peak for putrescine. Veening et al. [12] found 1,3-diaminopropane in some samples. When we ad-



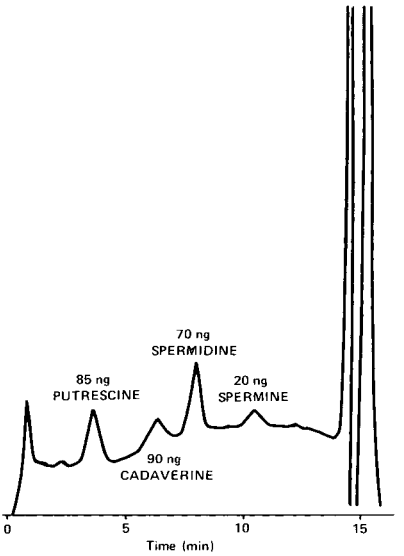


Fig. 6. Chromatogram of a hydrolyzed whole blood sample.

Fig. 7. Chromatogram of a standard with high-sensitivity settings.

ded this compound to standards, it gave a peak just before putrescine. In a few samples, the peak due to 1,3-diaminopropane has been larger than the putrescine peak and has interfered with its quantitation. Modifying the method by reducing the ionic strength of Buffer 1 would resolve these two peaks without greatly increasing total analysis time. We have also, quite rarely, observed an extra peak near the peak for cadaverine. This peak has not interfered with the determination of putrescine, spermine, or spermidine, and we have made no effort to identify the compound responsible.

Hydrolysis conditions

Hydrolysis with barium hydroxide was adopted as a means to avoid build-up of ionic strength of the hydrolysate, which would have interfered with the absorption of the polyamines in the ion-exchange resin in the sample cartridge. During the development of the method, it was found that recoveries of the polyamines were low when standard solutions were hydrolyzed with barium hydroxide, the base was neutralized with sulfuric acid, the clear supernatant was removed before the precipitate had aged. It was thought that the loss could be caused by either coprecipitation or by degradation of the polyamines during heating of the solution in the presence of the base. However, losses were found even when the heating step was eliminated; that is, when barium hydroxide was added to the polyamine solution and immediately neutralized with sulfuric acid.

Coprecipitation can be reversed by aging the barium sulfate precipitate. When the sample—precipitate mixture was held overnight at 100°, the polyamines were recovered quantitatively. Aging at room temperature is more convenient, and we found that overnight aging this way resulted in only small

losses (5—10%) that are rather constant from sample to sample and can thus be adequately compensated for in the calibration.

Other hydrolysis variables studied were the amount of barium hydroxide and the length of heating. Preliminary trials showed that hydrolysis is slow with only 0.5 g of base per 5 ml of sample. With 1 g of base per 5 ml of sample, heating for 4 h gave the best results. Hydrolysis was incomplete with 2 h heating, and with 6 h heating there was evidence of degradation of the polyamines; recoveries were found to be lower for putrescine and spermidine, and the cadaverine peak developed a shoulder.

Polyamine levels found

Only limited summary data are included in this report on analysis of samples from cancer patients and controls. The analysis results have been given to Dr. Hirshaut at Memorial Hospital for correlation with clinical information and eventual publication.

Urine. Analyses for free and total polyamines were done on 24-h urine collections from 21 ambulatory controls. The results (mg excreted per 24 h) are given here as the range of total amounts found and the mean. Putrescine: 0.02—2.55, 0.89; cadaverine: trace—2.98, 0.55; spermidine: 0.02—1.32, 0.53; spermine; not detected (2 samples)—0.38, 0.14. Statistical treatment of the analysis results showed that the data are not normally distributed, and perhaps have a log-normal distribution. Individuals who excreted larger amounts of one polyamine did not necessarily excrete large amounts of others. The total amount of the four polyamines excreted per day ranged from 0.28 to 6.5 mg, with a mean of 2.0 mg. The fraction in conjugated form was also highly variable from one person to another, and no relation was apparent between the fractions of different polyamines present as conjugates nor between the total amount excreted and the fractions conjugated. Many of the cancer patients, but not all by any means, excreted considerably larger amounts of the polyamines than were found in the urine of the ambulatory controls.

The amounts of individual polyamines found are similar to results reported for the analysis of normal urines by gas-chromatography [9], and somewhat lower than but overlapping the results by liquid chromatography [3]. Savory and Shipe [8] remarked: "Many more normal sera and urines need to be analyzed and a rigorous statistical analysis of the data made before a true normal range can be established."

Whole blood. We have analyzed whole blood from only a few ambulatory controls, besides the more extensive analyses on samples from hospital patients. Very little has been reported on polyamine levels in whole blood. Raina [16] reported the following means and standard deviations from the analysis of blood samples from 30 normal individuals: spermine, 1.34 ± 0.3, spermidine, 0.96 ± 0.16 μg/ml. (Note: Raina's publication lists the standard error of the mean, from which we have computed the standard deviation.) Iliev et al. [17] reported results nearly identical with those of Raina. Data from the present study are consistent with the earlier results. Spermine and spermidine were found in all blood samples. The concentrations for the few ambulatory controls were at the levels previously found, with considerable elevations in bloods from some of the cancer patients. The whole blood concentrations are so much

larger than what has been reported for serum [3—6] that it is apparent that polyamines in whole blood occur primarily in the cells. The data in the literature and our data have not been correlated with cell counts.

Plasma and serum. The results of analysis of plasma by this method are not in agreement with previously published results [3—6]. Analysis of synthetic solutions (Fig. 7) demonstrated that the method is adequately sensitive to detect the polyamines at the concentrations that have been reported in serum. Apparently, polyamines are lost during the sample preparation. We interpret the available evidence to suggest that polyamines in serum are bound to a specific protein — possibly an antibody or a carrier protein — firmly enough to be carried down with the protein when the sample is treated with sulfosalicylic acid.

We investigated non-specific coprecipitation of polyamines with protein during the development of the analytical method. Serum albumin was added to a standard containing all of the polyamines at normal urine levels, and the protein was then precipitated. The polyamines were recovered quantitatively in the supernatant. It is significant also that extraction with trichloroacetic acid is a common procedure to solubilize polyamines from tissue samples. Marton et al. [13] extracted polyamines from tissue samples with a solution of sulfosalicylic acid. We cannot account for the losses of the polyamines by assuming that volatilization occurs during hydrolysis, as this would have affected standards carried through the procedure.

It is well known that many small molecules in plasma are held by specific binding proteins, and this binding has been shown to cause serious errors in some radioimmunoassay procedures. Procedures are available to release small molecules from the binding proteins. For example, Fang and Refetoff [18] tested the release of triiodothyronine (T_3) from binding proteins by heat inactivation of the protein, by 8-anilino-1-naphthalenesulfonic acid, and by ethanol extraction. They also mentioned some other simple procedures that have been applied to release T_3 from binding proteins and that are presumably not specific to T_3. We have not been able to test the effectiveness of any of these procedures in the analysis of serum or plasma for polyamines.

CONCLUSIONS

We believe that the procedures described here meet the need for a more rapid analysis method than has previously been available for the determination of the polyamines, so that normal and pathological levels of polyamines in urine and blood can be studied in detail. Some further work is needed to adapt the method to the analysis of serum or plasma samples.

ACKNOWLEDGEMENTS

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REFERENCES

1 D.H. Russell, Nature (London), 233 (1971) 144.
2 D.H. Russell, C.C. Levy, S.C. Schimpff and I.A. Hawk, Cancer Res., 31 (1971) 1555.
3 L.J. Marton, J.G. Vaughn, I.A. Hawk, C.C. Levy and D.H. Russell, in D.H. Russell (Editor), Polyamines in Normal and Neoplastic Growth, Raven Press, New York, 1973, p. 367.
4 L.J. Marton, D.H. Russell and C.C. Levy, Clin. Chem., 19 (1973) 923.
5 K. Nishioka and M.M. Ramsdahl, Clin. Chim. Acta, 57 (1974) 155.
6 D.H. Russell and S.D. Russell, Clin.Chem., 21 (1975) 860.
7 D.H. Russell (Editor), Polyamines in Normal and Neoplastic Growth, Raven Press, New York, 1973.
8 J. Savory and J.R. Shipe, Ann. Clin. Lab. Sci., 5 (1975) 110.
9 C.W. Gehrke, K.C. Kuo, R.W. Zumwalt and T.P. Waalkes, in D.H. Russell (Editor), Polyamines in Normal and Neoplastic Growth, Raven Press, New York, 1973, p. 343.
10 M.T. Denton, H.S. Glazer, T. Walle, D.C. Zellmer and F.G. Smith, in D.H. Russell (Editor), Polyamines in Normal and Neoplastic Growth, Raven Press, New York, 1973, p. 373.
11 C.W. Gehrke, K.C. Kuo, R.W. Zumwalt and T.P. Waalkes, J. Chromatogr., 89 (1974) 231.
12 H. Veening, W.W. Pitt, Jr. and G. Jones, Jr., J. Chromatogr., 90 (1974) 129.
13 L.J. Marton, O. Heby, C.B. Wilson and P.L.Y. Lee, FEBS Lett., 41 (1974) 99.
14 G. Ertingshausen and H.J. Adler, Amer. J. Clin. Pathol., 53 (Suppl.) (1970) 860.
15 G. Ertingshausen and H.J. Adler, J. Chromatogr., 44 (1969) 620.
16 A. Raina, Scand. J. Clin. Lab. Invest., 14 (1962) 318.
17 V. Iliev, R.E. Nichols and C.C. Pfeiffer, Anal. Biochem., 25 (1968) 1.
18 V.S. Fang and S. Refetoff, Clin. Chem., 20 (1974) 1150.
19 L.R. Snyder, J. Chromatogr., 125 (1976) 287.

Journal of Chromatography, 143 (1977) 137—152
Biomedical Applications

CHROMBIO. 018

RECOMMENDED METHOD FOR THE ANALYSIS OF AMINO ACIDS IN BIOLOGICAL MATERIALS

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SUMMARY

Fifty-five ninhydrin-positive compounds in physiological fluids were determined with a Hitachi Model KLA-5 amino acid analyzer by a two-column chromatographic procedure. Both columns were packed with Hitachi Custom 2618 ion-exchange resin. The total analysis time was 9.5 h.

In this procedure, particularly glucosamine, mannosamine and galactosamine were separated completely from normal "protein" amino acids, and N^G-monomethylarginine, N^G,-N^G-dimethylarginine and N^G,N'^G-dimethylarginine, which were present in the myelin basic protein of several species and excreted in human urine, were separated from other basic amino acids. The method is useful for various applications with biological materials.

INTRODUCTION

Amino acid analysis has been applied to a wide variety of hydrolyzates of protein or glycoprotein, physiological fluids and tissue extracts. In such applications a combination of high resolution and speed of analysis is essential.

There are a large number of procedures available for the quantitative analysis of amino acids in physiological fluids [1—9]. Perry et al. [3] reported a versatile lithium buffer elution system for single-column chromatography, and 65 ninhydrin-positive substances were analyzed by this method. Although five steps with lithium buffers were used, all of the substances were not separated completely and the time of analysis was 21 h. On the other hand, Benson [8] reported a more rapid two-column chromatographic method for the analysis of amino acids in physiological fluids. He used two buffers for each analysis and 41 ninhydrin-positive compounds could be analyzed in 7.5 h. When the amino acid analysis was performed by this method using a different type of analyzer, unsatisfactory results were obtained. It was clear that the resolution of amino acids depends on the characteristic properties of the resin such as the degree of cross-linkage and the particle diameter.

We have used a Hitachi amino acid analyzer and have developed a two-column chromatographic procedure which gives a high resolution and is also widely appliciable.

MATERIALS AND METHODS

Reagents

Amino acid standard calibration mixture was a product of Takara Kosan Co., Tokyo, Japan. Other ninhydrin-positive compounds were purchased from Sigma (St. Louis, Mo., U.S.A.), Calbiochem (Los Angeles, Calif., U.S.A.), Fluka (Buchs, Switzerland), Wako (Osaka, Japan) and Tokyo Chemical Industry Co. (Tokyo, Japan). N^G-Monomethylarginine, N^G,N^G-dimethylarginine and N^G,N'^G-dimethylarginine were products of Calbiochem and N-ϵ-lysine was a product of Sigma. β-(2-Thienyl-DL-serine and S-β-(4-pyridylethyl)cysteine used as internal standards were purchased from Pierce (Rockford, Ill., U.S.A.).

Sample preparation

Synthetic mixture of ninhydrin-positive compounds. A standard solution wad made by diluting with 0.01 *N* HCl to give concentrations of 0.1 μmole/ml of ninhydrin-positive compounds, except for 0.04 μmole/ml of cystathionine, 0.2 μmole/ml of proline and anserine, 1.0 μmole/ml of urea and 5.0 μmole/ml of creatinine.

Deproteinized human plasma. Normal human plasma was deproteinized using sulphosalicylic acid [10].

Deammoniated human urine. Normal human urine was deammoniated by the method of Benson and Patterson [1].

Hydrolysis of human plasma and urine. Deproteinized human plasma (2 ml) and deammoniated human urine (4 ml) were hydrolyzed with an equal volume of concentrated HCl for 24 h at 110°. The hydrolyzates were dried with a rotary evaporator at 55° and dissolved in 0.01 *N* HCl.

Apparatus

A Hitachi Model KLA-5 automatic amino acid analyzer was used consisting of the main instrument, a tape-controlled programmer, an automatic sample injector and an integrator.

Column and resin

A 40 × 0.9 cm I.D. glass column was used for the separation of the acidic and neutral amino acids, and a 25 × 0.9 cm I.D. glass column for the separation of the basic amino acids. Both columns were filled with Hitachi Custom Ion-Exchange Resin 2618, which is sulphonated with a 10% degree of cross-linking. The particle diameter is 11.5 ± 2 μm with an exchange capacity of 4.5 mequiv./g.

Buffers

Two lithium citrate buffers were used for acidic and neutral amino acid analyses and two sodium citrate buffers for basic amino acid analysis (Table I). The pH of the eluting buffers must be controlled to within ±0.005 because the separation of the amino acids is sensitive to small differences in pH.

TABLE I

COMPOSITION OF BUFFERS

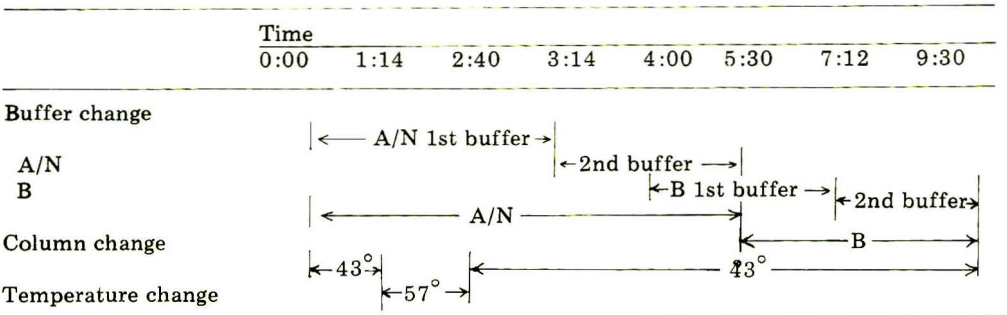
	Acidic and neutral amino acid analysis		Basic amino acid analysis	
	1st	2nd	1st	2nd*
Cation concentration				
Li (*N*)	0.25	0.25		
Na (*N*)			0.38	0.50
pH	2.90	4.30	4.10	6.09
Citric acid (g)	95.8	57.5	—	—
Lithium citrate (g)	25.2	80.1	—	—
Lithium chloride (g)	96.3	70.1	—	—
Sodium citrate (g)	—	—	373.0	490.0
Conc. HCl (ml)	—	—	150.0	25.0
Ethanol (ml)	300.0	—	300.0	—
Methanol (ml)	—	—	—	800.0
25% Brij-35 (ml)	40.0	40.0	40.0	40.0
Thiodiglycol (ml)	25.0	25.0	—	—
n-Caprylic acid (ml)	1.0	1.0	1.0	1.0
Final volume (l)	10.0	10.0	10.0	10.0

*This buffer was used for the rapid determination of glucosamine and galactosamine according to our previous paper [11].

TABLE II

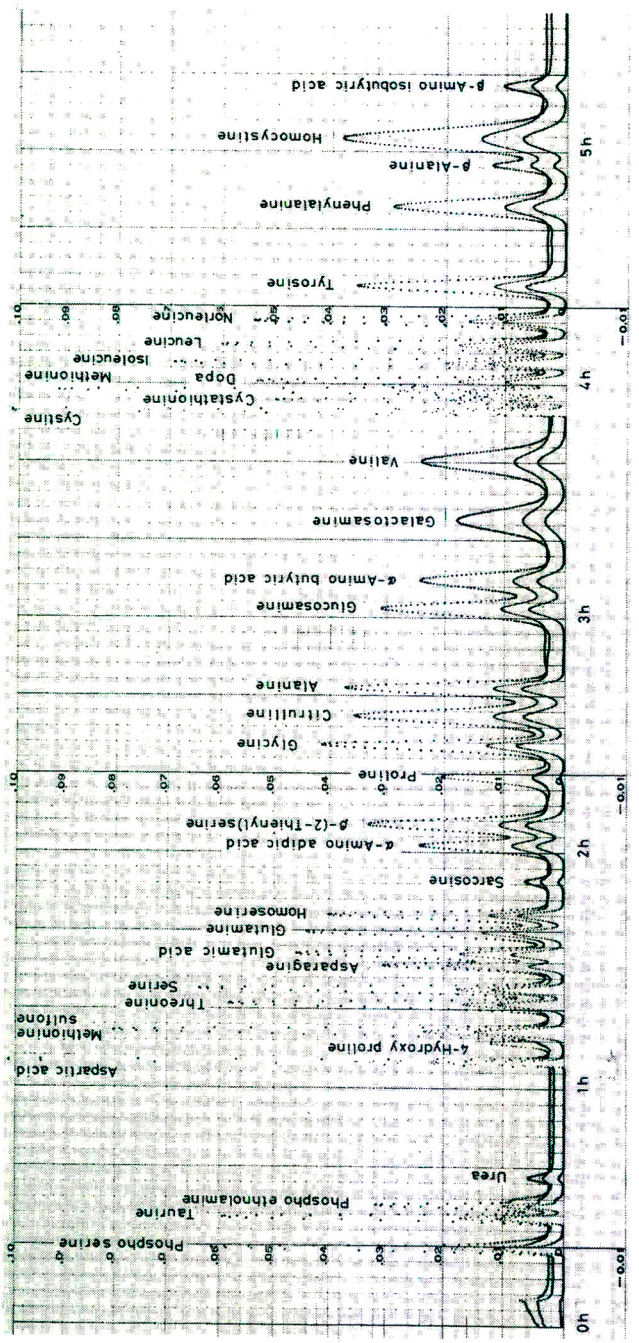
ELUTION PROGRAMME

A/N = Acidic and neutral amino acid analysis; B = basic amino acid analysis



Chromatographic conditions

The eluting buffer and ninhydrin solution were pumped at flow-rates of 60 and 30 ml/h, respectively. The operating back-pressure was 15 ± 1 kg/cm^2 for acidic and neutral amino acid analyses and 10 ± 1 kg/cm^2 for basic amino acid analysis. The elution programme is illustrated in Table II, and the column was operated at 43° and 57°, as shown in Table II.


0h
1h
2h
3h
4h
5h
Phospho serine
Taurine
Phospho ethanolamine
Urea
Aspartic acid
Methionine sulfone
4-Hydroxy proline
Threonine
Serine
Asparagine
Glutamic acid
Glutamine
Homoserine
Sarcosine
α-Amino adipic acid
β-(2-Thienyl)serine
Proline
Glycine
Citrulline
Alanine
Glucosamine
α-Amino butyric acid
Galactosamine
Valine
Cystine
Cystathionine
Dopa
Methionine
Isoleucine
Leucine
Norleucine
Tyrosine
Phenylalanine
β-Alanine
Homocystine
β-Amino isobutyric acid

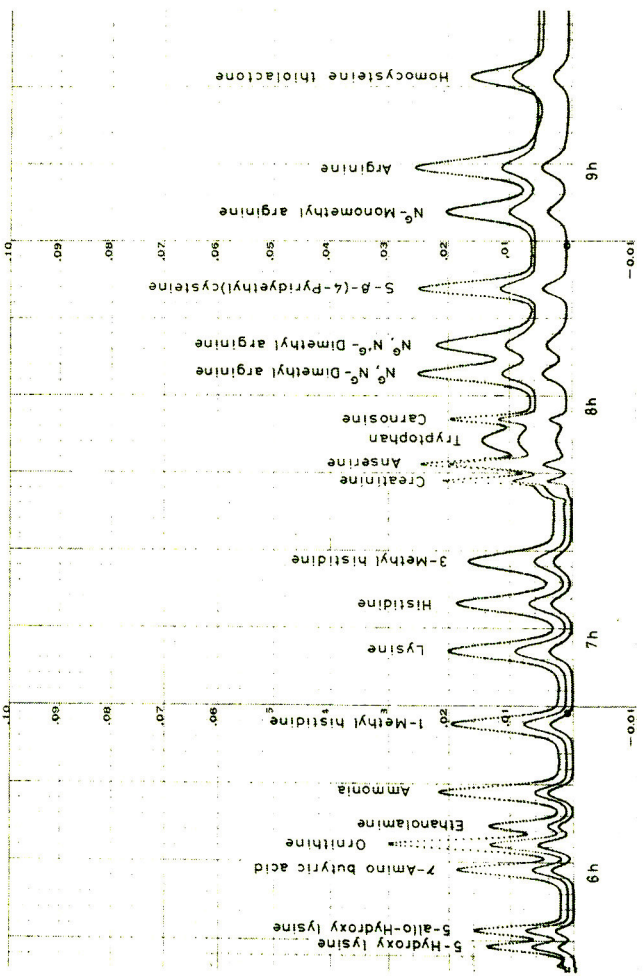


Fig. 1. Chromatogram from 0.5 ml of a synthetic mixture of 55 ninhydrin-positive compounds. The amount of each compound analyzed was 0.05 μmole. The following amino acids and their amounts were used: cystathionine (0.02 μmole), proline and anserine (0.1 μmole), urea (0.5 μmole) and creatinine (2.5 μmole).

TABLE III

RETENTION TIMES OF 79 NINHYDRIN-POSITIVE COMPOUNDS

Ninhydrin-positive compounds	Retention time (min)
Acidic and neutral amino acid analysis	
Cystic acid	17
Homocysteic acid	17
Phosphoserine	18
Taurine	25
Phosphoethanolamine	28
Laevulinic acid	28*
Urea	36
Aspartic acid	65
S-CMC	65
Glutathione (reduced)	66
Hydroxyproline	69
Methionine sulphone	73
Methionine sulphoxides	75, 78**
Threonine	80
Serine	83
Asparagine	90
Glutamic acid	93
Glutathione (oxidized)	97
Glutamine	98
Homoserine	102
Sarcosine	111
α-Aminoadipic acid	120
β-(2-Thienyl)-DL-serine	125
Proline	138
Glycine	146
Hippuric acid	146
Citrulline	153
Alanine	160
Glucosamine	181
α-Aminobutyric acid	188
Mannosamine	190
Galactosamine	204
Valine	219
Cystine	231
Norvaline	231
Pipecolic acid	232
Homocitrulline	234
Cystathionine	234
Methionine	236
Lanthionine	237
DOPA	239
Isoleucine	244
Leucine	248
Norleucine	255
β-(2-Thienyl)-DL-alanine	259
Tyrosine	264
Phenylalanine	284
β-Alanine	295
Homocystine	302
β-Aminoisobutyric acid	315

TABLE III *(continued)*

Ninhydrin-positive compounds	Retention time (min)
Basic amino acid analysis	
5-Hydroxylysine	100
allo-Hydroxylysine	104
δ-Aminolaevulinic acid	105
γ-Aminobutyric acid	120
Ornithine	126
Ethanolamine	131
Ammonia	140
Kynurenine	155
1-Methylhistidine	157
Lysine	175
N-ε-Methyllysine	176
Histidine	188
3-Methylhistidine	199
Creatinine	220
Creatine	221
Anserine	224*
Tryptophan	230
α-Amino-β-guanidinopropionic acid	232
Monoiodotyrosine	233
ε-Aminocaproic acid	234
Carnosine	236
Homocarnosine	236
Canavanine	239
N^G,N^G-Dimethylarginine	247
N^G,N'^G-Dimethylarginine	255
S-β-(4-Pyridylethyl)cysteine	268
N^G-Monomethylarginine	288
Arginine	300
Diiodotyrosine	318
Homocysteine thiolactone	322

*Ratio of 440 to 570 nm for laevulinic acid and anserine was higher than that of other amino acids and related compounds except for proline and hydroxyproline; for the former the ratio was 1.25 and for the latter 0.84.

**Methionine sulphoxide gave two peaks.

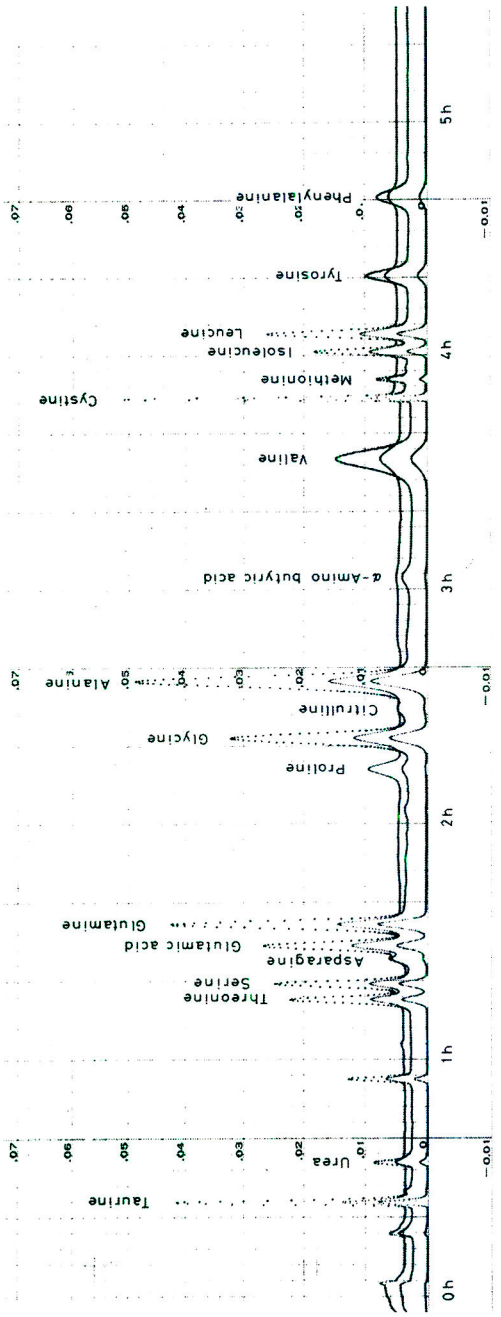
RESULTS

Analysis of a synthetic mixture

The results of the chromatography of a synthetic mixture containing 55 ninhydrin-positive compounds are shown in Fig. 1. A high resolution of all of these compounds was obtained and the total analysis time was 9.5 h.

A further 24 ninhydrin-positive compounds were investigated under the same conditions, and Table III lists the retention times of the 79 substances studied. It can be seen that not all of the latter group were separated from the first 55 ninhydrin-positive compounds.

The acidic and neutral amino acid analysis was improved so that 36 compounds containing methionine sulphone, homoserine, homocystine, DOPA, glu-


(a')
0 h
1 h
2 h
3 h
4 h
5 h
Taurine
Urea
Threonine
Serine
Asparagine
Glutamic acid
Glutamine
Proline
Glycine
Citrulline
Alanine
α-Amino butyric acid
Valine
Methionine
Isoleucine
Leucine
Tyrosine
Phenylalanine
Cystine
(a'')
6h
7h
8h
9h
Ornithine
Ethanolamine
Ammonia
1-Methyl histidine
Lysine
Histidine
Tryptophan
Arginine

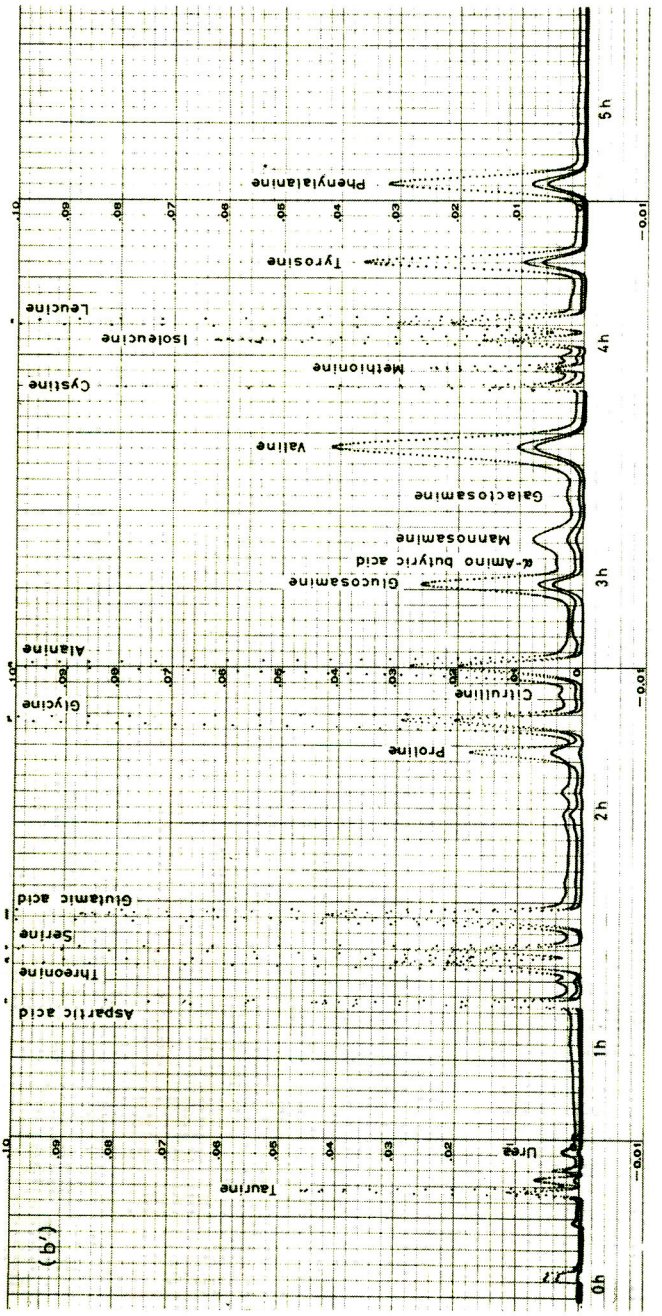


Fig. 2.

(Continued on p. 146)

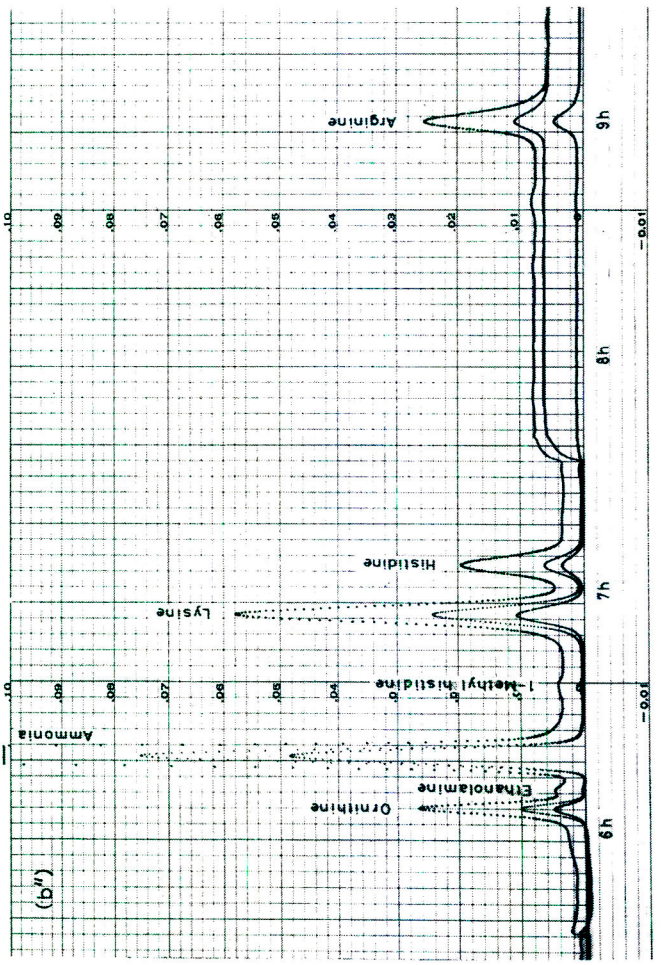


Fig. 2. (a) Chromatogram from 0.24 ml of normal human plasma; (b) chromatogram from 0.24 ml of hydrolyzate of normal human plasma.

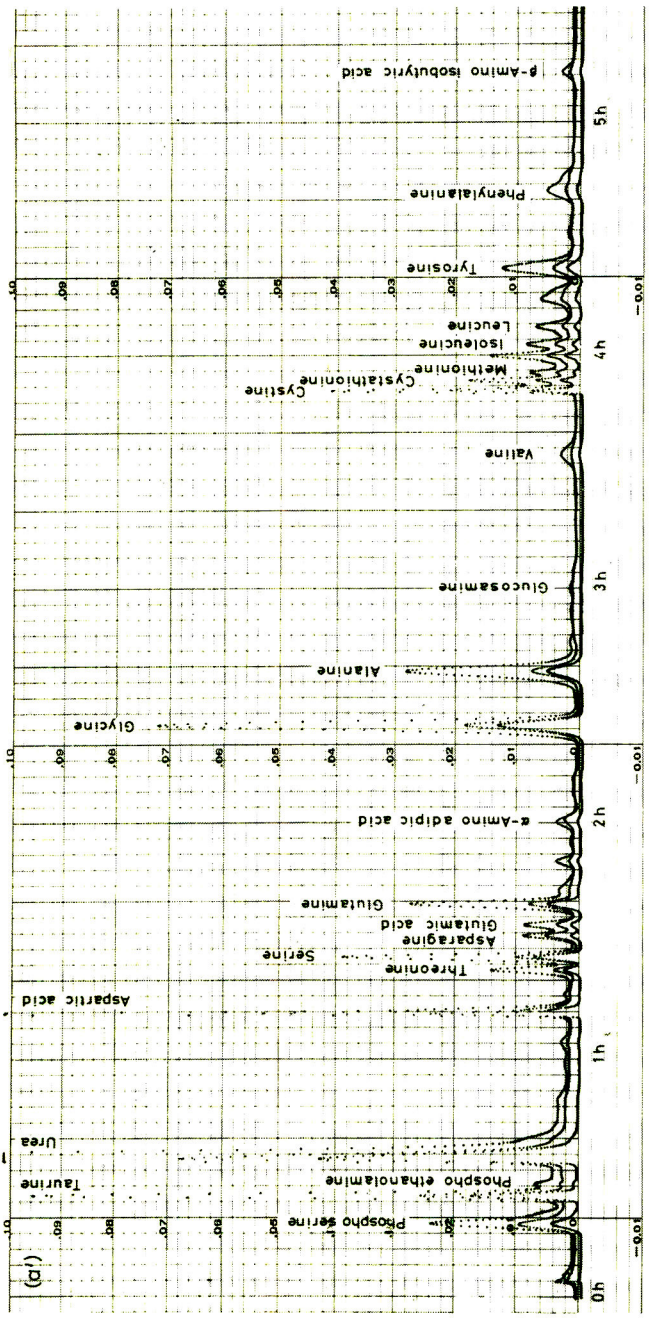
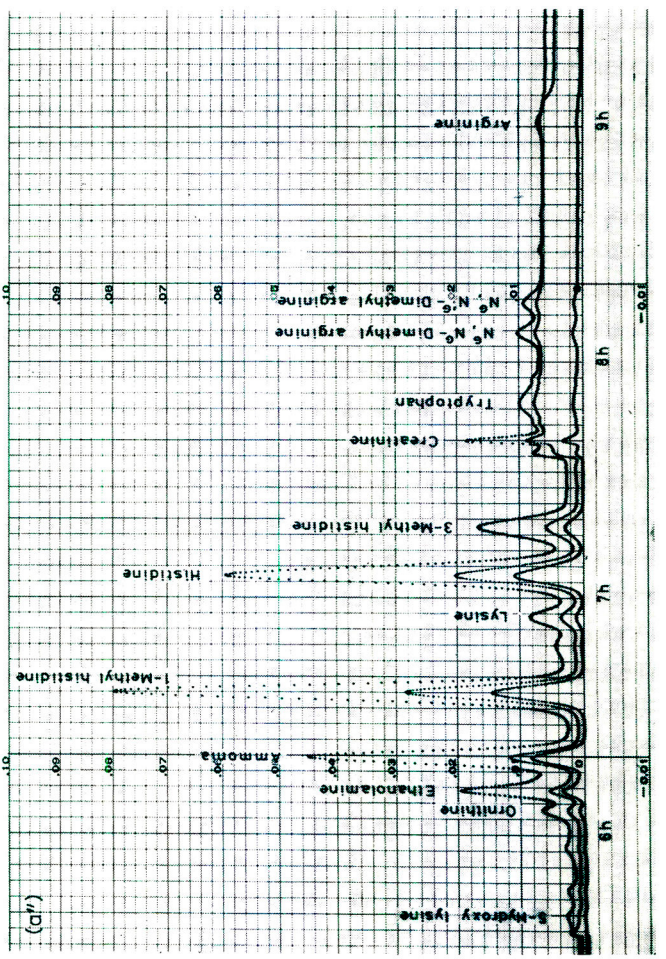


Fig. 3.

(Continued on p. 148)


5-Hydroxy lysine
Ornithine
Ethanolamine
Ammonia
1-Methyl histidine
Lysine
Histidine
3-Methyl histidine
Creatinine
Tryptophan
N6, N6- Dimethyl arginine
N6, N6- Dimethyl arginine
Arginine
9h
7h
8h
6h

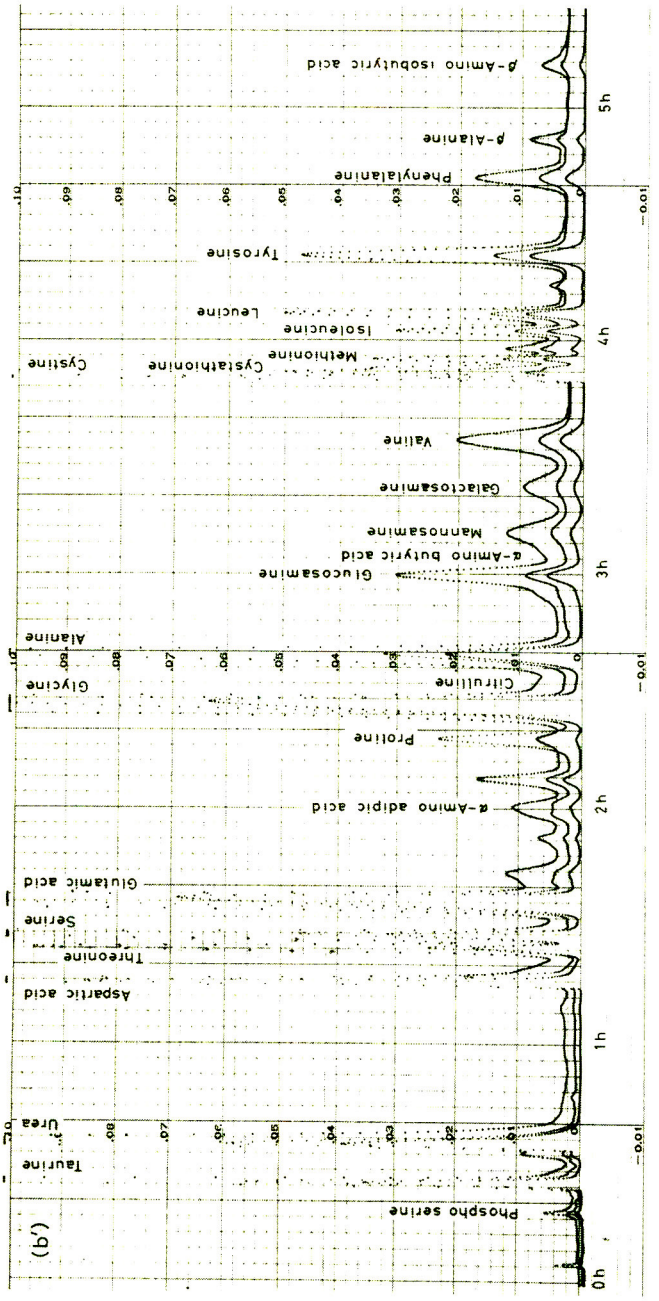


Fig. 3.

(Continued on p. 150)

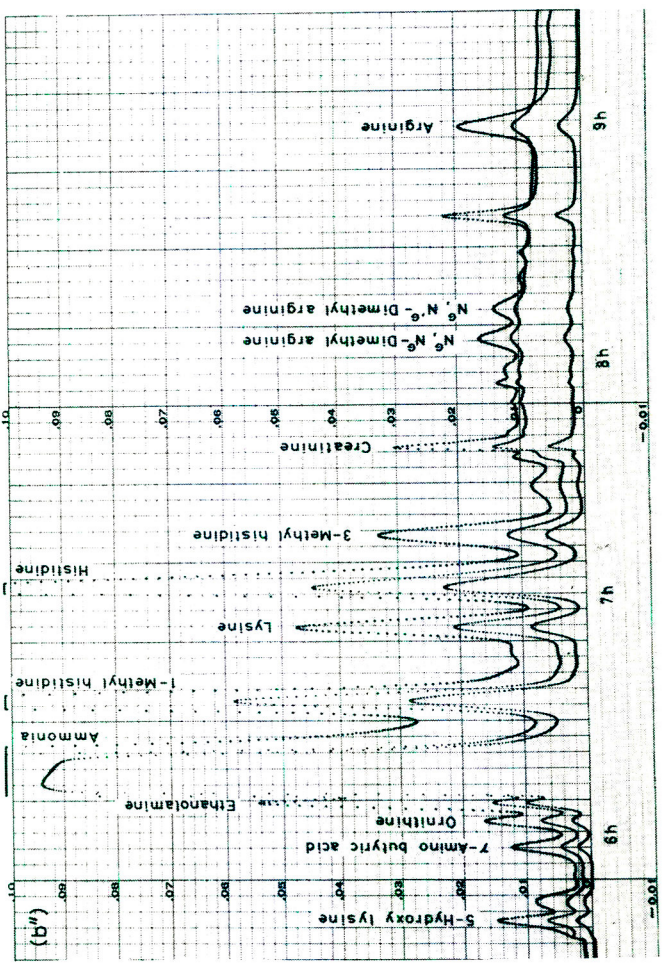


Fig. 3. (a) Chromatogram from 0.25 ml of normal human urine; (b) chromatogram from 0.5 ml of hydrolyzate of normal urine.

cosamine, galactosamine and β-(2-thienyl)serine were resolved by using two steps with lithium buffer for 5.5 h. Mannosamine occurred as a shoulder on the α-aminobutyric acid peak. Glucosamine, mannosamine and galactosamine were completely resolved from each other in the absence of α-aminobutyric acid. In order to separate citrulline from glycine and alanine, the column temperature was increased to 57° for 1 h 26 min.

In the basic amino acid analysis, the resolution of ornithine, γ-aminobutyric acid, ethanolamine and ammonia was very difficult. The column temperature and the concentration of alcohol in the buffer affected the chromatographic behaviour of these compounds; the optimal column temperature was 43° (isothermal) and 3% of ethanol was added to the basic first buffer. The most serious problem was to separate creatinine, anserine, tryptophan and carnosine completely from each other. In this instance, it was necessary to change the buffer at the correct moment; when the buffer was changed too late, anserine overlapped with creatinine and when it was changed too soon, carnosine was eluted simultaneously with tryptophan. We have also achieved the determination of methylated basic amino acids. In comparison with Deibler and Martenson's method [12], N^G,N^G-dimethyl-, N^G,N'^G-dimethyl-, and N^G-monomethylarginine were rapidly and completely separated. However, N-ε-methyllysine occurred as a shoulder on the lysine peak.

Analysis of normal human plasma

Fig. 2a is a chromatogram of deproteinized normal human plasma. Urea, glutamine, glycine and alanine were always present in relatively high concentrations. Although our method gave a very high resolution, two unknown peaks were evident, one of which was eluted immediately before aspartic acid and the other was eluted after the buffer change in the basic amino acid chromatogram. The latter was almost always present in plasma and could be confused with creatinine. The hydrolysis of deproteinized plasma was carried out in order to investigate whether these compounds were amino acid derivatives or peptides. It can be seen in Fig. 2b that these peaks disappeared in the hydrolyzate of deproteinized plasma and glutamine was also converted into glutamic acid. At the same time, the total amino acid content increased about 3-fold before hydrolysis. In addition, glucosamine and mannosamine occurred in relatively large amounts and galactosamine was observed in trace amounts. These results suggest that the two unknown peaks were peptides or possibly mucopolysaccharidepeptides.

Analysis of normal human urine

Fig. 3 shows the resolution of ninhydrin-positive compounds in normal human urine and the hydrolyzate of urine. As shown in Fig. 3a, N^G,N^G-dimethyl- and N^G,N'^G-dimethylarginine were identified in human urine. The normal occurrence of these methylarginines in human urine has been reported by Kakimoto and Akazawa [13]. The total amino acid contents in the hydrolyzate of urine were increased about 2-fold and amino sugars occurred at the same level as in the hydrolyzate of plasma. It can be concluded that glucosamine and galactosamine were derived from mucopolysaccharides that are a family of chondroitin sulphates characterized by a varying sulphate content [14].

DISCUSSION

Although a single-column system for the analysis of amino acids in physiological fluids has high resolution, it is time consuming and requires five or six different buffers [3, 4], and it is difficult to obtain reproducible chromatograms simply by such a method. The reproducibility and rapidity of analysis are greater with the two-column system described here.

In order to separate efficiently a large number of ninhydrin-positive substances, it is important to select the most suitable resin. Benson [8] recommended a 7% cross-linked polymer for this purpose, but we have found that a 10% linked polymer (2618) is more suitable than a 7% cross-linked polymer (2614). When the latter resin was used for acidic and neutral amino acid analyses, proline was not separated from α-aminoadipic acid.

The particle size of the resin also affected the resolution of amino acids. When a smaller particle size was used, a higher resolution was obtained on the chromatogram. In this instance, a higher pressure was generated in buffer pump, frequently resulting in various problems. We have overcome these problems by using a shorter column (40 cm) under an operating back-pressure of 15 kg/cm^2. The optimal particle size of the resin was 11.5 ± 2 μm (2618). In addition, uniformity of the particle size increased the resolution and also lowered the column pressure.

REFERENCES

1 J.V. Benson, Jr. and J.A. Patterson, Anal. Biochem., 13 (1965) 265.
2 J.V. Benson, Jr., M.J. Gordon and J.A. Patterson, Anal. Biochem., 18 (1967) 228.
3 T.L. Perry, D. Stedman and S. Hansen, J. Chromatogr., 38 (1968) 460.
4 A. Vega and P.B. Nunn, Anal. Biochem., 32 (1969) 446.
5 G.E. Atakin and W. Ferdinand, Anal. Biochem., 38 (1970) 313.
6 G. Ertingshausen and H.J. Adler, Amer. J. Clin. Pathol., 53 (1970) 680.
7 K. Efron and P.L. Wolf, Clin. Chem., 18 (1972) 621.
8 J.V. Benson, Jr., Anal. Biochem., 50 (1972) 477.
9 A. Niederwieser and G. Pataki, New techniques in amino acid, peptide and protein analysis, 1971, pp. 1—64.
10 A. Mondino, G. Bongiovanni, S. Fumero and L. Rossi, J. Chromatogr., 74 (1972) 255.
11 K. Murayama, N. Shindo and H. Koide, Anal. Biochem., 70 (1976) 537.
12 G.E. Deibler and R.E. Martenson, J. Biol. Chem., 248 (1973) 2387.
13 Y. Kakimoto and S. Akazawa, J. Biol. Chem., 245 (1970) 5751.
14 D.P. Varadi, J.A. Cifonelli and A. Dorfman, Biochim. Biophys. Acta, 141 (1967) 103.

Journal of Chromatography, 143 (1977) 153—160
Biomedical Applications

CHROMBIO. 026

QUANTITATIVE ANALYSIS OF HUMAN SERUM CHOLESTEROL BY THIN-LAYER CHROMATOGRAPHIC SPOT TEST

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SUMMARY

A fast, inexpensive, simple method is described for the determination of the total cholesterol concentration in human serum. The cholesterols (free and esterified) are extracted from serum by a double extraction procedure, using isopropanol—water—10 *N* sodium hydroxide (250:125:10) and then *n*-octane. An aliquot of the octane extract is spotted on a thin-layer plate and the cholesterol in the spot is rendered visible using an aqueous phosphomolybdic acid staining solution and a heat treatment for the color development. The colored spot is scanned with a densitometer.

INTRODUCTION

The significance of the cholesterol level in serum, due to its presumed relationship to atherosclerosis and other cardiovascular diseases, lead to the development of numerous laboratory tests for its quantitative determination. Among other methods, thin-layer chromatography (TLC) has also been applied for the analysis. In the direct method [1—7], cholesterol and its esters are separated from the other serum lipids on a silica gel coated thin-layer plate, the chromatogram is stained and the bands are scanned. In the indirect method [8—10], the separated cholesterol fractions are eluted from the silica gel and are quantitated by some conventional method. Both methods have their particular drawbacks: the direct method lacks the necessary precision and the indirect method is rather tedious. Both methods have an additional common drawback: the free and the esterified cholesterol fractions separate into two distinct bands and therefore both bands should be quantitated if the total cholesterol is to be determined.

The method described below permits the separation of the cholesterol and its esters from the other lipids by a double extraction, using a combination of alkaline isopropanol and *n*-octane. The octane extract is then applied to a thin-layer plate for the quantitative measurement of the total cholesterol.

EXPERIMENTAL

Procedure

Five hundred μl of the isopropanol—water—10 *N* sodium hydroxide mixture (250:125:10) are pipetted into a 12 × 75 mm glass tube. Twenty-five μl of serum are added to the tube and the contents are mixed on a Vortex-type mixer for 15 sec. After 2 min, 200 μl of *n*-octane are added and the contents are mixed again on a Vortex mixer for 30 sec. Octane separates from the lower isopropanol—water—sodium hydroxide mixture in 1 min. Ten μl of the upper octane phase are dispensed onto a 5 × 10 cm silica gel 60 coated glass plate with a 10-μl Eppendorf pipet. The octane forms a round spot on the plate with a diameter of about 10 mm. The spot dries in air in about 1 min, after which the plate is immersed in a staining solution. The staining solution is composed of 15 g of phosphomolybdic acid and 10 ml of perchloric acid dissolved in a mixture of 90 ml of water and 100 ml of ethanol. After the air bubbles have escaped (10 sec), the plate is taken out of the solution and blotted with a paper towel. The dried plate is placed in front of a forced air heater for 5 min at a distance at which the temperature does not exceed 50°. During the heat treatment the spot turns blue while the rest of the plate shows a pale yellow background. The color intensity of the spot is determined with a single-beam photodensitometer (Quick Scan; Helena Labs., Beaumont, Texas, U.S.A.). The spots are scanned and the readings are evaluated from a calibration curve. For the preparation of the calibration curve, solutions of 1, 2, 3, and 4 mg/ml cholesterol in isopropanol are prepared. The color of the spot and its intensity are stable for at least 1 h. To increase the sensitivity a 645-μm filter is used for the scanning.

RESULTS

The standard curve prepared from the spots of 1—4 mg/ml cholesterol solutions is shown in Fig. 1. The scanned values are the mean values of ten replicate measurements. The relative standard deviation (RSD) values are at 1 mg/ml: 8.7%, at 2 mg/ml: 7.9%, at 3 mg/ml: 8.6%, and at 4 mg/ml: 9.6%. The mean RSD is 8.7%.

Twenty serum specimens were analyzed by the conventional Liebermann—Burchard colorimetric method and by the micro-spot method, respectively. The scatter diagram is shown in Fig. 2.

It shows how well the scanning values compares with the Liebermann—Burchard test.

DISCUSSION

The color-producing reaction is not specific for cholesterol. Since the other lipid components of the serum (triglycerides, free fatty acids, phospholipids) also produce the same color, cholesterol must first be separated from these

components. The separation is carried out with a combination of an alkaline hydrolysis and an extraction with octane. When the serum is mixed with the alkaline isopropanol, the lipids are separated from their carrier proteins and simultaneously react with the alkali. Triglycerides and phospholipids are hydrolyzed and the unesterified and liberated fatty acids are neutralized. Accordingly, the alkaline isopropanolic extract contains sodium salts of the fatty acids, sodium phosphate, free glycerol and the free and esterified cholesterol

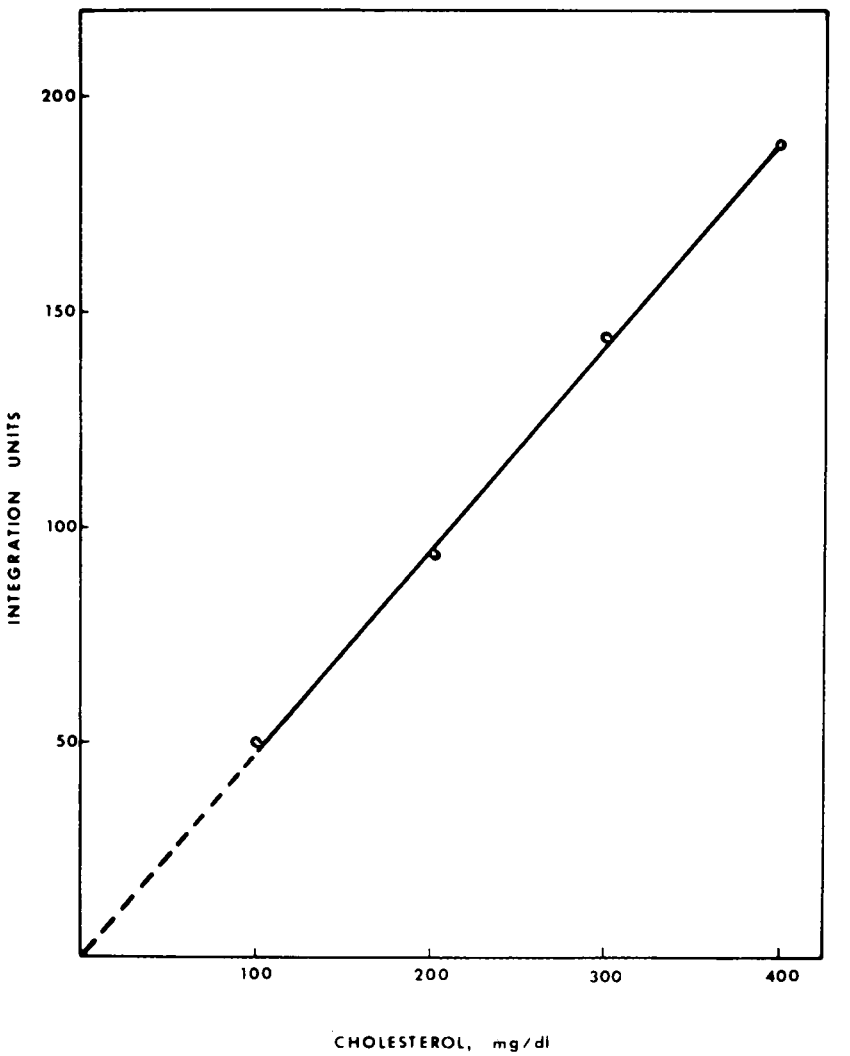

Fig. 1. Calibration curve constructed from mean values of 10 replicate densitometric measurements in reference solutions with 1—4 mg/ml cholesterol concentration. Scanner, Quick Scan; slit width, 5 mm.

Octane extraction of this mixture achieves the separation of free and esterified cholesterol from the other components, since only the cholesterol compounds are soluble in the apolar solvent. Octane separates rapidly from the aqueous isopropanolic phase, which contains all the other compounds present after the hydrolysis.

The effect of the alkaline treatment and of the octane extraction can be

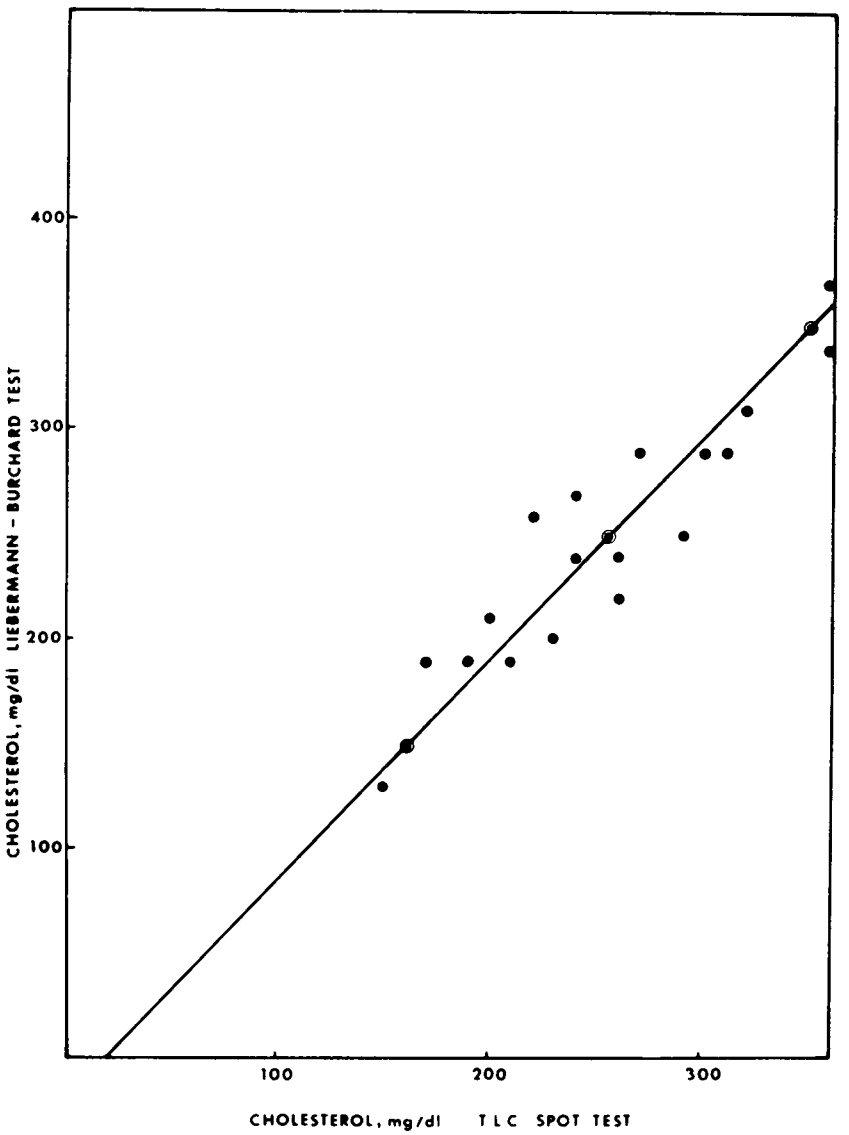

Fig. 2. Comparison of total serum cholesterol values obtained by the TLC-spot method and the Liebermann—Burchard test.

followed by TLC. Fig. 3 shows the composition of a conventional isopropanolic extract. Starting from the round shaped spot reading upwards, the chromatogram shows the five main lipid classes of serum; phospholipids, free cholesterol, free fatty acids, triglycerides, and cholesterol esters.

This chromatogram has been prepared from the extract of a pooled serum. Five hundred μl of isopropanol and 25 μl of serum were mixed on a Vortex mixer for 5 sec and the precipitated protein was separated by centrifugation. A 10-μl aliquot of the supernatant was applied to the TLC plate.

Two solvent mixtures were used for the separation. The development in the first mixture moved all the lipids except the phospholipids to the solvent front, concentrating them into a thin line 1 cm above the starting point. This

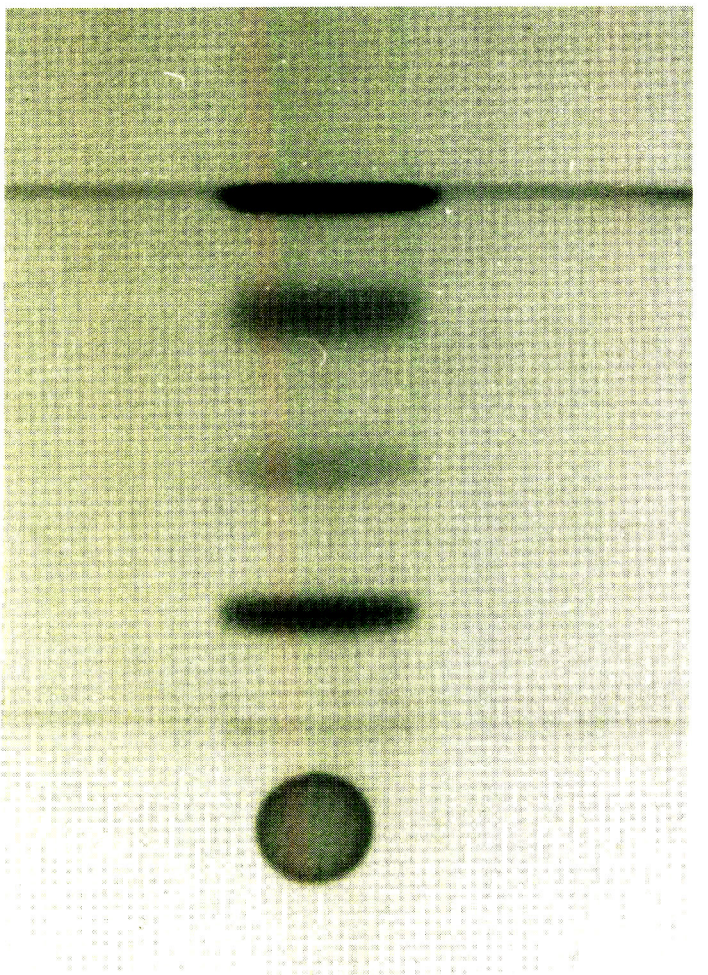

Fig. 3. Chromatogram of the isopropanolic extract of a pooled serum.

separation took 1 min. This made it possible to perform a fast and easy separation with the second solvent, for a 2-cm distance in 4 min. After it was removed from the first solvent, the plate was air dried and returned to the second solvent. This separated the four other lipids into distinct bands. The first solvent system contained methanol—chloroform—glacial acetic acid—water (50:50:5:5). The second solvent system consisted of light petroleum (b.p. 20—40°)—diethyl ether—glacial acetic acid (80:20:1).

Fig. 4 shows the effect of the octane extraction when there is no alkali present in the isopropanol. Twenty-five μl of the serum were added to 500 μl of isopropanol and the mixture was agitated for 15 sec. Two hundred μl octane were added and the contents were mixed on a Vortex again for 30 sec.

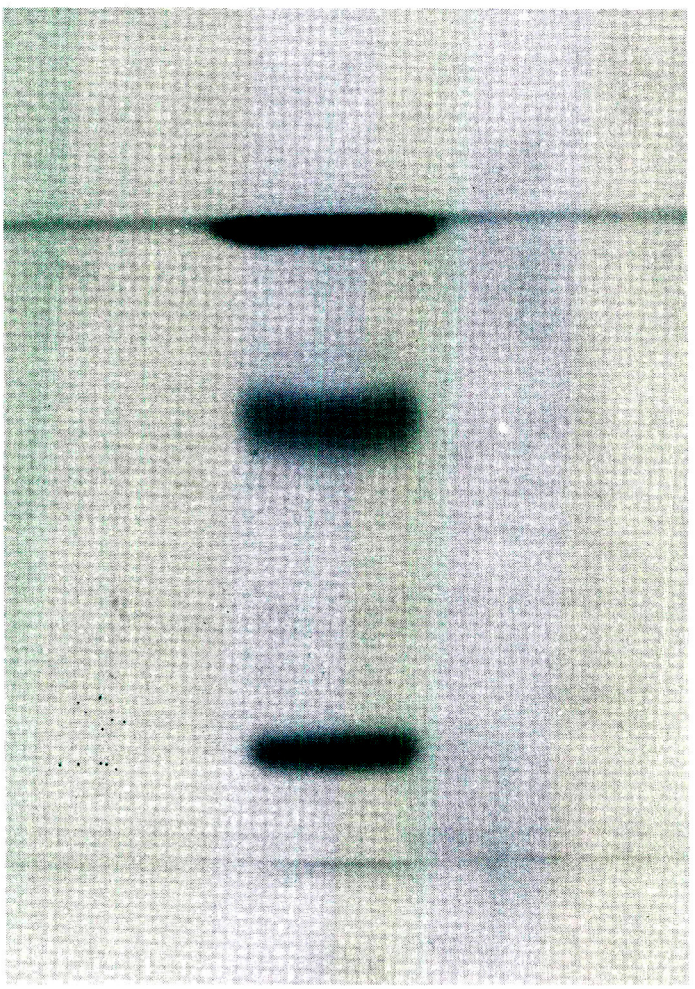

Fig. 4. Chromatogram of the octane extract of the isopropanolic solution of serum lipids.

After the two phases separated (2 min), 10 μl of the upper octane phase were applied to a TLC plate. The same two-step separation was carried out on the plate with the first and then with the second solvent system as before. The chromatogram shows that, although the octane extraction eliminates both the phospholipids and the free fatty acids (the two polar lipid species) in the absence of alkali, triglycerides are still present in the octane in addition to the cholesterols.

Fig. 5 shows the effect of alkali in the elimination of triglycerides. The alkaline isopropanolic extracting solvent hydrolyzed the triglycerides in 2 min and split them into free fatty acids and glycerol. Both the neutralized fatty

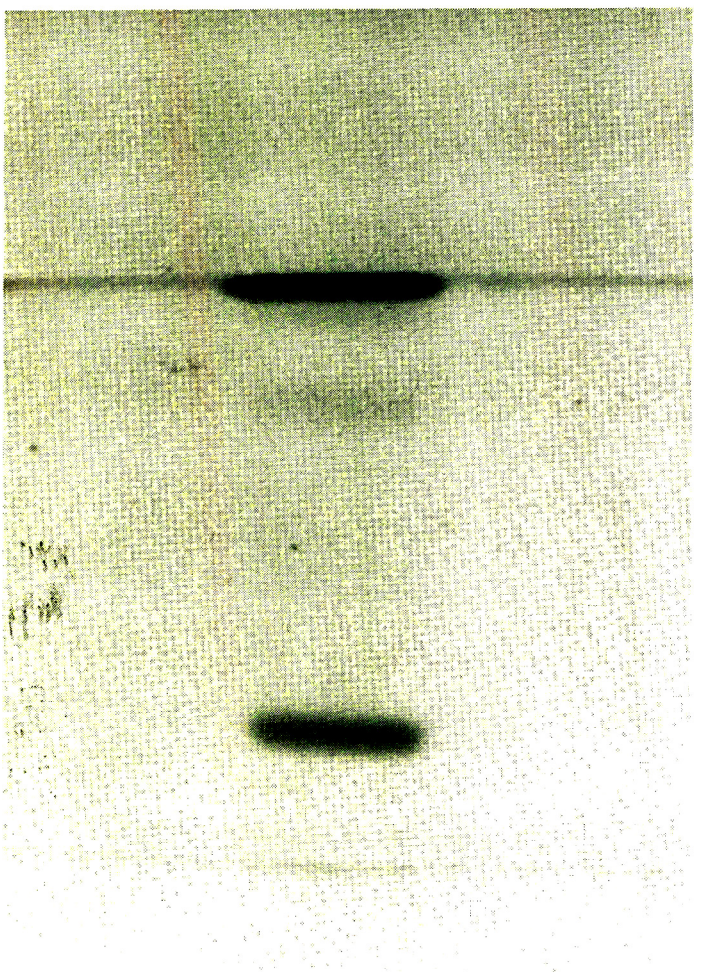

Fig. 5. Chromatogram of the octane extract of the alkaline isopropanolic solution of serum lipids.

acids and the glycerol remained in the isopropanolic phase after the octane extraction, as indicated in the chromatogram, which now shows the bands of free and esterified cholesterol and a very faint band representing an insignificant amount of unhydrolyzed triglyceride.

The advantage of the method is that it eliminates the need for separation of the lipids on the plate and the spots are much more uniformly stained. Therefore, the densitometric readings are much more reproducible and the results more reliable. The determination is rapid. It takes less than 10 min, including the evaluation and can be carried out with common laboratory equipment. It is relatively inexpensive and suitable for handling a large number of specimens, and thus can be used for screening purposes. If a semiquantitative evaluation is sufficient, the intensity of the blue color can be compared with the color intensity of a reference spot (e.g. an extract of a 2.5 mg/ml cholesterol solution) to determine whether the unknown spot has a higher or lower concentration than the reference spot.

REFERENCES

1 G.C. Buckley, J.A. Little, M.A. Antar and A. Csima, Clin. Chim. Acta, 23 (1969) 53.
2 R.J. Nicolosi, S.C. Smith and R.F. Santerre, J. Chromatogr., 60 (1971) 111.
3 S.A. Feldman and E. Fosslien, J. Chromatogr., 63 (1971) 63.
4 B. Wiklund and R. Eliasson, J. Chromatogr., 68 (1972) 153.
5 D. Kritchevsky, L.M. Davidson, H.K. Kim and S. Malhotra, Clin. Chim. Acta, 46 (1973) 63.
6 W. Mlekusch, W. Truppe and P. Paletta, Clin. Chim. Acta, 49 (1973) 73.
7 T. Castellani, P. Kjelgaard-Nielsen and J. Wolff-Jensen, J. Chromatogr., 104 (1975) 123.
8 J.S. Amenta, Clin. Chem., 16 (1970) 339.
9 M.J. Manners, D.E. Kidder and P.P. Parsons, J. Chromatogr., 43 (1969) 276.
10 T. Kremmer, E. Ferenczy and E. Posch, Chromatographia, 2 (1969) 142.

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Biomedical Applications

CHROMBIO. 016

MASS FRAGMENTOGRAPHIC QUANTITATION OF ETHOTOIN AND SOME OF ITS METABOLITES IN HUMAN URINE

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SUMMARY

A method for the determination of ethotoin and its *p*-hydroxylated and dealkylated metabolites in urine has been developed. Ethotoin and the metabolites were extracted from acidified urine with ethyl acetate and silylated before injection into a combined gas chromatograph—mass spectrometer. Four partly identified metabolites were recorded, but their exact quantitation was not possible as pure reference substances were not available.

The limit of sensitivity was far below the amounts of ethotoin and of its metabolites found in urine from patients treated with therapeutic doses of ethotoin.

INTRODUCTION

Recently, the antiepileptic drug ethotoin (Peganone) was shown by our group to exhibit dose-dependent kinetics [1]. The purpose of the present investigation was to develop a method for the quantitative determination of ethotoin and its metabolites excreted in urine in order to clarify the mechanism behind the dose-dependent kinetics of ethotoin.

EXPERIMENTAL

Samples

Urine samples were collected from patients undergoing continuous treatment with ethotoin in therapeutic doses. Blank urines were collected from the same patients before ethotoin treatment was started.

Reagents and solvents

Ethyl acetate and toluene from E. Merck (Darmstadt, G.F.R.) were of analytical-reagent grade. N,O-Bis(trimethylsilyl)acetamide (BTSA) from Pierce

(Rockford, Ill., U.S.A.) was of specially purified grade. Glucuronidase/arylsulphatase was obtained from Boehringer (Mannheim, G.F.R.).

Reference substances

The structures of ethotoin and its metabolites are shown in Fig. 1. Ethotoin and its dealkylated metabolite (5-phenylhydantoin) were donated by Abbott Labs. (North Chicago, Ill., U.S.A.). The two internal standards, mephenytoin and heptamal, were donated by Sandoz (Basle, Switzerland) and Ciba-Geigy (Basle, Switzerland), respectively. The *p*-hydroxylated metabolite was isolated from human urine and purified. Metabolites III, IV, V and VI were extracted from urine and purified, but were not isolated in a pure state.

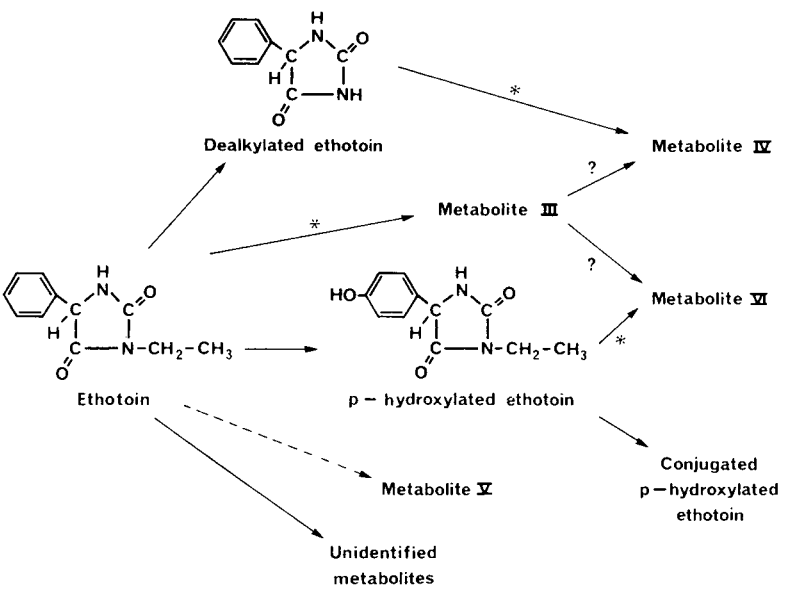

Fig. 1. Metabolic pathways of ethotoin in man.

Isolation and purification. Metabolites III, IV, V and VI were extracted from urine samples with ethyl acetate and cleaned by column chromatography on silica gel with ethyl acetate—*n*-hexane (1:1).

The urine remaining after this extraction was hydrolysed with glucuronidase/arylsulphatase. The *p*-hydroxylated metabolite released was extracted with ethyl acetate and cleaned by column chromatography on silica gel with ethyl acetate—*n*-hexane (1:1), followed by two recrystallizations from ethyl acetate—*n*-hexane (1:1). The melting point of the compound and its UV spectrum in ethanol, as well as the acid properties and the mass spectra of the compound and some of its derivatives, were determined.

Identification. The identity of the isolated *p*-hydroxylated ethotoin was confirmed by the following analytical data. The main peaks in the mass spectrum were 16 *m/e* higher than the corresponding peaks in the mass spectrum of ethotoin. The compound could be silylated at two positions and acetylated at one. The unchanged compound was a weak acid, but its acetylated derivative was neutral. The UV spectrum and the melting point were in good agreement with those of synthetic 3-ethyl-5-(*p*-hydroxyphenyl)hydantoin [2]. No definite proof was obtained that the hydroxylation takes place in the *para*-position, but *p*-hydroxylation has been shown to be a major metabolic pathway in man for phenytoin [3,4] and other 5-phenylhydantoin derivatives [5], the *p*-hydroxylated metabolite was found in urine from dogs treated with ethotoin [2] and *m*-hydroxylation of phenytoin occurs to a slight extent [6].

The exact structural formulae of metabolites III, IV and VI have not yet been proved. The compounds could be formed in small amounts in vitro from ethotoin, dealkylated ethotoin and *p*-hydroxylated ethotoin, respectively, in alkaline solution. The mass spectra of the unchanged compounds (III, IV and VI) and of their silylates may point to an oxidation in position 5 in the hydantoin ring system [2].

The mass spectra of metabolite V and of its silylated derivative could be explained by the assumption that the metabolite had arisen from ethotoin in which the phenyl group had been converted into the 3,4-dihydroxy-1,5-cyclohexadien-1-yl group. This metabolic pathway has been shown to be open for phenytoin [7]. Furthermore, this would be in agreement with the fact that the metabolite had no acidic properties.

No compound having two phenolic hydroxyl groups, as shown for phenytoin [8] was observed.

Mass spectrometry. A combined gas chromatograph—mass spectrometer (LKB 9000) was used. The conditions used for the mass spectra were as follows: ionization energy, 70 eV; trap current, 60 μA; accelerating voltage, 3500 V. Tables I and II give the mass spectra of ethotoin, its metabolites and the internal standard as unchanged and as silylated compounds, respectively.

Quantitation

Extraction and derivatization of non-conjugated metabolites. To a 50-μl urine sample in a centrifuge tube 1 ml of 0.1 *N* acetic acid and 100 μl of mephenytoin solution (0.1 g/l in ethanol) as internal standard were added. The sample was mixed with 3 ml of ethyl acetate for 5 min. After centrifugation, the organic phase was transferred into a tapered tube and evaporated to dryness in a stream of nitrogen. The residue was derivatized with 50 μl of BTSA for 15 min and diluted with 100 μl of toluene. A volume of 1 μl was injected into the combined gas chromatograph—mass spectrometer.

Extraction and derivatization of total p-hydroxylated metabolites. To a 50 μl urine sample in a centrifuge tube, 200 μl 0.1 *N* acetic acid were added. After the addition of 5 μl of glucuronidase/arylsulphatase, the tube was left for 18 h at 37°. The subsequent procedure was as described for the non-conjugated compounds.

If only the *p*-hydroxylated metabolite was to be measured, gas chromatography with a flame-ionization detector could be used. The procedure was then

TABLE I

MASS SPECTRA OF ETHOTOIN (ETO), DEALKYLATED ETHOTOIN (DA-ETO), *p*-HYDROXYLATED ETHOTOIN (*p*-H-ETO) AND METABOLITES III, IV AND V

Fragments below 100 *m/e* and/or with intensities below 10% have been omitted. The results given are relative intensities.

m/e	ETO (mol.wt. 204)	DA-ETO (mol.wt. 176)	*p*-H-ETO (mol.wt. 220)	Metabolite III	Metabolite IV	Metabolite V
103				100	100	89
104	100	100		37	35	100
105	96	62				87
119						32
120			86			
121			100			99
132	10					
133	20	18				24
135						41
148			25			
149						29
174					13	
176		40				
191						26
192						30
202				22		25
204	56					59
220			59			
238						19

the same as described above, except that heptamal was used as the internal standard and after the extraction the residue was dissolved in 50 μl of ethyl acetate. Derivatization with BTSA was omitted. A volume of 3 μl was injected into the gas chromatograph.

Mass fragmentography and gas chromatography. The conditions for mass fragmentography were as follows: ionization energy, 20 eV; trap current, 60 μA; column, glass, 0.6 m × 2 mm I.D.; 3% OV-1 on Celite J.J. CQ, 100—120 mesh; pre-heater temperature 250°, column oven 180°, separator 270°, ion source 290°; helium flow-rate, 15 ml/min. The mass fragments used for analysis are given in Table II.

The gas chromatographic measurements were performed on a Pye Series 104 gas chromatograph equipped with a flame-ionization detector. The conditions were as follows: column, glass, 0.9 m × 4 mm I.D.; 1% OV-17 on Celite J.J. CQ, 100—120 mesh; pre-heater temperature 250°, detector oven 350°, column oven 230°; carrier gas, nitrogen at a flow-rate of 60 ml/min.

Samples for standard curves. Known amounts of ethotoin and its metabolites were added in increasing amounts to urine blanks.

For dealkylated ethotoin, for metabolite IV and for total *p*-hydroxylated ethotoin, standard curves from 100 to 1000 mg/l were used. For ethotoin, for non-conjugated *p*-hydroxylated ethotoin and for metabolites III, V and VI, standard curves from 5 to 50 mg/l were used. If higher concentrations were present, the urine samples were diluted with water.

Relative standard solutions of metabolites III, IV, V and VI were prepared,

because complete isolation of the compounds was not feasible. The concentrations were estimated by comparison of the areas under the peaks in the gas chromatograms of the metabolites with the area under the peak in a gas chromatogram of the dealkylated metabolite of ethotoin (5-phenylhydantoin). A flame-ionization detector was used.

RESULTS AND DISCUSSION

Extraction and formation of derivatives

Ethotoin itself could be extracted quantitatively with dichloromethane [9]. However, it was necessary to use a more polar extraction medium such as ethyl acetate, as some of the ethotoin metabolites are rather polar. The fact that several of the metabolites are weak acids could not be utilized in a cleaning procedure as ethotoin and most of its metabolites are unstable in alkaline solution.

For most of the compounds, the extraction recovery was approximately 100% (only 75% for metabolite V).

Silylation of ethotoin, of the internal standard, of *p*-hydroxylated ethotoin and of metabolite III proceeded readily at room temperature, but the dealkylated ethotoin and metabolites IV and V reacted more slowly, especially in low concentrations. This effect might be due to adsorption of the non-silylated compounds on the glass walls, but after 20 min the derivatizations were found to be almost quantitative.

Hydrolysis of conjugated metabolites

The only conjugated metabolite found in noticeable amounts was *p*-hydroxylated ethotoin. Enzymatic hydrolysis of conjugated *p*-hydroxylated ethotoin under the conditions described was found to be quantitative (or at least maximal) after 12—15 h. Acid hydrolysis with concentrated hydrochloric acid was not possible as it gave rise to some decomposition of the *p*-hydroxylated ethotoin released.

Urine analysis

Typical mass fragmentograms for the determination of ethotoin, dealkylated metabolite, metabolite IV and non-conjugated *p*-hydroxylated ethotoin, of standards and of urine extracts from blank urine and from urine from a patient treated with ethotoin are shown in Fig. 2. Metabolites III, IV, V and VI could not be measured exactly as no reference with known concentrations was available. The concentrations were estimated from the areas of the peaks in the gas chromatograms as described above.

OV-1 or OV-101 could be used as the column material. OV-17 yielded no separation of the silyl derivatives of ethotoin, of dealkylated ethotoin and of metabolite IV. Although they were measured at different channels, slight interference from the latter was observed at the channels measuring ethotoin and dealkylated ethotoin.

Concerning the conjugated metabolites, only *p*-hydroxylated ethotoin was found in noticeable amounts (a trace amount of conjugated metabolite V was observed). It was measured as total minus free compound (the free compound

TABLE II

MASS SPECTRA OF SILYLATED COMPOUNDS OF ETHOTOIN (ETO), DEALKYLATED ETHOTOIN (DA-ETO), *p*-HYDROXYLATED ETHOTOIN (*p*-H-ETO), METABOLITES III, IV, V AND VI AND MEPHENYTOIN (MPT)

Fragments below 100 *m/e* (except 73) and/or with intensities below 10% (except molar fragments or fragments used for mass fragmentography) have been omitted. The results given are relative intensities (I).

ETO		DA-ETO		*p*-H-ETO		Metabolite III		Metabolite IV		Metabolite V		Metabolite VI		MPT	
m/e	*I*	*m/e*	*I*	*m/e*	*I*	*m/e*	*I*	*m/e*	*I*	*m/e*	*I*	*m/e*	*I*	*m/e*	*I*
73	50	73	98	73	100	73	100	73	100	73	100	73	100	135	29
135	10	162	100	223	22	105	33	105	21	191	15	147	21	176	13
162	100	163	15	250	31	147	47	147	20	292	4*	193	10	190	15
163	16	177	69	264	16*	162	14	264	13*	454	0.4	322	8*	261	100*
176	15	178	12	265	10	234	42	265	11			452	2	262	22
177	23	188	23	349	15	264	12	408	0.3					263	60
261	51*	190	15	364	55	287	28*							275	15
262	11	205	46			335	36							290	2
276	23	305	16*			336	11								
		320	3			349	12								
						364	5								

*Mass fragments used for mass fragmentographic measurements.

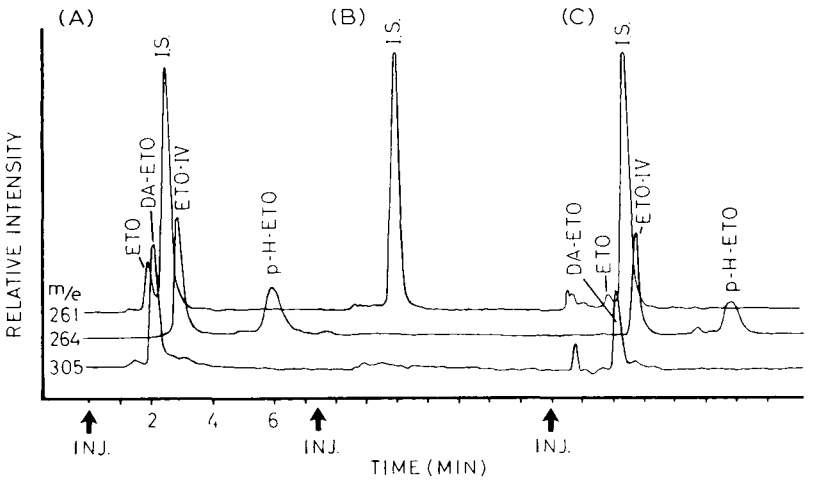

Fig. 2. Mass fragmentograms of urine extracts. (A), blank urine with added standards: ethotoin (ETO), non-conjugated *p*-hydroxylated ethotoin (*p*-H-ETO), dealkylated ethotoin (DA-ETO), metabolite IV (ETO-IV) and mephenytoin (internal standard, I.S.). (B), blank urine with added internal standard. (C), urine from patient treated with 2 g of ethotoin; the concentrations found 3.5, 5.5, 280 and 450 mg/l were for ethotoin, non-conjugated *p*-hydroxylated ethotoin, dealkylated ethotoin and metabolite IV, respectively.

being about 2% of the total). As no interfering compounds were found with the same retention time as *p*-hydroxylated ethotoin, it could be measured as accurately with a gas chromatograph equipped with a flame-ionization detector (see below).

Specificity

Interfering material in urine extracts from patients not treated with ethotoin was never observed.

Mass spectra of reference materials and mass spectra of peaks in gas chromatograms from urine extracts were in agreement (identical after subtraction of the background).

The specificity of the gas chromatographic method for the determination of total *p*-hydroxylated ethotoin was checked analyzing 20 urine samples by both gas chromatography and mass fragmentography [concentration range 300—1600 mg/l (1—5 mmole/l); coefficient of correlation 0.975; the slope was not significantly different from unity, and no sample concentration differed by more than 8% as determined by the two methods].

Accuracy

Urine from a patient treated with 2125 mg of ethotoin per day was analyzed 20 times by mass fragmentography and by gas chromatography. The standard deviation found by mass fragmentography was 4—5% for non-conjugated metabolites and ethotoin itself and 6% for total *p*-hydroxylated ethotoin (both by mass fragmentography and by gas chromatography).

Recovery

Recoveries of ethotoin and its non-conjugated metabolites through the whole procedure were not investigated intensively as they varied in parallel with the recovery of the internal standard (range 75—90%), and no effort was made to increase the recoveries. The recovery of the conjugated *p*-hydroxylated metabolite could not be determined exactly as no reference material was available, but the same amounts were found whether the urine samples were hydrolyzed for 15, 18 or 24 h under the conditions described.

Sensitivity

Less than 1 ng (5 nmole), corresponding to 5 mg/l (25 μmole/l), in 50 μl of urine could easily be measured quantitatively by mass fragmentography for ethotoin and all of the metabolites mentioned. The gas chromatographic method was reliable down to 50 mg/l (lower concentrations, in some urine samples after single doses, were measured by mass fragmentography).

Quantitative determination

Standard curves were prepared from gas chromatograms or mass fragmentograms of urine extracts to which known amounts of ethotoin and its metabolites had been added. The ratio of the peak height of the compound determined to that of the corresponding internal standard was plotted against the concentration. A linear relationship was obtained for all of the compounds (see Fig. 3).

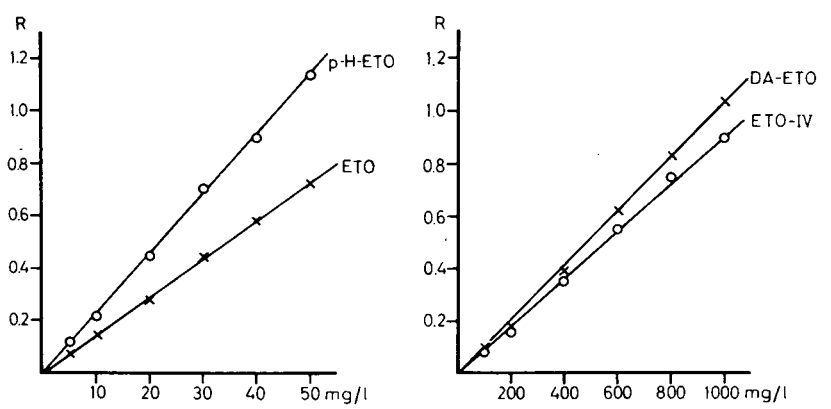

Fig. 3. Standard curves for ethotoin (ETO), non-conjugated *p*-hydroxylated ethotoin (*p*-H-ETO), dealkylated ethotoin (DA-ETO) and metabolite IV (ETO-IV). R = ratio between the peak heights of the compounds analysed and that of the internal standard.

The concentration range found after application of the dose range 1.75—4.25 g of ethotoin per day is shown in Table III.

The details and their significance for dose-dependent kinetics of ethotoin will be published elsewhere [10].

TABLE III

EXCRETION RANGE OF ETHOTOIN AND ITS METABOLITES IN URINE FROM FIVE PATIENTS UNDERGOING CONTINUOUS TREATMENT WITH ETHOTOIN (2—4 g PER DAY)

Compound	Concentration range (mg/l)	Excreted amounts (% of applied dose)
Ethotoin	3— 38	0.2— 1.9
Total *p*-hydroxylated ethotoin	300—1600	14 —32
Non-conjugated *p*-hydroxylated ethotoin	8— 30	0.4— 0.8
Dealkylated ethotoin	90—1445	5.4—14.3
Metabolite IV	300—1200	17 —34
Metabolites III + V + VI		0.8— 2.2
Total		44 —76

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REFERENCES

1 O. Sjö, E.F. Hvidberg, N.-E. Larsen, M. Lund and J. Næstoft, Clin. Exp. Pharmacol. Physiol., 2 (1975) 185.
2 K.H. Dudley, D.L. Bius and T.C. Butler, J. Pharmacol. Exp. Ther., 175 (1970) 27.
3 T.C. Butler, J. Pharmacol. Exp. Ther., 119 (1957) 1.
4 E.W. Maynert, J. Pharmacol. Exp. Ther., 130 (1960) 275.
5 T.C. Butler, J. Pharmacol. Exp. Ther., 117 (1956) 160.
6 A.J. Atkinson, Jr., J. MacGee, J. Strong, D. Garteiz and T.E. Gaffney, Biochem. Pharmacol., 19 (1970) 2483.
7 T. Chang, A. Savory and A. Glazko, Biochem. Biophys. Res. Commun., 38 (1970) 444.
8 O. Borgå, M. Garle and M. Gutova, Pharmacology, 7 (1972) 129.
9 N.-E. Larsen and J. Næstoft, J. Chromatogr., 92 (1974) 157.
10 J. Næstoft, E.F. Hvidberg and O. Sjö, Clin. Exp. Pharmacol. Physiol., 3 (1976) 453.

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Biomedical Applications

CHROMBIO. 013

MICRO-DETERMINATION OF PLASMA DIPHENYLHYDANTOIN BY GAS—LIQUID CHROMATOGRAPHY

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SUMMARY

A selective, sensitive and precise gas—liquid chromatographic method for the determination of diphenylhydantoin in micro samples of blood plasma is described. After a double extraction with chloroform containing an analogue of diphenylhydantoin as an internal standard, the drug and standard are N,N-dimethylated in alkaline aqueous solution with methyl iodide followed by extraction into acetone. The methylated derivatives are separated gas chromatographically and measured using a flame-ionization detector. The lowest concentration of diphenylhydantoin in plasma which can be measured in a 100-μl sample is 1 μg/ml, which is well below the normal therapeutic concentration of 10—20 μg/ml in plasma. The methylated derivatives of diphenylhydantoin and the internal standard have been identified by their proton magnetic resonance spectra and mass spectra.

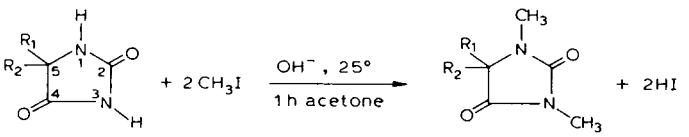
INTRODUCTION

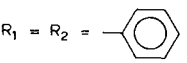
Diphenylhydantoin (5,5-diphenylhydantoin, phenytoin, DPH) is used in the therapy of epilepsy and it has been shown that knowledge of the blood level of this drug is helpful in the control of seizures in patients. Various methods for the determination of DPH and 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (HPPH), the principal metabolite of DPH in man, as well as of other anticonvulsant drugs in biological material, have been reported [1—35]. Analyses using colorimetry, ultraviolet spectrophotometry and fluorimetry [1—4], thin-layer chromatography [1, 5], radioimmunoassay [6, 7], spin immunoassay [8] and polarography [9] have been carried out.

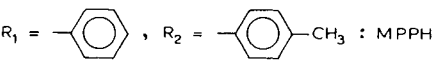
Gas—liquid chromatographic (GLC) determinations [10—35] have the advantage of being sufficiently sensitive and specific, so that several anticonvulsants can be determined simultaneously. In some of these procedures, the unchanged drugs are chromatographed [10—20], whereas in others deriv-

atives are prepared prior to GLC. Some workers [21] prepare trimethylsilyl (TMS) derivatives, but the most common approach appears to be the conversion of the antiepileptics into methylated products with diazomethane [22, 23], dimethyl sulphate [24,25] or the flash-methylating reagents tetramethylammonium hydroxide [26—29] and trimethylanilinium hydroxide [19, 30—35].

Recently, heptabarbital and cyclobarbital have been converted into their dimethyl derivatives by alkaline extraction of biological fluid with methyl iodide in acetone at room temperature [36]. In our work, an adaptation of this methylation procedure is used for the GLC determination of DPH in plasma. With known amounts of 5-(*p*-methylphenyl)-5-phenylhydantoin (MPPH) as an internal standard in the extraction solvent, there is no need for accurate aliquot measurements during extraction, derivative formation and chromatography. After a double extraction of 100 μl of plasma sample containing the drug, DPH and MPPH are dissolved in aqueous alkaline solution, methylated with methyl iodide in acetone and extracted into acetone.







These compounds are gas chromatographed and identified as the N,N-dimethylated derivatives of DPH and MPPH, 1,3-dimethyl-5,5-diphenylhydantoin (DPH-Me_2) and 1,3-dimethyl-5-(*p*-methylphenyl)-5-phenylhydantoin (MPPH-Me_2), respectively.

OPTIMIZATION OF THE PROCEDURE

Extraction

DPH and MPPH are best extracted from plasma and aqueous buffer solutions into chloroform at pH 5—7 (for DPH pK_a' = 8.33 [37]). A peak of an unidentified physiological component with a retention time of approximately 12 min under the GLC conditions outlined below disappears when the plasma is extracted with chloroform (pH 6.8), the organic extract re-extracted into aqueous solution (pH 13) and the aqueous extract back-extracted into chloroform (pH 7.2) (Fig. 1).

Derivative formation

The methylation reaction of DPH and MPPH in a mixture of water (buffer) and methyl iodide in acetone followed by extraction of the methylated derivatives into the organic phase is pH-dependent. By shaking 20 μg of DPH with 0.5 ml of aqueous buffer solution of variable pH and 1 ml of a 4% (v/v)

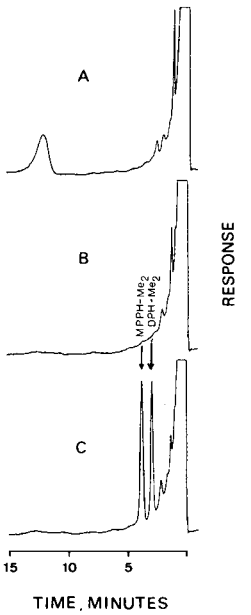


Fig. 1. Gas chromatograms of 100-μl plasma extracts subjected to the procedure described. A, Drug-free plasma, single extraction; B, drug-free plasma, double extraction; C, peaks of DPH-Me_2 and MPPH-Me_2 from plasma containing 15 μg/ml of DPH (2 μg of MPPH per sample). GLC conditions: 3% OV-225 on Chromosorb W HP; nitrogen flow-rate, 35 ml/min; column temperature, 234°; a 3-μl aliquot of 200 μl of chloroform extract was injected.

solution of methyl iodide in acetone at room temperature for 1 h followed by GLC analysis of the evaporated organic phase at pH > 13, only one methylated derivative is produced. At pH 11.5, two reaction products are observed, whereas at pH < 11 DPH is not methylated. A single chromatographic peak appears after methylation of DPH and MPPH with buffer of pH 13 (0.47 *M*). These reaction products have been identified as the N,N-dimethylated derivatives of DPH and MPPH (DPH-Me_2 and MPPH-Me_2, respectively). The rate of formation of DPH-Me_2 and MPPH-Me_2 is not increased by shaking the reaction mixture in a water bath at elevated temperature (22—70°) and is almost completed within 30 min at room temperature. Optimal reaction yields are obtained with a reaction time between 1 and 2 h. The ratio of aqueous buffer solution to acetone in the reaction mixture does not seem to be critical. For the methylation of micro amounts (0—3 μg of DPH and 2 μg of MPPH) there is no change in reaction yield and reproducibility when using 50—300 μl of buffer of pH 13 (0.47 *M*) and 0.6—2 ml of a 4% solution of methyl iodide in acetone (10^5—10^6-fold in excess).

Recovery

GLC response curves which correlate peak area with the amount of DPH-Me_2 and MPPH-Me_2 per sample offer the possibility of determining total yields (double extraction and derivative formation) for DPH and MPPH in our procedure. For 2 μg of DPH in 100 μl of plasma and 2 μg of MPPH extracted,

methylated and chromatographed under standard optimal conditions, the total recoveries are 64% (coefficient of variation, CV = 3.5%) for DPH and 68% (CV = 4.5%) for MPPH (nine determinations).

Stability

A freshly prepared plasma standard solution of 20 μg/ml of DPH was compared with a standard solution of the same drug level, kept at −18° for 2 months and with a similar standard solution produced by diluting an aqueous alkaline (0.1 *N* NaOH) solution of DPH, which had been refrigerated for 2 months, with fresh plasma. The variations in the GLC peak area ratios were insignificant.

Solutions of DPH-Me_2 and MPPH-Me_2 in chloroform awaiting GLC were examined by repeated injection of aliquots of the same extract. It was found that these extracts, refrigerated when not in use, are stable for several days.

Interference from other drugs and the metabolite HPPH

So far, no interference has been observed from other antiepileptic drugs, including phenobarbital and primidone, by analyzing plasma samples of patients receiving anticonvulsant therapy. A mixture of plasma from 50 patients receiving a large number of commonly prescribed drugs has been examined by our procedure, and no drug has been found to interfere.

In addition, micro amounts of barbital, allobarbital, allylisobutylbarbital, cyclobarbital, heptabarbital, phenobarbital and HPPH together with DPH and MPPH were methylated with methyl iodide. The methylated derivatives of the compounds investigated did not interfere in the DPH assay and were separated chromatographically from each other by using a temperature programme. Therefore, a simultaneous quantitative determination of these drugs and the metabolite HPPH should be possible.

Extraction from urine

The method for the determination of DPH in plasma was applied to urine samples. Volumes of 100 μl of drug-free urine and 100 μl of urine containing 2 μg of DPH (2 μg of MPPH per sample in the extraction solvent) were extracted, methylated and chromatographed. There was no interfering peak in the blank and the peak area ratio was approximately unity.

MATERIALS AND METHODS

Human blood and plasma

Fresh human blood, mixed with CPD anticoagulant (Fenval Division, Travenol Labs., Brussels, Belgium) is centrifuged for 20 min at 3000 rpm(1250 *g*). Plasma and plasma standard solutions of DPH are stored at −18°.

Reagents

DPH and MPPH were obtained from Parke, Davis & Co., Detroit, Mich., U.S.A.; and chloroform, acetone and methyl iodide from Merck, Darmstadt, G.F.R. All of the solvents and reagents used were of analytical grade and were specially tested for purity by carrying out blank runs.

Buffer of pH 7 contained 35.22 g of KH_2PO_4 (0.26 *M*) and 72.65 g of $Na_2HPO_4 \cdot 2H_2O$ (0.41 *M*) in 1000 ml of distilled water (Titrisol, Merck).

Buffer of pH 13 (0.47 *M*) contained 37.28 g of KCl (0.50 *M*) and 18.84 g of NaOH (0.47 *M*) in 1000 ml of distilled water (Titrisol, Merck).

Buffer of pH 13 (0.047 *M*) was obtained by diluting buffer of pH 13 (0.47 *M*) 1:10 with distilled water.

0.1 *N* NaOH solution (Titrisol, Merck) was used.

OV-225, 3% on Chromosorb W HP, 100—120 mesh, was obtained from Supelco, Bellefonte, Pa., U.S.A.

Plasma standard solutions of DPH

Plasma standards containing 1—30 μg/ml of DPH are prepared by adding 0.25 ml of a solution of DPH containing 40—1200 μg/ml in 0.1 *N* NaOH solution to drug-free plasma to a total volume of 10 ml.

Extraction procedure and derivative formation

To 100 μl of plasma standard solution of DPH are added 100 μl of buffer of pH 7 and 2 ml of chloroform containing 2 μg of the internal standard (MPPH). The glass-stoppered tube (100 × 18 mm) is shaken for 10 min on a mechanical shaker at 200 rpm and centrifuged for 5 min at 4500 rpm (2700 *g*). The aqueous phase is removed by aspiration and discarded. The organic phase is transferred into a similar glass tube and shaken with 1 ml of buffer of pH 13 (0.047 *M*) for 10 min at 200 rpm. After centrifugation for 5 min at 4500 rpm, the aqueous phase is transferred into a third glass tube and the organic layer is discarded. The aqueous extract is neutralized by the addition of 600 μl of buffer of pH 7 and shaken for 10 min at 200 rpm with 4 ml of chloroform. The mixture is centrifuged for 5 min at 4500 rpm, the aqueous phase removed by aspiration and the organic extract transferred into a glass-stoppered conical centrifuge tube (100 × 9—22 mm) and evaporated to dryness under a stream of dry nitrogen at room temperature. To the dry residue are added 100 μl of buffer of pH 13 (0.47 *M*) and 600 μl of a 4% (v/v) solution of methyl iodide in acetone. The sample in the stoppered tube is mixed on a Vortex mixer for a few seconds and then shaken mechanically for 1 h at 200 rpm and room temperature. The supernatant organic layer is transferred into a similar centrifuge tube using a capillary Pasteur pipette and evaporated to dryness under a stream of dry nitrogen at room temperature. Chloroform (200 μl) is added to the dry residue and, after mixing for a few seconds, a 3-μl aliquot is injected "on-column" into the GLC unit.

Gas–liquid chromatography

A Pye Unicam GCV gas chromatograph with flame-ionization detector (FID), an Infotronics Model CRS 204 integrator and a W + W Model 1100 recorder were used. The column was a 5 ft. × 2 mm I.D. glass column packed with 3% OV-225 (phenylcyanopropylmethylsilicone) on Chromosorb W HP, 100—120 mesh, conditioned for 24 h at 245° with nitrogen at a flow-rate of 20 ml/min. The following flow-rates were used in the GLC analysis: nitrogen (carrier gas), 35 ml/min; hydrogen, 30 ml/min; air, 330 ml/min. Temperatures

were 234° in the column, 200° in the injector and 300° in the detector. Under these conditions, the retention times of N,N-dimethylated DPH and MPPH were approximately 3 and 4 min, respectively (Fig. 1C).

Quantitation

A standard curve for DPH in plasma was prepared by analyzing plasma standard solutions according to the procedure described above. Ratios of the area of the drug peak (DPH-Me_2) to that of the internal standard peak (MPPH-Me_2) were plotted against concentrations of DPH (Fig. 2). Peak areas were measured automatically with an Infotronics Model 204 integrator, which corrects for baseline variations. The standard curve was linear for DPH plasma concentrations in the range 1—30 μl/ml (2 μg of MPPH per sample) and therefore allows the use of peak-area ratios for the analysis of unknown samples.

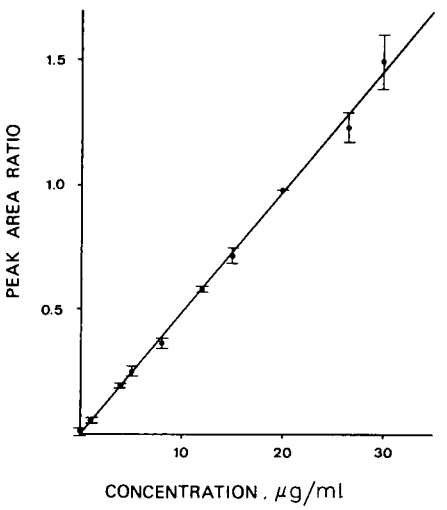


Fig. 2. Standard curve for DPH in plasma (2 μg of MPPH per sample): peak-area ratio of DPH-Me_2 to MPPH-Me_2 as a function of DPH plasma concentration. Points and vertical bars represent the mean ± standard deviation of three separate determinations at each concentration. The straight line was calculated by the method of least squares (correlation coefficient = 0.9988).

RESULTS

Identification of the derivatives

Milligram amounts of DPH and MPPH were methylated separately with methyl iodide in aqueous alkaline solution at room temperature, followed by extraction of the reaction products into acetone.

The recrystallized compounds were identified by their proton magnetic resonance (PMR) spectra and mass spectra (MS) as 1,3-dimethyl-5,5-diphenylhydantoin (DPH-Me_2) and 1,3-dimethyl-5-(*p*-methylphenyl)-5-phenylhydantoin (MPPH-Me_2). The PMR spectra of DPH-Me_2 and MPPH-Me_2 in deuterated chloroform were run on a Varian HA-100 spectrometer with tetramethyl-

silane as internal reference. Mass spectra were recorded on an AEI-MS 30 double-beam mass spectrometer with a direct inlet probe at 50° and electron energy 75 eV with perfluorokerosene as mass marker.

DPH-Me_2 : m.p. 190—192°.
PMR: 2.81 ppm (δ), N(1)-CH_3 ; 3.14 ppm, N(3)-CH_3 ; 7.2—7.5 ppm, aromatic protons.
MS: *m/e* 280, M^+; peaks for $M - CH_3NCO$, $M - C_6H_5$, $M - CH_3NCONCH_3$ and $C_6H_5CNCH_3^{+\cdot}$.

MPPH-Me_2 : m.p. 114—116°.
PMR: 2.36 ppm (δ), *p*-C_6H_4-CH_3 ; 2.80 ppm, N(1)-CH_3 ; 3.12 ppm, N(3)-CH_3 ; 7.1—7.45 ppm, aromatic protons.
MS: *m/e* 294, M^+; peaks for $M - C_6H_5$, $CH_3C_6H_4CNCH_3^+$ and $C_6H_5CNCH_3^+$.

These data are in good agreement with those reported by other workers for methylated derivatives of DPH [23,25,29,38].

Response curve

The linearity of the detector (FID) response was demonstrated by injecting mixtures of various amounts of both DPH-Me_2 and MPPH-Me_2 into the gas chromatograph. Peak-area ratios of DPH-Me_2 to MPPH-Me_2 are plotted against the ratio of the amount of DPH-Me_2 to that of MPPH-Me_2 in the sample. The response curve was linear in the ranges 0.26—2.6 μg of DPH-Me_2 and 0.58—2.3 μg of MPPH-Me_2 in 200 μl of chloroform (3 μl injected).

Standard curve and working standard curve: precision and reproducibility

A standard curve prepared by analyzing plasma standard solutions of DPH is shown in Fig. 2. For three independent determinations at each level, the coefficient of variation was less than 10% in the concentration range of 1—30 μg/ml of DPH in plasma (Fig. 2). The reproducibility of standard curves over the course of 2 months was examined by performing control analyses with concentrations of 5, 15 and 30 μg/ml of DPH in plasma simultaneously with the determination of unknown samples. Each individual working standard curve showed a linear relationship between the peak-area ratio and DPH plasma

TABLE I

PEAK-AREA RATIO OF DPH-Me_2 TO MPPH-Me_2, STANDARD DEVIATION AND COEFFICIENT OF VARIATION OF WORKING STANDARD CURVES PREPARED BY ANALYZING PLASMA SAMPLES OF 5, 15 AND 30 μg/ml OF DPH OVER THE COURSE OF 2 MONTHS (2 μg OF MPPH PER SAMPLE)

Concentration (μg/ml)	Peak-area ratio (mean)	Number of determinations	Standard deviation	Coefficient of variation (%)
5	0.251	17	0.030	12.0
15	0.728	18	0.041	5.6
30	1.459	17	0.096	6.6

TABLE II

DETERMINATION OF DPH PLASMA LEVELS IN UNKNOWN SAMPLES

Sample	Actual concentration (μg/ml)	Found concentrations (single analysis) (μg/ml)	Found concentration (mean) (μg/ml)	Coefficient of variation (%)	Difference between mean found and actual concentration (%)
1	15.0	13.8, 15.1, 15.1	14.7	5.1	−2
2	4.0	4.1, 3.8, 4.1	4.0	4.3	0
3	8.0	7.0, 7.7, 7.9	7.5	6.3	−6
4	33.1	31.5, 34.0, 34.7	33.4	5.0	+1
5	0.0	0.5, 0.0, 0.0	0.2		—
6	1.0	1.5, 1.5, 1.2	1.4	12.4	+40
7	26.5	23.6, 25.8, 25.4	24.9	4.7	−6
8	12.0	11.8, 12.0, 11.6	11.8	1.7	−2
9	20.1	19.8, 19.8, 19.8	19.8	0.0	−1

concentration, but there were minor day-to-day variations in this ratio. The results are given in Table I.

Sensitivity and accuracy

The lowest concentration of DPH that can be quantitatively determined in plasma using a 100-μl sample is 0.5—1 μg/ml. Nine test samples of DPH in plasma with concentrations unknown to the analyst were prepared in the same way as plasma standard solutions. Three separate determinations were made of the unknown samples and of three control samples with plasma levels of 5, 15 and 30 μg/ml of DPH. Plasma concentrations were evaluated by means of the simultaneously produced working standard curve. The results are listed in Table II.

Comparison of two GLC methods

The procedure described here for the determination of DPH levels in plasma was compared with another GLC method by analyzing plasma from nine patients undergoing treatment with DPH by means of the two methods. The results were in good agreement (Table III).

TABLE III

COMPARISON OF TWO GLC METHODS FOR THE DETERMINATION OF DPH PLASMA CONCENTRATIONS

Method 1 is a routine determination of the DPH plasma level in the management of epileptics performed at the Department of Clinical Pharmacology, University of Berne: double extraction of DPH and MPPH is followed by flash-heater methylation with trimethylanilinium hydroxide [39] and chromatography of drug and internal standard. Concentrations are rounded off. Coefficient of variation is less than 5% for three separate determinations of each sample in the range 4—40 μg/ml of DPH. Method 2 is the procedure described in this paper with three separate determinations on each sample.

Sample	Concentration determined by Method 1 (μg/ml)	Mean concentration ± standard deviation determined by Method 2 (μg/ml)
1	7	8.0 ± 0.5
2	32	31.4 ± 0.8
3	26	26.6 ± 0.8
4	10	10.6 ± 0.6
5	9	9.4 ± 0.3
6	42	39.7 ± 1.2
7	9	9.6 ± 0.8
8	22	20.8 ± 0.2
9	1	1.9 ± 0.1

CONCLUSIONS AND DISCUSSION

This procedure is sufficiently selective, sensitive and accurate for the measurement of DPH plasma levels of patients following therapeutic doses (therapeutic range 10—20 μg/ml). For a single assay, 100 μl of plasma are required, which is of great value in the control of antiepileptic therapy in children.

Forty samples can be extracted and chromatographed in 2 days. It is planned to make the method more rapid by means of automatic injection of extracts awaiting gas chromatography.

Most of the reported spectrophotometric, thin-layer and gas chromatographic methods of analysis for DPH and other anticonvulsant drugs use 1 ml or more of plasma for a single determination, whereas our method requires micro amounts of biological material. In addition to the aspect of sensitivity, "pre-column" derivative formation is performed in our procedure. Some of the advantages of "pre-column" compared with "on-column" methylation of DPH and MPPH are that the reaction conditions can be controlled, excess of reagents can be eliminated prior to GLC, extractive methylation with methyl iodide in acetone is a further clean-up step and chloroform extracts of the methylated derivatives awaiting GLC are stable.

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REFERENCES

1 A.J. Glazko, in D.M. Woodbury, J.K. Penry and R.P. Schmidt (Editors), Antiepileptic Drugs, Raven Press, New York, 1971, Ch. 10, p. 103.
2 W.A. Dill and A.J. Glazko, Clin. Chem., 18 (1972) 675.
3 J.E. Wallace and H.E. Hamilton, J. Pharm. Sci., 63 (1974) 1795.
4 A.J. Fellenberg, A. Magarey and A.C. Pollard, Clin. Chim. Acta, 59 (1975) 155.
5 C. Gardner-Thorpe, M.J. Parsonage and C. Toothill, Clin. Chim. Acta, 35 (1971) 39.
6 R.E. Tigelaar, R.L. Rapport, J.K. Inman and H.J. Kupferberg, Clin. Chim. Acta, 43 (1973) 231.
7 C.E. Cook, J.A. Kepler and H.D. Christensen, Chem. Pathol. Pharmacol., 5 (1973) 767.
8 M.R. Montgomery, J.L. Holtzman, R.K. Leute, J.S. Dewees and G. Bolz, Clin. Chem., 21 (1975) 221.
9 W. Wiegrebe and L. Wehrhahn, Arzneim.-Forsch., 25 (1975) 517.
10 K. Sabih and K. Sabih, Anal. Chem., 41 (1969) 1452.
11 C.E. Pippenger and H.W. Gillen, Clin. Chem., 15 (1969) 582.
12 M.A. Evenson, P. Jones and B. Darcey, Clin. Chem., 16 (1970) 107.
13 J.C. Van Meter, H.S. Buckmaster and L.L. Shelley, Clin. Chem., 16 (1970) 135.
14 J.W.A. Meijer, Epilepsia, 12 (1971) 341.
15 D. Sampson, I. Harasymiv and W.J. Hensley, Clin. Chem., 17 (1971) 382.
16 P.A. Toseland, J. Grove and D.J. Berry, Clin. Chim. Acta, 38 (1972) 321.
17 C. Gardner-Thorpe, M.J. Parsonage, P.F. Smethurst and C. Toothill, Clin. Chim. Acta, 36 (1972) 223.
18 D. Chin, E. Fastlich and B. Davidow, J. Chromatogr., 71 (1972) 545.

19 H.M.H.G. Cremers and P.E. Verheesen, Clin. Chim. Acta, 48 (1973) 413.
20 P.A. Toseland, M. Albani and F.D. Gauchel, Clin. Chem., 21 (1975) 98.
21 T. Chang and A.J. Glazko, J. Lab. Clin. Med., 75 (1970) 145.
22 D.H. Sandberg, G.L. Resnick and C.Z. Bacallao, Anal. Chem., 40 (1968) 736.
23 G. Grimmer, J. Jacob and H. Schäfer, Arzneim.-Forsch., 19 (1969) 1287.
24 E.M. Baylis, D.E. Fry and V. Marks, Clin. Chim. Acta, 30 (1970) 93.
25 K. Sabih and K. Sabih, J. Pharm. Sci., 60 (1971) 1216.
26 J. MacGee, Anal. Chem., 42 (1970) 421.
27 E.B. Solow and J.B. Green, Neurology, 22 (1972) 540.
28 P. Friel, J.R. Green and H.J. Kupferberg, Epilepsia, 13 (1972) 273.
29 A. Estas and P.A. Dumont, J. Chromatogr., 82 (1973) 307.
30 H.J. Kupferberg, Clin. Chim. Acta, 29 (1970) 283.
31 R.H. Hammer, B.J. Wilder, R.R. Streiff and A. Mayersdorf, J. Pharm. Sci., 60 (1971) 327.
32 A. Berlin, S. Agurell, O. Borgå, L. Lund and F. Sjöqvist, Scand. J. Clin. Lab. Invest., 29 (1972) 281.
33 R.J. Perchalski, K.N. Scott, B.J. Wilder and R.H. Hammer, J. Pharm. Sci., 62 (1973) 1735.
34 J.H. Goudie and D. Burnett, Clin. Chim. Acta, 43 (1973) 423.
35 H.L. Davis, K.J. Falk and D.G. Bailey, J. Chromatogr., 107 (1975) 61.
36 U.P. Geiger, Internal Report, Ciba-Geigy, Basle, Switzerland.
37 S.P. Agarwal and M.I. Blake, J. Pharm. Sci., 57 (1968) 1434.
38 H. Biltz and O. Behrens, Ber. Deut. Chem. Ges., 43 (1910) 1984.
39 E. Brochmann-Hanssen and T.O. Oke, J. Pharm. Sci., 58 (1969) 370.

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Biomedical Applications

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RAPID TRACE ANALYSIS OF BARBITURATES IN BLOOD AND SALIVA BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY*

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SUMMARY

The performances of a number of liquid—solid systems, consisting of mixtures of water and methanol as liquid phase and methyl silica as solid phase, were investigated with respect to their use in the separation of barbiturates by high-pressure liquid chromatography (HPLC). Phase system selectivities and column efficiencies were determined.

The results were applied to the development of a rapid method for the determination of trace amounts of barbiturates in blood. The first step in the analysis, the extraction of barbiturates from blood, was also investigated and good recoveries were achieved. The extracts were analyzed by HPLC using ultraviolet detection at 220 nm. A low detection limit and high precision were obtained. An amount of 5 ng hexobarbital, for example, can be determined with a precision of ±15% and 5 μg with a precision of ± 0.3%. The time course of the concentration of hexobarbital in the serum and saliva of man after an oral administration of 400 mg is demonstrated.

INTRODUCTION

Insomnia, which is mostly a symptom of a physical or emotional disorder of man, has been successfully treated for many years with hypnotic drugs such as barbiturates. In order to obtain a greater insight into the pharmacokinetics [1] and the determination of the minimal effective and toxicological concentrations, the analysis of the drug itself and its metabolites is of great importance.

Previously, a number of analytical methods such as UV spectrophotometry and gas chromatography with flame-ionization detection [2—4] have been ap-

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plied. The former method is non-specific while the latter is mostly restricted to the determination of the drug itself at overdose levels. Some papers have described the thermionic detection of barbiturates, by means of which analyses at therapeutic levels became possible [5,6]. In order to determine the more hydrophilic metabolites, derivatization steps [7] are necessary. Such steps, however, seriously invalidate the quantitative analyses.

The low working temperatures and the many possibilities of adjusting the phase system selectivity combined with high efficiencies make high-pressure liquid chromatography (HPLC) very suitable for the analysis of thermally labile. hydrophilic and hydrophobic compounds. Because the metabolites of a drug are more hydrophilic than the drug itself, it is appropriate to choose a phase system with a hydrophobic stationary phase, in which the more hydrophilic compounds are eluted first, which permits easier detection and determination of low concentrations of metabolites.

The separation and quantitation of barbiturates in pharmaceuticals by HPLC on a strong ion exchanger has been reported previously [8]. This paper describes the separation and quantitation of barbiturates at therapeutic levels in serum and saliva by high-pressure liquid—solid chromatography with UV detection using a highly selective hydrophobic adsorbent. An improved extraction procedure for barbiturates, which is a modification of a procedure described elsewhere [6], is also presented.

EXPERIMENTAL

Apparatus

A high-pressure liquid chromatograph (Siemens SP 100) equipped with a UV detector (DuPont, Model 837), a high-pressure sampling valve (Valco CV-6-UHPa), a linear potentiometric recorder (Goerz, Servogor RE 542) and an electronic integrator (Spectra-Physics, Autolab System I) was used. Stainless-steel 316 tubing with an I.D. of 2.8 mm, an O.D. of 6.35 mm and a length of 10 cm were used for the construction of the column. In order to prevent contamination of the separation column, a pre-column (500 × 9 mm) was installed.

Chemicals and materials

In all experiments, double-distilled water and organic solvents of analytical grade (Merck, Darmstadt, G.F.R.) were used. The components for the preparation of the extraction solvent were freshly distilled.

The methyl silica was prepared from narrow-sized silica (LiChrosorb SI 60, Merck) treated with dimethyldichlorosilane, as described previously [9]. The pre-column was filled with 63—200 μm silanized silica (SI 60, Merck).

Procedures

Chromatography. In order to pack the column, 0.8 g of methyl silica was added to 5 ml of a mixture of tetrabromoethane and chloroform (sp. gr. 1.82) and dispersed ultrasonically in the liquid. The slurry was then placed in a wide-bore tube to which the column, closed at the bottom by a frit, was attached. 2,2,4-Trimethylpentane was then pumped into the tube with a high flow-rate at 1000 bar, displacing the slurry into the column. After filling, the column

was successively eluted with 100 ml of acetone and 100 ml of ethanol in order to remove the dispersion liquid and finally with 100 ml of eluent. The precolumn was filled by using a dry packing technique.

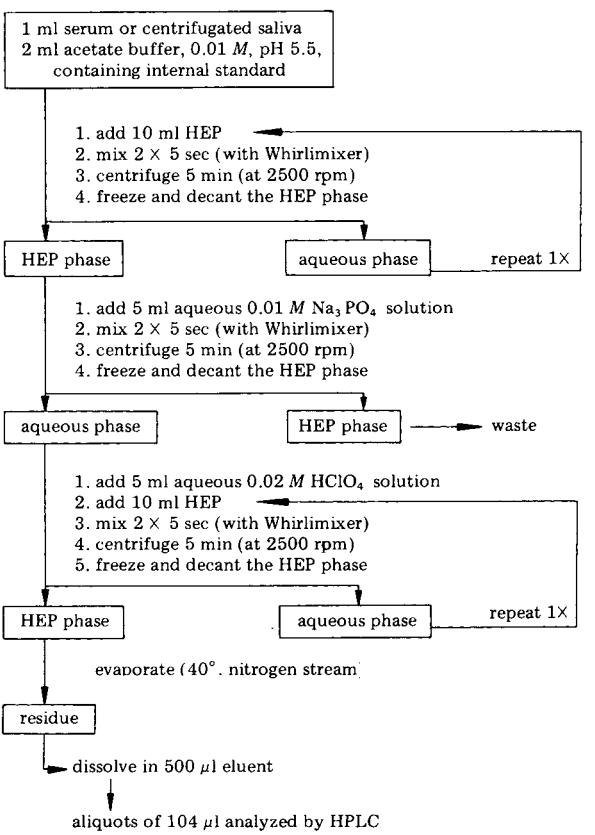
The capacity ratios were calculated from the retention times of the barbiturates and of an unretarded compound, for which potassium chromate was used. The selectivity coefficients of pairs of compounds were calculated as the ratio of their capacity ratios.

The theoretical plate height for a compound was calculated from its retention time and half the peak width at 60% of the peak height.

The samples were dissolved in the eluent and injected by means of a high-pressure sampling valve with a sample loop of 13.6 μl for the theoretical plate height measurements and of 104 μl for the quantitative determinations in serum and saliva.

Extraction. The full scheme for the extraction of barbiturates from serum and saliva with *n*-hexane—diethyl ether—*n*-propanol (49 : 49 : 2) (HEP) is outlined in Scheme 1.

In order to obtain a rapid and efficient separation of the organic and aqueous phases after mixing, the extraction mixture is centrifuged and then placed



Scheme 1. The extraction of barbiturates from serum and saliva.

in liquid nitrogen. The aqueous phase freezes within 60 sec and the organic phase can be decanted easily. The total extraction procedure takes less than 1 h.

RESULTS AND DISCUSSION

The analysis of closely related compounds such as a drug and its metabolites, present in very low concentrations in a small amount of sample, by HPLC places great demands on the magnitude of the selectivity coefficient, the capacity ratio, the theoretical plate height and the extraction procedure [10]. High selectivity coefficients, relatively small capacity ratios and small theoretical plate heights, combined with an extraction procedure with high selectivity and high recoveries for the removal of interfering compounds, are the requirements for achieving low detection limits with adequate resolution. In practice, a compromise between detection limit and resolution has to be found.

In earlier work [9], the remarkable selectivity of methyl silica towards compounds with closely related structures such as psychopharmaceuticals and sulpha drugs was reported. In order to test this type of adsorbent for the separation and determination of barbiturates, a systematic investigation with respect to column efficiency and selectivity was made. The precision of the quantitative determination of barbiturates by HPLC and the influence of the extraction procedure on the determination of barbiturates in serum were also investigated.

Phase system selectivity

The capacity ratios and selectivity coefficients of successively eluted barbiturates were measured as a function of the percentage of methanol in the mixture with water used as the mobile phase. The retention behaviour of barbiturates is dependent on pH and therefore the use of a buffer should be favourable. It was found, however, that this technique leads to non-reproducible capacity ratios and to low selectivity coefficients. Therefore, no buffer but distilled water alone was used.

The results are shown in Table I and Fig. 1. The effect of the methanol content on the capacity ratio and selectivity coefficient of barbiturates is two-fold: (i) owing to the greater lipophility of methanol compared with water, the capacity ratio decreases with increasing methanol content, and (ii) the selectivity coefficients change irregularly with the methanol content. In general, the selectivity coefficient decreases with increasing methanol content. For some pairs of compounds, however, the selectivity coefficients has a pronounced maximum or minimum at a given methanol content.

No correlation between the pK_a values of the barbiturates and their retention behaviour could be found. The systematic effect of structural increments on the log κ_i values cannot be observed clearly, mainly because of the lack of the number of systematic changes in one of the substituents. Some effects are clearly systematic: the methylene increment, according to $\log \kappa_i = a + bn$, where a and b are constants and n is the number of carbon atoms in the alkyl chain, as discussed in a previous paper [9], and the effect of a double bond in the alkyl chain, not attached directly to the ring.

The optimal composition of the eluent required to fulfil the demands of

TABLE I

CAPACITY RATIOS AND SELECTIVITY COEFFICIENTS OF BARBITURATES MEASURED WITH DIFFERENT METHANOL—WATER MIXTURES AS ELUENT AND METHYL SILICA AS STATIONARY PHASE

Barbiturate	Eluent							
	25 : 75		30 : 70		40 : 50		50 : 50	
	κ_i	r_{ji}	κ_i	r_{ji}	κ_i	r_{ji}	κ_i	r_{ji}
Barbital	3.58	—	2.49	—	1.57	—	1.05	—
Heptobarbital	5.79	1.62	3.82	1.53	2.02	1.29	1.21	1.15
Phenobarbital	6.06	1.05	3.85	1.01	2.05	1.01	1.27	1.05
Allobarbital	7.35	1.21	5.25	1.36	2.72	1.33	1.61	1.27
Barotal	9.17	1.25	7.16	1.36	3.57	1.31	1.69	1.05
Brallobarbital	11.1	1.21	7.29	1.02	3.65	1.02	1.77	1.05
Butobarbital	14.8	1.34	10.3	1.41	4.71	1.29	2.25	1.27
Cyclobarbital	15.5	1.04	10.4	1.01	4.78	1.01	2.30	1.02
Hexobarbital	20.4	1.32	14.4	1.38	6.36	1.35	2.85	1.24
Mephobarbital	26.6	1.30	17.6	1.22	7.06	1.11	2.98	1.05
Heptabarbital	29.1	1.09	19.8	1.13	7.81	1.11	3.29	1.10
Vinylbital	32.3	1.11	22.3	1.13	9.03	1.16	3.61	1.10
Pentobarbital	33.6	1.04	22.9	1.03	9.39	1.04	3.69	1.02
Amobarbital	35.0	1.04	24.3	1.06	9.49	1.01	3.80	1.03
Secobarbital	48.8	1.39	34.3	1.41	12.6	1.33	4.67	1.23

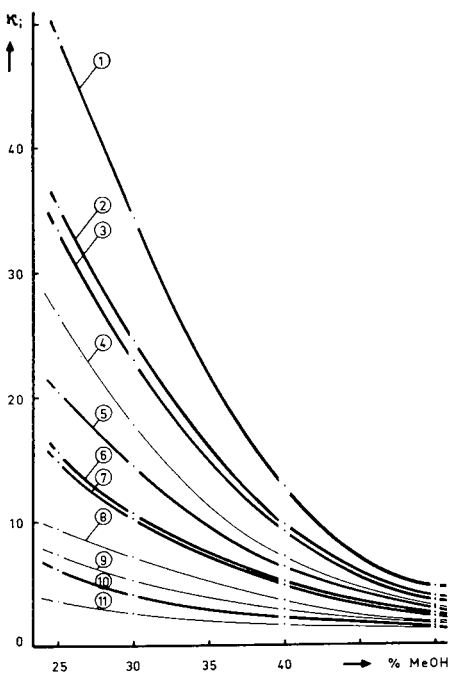


Fig. 1. Plot of the capacity ratio, κ_i, of barbiturates against the methanol content on the phase system methanol—water/methyl silica. The heavy lines represent the most common used barbiturates. 1 = Secobarbital; 2 = amobarbital; 3 = pentobarbital; 4 = methylphenobarbital; 5 = hexobarbital; 6 = cyclobarbital; 7 = butobarbital; 8 = barotal; 9 = allobarbital; 10 = phenobarbital; 11 = barbital.

small capacity ratios and high selectivity coefficients can easily be found from Table I and Fig. 1. A methanol content of about 40% seems to be a good compromise in this respect. Some pairs of barbiturates cannot be separated with the phase system investigated. However, in practice these barbiturates are seldom administred together in one dose. On the other hand, all barbiturates should be separated with sufficient resolution in order to allow accurate chromatographic identification.

Column efficiency

Some workers [11, 12] predicted an enormous improvement in column efficiency if small particles, of the order of a few micrometres, and a narrow size distribution were used. Small particles lead to large pressure drops and the lack of high-pressure pumping and injection systems delayed the development of HPLC until about 10 years ago. Since then, the improvement in column technology reported in several papers confirmed the effect of small particles on the theoretical plate height [13, 14]. In practice, good column packings can be obtained with particle sizes down to about 5 μm.

For adsorption chromatography on hydrophilic surfaces, the reported column efficiencies are generally better than those for adsorption chromatography on hydrophobic surfaces, e.g., with chemically bonded materials such as C_8 and C_{18} [15]. This effect was mainly attributed to the larger particles used on the one hand and to a slow mass transfer in the hydrophobic layer on the other. Recently, the synthesis of a short-chain modified silica, which shows excellent selectivity and efficiency, was reported [16].

In this work, a methyl silica of small particle size was used. For a number of barbiturates with capacity ratios ranging from 2 to 12, the theoretical plate height, H, was measured as a function of the mobile phase velocity, u. The results are plotted in Fig. 2.

The flatness of the H versus u curves indicates a rapid mass transfer on this type of support, opposite to the effect observed on C_8 and C_{18} bonded phase materials [15]. As can be seen, the convective mixing is also small, owing to the use of small particles and an appropriate packing procedure.

This investigation demonstrates that with methyl silica, highly efficient, small-diameter columns can be prepared, which are very suitable for trace analysis. The ability of methyl silica columns to separate barbiturates is illustrated by Fig. 3, which demonstrates the separation of six barbiturates in about 2 min.

Composition of the extraction solvent

The extraction of barbiturates from serum and saliva in order to remove interfering substances and to enrich the barbiturates is an important step in this type of trace analysis.

Barbiturates are weakly acidic compounds with pK_a values ranging from 7.4 to 8.3. The distribution of acidic compounds between an organic solvent and an aqueous solution depends on the pH of the aqueous phase and on the pK_a value of the acids. The total distribution coefficient of an acid HX, defined as the ratio of the concentrations in the organic and the aqueous phases, excluding side reactions, can be expressed by

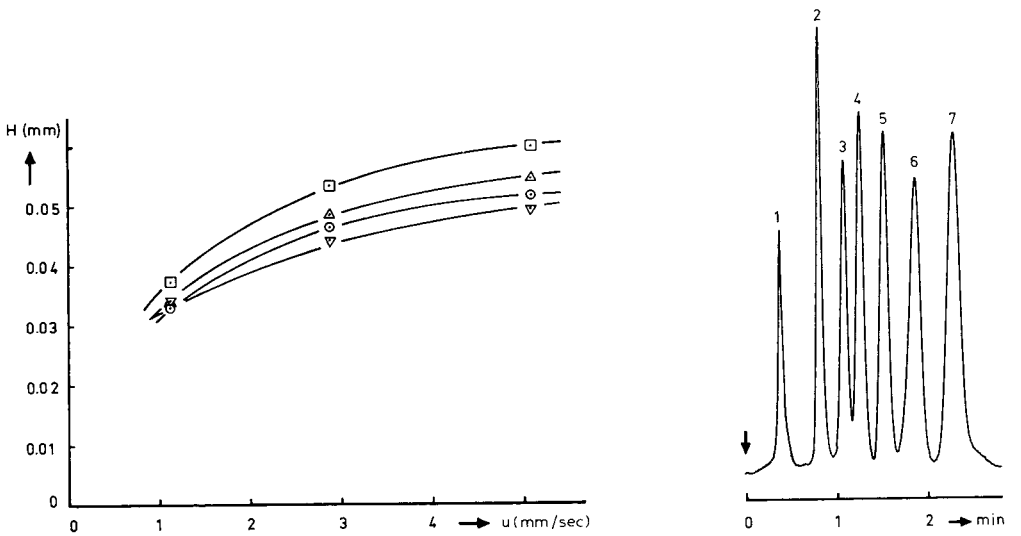


Fig. 2. H versus u curve for several barbiturates, measured on a column filled with 5-μm methyl silica as stationary phase and methanol—water (2 : 3) as mobile phase. □, Phenobarbital; △, butobarbital; ○, hexobarbital; ▽, secobarbital.

Fig. 3. HPLC separation of a test mixture of six barbiturates on methyl silica. Column, 100 × 2.8 mm; packing, methyl silica; eluent, methanol—water (1 : 1); UV detection at 205 nm. 1 = Potassium chromate; 2 = phenobarbital; 3 = barotal; 4 = butobarbital; 5 = hexobarbital; 6 = vinylbital; 7 = secobarbital.

$$K_X = \frac{[HX]_{org}}{[HX]_{aq} + [X^-]_{aq}} = \frac{1}{1 + K_a/[H^+]_{aq}} \cdot K_{HX} \qquad (1)$$

where the subscripts org, and aq refer to the organic and aqueous phases, respectively, K_{HX} is the partition coefficient to the undissociated acid HX.

The composition of the extraction solvent determines the value of K_{HX}. From eqn. 1, it can be seen that pH $\ll$ pK_a is favourable for obtaining high distribution coefficients, i.e., high recoveries. From the literature, many data [17, 18] are available on the effect of the composition of the organic phase on the distribution of barbiturates. These data show that highly lipophilic barbiturates can be extracted quantitatively from acidified solutions with non-polar solvents such as hexane and light petroleum. For less lipophilic barbiturates and the metabolites, more polar solvents such as diethyl ether and chloroform or mixtures of non-polar and polar solvents have to be used in order to obtain good recoveries.

Therefore, it was decided to use HEP as the extraction solvent in order to achieve the extraction of both the highly lipophilic and the less lipophilic barbiturates.

Blank serum extractions of untreated subjects with HEP, however, showed the presence of substances with a chromatographic retention behaviour within the group of barbiturates, as shown in Fig. 4a and 4b. In order to remove

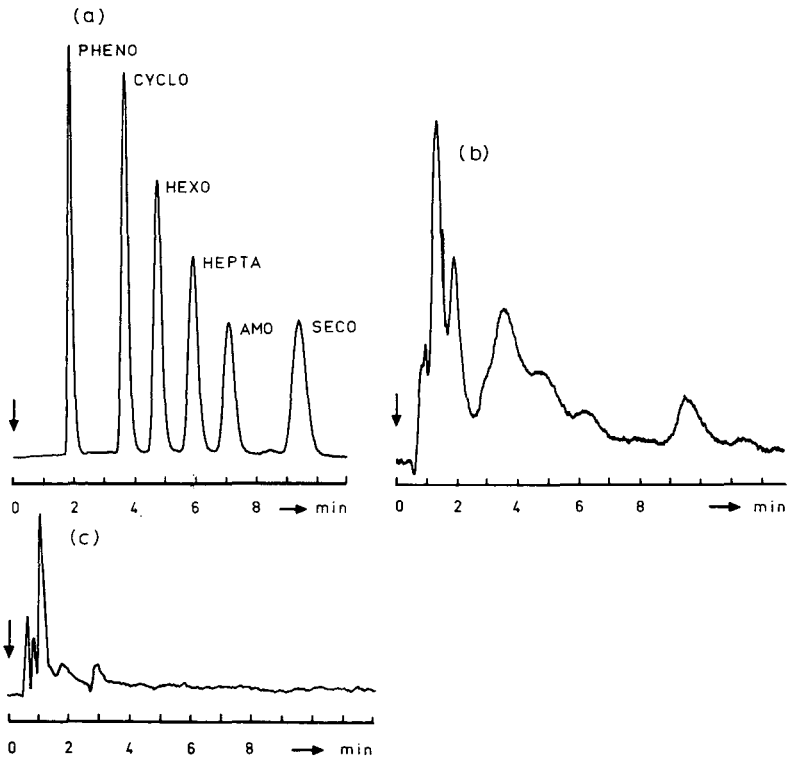


Fig. 4. Influence of the extraction procedure on the background. Column, 100 × 2.8 mm; packing, methyl silica; eluent, methanol—water (2 : 3); UV detection at 205 nm. (a) Test mixture of six barbiturates; (b) serum background before back-extraction; (c) serum background after back-extraction.

these interfering serum constituents, the extraction procedure was amended. The barbiturate-containing HEP phase was shaken with a sodium phosphate solution of pH 11.7. According to eqn. 1, a high pH value promotes the distribution of acids towards the aqueous phase. Owing to their high pK_a values, the barbiturates will be extracted completely from the organic into the aqueous phase, while a number of non-acidic compounds will remain in the organic phase and can be removed. The aqueous phase is acidified and the barbiturates are back-extracted with HEP. This extension to the extraction procedure results in a clean extract, almost without interfering substances in the elution range of the barbiturates, as shown in Fig. 4c.

Precision and linearity of the method

The precision of the quantitative determination of barbiturates by HPLC was investigated by injecting a constant volume (104 μl) of solutions of barbiturates of different concentrations (1—12 μg/ml). The UV spectra of barbiturates show a maximum at about 205 nm, but the linear dynamic range of the detector was found to be greater at 220 nm. In order to be able to cover a wide range of concentrations of barbiturates, it was decided to measure at 220 nm.

Fig. 5 shows the proportionality of peak area and injected amount of hexo-

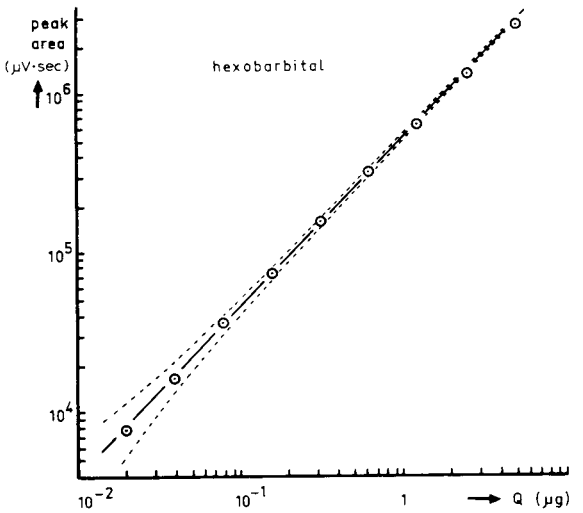


Fig. 5. Linearity and precision. The dashed lines show 3 × the standard deviation. $y = 5.18 \cdot 10^5 \cdot x - 6.20 \cdot 10^3$, where x = amount injected (μg) and y = peak area (μV·sec).

barbital measured over the range 20—5000 ng. The dashed lines show the confidence limits for ± 3 times the standard deviation (99.7% reliability). The relative standard deviation is 0.3% for 50 μg/ml, 3% for 5 μg/ml and 15% for 0.05 μg/ml. The sensitivity of the whole system, defined as the slope of the curve of the peak area versus injected amount of compound (hexobarbital), expressed in integration units (I.U.), is 5.18 μV·sec/ng, calculated by linear regression.

The linearity of the calibration graph is characterized by the correlation coefficient, which was determined to be 0.99984, indicating the very high linearity.

The standard deviation of the baseline noise, measured during the same period of time as the peak integral, was 385 μV·sec, corresponding to about 0.8 ng of hexobarbital. The detection limit of hexobarbital for a signal to noise ratio of 3 is about 2.4 ng.

The recovery and reproducibility of the extraction procedure were tested by extraction of known amounts of barbiturates of different lipophilicity added to distilled water and to blank serum. The relationship between the added and determined amounts of a number of barbiturates after extraction from distilled water and blank serum is shown in Fig. 6. The recoveries of the highly lipophilic hexobarbital and secobarbital for both water and serum ranges from 90 to 95%. For the less lipophilic phenobarbital, a smaller recovery (water 70% and serum 50%) was found.

The reproducibility in all extractions was about 3% at 10 μg/ml and 10% at 1 μg/ml, indicating that the precision of the total determination is mainly determined by the extraction. Fig. 7 shows the separation of six widely used barbiturates (300 ng of each), added to blank serum and extracted as described above.

In order to eliminate possible errors during extraction, one of these barbiturates can be chosen as an internal standard for the determination of the others.

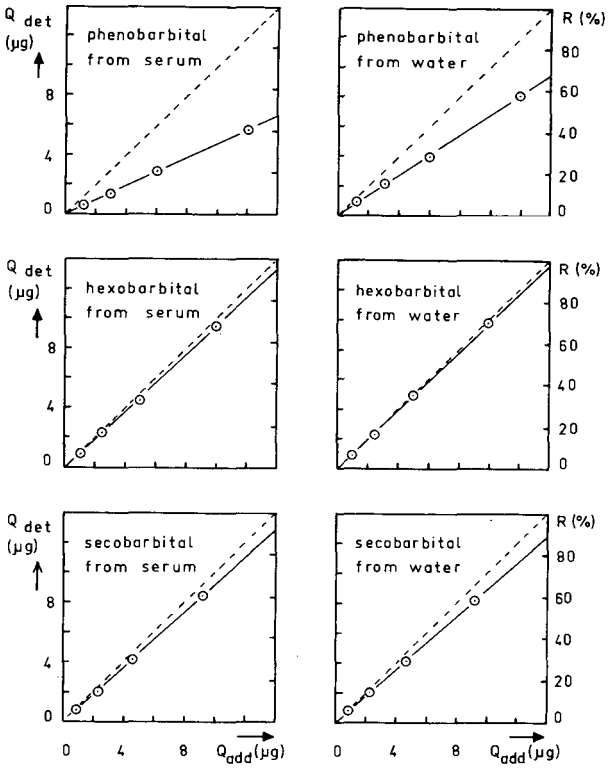


Fig. 6. Recovery of some barbiturates extracted from serum and water. The dashed lines represent 100% recovery.

Time course of concentration of hexobarbital in blood and saliva after oral administration

In order to test the method with natural samples, blood and saliva from man were examined after oral administration of 400 mg of hexobarbital in solid gelatine capsules. Blood and saliva samples were taken at increasing time intervals.

To 1 ml of serum or centrifuged saliva (5 min, 2500 rpm), 3.5 µg of amobarbital were added as internal standard and the sample was then treated as described above. The extract was analyzed by HPLC.

Figs. 8 and 9 show the chromatograms of the extracts of serum and saliva samples, respectively, from the same subject. The concentration curves obtained from another subject are shown in Fig. 10 and agree well with results obtained by other workers in similar experiments [5] using gas chromatography. From Figs. 8 and 9, it can be seen that the determination of metabolites, which are expected in the chromatogram before the barbiturate, is hindered by interfering compounds present in the extract.

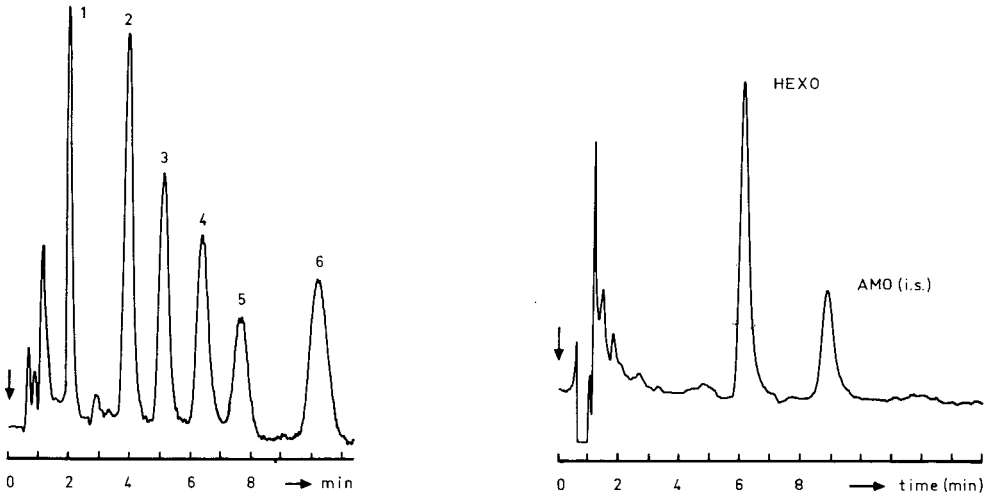


Fig. 7. Analysis of six barbiturates extracted from serum. Injection: 104 µl from 500 µl of solution obtained from 1 ml serum, to which 1.5 µg of each component was added. Conditions as in Fig. 4. 1 = Phenobarbital; 2 = cyclobarbital; 3 = hexobarbital; 4 = heptabarbital; 5 = amobarbital; 6 = secobarbital.

Fig. 8. Chromatogram of an extract from serum of man after oral administration of 400 mg of hexobarbital in solid gelatine capsules. Sample taken at 6 h after administration. Conditions as in Fig. 4. Hexobarbital peak corresponds to 160 ng; amobarbital peak corresponds to 700 ng.

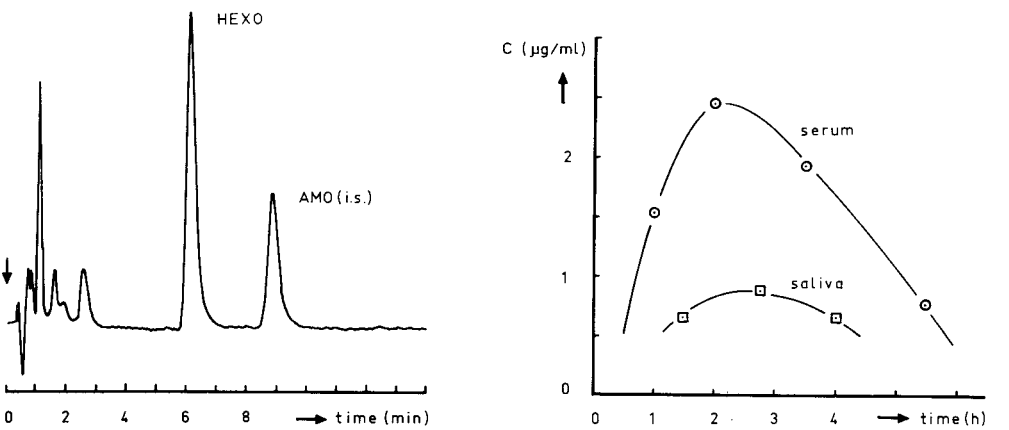


Fig. 9. Chromatogram of an extract from saliva of man after oral administration of 400 mg of hexobarbital in solid gelatine capsules. Sample taken 2.5 h after administration. Conditions as in Fig. 4. Hexobarbital peak corresponds to 140 ng; amobarbital peak corresponds to 700 ng.

Fig. 10. Hexobarbital serum and saliva concentration curves for one subject after oral administration of 400 mg of hexobarbital in solid gelatine capsules.

CONCLUSIONS

The main conclusions can be summarized as follows:

(i) Methyl silica shows excellent selectivity towards the barbiturates.
(ii) Highly efficient columns can be prepared with methyl silica as adsorbent.
(iii) Rapid trace analysis of barbiturates in body fluids at submicrogram levels is possible.
(iv) In order to determine metabolites, the extraction procedure has to be altered in order to remove interfering compounds.

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REFERENCES

1 G. Levy, in D.S. Davies and B.N.C. Prichard (Editors), Biological Effects of Drugs in Relation to their Plasma Concentrations, MacMillan, London, 1973, Ch. 6.
2 D.J. Berry, J. Chromatogr., 86 (1973) 89.
3 H.V. Street, Clin. Chim. Acta, 34 (1971) 357.
4 R.G. Cooper, M.S. Greaves and G. Owen, Clin. Chem., 18 (1972) 1343.
5 D.D. Breimer and J.M. van Rossum, J. Chromatogr., 88 (1974) 235.
6 D.D. Breimer, Thesis, University of Nijmegen, Nijmegen.
7 M. Ehrnebo, S. Agurell and L.O. Boréus, Eur. J. Clin. Pharmacol., 4 (1972) 191.
8 R.W. Roos, J. Pharm. Sci., 61 (1972) 1979.
9 J.F.K. Huber and U.R. Tjaden, in preparation; U.R. Tjaden, Thesis, University of Amsterdam, Amsterdam, 1976, Ch. 1.
10 C.A.M. Meijers, J.A.R.J.Hulsman and J.F.K. Huber, Z. Anal. Chem., 261 (1972) 347.
11 J.F.K. Huber and J.A.R.J. Hulsman, Anal. Chim. Acta, 38 (1967) 305.
12 J.H. Knox and M. Saleem, J. Chromatogr. Sci., 7 (1969) 614.
13 J.F.K. Huber, Ber. Bunsenges. Physik. Chem., 77 (1973) 159.
14 R.E. Majors, J. Chromatogr. Sci., 11 (1973) 88.
15 J.N. Done and J.H. Knox, J. Chromatogr. Sci., 10 (1972) 606.
16 J.H. Knox and A. Pryde, J. Chromatogr., 112 (1975) 171.
17 M.T. Bush, G. Berry and A. Hume, Clin. Pharmacol. Ther., 7 (1966) 373.
18 J.R. Cooper and B.B. Brodie, J. Pharmacol. Exp. Ther., 114 (1955) 109.

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Biomedical Applications

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THE DETERMINATION OF ALLOPURINOL AND OXIPURINOL IN HUMAN PLASMA AND URINE

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SUMMARY

A method is described for allopurinol and oxipurinol assay within human plasma and urine in the range expected during therapy. The method is based on high-performance ion-exchange chromatography following an efficient sample purification step using Chelex-100 resin in the Cu^{2+} form. Linear calibration curves are produced for allopurinol over the range 0.05—10 μmole/l (0.068—1.36 μg/ml) in plasma and 0.005—1 mmole/l (0.68—136 μg/ml) in urine and for oxipurinol 0.5—100 μmole/l (0.076—15.2 μg/ml) in plasma and 0.1—2 mmole/l (15.2—304 μg/ml) in urine.

INTRODUCTION

Formation of uric acid, which is harmful in excess in humans, is much reduced by purine metabolism inhibition by allopurinol (4-hydroxy-3,4-*d*-pyrazolopyrimidine) and its major metabolite oxipurinol (3,4-dihydroxy-3,4-*d*-pyrazolopyrimidine)[1]. Assay procedures other than radioactive dilution [2] suitable for clinical studies of the two compounds had not been published until the preliminary note on an assay procedure based on high-pressure liquid chromatography (HPLC) by Endele and Lettenbauer [3]. A suitable assay, also using HPLC, is described which also discriminates between the naturally occurring purines xanthine and hypoxanthine. The principle is to subject samples, after forced filtration, to a preliminary step of low-pressure ligand-exchange chromatography. HPLC follows but is confined mainly to detection of allopurinol (A), oxipurinol (O), hypoxanthine (H) and xanthine (X). The analytical procedure is equally applicable to urine and plasma.

EXPERIMENTAL

Materials and methods

Ammonia solution (35%, w/v) (Hopkin & Williams, Chadwell Heath, Rom-

ford Great Britain), ammonium acetate, copper(II) sulphate pentahydrate, glycine, sodium carbonate, sodium hydrogen carbonate and sodium hydroxide solution (2 mole/l) (BDH, Poole, Great Britain) were analytical reagent grade. Brij-35 (polyoxyethylene lauryl ether; BDH) was general purpose reagent grade. Standard pH buffer solutions (Fisons Scientific Apparatus, Loughborough, Great Britain) were used to calibrate the pH meter (Radiometer, Model 26; V.A. Howe, London, Great Britain) in the expanded range. Hypoxanthine and xanthine (Sigma London, Kingston upon Thames, Great Britain) were used as purchased. Allopurinol and oxipurinol (Burroughs Wellcome & Co., Dartford, Great Britain) were obtained in pure form, that is without tablet excipients.

Chelex-100 resin (37—74 μm, 200—400 mesh) (Bio-Rad Labs., Bromley, Great Britain) in the sodium form was first suspended in excess distilled water and acidified with dilute nitric acid to below pH 2.0. The resin was filtered, washed and resuspended in the minimum of 1 mole/l ammonia solution. The resin, now in the ammonium form, was then converted to the Cu^{2+} form by stirring with excess cuproammonium sulphate solution (200 g copper(II) sulphate per litre of 35% (w/v) ammonia solution) for 30 min. The resin was then washed repeatedly with 1 mole/l ammonia solution to remove excess Cu^{2+} ions and then packed in the glass column of the low-pressure ligand-exchange chromatograph as a slurry in this solution.

Aminex A-27 resin in the chloride form (Bio-Rad Labs) was packed in HPLC columns using double-distilled water as the solvent for slurry packing. It was converted to the acetate form by running the chromatographic buffer (1 mole/l ammonium acetate pH 8.70) through the column at normal flow-rate (1 ml/min) for 30 min before use.

Preparation of standards

For HPLC a stock solution of 400 μg/ml of A, O, H and X was prepared. The working HPLC standard solution was made by diluting 25 μl of the stock solution with 25 ml ammonia solution (10 mole/l).

For plasma standards of A and O, stock solutions (34 mg A per 100 ml water and 38 mg O per 100 ml water) were prepared. The calculated volumes of each were added to normal human plasma from volunteers not receiving A or O so that a range of working standards was produced. Standard I contained 20 μmole/l (2.72 μg/ml) A and 100 μmole/l (15.2 μg/ml) O. By serial dilution, standards II, III, IV and V were produced containing the range 10—1.25 μmole/l A and 50—6.25 μmole/l O.

Urine standards were prepared similarly so that the final range was 1—0.05 mmole/l (136—6.8 μg/ml) A and 2.0—0.125 mmole/l (304—19 μg/ml) O.

Apparatus and assay procedure

Plasma and urine filtration. A sample clarification kit (Waters Assoc., Stockport, Great Britain) was assembled omitting the filter but retaining the prefilter; a disposable plastic 2-ml syringe (Brunswick) was substituted for the glass one provided.

One ml plasma diluted to 2 ml with carbonate buffer pH 11.4 containing 1% v/v Brij-35 (30% w/v) was forced through the filter using the syringe. The syringe was disconnected, filled with a further 1 ml of carbonate buffer and again

forced through the filter. Both the initial filtrate and the washings were collected in a 5-ml volumetric flask and their combined volume made up to the mark with the buffer solution (final pH 11.0).

Urine filtration was carried out in the same way as plasma filtration but using 0.5—2.5 ml sample (depending on the expected drug concentration) and omitting Brij-35 from the buffer (final pH 11.0).

Low-pressure ligand-exchange chromatograph. A pressure controller, Superfine Pressure-Stat (Biolabs, Cambridge, Great Britain) introduced nitrogen or compressed air at 1.3—3.5 MN/m^2 (2—5 p.s.i.) into two 2.5-l brown glass bottles each containing a solvent, i.e. 0.01 mole/l carbonate buffer and 10 mole/l ammonia, respectively. The pressurised solvents went via a solvent switching valve (LV4, Pharmacia (GB), Uxbridge, Great Britain) to two more LV4 valves connected as a loop injector system (4-ml volume for plasma or 0.5-ml for urine). The loop injector in turn fed a glass column 65 mm × 6.5 mm I.D. of Chelex-100 Cu^{2+} resin. Interconnecting tubing was 1.5 mm O.D. Buffer flow-rate was 1 ml/min. The column system was triplicated so that three samples could be prepared together.

Injection technique for plasma and urine. The 4-ml sample loop was filled using a 5-ml plastic syringe containing 5 ml filtered plasma. The loop was switched into the carbonate buffer flow. After 15 min the flow was diverted past the loop and 5 min later the solvent valve was switched to the ammonia solution. The next 1 ml eluting from the column was discarded and the following 5 ml which contained the compounds of interest were collected by means of a volumetric flask. Urine was treated in the same way except that a smaller loop was used (usually 500 µl).

The samples purified by ligand-exchange chromatography in the manner described were stored at 2° and generally analysed by HPLC within 24 h by the method described below.

High-pressure liquid chromatography. A Varian 4200 chromatograph (Varian Walton on Thames, Great Britain) was fitted with a fixed-wavelength (254 nm) absorbance detector. The injector was a stop-flow loop injector with 1-ml, 500-µl or 200-µl sample loops. The HPLC pre-column and columns were stainless-steel tubes 6.35 mm (¼ in.) O.D., 4.5 mm I.D., 30 mm and 70 mm long respectively, packed with Aminex A-27 (12—15 µm) anion-exchange resin. The column was totally enclosed in a water jacket and low-dead-volume reducing unions were used at the top and bottom of the columns, and for connecting the pre-column and column with the shortest possible length of 1.5 mm O.D. tubing.

The loop injector was filled with the ligand-exchange treated sample. The size of the loop depended on the concentration of drugs expected but generally 1 ml was used for plasma derived samples and 200 µl for urine derived samples.

The chromatography was then carried out isocratically using 1 mole/l ammonium acetate pH 8.7 at 1 ml/min at 71° as eluting solvent and an inlet pressure of 10—11.3 MN/m^2 (1500—1700 p.s.i.). Peak areas were integrated and recorded by a HP 3352 computer (Hewlett-Packard, Winnersh, Great Britain).

Calibration method. The HPLC chromatograph performance was always checked by running the HPLC H, A, X, O stock solution before any samples. The three Chelex columns were calibrated by running the range of prepared standards through the described procedure. Unknown samples were analysed in the same way as the prepared standards.

RESULTS

Fig. 1a shows a typical chromatogram of the stock H (R_t = 4.45 min), X (9.10 min), A (5.98 min) and O (17.08 min) solution. The elution order was always reproducible with good resolution, and readily interpreted by the integrator system. No major peaks were seen after the elution of O. The HPLC column (including pre-column) usually gave a height equivalent to theoretical plate (HETP) value of 0.125—0.0625 mm. When distortion of the X and O peaks oc-

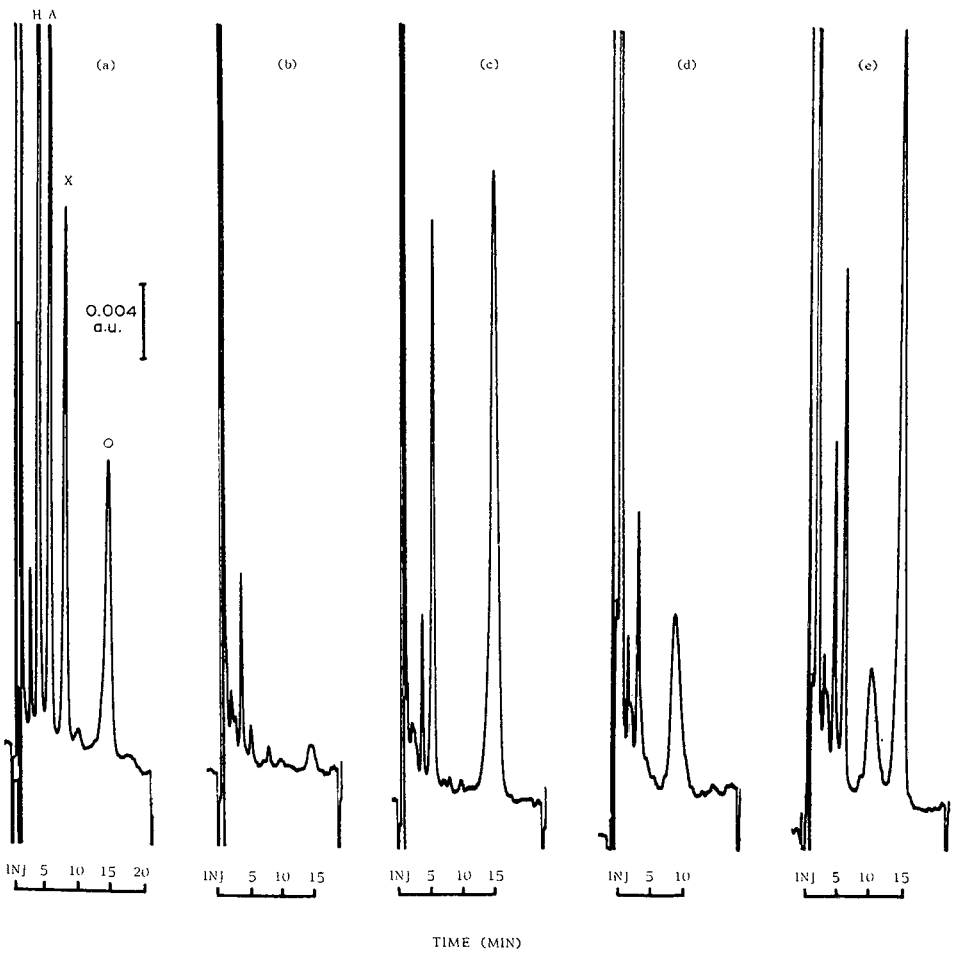

Fig. 1. Typical chromatograms of (a) a standard containing hypoxanthine (H), xanthine (X), allopurinol (A) and oxipurinol (O), (b) blank urine, (c) standard urine, (d) blank plasma and (e) standard plasma.

curred, changing the pre-column restored column performance.

Figs. 1b, c, d and e are typical chromatograms of blank urine, standard urine, blank plasma and standard plasma, respectively, showing an absence of interfering peaks.

When the plasma and urine standards were analysed, the data were collected and used to prepare calibration curves which were subjected to least squares regression analysis and tested for linearity. When the intercepts of the calibration lines did not significantly differ from zero, the least squares line, forced through the origin, was used. Urine calibration lines were all linear and no intercept differed significantly from zero ($2P > 0.05$). Table I summarises the statistical results for urine. The three slopes for A on Chelex columns 1, 2 and 3 did not differ significantly from each other and their common slope was highly significant ($2P < 0.001$). Fig. 2 shows the common line together with its 95% confidence limits. The three slopes for O differed significantly but consistently from each other and this was taken into account in the quantitation of O in urine.

TABLE I

CALIBRATION LINES FROM URINE FOR ALLOPURINOL AND OXIPURINOL, FORCED THROUGH THE ORIGIN, FROM CHELEX COLUMNS 1, 2 AND 3

	Chelex column	Calibration line*	R^2
Allopurinol	1	$y = 60.839x$	0.990
	2	$y = 64.007x$	0.989
	3	$y = 61.862x$	0.995
Common regression line		$y = 62.329x$	0.991
Oxipurinol	1	$y = 40.507x$	0.974
	2	$y = 45.223x$	0.976
	3	$y = 41.869x$	0.993

No common regression derived as slopes are significantly different.

*y = Integrated peak area (mV·sec); x = concentration (mmole/l).

Plasma calibration lines were all linear and no intercept differed significantly from zero. Three slopes each for A and O were all linear and no intercept differed significantly from zero and the common slope was highly significant ($2P < 0.001$). Table II summarises the statistical results for plasma.

Inclusion of the standard of the highest concentration reduced the slope of the A calibration lines, which did not then always pass through the origin. Hence the method was only valid for concentrations up to 10 μmole/l where the calibration was linear. Samples with original concentrations higher than this must first be diluted.

A 0—12-h urine sample from a healthy female volunteer who had taken 300 mg A (as a standard tablet) was repeatedly analysed. For each analysis 0.5 ml of urine was filtered, 5 ml were collected from the Chelex column and 500 μl of this eluate were injected on to the HPLC column. The results show a mean

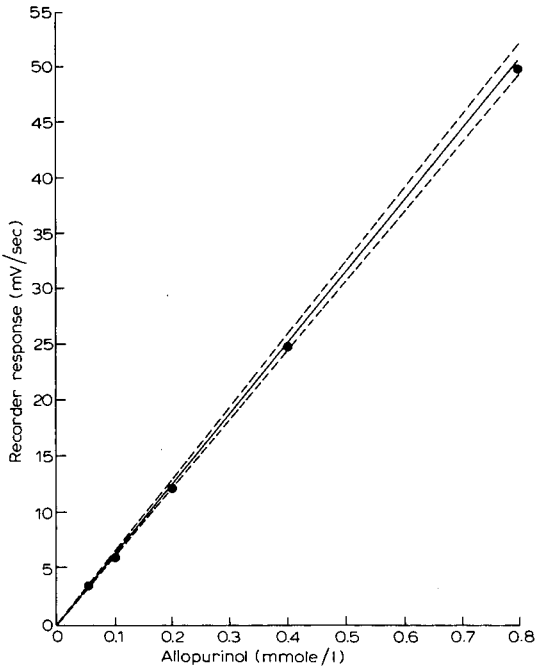

Fig. 2. A typical calibration line showing the common slope (with 95% confidence limits) from Chelex columns 1, 2 and 3 for allopurinol in repeatedly analysed (n = 10) urine standards.

TABLE II

CALIBRATION LINES FROM PLASMA FOR ALLOPURINOL AND OXIPURINOL. FORCED THROUGH THE ORIGIN FROM CHELEX COLUMNS 1, 2 AND 3

	Chelex column	Calibration line*	R^2	
Allopurinol	1	y = 2.012x	0.998	n.s. diff.**
	2	y = 2.043x	0.996	
	3	y = 1.960x	0.992	
Common regression line		y = 2.010x	0.996	
Oxipurinol	1	y = 1.580x	0.995	n.s. diff.
	2	y = 1.528x	0.996	
	3	y = 1.574x	0.997	
Common regression line		y = 1.559x	0.996	

*y = integrated peak area (mV·sec); x = concentration (mmole/l).
**ns diff. = not significantly different.

value of 0.3133 mmole/l (S.D. 0.01451) for A and 0.6552 mmole/l (S.D. 0.0226) for O. Fig. 3 is a typical plasma profile from a patient who had received 300 mg A showing the rapid absorption and elimination of A, the formation and slow elimination of O and the concentration of each drug usually encountered after a single oral dose in man.

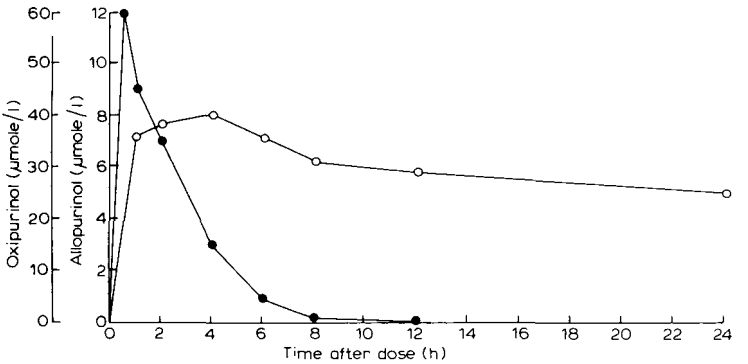

Fig. 3. Typical plasma profile of allopurinol (●) and oxipurinol (○) from a patient who received one 300-mg dose of allopurinol orally.

DISCUSSION

Several methods of determining A and O have been published but each has limitations as a routine analytical technique in biological fluids. Methods based on electrophoresis and polarography lack the requisite sensitivity and selectivity. Anion-exchange chromatography of purine nucleotides is well documented [4] and was exploited successfully with HPLC [3, 6]. However, when applied to the purine bases H, A, X and O there was the complication caused by the many other anions normally present in biological fluids. The peaks of interest elute soon after the void volume so that an unnecessary delay of about 6 h is encountered while waiting for the elution of the later peaks (nucleotides, etc.). Even the use of complex gradients with expensive high-performance ion-exchange packings gave an analysis time of 40 min but reproducibility and column life suffered. Attempts to simplify these procedures proved unsuccessful as no way of selectively removing the later eluting peaks was found. Attempts to concentrate the sample after removing protein (e.g., ultrafiltration, trichloroacetic acid, etc.) led to variable recovery. An internal standard as recently described by Endele and Lettenbauer [3] overcame this variability to some extent but the basic problem of the numerous other anions persists with the associated problems such as short column life.

These shortcomings prompted the search for selective purification for A and O by adsorption. A xanthine oxidase competitive binding technique was rejected because labelled material would be needed routinely and A would be a substrate for the production of O. The method now recommended originated from the observation that purines form an almost insoluble Cu^{2+} complex. As described above, when diluted raw plasma or urine passes down a column of Cu^{2+} Chelex-100 in an analogous manner to that described by Siegel and Degens [6], followed by a stepwise elution with ammonia, H, A, X and O are exclusively retained in the 10 mole/l ammonia fraction. On directly applying this fraction to the ion-exchange column no major peaks eluted after O showing that neither uric acid [7] nor the other late peaks were retained with H, A, X or O by the Cu^{2+} Chelex. The HPLC ion-exchange system was so simplified by the inclusion of ligand exchange that a total analysis time of 25—30 min was possible in the

isocratic mode as opposed to a minimum of 40 min with a gradient elution system. Likewise the ligand-exchange system was straightforward and the overall throughput using the two-column system was better than that of either system alone.

The use of copper Chelex-100 is not itself without problems; other authors [6, 8, 9] have complained of Cu^{2+} ion bleed from the columns especially after the running of biological samples. However the extent of the Cu^{2+} ion bleed diminishes with use and can be attenuated to an acceptable level if the column is initially treated with 0.5 ml injection of 10 μg/ml glycine solution. The recoveries of H, X, A and O were only quantitative on glycine-treated or "old" columns and the recoveries of X and O were only quantitative at pH 11.0.

Some system overloading can be detected (>10 μmole/l A) but in practice these higher levels can be measured by simply using less sample volume in the filtration step. When the lower sample level of 0.5 ml was used for urine, Chelex column life was longer and gave fewer problems (e.g. blocked frits) than the higher level.

The binding characteristics of H and X to Chelex were observed to be similar to A and O; this suggests that the method can be adapted to measure these purines. Indeed, where H and X are to be measured in biological fluids not containing A or O, these latter compounds may be used as internal standards. However, as H and X are always present in biological fluids, "blank" samples would be difficult to obtain and such a method would only detect changes in level.

In conclusion, the method is suitable for analysing A in the range 0.05—10 μmole/l (0.0068—1.36 μg/ml) in plasma and 0.005—1 mmole/l (0.68—136 μg/ml) in urine and O in the range 0.5—100 μmole/l (0.076—15.2 μg/ml) in plasma and 0.1—2 mmole/l (15.2—304 μg/ml) in urine.

REFERENCES

1 G.B. Elion, Ann. Rheum. Dis., 25 (1966) 608.
2 C.P. Rodnan, J.A. Robin, S.F. Tolchin and G.B. Elion, J. Amer. Med. Ass., 231 (1975) 1143.
3 R. Endele and G. Lettenbauer, J. Chromatogr., 115 (1975) 228.
4 P.R. Brown, S. Bobick and F.L. Hanley, J. Chromatogr., 99 (1974) 587.
5 D.J. Nelson, C.J.L. Bugge, H.C. Krasny and G.B. Elion, Biochem. Pharmacol., 22 (1973) 2003.
6 A. Siegel and E.T. Degens, Science, 151 (1966) 1098.
7 J.C. Wolford, J.A. Dean and G. Goldstein, J. Chromatogr., 62 (1971) 148.
8 N.R.M. Buist and D. O'Brien, J. Chromatogr., 29 (1967) 398.
9 G. Goldstein, Anal. Biochem., 20 (1967) 477.

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Biomedical Applications

CHROMBIO. 017

Note

Rapid analysis of nicotine and cotinine in the urine of smokers by isocratic high-performance liquid chromatography

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(Received April 14th, 1976)

Nicotine and cotinine can be detected using ultra-violet spectroscopy after basic extraction [1], and may be separated by thin-layer chromatography [2]. There are several methods utilising gas chromatography (GC) in use [3—5]. Thin-layer chromatography is a good qualitative method, ultra-violet spectroscopy will not differentiate between nicotine and cotinine, GC, although rapid and sensitive, does not allow rapid analysis of nicotine and cotinine, temperature programming being required, or replicate analysis of samples at a higher temperature [3].

Distillation [6] and multiple extraction [3] have been used to obtain complete recovery prior to estimation. Using the modification of Bell and Stewart [7] of the extraction procedure for amphetamine estimation [8], and an appropriate internal standard, a rapid, quantitative method for estimation of urinary nicotine and cotinine was evolved using high-performance liquid chromatography (HPLC).

MATERIALS AND METHODS

Nicotine was obtained from BDH (Poole, Great Britain) and a standard of 61.7 μmole/l in 0.1 *M* hydrochloric acid was prepared. Quinoline was also obtained from BDH, desmethylimipramine was donated by Mr. R. Sparks (Department of Pharmacology, Ninewells Hospital and Medical School, Dundee, Great Britain). Cotinine was prepared by the Dundee City Analyst. The bromothymol blue and the sodium hydroxide were also obtained from BDH.

CHROMATOGRAPHY CONDITIONS

A Varian 8500 chromatograph, with a Varian 635 series spectrophotometer was used. The stainless-steel column (25 cm × 2 mm I.D.) was packed with Micropak SI-10 silica gel. The operating conditions were as follows: temperature, ambient; pressure, 550 p.s.i.; flow-rate, 40 ml/h; chart speed, 25 cm/h; slit width, 1 nm; wavelength, 260 nm; solvent, ethyl acetate—propan-2-ol—ammonia (80:3:0.4).

EXTRACTION PROCEDURE

A 3-ml volume of urine in a conical centrifuge tube was made alkaline to 1% bromothymol blue using 5 *M* NaOH. 0.3 ml of the desmethylimipramine or quinoline internal standard was added and the sample was mixed for 30 sec on a whirlimixer. The tubes were centrifuged to clear any emulsions formed and 10 μl injected onto the column.

RESULTS

Recovery

Recoveries of 98—104% relative to the internal standard were obtained on standard samples.

Standards

A calibration curve for nicotine showed linearity over the range 0.6—60 μmole/l.

Desmethylimipramine was the internal standard for HPLC work. Quinoline was the internal standard for the GC method used to confirm the results obtained by HPLC.

10 μg/ml of desmethylimipramine and quinoline were dissolved in dichloromethane. This is equivalent to a concentration of 77.5 μmole/l of desmethylimipramine and 37.5 μmole/l of quinoline. As the available cotinine was not pure, cotinine peaks were calculated as nicotine.

Sensitivity

By suitable dilution of a standard solution the minimum detectable level was found to be 5 ng on column, i.e. equivalent to a concentration of 300 nmole/l.

Separation

Fig. 1 is a trace obtained from an extract of a smoker's urine, showing nicotine and cotinine with the internal standard desmethylimipramine. Fig. 2 shows the trace obtained when an extract from a non-smoker is chromatographed.

Precision

Replicate analysis of a smoker's urine showed a coefficient of variation of 7.3% for nicotine, 9.0% for cotinine, and the coefficient of variation of the calculated nicotine:cotinine ratios was 11.1%.

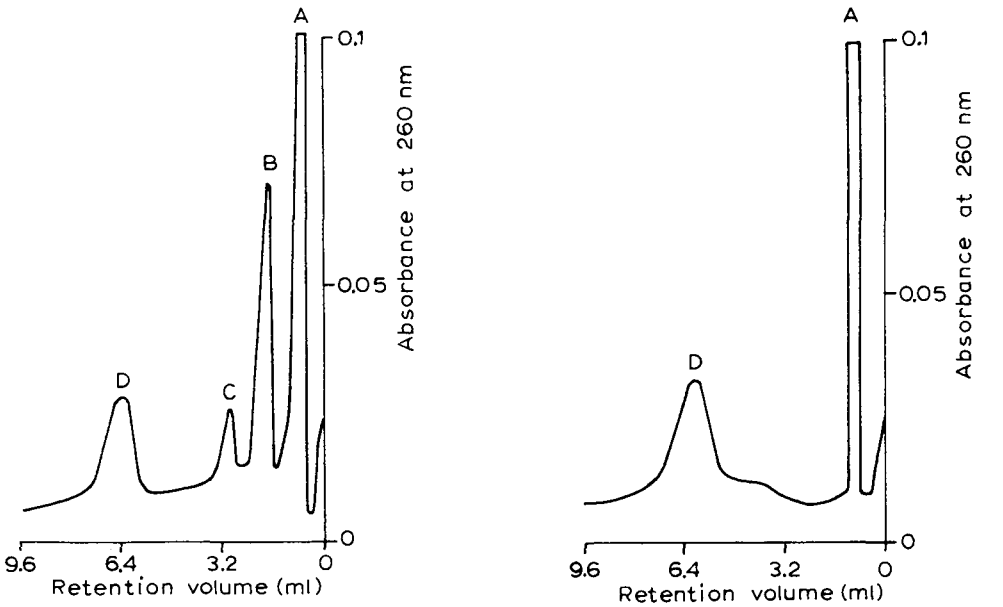

Fig. 1. Chromatogram of a smoker's urine. A = Solvent front, B = nicotine, C = cotinine, D = desmethylimipramine. Absorbance range, 0—0.1.

Fig. 2. Chromatogram of a non-smoker's urine. A = Solvent front, D = desmethylimipramine. Absorbance range, 0—0.1.

Specificity

As a tricyclic antidepressant is used as the internal standard, it is to be expected that such drugs might interfere with the analysis. No interference was noted from the common tricyclic antidepressants. Interference when found in HPLC, did not occur in the GC method. Similarly interference in the GC method was not observed in the HPLC method.

Correlation

Nicotine levels using the HPLC method were correlated with the GC method of Bell and Stewart [7]. Using 38 specimens, a correlation coefficient of 0.986 was found.

DISCUSSION

The major advantage of HPLC over the GC methods available, is the rapidity of elution of nicotine and cotinine (within 5 min), although if precise results are required a further 5 min are needed for elution of the HPLC internal standard. Work up time of specimens is fast, being based on the method of Ramsay and Campbell [8] for amphetamine. Previous published methods for nicotine and cotinine have involved lengthy extraction procedures [3, 6].

Solvent concentrations were found to be critical for peak shape and retention times, the latter is particularly sensitive to the degree of deactivation caused by propan-2-ol.

It has been reported that nicotine levels of 100—3000 μg/l (617 nmole/l—18.5 μmole/l) have been found in smokers' urine [9]. The results obtained in this investigation agree with these levels, nicotine concentrations ranging from 300 nmole/l—18.3 μmole/l, cotinine levels ranging from 250 nmole/l—3.45 μmole/l, and nicotine:cotinine ratios varying from 0.54—22.9. Most variation in the ratios appears to be attributable to the wider range of nicotine levels found. Cotinine is less frequently detected than nicotine.

The same authors claim that levels of 150 nmole/l of nicotine are seen in the urine of non-smokers, rising to 1.54 μmole/l when exposed to a smoky atmosphere. It is doubtful if the method presented here would have sufficient sensitivity to detect the lower limit precisely.

The chief advantage of this method is its rapidity in the estimation of nicotine and cotinine. Simultaneous nicotine and cotinine estimations could be performed by GC, but require temperature programming and are time consuming.

The use of HPLC eliminates the need for lengthy analysis times. Thus specimens from an anti-smoking clinic can be analysed as a patient waits, and objective evidence can be obtained of the truth of their statements that they have stopped or cut down, based on the nicotine levels measured. Nicotine metabolism may relate to the amounts detected.

The estimation of cotinine simultaneously with nicotine might be of use in metabolic studies in smokers.

ACKNOWLEDGEMENTS

I would like to thank Dr. M.J. Stewart for supplying the samples and for checking the manuscript.

REFERENCES

1 F. Schmidt, Int. Z. Klin. Pharm. Ther. Tox., 1 (1968) 461.
2 D.J. Berry and J. Grove, J. Chromatogr., 61 (1971) 111.
3 A.H. Beckett and E.J. Triggs, Nature (London), 211 (1966) 1415.
4 J.P. Cano, J. Catalin, R. Badre, C. Dumas, A. Viala and R. Guillerne, Ann. Pharm. Fr., 11 (1970) 633.
5 C. Feyerabend, T. Levitt and M.A.H. Russel, J. Pharm. Pharmacol., 27 (1975) 434.
6 Chemistry and Pharmacy of Nicorette, Technical Bulletin, Leo Laboratories Ltd. Halsävagen, Helsingborg, Sweden.
7 J. Bell and M.J. Stewart, unpublished results.
8 J. Ramsay and B.B. Campbell, J. Chromatogr., 63 (1971) 303.
9 M.A.H. Russel and C. Feyerabend, Lancet, i (1975) 179.

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Biomedical Applications

CHROMBIO. 015

Note

Probenecid, a possible interferent in the gas chromatographic determination of diphenylhydantoin

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The on-column alkylation of barbiturates and related compounds with reagents such as tetramethylammonium hydroxide (TMAH) or triethylanilinium hydroxide (TEAH) is a frequently used technique for the quantitative analysis of these compounds [1—6]. As these determinations are usually performed on sera from patients suffering from epilepsy with the object of helping the clinician to establish the optimal dose regimen, it is imperative that one should be aware of any drugs that may influence the quantitation of the antiepileptic drugs or produce a false positive result. We have found probenecid to be such a drug.

METHOD FOR THE ANALYSIS OF BARBITURATES AND DIPHENYLHYDANTOIN

Our laboratory is engaged in the routine analysis of phenobarbitone and diphenylhydantoin for an epilepsy clinic and we make use of the following method. To 1 ml serum are added secobarbitone and tolylphenylhydantoin as internal standards. The serum is acidified with 0.5 ml of 1 *M* H_3PO_4 and extracted with 3 ml of toluene by shaking for 2 min on a Whirlimixer. After centrifuging to separate the layers, the toluene phase is transferred into a conical centrifuge tube and 100 μl of 0.5 *M* TEAH solution in ethanol and 20 μl of water are added. After vigorous shaking for 2 min on a Whirlimixer, the tube is centrifuged and 4 μl of the bottom layer are injected during 7 sec into the gas chromatograph.

The ratios of the peak areas of N-ethylphenobarbitone and N-ethyldiphenylhydantoin to those of the two internal standards are used to quantitate the drugs.

EXPERIMENTAL

Conventional gas chromatography

A Hewlett-Packard 5700A gas chromatograph equipped with a flame-ionization detector was used. The peak areas were recorded on a Hewlett-Packard 3380A reporting integrator. Conditions: column length, 180 cm; temperatures: column, 150°, programmed at 8°/min to 270°; injector port and detector, 300°; stationary phase, 3% SE-30 on Chromosorb W (Supelco, Bellefonte, Pa., U.S.A.); carrier gas, nitrogen, at a flow-rate of 60 ml/min.

Gas chromatography—mass spectrometry (GC—MS)

A Varian CH5 mass spectrometer coupled to a Varian 2700 gas chromatograph and a Varian SS 100 computer was used for GC—MS analysis. A double-stage Watson-Biemann separator was used for enrichment of the eluting sample. Conditions: column length, 120 cm; temperatures: column, 120°, programmed at 8°/min to 270°; injector port, 270°; detector and separator, 280°; stationary phase, 3% Dexsil 300 on Supelcon AW DMCS, 80—100 mesh (Supelco, Bellefonte, Pa., U.S.A.); carrier gas, helium, at a flow-rate of 30 ml/min.

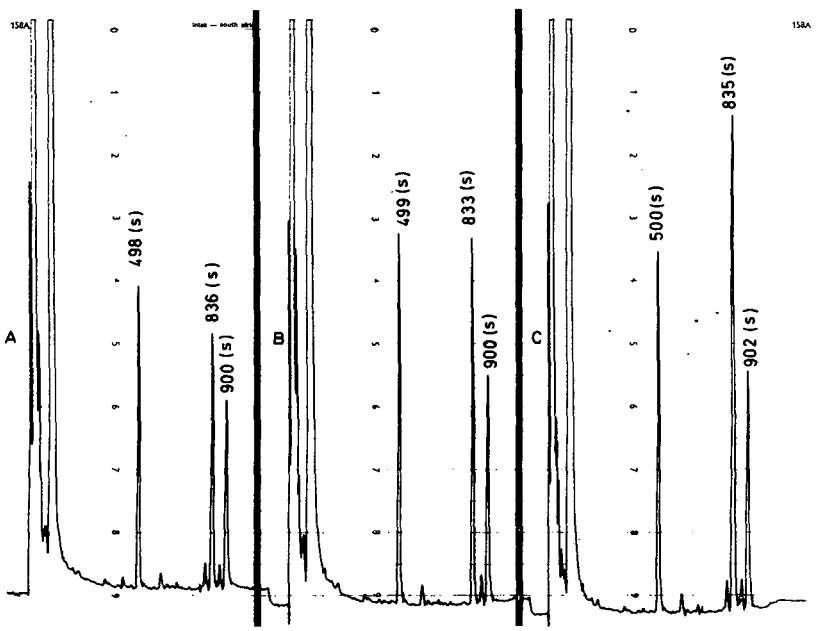

Fig. 1. A, Gas chromatogram of N-ethyldiphenylhydantoin (836 S), N-ethylsecobarbitone (498 S) and N-ethyltolylphenylhydantoin (900 S). B, Gas chromatogram of probenecid ethyl ester (833 S), N-ethylsecobarbitone (499 S) and N-ethyltolylphenylhydantoin (900 S). C, Gas chromatogram of a mixture of N-ethyldiphenylhydantoin and probenecid ethyl ester (835 S), N-ethylsecobarbitone (500 S) and N-ethyltolylphenylhydantoin (902 S). Column, 180 cm, glass, packed with 3% SE-30 on Chromosorb W, 80—100 mesh; column temperature, 150°, programmed at 8°/min to 270°; injector and detector temperatures, 300°; carrier gas (nitrogen) flow-rate, 60 ml/min; flame-ionization detection.

RESULTS AND DISCUSSION

During the investigation of a case of attempted suicide, we identified diphenylhydantoin as one of the drugs involved by using the method described above. As the gas chromatogram also showed peaks that could not be attributed to the more commonly used barbiturates, we decided to submit the sample to a GC—MS investigation. A 2-μl volume of the TEAH extract, obtained by the method described above, was injected and mass spectra were obtained during the entire analysis and the computer used to reconstruct the gas chromatogram. An investigation of the spectra of the eluates disclosed that the product identified previously as diphenylhydantoin was in fact some other substance, as its mass spectrum differed from that expected from N-ethyldiphenylhydantoin.

A computer-assisted library search identified the unknown product as probenecid ethyl ester, which was confirmed by subjecting an authentic sample of probenecid to the procedure described above, followed by GC—MS analysis.

A further investigation revealed that N-ethyldiphenylhydantoin and probenecid ethyl ester could not be separated gas chromatographically (Fig. 1) when 3% SE-30, 3% SP-2100, 3% Dexsil 300 or 3% OV-17 stationary phases were used.

We found that the detector responses for probenecid ethyl ester and N-ethyldiphenylhydantoin were virtually identical and also that probenecid taken therapeutically procudes blood levels equivalent to those expected to be found for diphenylhydantoin in therapeutic doses.

Probenecid is, therefore, a potential source of error when the method of on-column alkylation of diphenylhydantoin is applied, a fact which needs to be heeded when determining the latter drug for therapeutic or toxicological purposes.

REFERENCES

1 G. Kananen, R. Osiewicz and I. Sunshine, J. Chromatogr. Sci., 10 (1972) 283.
2 J. MacGee, Anal. Chem., 42 (1970) 431.
3 M.J. Barret, Clin. Chem. Newsletter, 3 (1971) 16.
4 M. Kowblansky, B.M. Scheintal, G.D. Gravallo and L. Chafetz, J. Chromatogr., 76 (1973) 467.
5 N. Kallberg, S. Agurell, B. Jalling and L.O. Boréns, Eur. J. Clin. Pharmacol., 3 (1971) 185.
6 E. Brochman-Hanssen and T. Olaquyi Oke, J. Pharm. Sci., 85 (1969) 370.

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Biomedical Applications

CHROMBIO. 012

Note

Thin-layer chromatographic determination of mafenide [(*p*-aminomethyl) benzenesulphonamide] in human serum

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(Received April 2nd, 1976)

Mafenide (Sulfamylon) is often used to combat infection in patients with burn wounds [1, 2], but it has been found [1—3] that an undesirable side-effect is the inhibition of carbonic anhydrase. As this enzyme is important with regard to the buffering system by which the kidney rids the body of excess of hydrogen ions, its inhibition would have the effect of reducing the kidney's efficiency as a protective organ against metabolic acidosis. High blood levels of mafenide may lead to acidosis and therefore the ability to monitor the blood levels of this drug may be of benefit to the patient.

This paper describes a simple, accurate method for the determination of mafenide in human serum. The method consists in partially deproteinating a small volume of serum, spotting the supernatant on a thin-layer chromatographic (TLC) plate together with serum standards, developing the plate and subjecting the mafenide to reaction in situ with fluorescamine to induce fluorescence. The intensities of the fluorescence of the various spots are then measured and peak heights of the standards are used to calculate the concentration of mafenide in unknown samples.

EXPERIMENTAL

Reagents

All reagents and solvents were of guaranteed reagent grade (E. Merck, Darmstadt, G.F.R.). Mafenide (Sulfamylon) was supplied by the Winthrop Laboratories Division of Sterling Drugs (Mobeni, Durban, South Africa). Fluorescamine was obtained from Hoffman-La Roche Diagnostica (Basle, Switzerland).

The spray reagent was 15 mg of fluorescamine dissolved in 200 ml of

acetone. The thin-layer developing solvent was an ethyl acetate—methanol—ammonia (75 : 20 : 5) solution.

Apparatus

A Perkin-Elmer MPF 3 spectrofluorimeter equipped with a thin-layer scanning attachment was used to measure the fluorescence of the spots on the thin-layer plates using the following operating conditions: light source, xenon lamp; excitation wavelength, 390 nm; excitation slit width, 10 nm; emission wavelength, 490 nm; scan speed, "high"; paper speed, 2.5 cm/min. The emission slit width, amplifier sensitivity and sample adjustment were set to obtain about 80% of full-scale deflection on the recorder when the strongest spot in the chromatogram was being scanned.

Other equipment included silica gel 60 TLC plates (Merck) and 5-μl disposable glass micropipettes (Clay-Adams, Division of Becton, Dickinson & Co., Parsippany, N.Y., U.S.A.).

Stock solutions

Stock solutions were made up in absolute methanol and stored at −20°. Stock solutions containing 1, 2, 4, 8, 10, 15, 20 and 40 μg mafenide per 100 μl of methanol were prepared as follows. The mafenide was weighed on a Mettler ME 22 electronic microbalance and dissolved in absolute methanol to yield a solution containing 10 mg of mafenide per 10 ml of methanol. By further appropriate dilutions, the above stock solutions were prepared. These stock solutions can be stored at −20° for at least a month without deterioration.

Standard solutions

Eight standard solutions were prepared by evaporating under nitrogen 100 μl of each of the above stock solutions in screw-capped bottles and then adding 1 ml of fresh, drug-free human serum. The containers were tightly closed and allowed to stand at room temperature for 1 h, with agitation at regular intervals to ensure complete dissolution of the mafenide. These standard solutions were freshly prepared on each occasion when a series of analyses were to be performed.

Preparation of serum

A 50-μl volume of serum (standard or unknown) was measured accurately into a small, stoppered, conical centrifuge tube, then 150 μl of methanol were added in order to precipitate the proteins. The contents of the tubes were mixed thoroughly by means of a Whirlimixer and centrifuged for 2 min to produce a clear supernatant liquid.

Spotting the plates

A 10-μl volume of the clear supernatant liquid was applied to the thin-layer plate in two equal portions of 5 μl. The liquid was applied in one smooth application and it was allowed to run on to the plate by the natural capillary action of the plate and gravity alone. The spot was dried with a hair-drier between applications. In this fashion, standard sera and unknown sera,

alternating in duplicate, were applied to a 10 × 20 cm plate. This procedure allowed four determinations on unknown sera to be carried out in duplicate.

Spraying the plate

The solvent was allowed to migrate to a height of 5 cm above the point of application, whereupon the plate was dried in an oven. After cooling, the plate was sprayed with the fluorescamine solution. The spraying apparatus must deliver a very fine mist and for optimal results great care must be taken to spray the plate evenly.

RESULTS AND DISCUSSION

The fluorescence peak heights measured for the serum standards were used to plot a standard graph of peak height versus mafenide concentration. Mafenide concentrations of unknown sera could be obtained by interpolation from this graph.

When determining the concentration of mafenide from unknown sera, standards between 1.0 and 8.0 μg/ml were applied to the plate. Whenever a sample with a higher concentration than 8.0 μg/ml was found, the determination was repeated with serum standards between 10 and 40 μg/ml. In these instances it was sufficient to apply only 5 μl of the supernatant.

Reproducibility and accuracy

The accuracy and reproducibility of the method were determined by preparing sera containing known amounts of mafenide and having these sera analyzed by a technician to whom the actual concentrations had not been revealed. The results were very satisfactory and are presented in Table I.

TABLE I

REPRODUCIBILITY AND ACCURACY OF THE DETERMINATION OF MAFENIDE IN SERUM

Mafenide added (μg/ml)	Mafenide recovered (μg/ml)	Mean ± S.D. (μg/ml)
1.6	1.45	1.49 ± 0.03
	1.52	
	1.48	
	1.50	
8.5	8.0	8.39 ± 0.54
	7.85	
	8.90	
	8.80	

Specificity

A variety of sulpha compounds (sulphisoxazole, sulphasomidine, sulpha-

dimethoxine, sulphafurazole, sulphamoxole, sulphatolamide and sulphanilamide) were tested for possible interferences, but none was found.

Limit of detection

The lower limit of detection that was possible without losing accuracy was 1 μg/ml. The response of the fluorimeter to the fluorescence was found to be linear up to 600 ng of mafenide.

REFERENCES

1 J.M. Shuck and J.A. Moncrief, *Amer. J. Surg.*, 118 (1969) 864.
2 Editorial Staff, *Drugs*, 1 (1971) 435.
3 H.N. Harrison, H.W. Bales and F. Jacoby, *J. Trauma*, 12 (1972) 994.

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Biomedical Applications

CHROMBIO. 019

Note

Simplified thin-layer chromatographic method for the simultaneous determination of clonazepam, diazepam and their metabolites in serum

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(First received May 13th, 1976; revised manuscript received June 25th, 1976)

When epileptic patients are admitted as emergency cases, they are usually given diazepam and, if the desired response is not observed, then clonazepam is often administered. This clinical procedure necessitates a method for the simultaneous and quantitative measurement of the two drugs and their metabolites.

Clonazepam [1], diazepam [2] and their metabolites are usually quantitatively measured separately by gas chromatography using electron-capture detection. As an alternative to this lengthy procedure, we have applied the method of Wad et al. [3], in which the diffuse light reflectance of both substances and their metabolites which have been simultaneously extracted and separated are measured directly on an unstained thin-layer chromatographic (TLC) plate.

METHOD

The extraction and separation were carried out according to the method of Wad et al. [3]. We are listing some precautions that are necessary for the optimal separation of the eight drugs when using this method.

The drugs are applied to the plate at a width of 0.8 cm and to a height of 2—3 mm. An application of height less than 2 mm will result in incomplete separation of the two metabolites of clonazepam.

The first separation solvent, chloroform—diethyl ether (60:40), is used primarily for the removal of natural interfering substances, but it also initiates the separation of the drugs. Thus development of this first separation phase by more than 14 cm will result in the merging of oxazepam with caffeine

and of N-desmethyldiazepam with 3-hydroxydiazepam.

The final solvent, chloroform—*n*-heptane—ethanol (50:50:5), must be developed to the top of the plate; if not, incomplete separation of oxazepam and 7-aminoclonazepam will occur.

To the stock standard solution (25 mg per 100 ml) were added 25 mg each of clonazepam, 7-aminoclonazepam and 7-acetamidoclonazepam (donated by F. Hoffmann-La Roche, Basle, Switzerland). This solution was then diluted 1:10 with absolute ethanol to give a 2.5 mg per 100 ml working standard solution. The standard curves for these additional drugs are shown in Fig. 1.

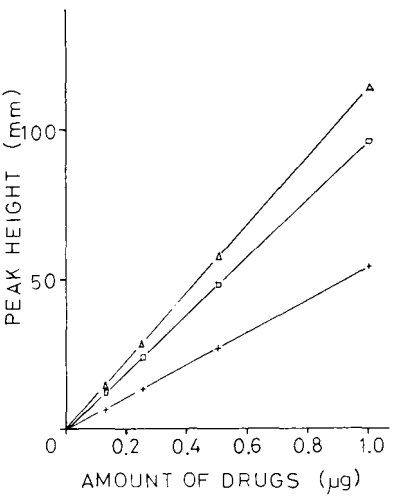

Fig. 1. Standard curves for clonazepam (△), 7-aminoclonazepam (□) and 7-acetamidoclonazepam (+) extracted from serum, expressed as peak height versus amount.

The scanning for diffuse light reflectance was executed at 230 nm for diazepam and its metabolites and at 250 nm for clonazepam and its metabolites by means of a Zeiss chromatogram-spectrophotometer.

RESULTS AND DISCUSSION

One of the advantages of our TLC method compared with GC methods is the ease of extraction of the native medicaments and their metabolites from serum [1, 2]. Only one extraction is necessary and the extracted substances are chromatographed without any derivatization. A major advantage over GC methods is the possibility of scanning the spots on the TLC plate directly in the UV range in order to obtain the absorption spectrum and then comparing this pattern with known absorption spectra for positive identification. Diazepam and its metabolites exhibit the same absorption spectrum whereas clonazepam and its metabolites exhibit dissimilar absorption spectra. Absorption spectra of clonazepam, diazepam and their metabolites are shown in Fig. 2.

Time is employed more efficiently in our TLC method than in GC methods: there is approximately 2.5 h of free time during the two developments of

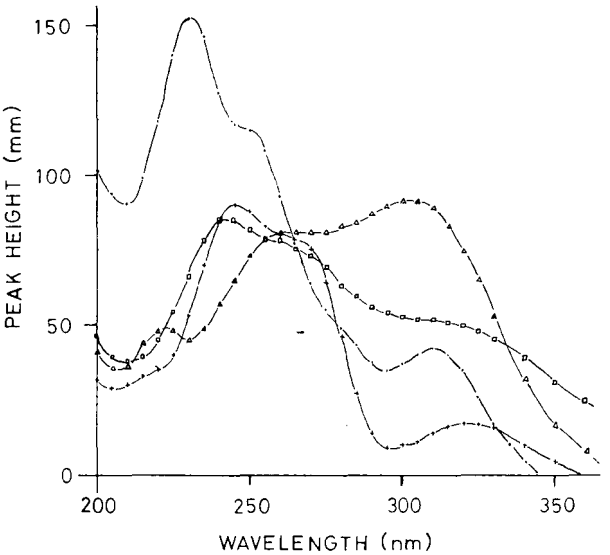

Fig. 2. Ultraviolet absorption spectra obtained by scanning a TLC plate containing 0.5 μg each of clonazepam (△), 7-aminoclonazepam (□), 7-acetamidoclonazepam (+), diazepam and the three metabolites of diazepam (•) which were applied directly on to a plate, chromatographically separated and measured in situ by means of a Zeiss chromatogram-spectrophotometer.

an eight-sample TLC plate, compared with 22 min for one GC separation of diazepam and its metabolites [2]. Clonazepam and its metabolites must be measured separately with GC [1]. Clonazepam is hydrolyzed into its corresponding benzophenone and then measured by GC, whereas the metabolites of clonazepam are measured directly without hydrolysis. The total time for these two GC runs is approximately 12 min.

The TLC separation of the working standard solution which was added to 1 ml of serum is shown in Fig. 3. The TLC separations of serum extracts from most of our patients (Fig. 4) exhibit a peak at R_F 0.25, which was found to be caffeine.

We have analyzed sera from 39 patients for therapeutic control of clonazepam using our TLC method. Only 12 of these sera exhibited a calculable peak for all three serum constituents of clonazepam. The mean values for these 12 sera were 62 ng/ml for clonazepam, 243 ng/ml for 7-aminodiazepam and 420 ng/ml for 7-acetamidodiazepam.

Interference from other anticonvulsive drugs administered in our hospital has not been observed. We have found only carbamazepine and sulfamethoxazol (a component of Bactrim) to be extracted and separated by this method, but they create no problems as they both have the same R_F value as caffeine.

The recovery and reproducibility of the method are presented in Table I. The recoveries were obtained by comparing the drugs directly applied in the same amounts as the drugs being extracted from serum, applied and separated. The reproducibility is the result of 30 analyses of the same serum sample to which the five drugs were added.

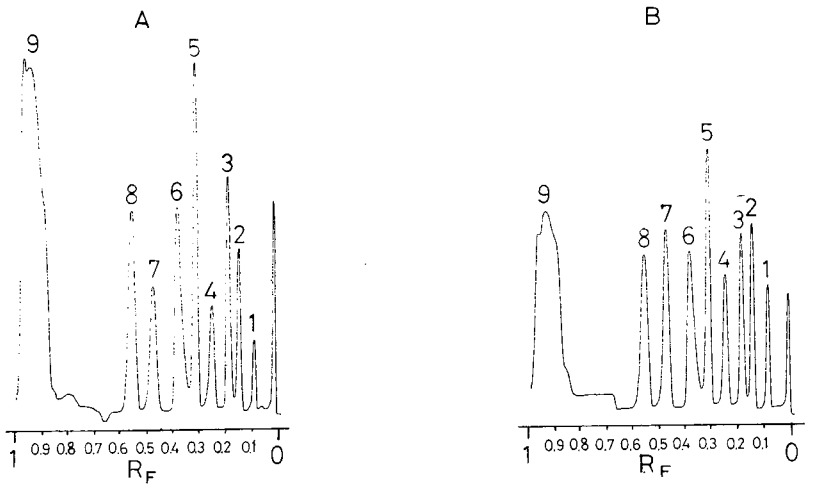

Fig. 3. Results obtained from scans at 230 nm (A) and 250 nm (B) of a TLC plate after the separation of 1 ml of a drug-free serum to which had been added 0.5 μg/ml each of diazepam, clonazepam, and their metabolites. The solvent system was chloroform—diethyl ether (60:40) followed by a second separation in chloroform—*n*-heptane—ethanol (50:50:5) [3]. Peaks:1 = 7-acetamidoclonazepam; 2 = 7-aminoclonazepam; 3 = oxazepam; 4 = caffeine; 5 = N-desmethyldiazepam; 6 = 3-hydroxydiazepam; 7 = clonazepam; 8 = diazepam; 9 = solution front containing the naturally occurring substances in serum.

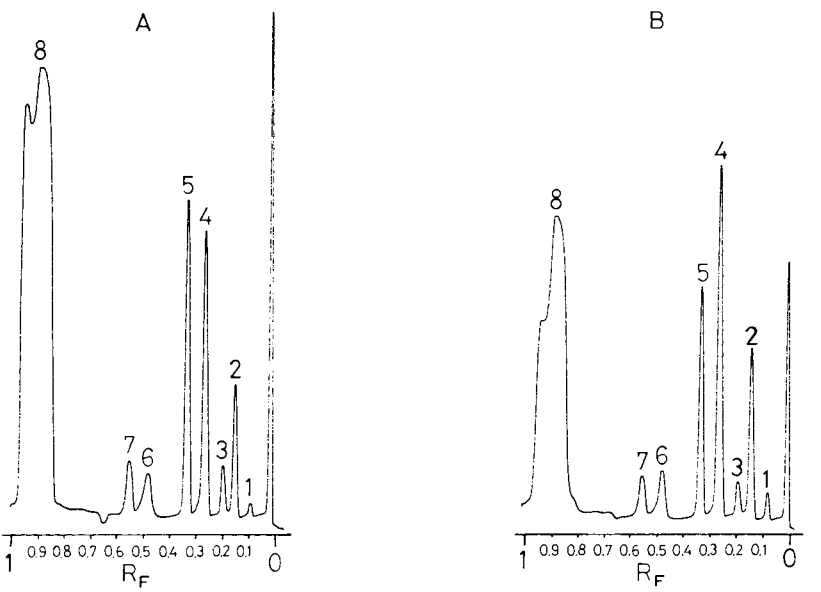

Fig. 4. Results obtained from scans at 230 nm (A) and 250 nm (B) of a TLC plate after the separation of 1 ml of serum of a patient. The solvent system was chloroform—diethyl ether (60:40) followed by a second separation in chloroform—*n*-heptane—ethanol (50:50:5) [3]. Peaks: 1 = 7-acetamidoclonazepam; 2 = 7-aminoclonazepam; 3 = oxazepam; 4 = caffeine; 5 = N-desmethyldiazepam; 6 = clonazepam; 7 = diazepam; 8 = solution front containing the naturally occurring substances in serum.

TABLE I

RECOVERY OF THE DRUGS FROM SERUM AND REPRODUCIBILITY OF THE METHOD

Drug	Recovery (%)	Reproducibility (30 samples)	
		Mean ± S.D. (μg/ml)	C.V. (%)
Diazepam	92.7	0.80 ± 0.04	5.4
N-Desmethyldiazepam	93.9	0.47 ± 0.02	4.7
3-Hydroxydiazepam	99.0	0.42 ± 0.01	2.4
Oxazepam	86.8	0.51 ± 0.04	8.5
Clonazepam	98.6	0.56 ± 0.03	5.2
7-Aminoclonazepam	77.1	0.59 ± 0.04	6.8
7-Acetamidoclonazepam	40.3	0.51 ± 0.03	4.8

It can be concluded that our quantitative TLC method is rapid and precise and the results are well within the accepted limits of deviation.

REFERENCES

1 J. Naestoft and N.E. Larsen, J. Chromatogr., 93 (1974) 113.
2 J.A.F. de Silva and C.V. Puglisi, Anal. Chem., 42 (1970) 1725.
3 N. Wad, H. Rosenmund and E. Hanifl, J. Chromatogr., 128 (1976) 231.

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Biomedical Applications

CHROMBIO. 033

Book Review

High pressure liquid chromatography in clinical chemistry, edited by P.F. Dixon, C.H. Gray, C.K. Lim and M.S. Stoll, Academic Press, New York, London, 1976, XXV + 224 pp., price US$ 12.25, ISBN 0-12-218450-5.

This book presents the Proceedings of a Symposium held at King's College Hospital Medical School, London, December 15—16, 1975. As claimed by the authors in the Preface, the aims of the Symposium were (1) to find out what has been done in high-pressure liquid chromatography (HPLC) in clinico-chemical laboratories in Great Britain and (2) to discuss the pathways of further development of this technique. Although the participants were mainly from Great Britain, 28 came from other countries. The attendance of local workers was surprisingly high (about 170), which illustrates clearly the interest in the applications of modern liquid column chromatography in clinical chemistry.

Twenty-nine lectures are included, three being only abstracts. About one third of papers are devoted to the area that has become very popular in routine analysis, viz. the analysis of drugs for drug monitoring purposes. The main part deals with compounds that are routinely analyzed in clinical laboratories, the main attention being focused on porphyrins (4), steroids (3) and biogenic amines and their metabolites (3). In many instances, however, the technique has been developed with pure substances, which suggests future possibilities for HPLC. About 10 papers are immediately applicable in clinical analysis; these are devoted to the HPLC of lipids (Worth, Carter et al.), porphyrins (Evans et al., Gray et al., Carlson and Dolphin), steroids (Fantl et al., Butler et al.) and biogenic amines and their metabolites (Jurand, Leppard et al.). The subjects of some of the papers presented lie beyond the limits of interest of clinical chemists (pollutants, triterpenes, bitter principles).

For a final judgement on this book, the routine criteria used for monographs are not applicable: firstly, the book deals with the Proceedings of a Symposium and secondly, it is the first volume to be devoted solely to the application of HPLC in clinical chemistry. This book has appeared when the technique of HPLC is only beginning to penetrate clinical laboratories and the Editors have tried to show the possibilities offered by this technique. The optimal time for a critical monograph will come in about 5 years, while on the other hand one has to agree with the Editors that in 10 years HPLC will seem much more at home

in clinical laboratories than does GC today.

In conclusion, Academic Press must be congratulated for producing these Proceedings within 5 months after the Symposium (the book was available in May, 1976) and for giving the book a very reasonable price.

Prague (Czechoslovakia) K. MACEK

Vol. 143, No. 2

JOURNAL OF CHROMATOGRAPHY
BIOMEDICAL APPLICATIONS

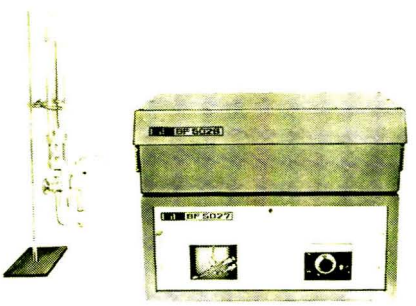
NEWS SECTION

APPARATUS

N-949

RADIOMETRIC DETECTOR FOR LC

The BF 5025 from ESI Nuclear is a new continuous radioactivity measuring system for column liquid chromatography. In use with HPLC columns the measuring volumes can be as small as 20 μl. The liquid scintillator is mixed directly with the column effluent, the homogeneous mixture then being measured continuously in a flow-type scintillation detector. A variable splitter unit is available for those cases where some of the effluent is required uncontaminated by scintillant. Sensitivity: 95% for ^{14}C, 50% for $^{3}H_2$. The instrument offers a means of separating and identifying the metabolites of new drugs, pesticides and herbicides using LC.



N-957

ELECTROPHORESIS SYSTEMS

A 12-page colour brochure from Beckman Instruments describes the Microzone electrophoresis systems and features the recently developed fluorescence scanning capability for the CDS-100 computing densitometer and R-112 scanning densitometer, along with details of electrophoresis instrumentation, accessories, supplies and technical support.

CHEMICALS

N-944

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Gelman's new Super Sepraphore cellulosic electrophoresis strips consist of a microporous electrophoresis membrane laminated to a thin mylar support, and are particularly suitable for the electrophoresis of serum proteins, hemoglobins and isozymes of LDH and CPK. The strips measure 5.7 x 14.4 cm (suitable both for the Gelman Sepratek System and the Beckman Microzone System).

N-946

METHYLATING AGENT FOR GLC DRUG DETERMINATIONS

MethElute™ is an on-column methylating agent being offered by the Pierce Chemical Co. The reagent is used for the quantitative methylation and detection of barbiturates, sedatives, xanthine bases, phenolic alkaloids, and dilantin, by gas chromatography. MethElute is available in Hypo-Vials™ of 10 ml or 1 ml.

N-971

DISPOSABLE, PREPACKED SEPHADEX COLUMNS

Pharmacia Columns PD-2 and PD-10 are polypropylene columns prepacked with Sephadex G-25 Medium. The PD-2 would be suitable in assay systems where it is necessary to separate small molecules from large ones. The bed volume is 1.5 ml and the columns are equilibrated with 0.08 *M* NaOH (pH 12), for serum T_3 or T_4 analysis for example, or are equilibrated with citrate : phosphate buffer (pH 5) containing 0.1% formaldehyde.

The larger PD-10 columns (bed volume 9.1 ml) are equilibrated in 0.9% NaCl containing 0.01% of Merthiolate, and are suitable for routine group separations with sample volumes up to 2.5 ml, such as desalting protein solutions, buffer exchange and purification of small samples. The columns may be readily equilibrated with other aqueous buffer solutions or with water.

For further information concerning any of the news items, apply to the publisher, using the reply cards provided, quoting the reference number printed at the beginning of the item.

N-945

POLYACRYLAMIDE GRADIENT GELS FOR ELECTROPHORESIS

Pharmacia Fine Chemicals has introduced a preformed polyacrylamide gradient gel with a concave monomer concentration gradient of 2–16%. The gradient gels PAA 2/16 are particularly suitable for the analysis of complex mixtures of proteins in the M.W. range 100,000–5,000,000, and high molecular weight nucleic acids up to approximately 30 S. The gels are supplied in boxes of 8 with a stability of 18 months when refrigerated.

N-951

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Pharmacia Fine Chemicals has introduced Protein A, which is isolated from the cell wall of *Staphylococcus aureus*. Protein A has the biological property to interact with, and form precipitates with, a wide variety of IgG molecules from several species. It is available in 5-mg packs and also covalently bound to Sepharose CL-4B.

PROCEDURES

N-964

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NEW JOURNAL

Immunology Abstracts, edited by E.S. Krudy (Editor in Chief) and H. Whelan, Information Retrieval Limited, London. Subscription price: Vol. 1 (Nos. 1–12, 1976 + subject index), £ 75.00.

MEETINGS

10TH INTERNATIONAL CONGRESS OF CLINICAL CHEMISTRY

The 10th International Congress of Clinical Chemistry will be held in Mexico City from February 26–March 3, 1978.

The scientific program covers all aspects considered of great interest at the present time, as well as those in which interesting developments are expected at the time of the meeting. All scientific sessions will be held in the modern Congress Unit of the IMSS National Medical Center in halls with simultaneous translation equipment.

Preliminary topics for the scientific program include: plenary sessions; newer technology for clinical analytical methodology; nutrition; selection of tests, interpretation and use of clinical chemical data; cellular receptors; genetic aspects of perinatal problems.

The Congress Unit has a special exhibition area where the latest developments in equipment, instruments and presentation methods will be shown.

Further information can be obtained from Ms. Ana Laura Pulido, 10th International Congress of Clinical Chemistry, Apartado Postal 24-498, México 7, D.F.

4TH INTERNATIONAL SYMPOSIUM ON MASS SPECTROMETRY IN BIOCHEMISTRY AND MEDICINE

This Symposium will be organized by the Italian Group for Mass Spectrometry in Biochemistry and Medicine, and will be held in Riva del Garda, Lake of Garda, Italy, on June 20–22, 1977.

As in the past, the Symposium will be devoted to topics such as gas chromatography–mass spectrometry, mass fragmentography, stable isotope measurements, field ionization, field desorption, chemical ionization, high-resolution studies and data of acquisition and processing. The areas of application will include biochemistry, medicine, toxicology, drug research, forensic science, clinical chemistry and pollution. The Symposium will consist of presentations from invited speakers and free communications.

Those wishing to present a communication (approx. 20 min) are requested to submit the title and an abstract of no more than 200 words written in English, before March 1, 1977.

For further details please contact: Dr. Alberto Frigerio, Secretary of the Group, Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea 62, 20157 Milan, Italy.

CALENDAR OF FORTHCOMING MEETINGS

February 28–March 4, 1977 Cleveland, Ohio, U.S.A.	**28th Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy** Contact: John E. Graham, Program Chairman, Koppers Company, Inc., 440 College Park Drive, Monroeville, Pa. 15146, U.S.A.
April 26–28, 1977 Bratislava, Czechoslovakia	**5th International Symposium. Improvements and Application of Chromatography in the Chemical Industry** Contact: Ing. J. Remeň, Analytická sekcia ČS VTS, pri. n. p. Slovnaft, 82300 Bratislava, Czechoslovakia
May 2–6, 1977 Brugge, Belgium	**25th Annual Colloquium on Protides of the Biological Fluids** Contact: Simon Stevin Institute, Jerusalemstraat 34, B-8000 Brugge, Belgium

May 2–6, 1977 Hindelang, G.F.R.	**2nd International Symposium on Glass Capillary Chromatography** Contact: Dr. R.E. Kaiser, Institut für Chromatographie, Postfach 1308, D-6702 Bad Dürkheim-1, G.F.R.
May 8–20, 1977 Brugge, Belgium	**The Lipoprotein Molecule** Contact: Dr. Hubert Peeters, Simon Stevin Institute, Jerusalemstraat 34, B-8000 Brugge, Belgium
May 22–27, 1977 Davos, Switzerland	International Symposium on Microchemical Techniques 1977 Contact: Dr. W. Merz, BASF A.G. Untersuchungslaboratorium, WHU, D-6700 Ludwigshafen, G.F.R. (Further details published in Vol. 117, No. 1)
June 20–22, 1977 Riva del Garda, Italy	**4th International Symposium on Mass Spectrometry in Biochemistry and Medicine** Contact: Dr. A. Frigerio, Secretary of the Group, Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea 62, 20157 Milan, Italy
July 17–22, 1977 Birmingham, Great Britain	**4th SAC Conference** Contact: Dr. A. Townshend or Dr. A.M.G. Macdonald, Chemistry Department, The University, P.O. Box 363, Birmingham B15 2TT, Great Britain (Further details published in Vol. 125, No. 3)
September 27–30, 1977 Salzburg, Austria	3rd International Symposium on Column Liquid Chromatography Contact: Verein Österreichischer Chemiker, Eschenbachgasse 9, A-1010 Vienna 1, Austria (Further details published in Vol.132, No. 1
November 7–10, 1977 Amsterdam, The Netherlands	**Chromatography '77. 12th International Symposium on Advances in Chromatography** Contact: Professor A. Zlatkis, Chemistry Department, University of Houston, Houston, Texas 77004, U.S.A. (Further details published in Vol. 131)
Feb. 26–March 3, 1978 Mexico City, Mexico	**10th International Congress of Clinical Chemistry** Contact: Ana Laura Pulido, P.O. Box 24-498, Mexico 7 D F
April 18–22, 1978 Munich, G.F.R.	**Analytica 78** Contact: Münchener Messe- und Ausstellungsgesellschaft, Messegelände, P.O. Box 121009, D-8000 Munich 12, G.F.R.
September 25–29, 1978 Baden-Baden, G.F.R.	**12th International Symposium on Chromatography** Contact: Gesellschaft Deutscher Chemiker, P.O. Box 900440, D-6000 Frankfurt/Main 90, G.F.R.

GENERAL INFORMATION

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

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

Manuscripts. Manuscripts should be typed in double spacing on consecutively numbered pages of uniform size. The manuscript should be preceded by a sheet of manuscript paper carrying the title of the paper and the name and full postal address of the person to whom the proofs are to be sent. Authors of papers in French or German are requested to supply an English translation of the title of the paper. As a rule, papers should be divided into sections, headed by a caption (*e.g.*, Summary, Introduction, Experimental, Results, Discussion, etc.). All illustrations, photographs, tables, etc. should be on separate sheets.

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Illustrations. The figures should be submitted in a form suitable for reproduction, drawn in Indian ink on drawing or tracing paper. One original and two photocopies are required. Attention should be given to any lettering (which should be kept to a minimum) and to spacing on axes of graphs in order to ensure that numbers etc. remain legible after reduction. Axes of a graph should be clearly labelled. The figures should preferably be of such a size that the same degree of reduction can be applied to all of them. Photographs should have good contrast and intensity. Sharp, glossy photographs are required to obtain good halftones. References to the illustrations should be included in appropriate places in the text using arabic numerals. Each illustration should have a legend, all the *legends* being typed (with double spacing) together on a *separate sheet*. If structures are given in the text, the original drawings should be supplied. Coloured illustrations are reproduced at the authors' expense, the cost being determined by the number of pages and by the number of colours needed. The written permission of the author and publisher must be obtained for the use of any figure already published. Its source must be indicated in the legend.

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1 A. T. James and A. J. P. Martin, *Biochem. J.*, 50 (1952) 679.
2 L. R. Snyder, *Principles of Adsorption Chromatography*, Marcel Dekker, New York, 1968, p. 201.
3 Z. J. Zmrhal, J. G. Heathcote and R. J. Washington, in Z. Deyl, K. Macek and J. Janák (Editors), *Journal of Chromatography Library, Vol. 3, Liquid Column Chromatography — A Survey of Modern Techniques and Applications*, Elsevier, Amsterdam, Oxford, New York, 1975, p. 665.
4 R. H. Doremus, B. W. Roberts and D. Turnbull (Editors), *Growth and Preparation of Crystals, Proc. Int. Conf. Crystal Growth, Coopertown, N.Y., August 27–29, 1958*, Wiley, New York, 1958.

Abbreviations for the titles of journals should follow the system used by *Chemical Abstracts*. Articles not yet published should be given as "in press", "submitted for publication", "in preparation" or "personal communication". The *Journal of Chromatography; Journal of Chromatography, Biomedical Applications* and *Chromatographic Reviews* should be cited as *J. Chromatogr.*

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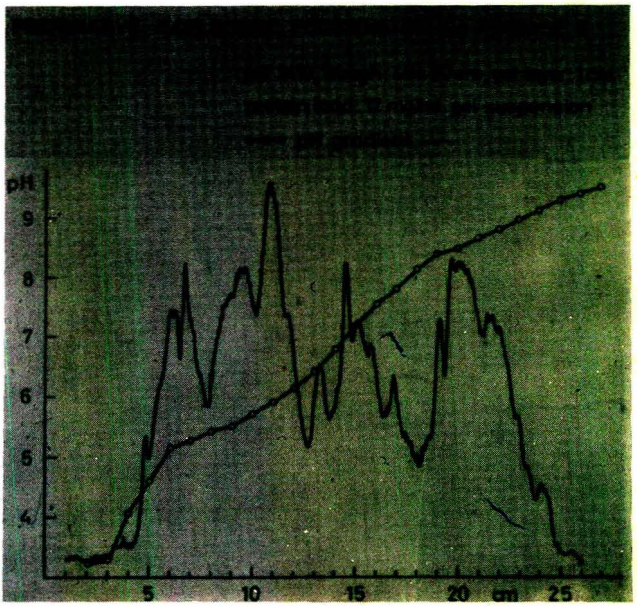

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