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Journal of Chromatography (incorporating *Biomedical Applications* and *Chromatographic Reviews*)
In the course of 1977, also the cumulative indexes for Vols. 121–130 and 131–140 will appear.

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
Biomedical Applications	143/1		143/2		143/3		143/4		143/5		143/6	
Chromatographic Reviews				141/1				141/2				141/3

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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or to:
The Editor of *Journal of Chromatography, Biomedical Applications*, P.O. Box 681, Amsterdam, The Netherlands.
Reviews are invited or proposed by letter to the Editors and will appear in *Chromatographic Reviews* or *Biomedical Applications*. An outline of the proposed review should first be forwarded to the Editors for preliminary discussion prior to preparation.

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Publication. The *Journal of Chromatography* (including *Biomedical Applications* and *Chromatographic Reviews*) has 15 volumes in 1977. The subscription price for 1977 (Vols. 130–144) is Dfl. 1650.00 plus Dfl. 210.00 (postage) (total *ca.* US$ 744.00). The subscription price for the *Biomedical Applications* section only (Vol. 143) is Dfl. 110.00 plus Dfl. 14.00 (postage) (total *ca.* US$ 49.60). Journals are automatically sent by air mail to the U.S.A. and Canada at no extra costs, and to Japan, Australia and New Zealand with a small additional postal charge. Back volumes of the *Journal of Chromatography* (Vols. 1 through 129) are available at Dfl. 100.00 (plus postage). Claims for issues not received should be made within three months of publication of the issue. If not, they cannot be honoured free of charge.

For further information, see page 3 of cover.

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(Continued overleaf)

Bibliography Section

RS Solvents for the analysis of pesticide residues

On the recommendation of international organisations such as FAO and WHO, many nations throughout the world have felt the necessity to regulate the use and the control of pesticides in foodstuffs.
The analysis of pesticide residues presents unusual problems because of the small amounts of substances to be determined and the large number of possible interfering substances which must be first eliminated. In order to determine extremely small quantities, very sensitive analytical methods are required, which however cannot be applied directly to the substances under examination. The general procedure is:

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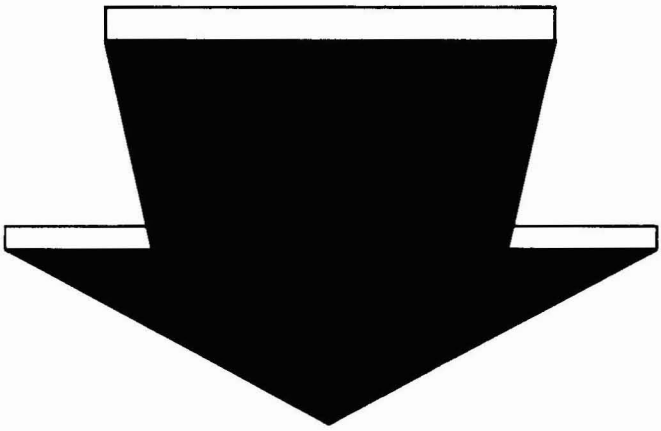

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Journal of Organometallic Chemistry Library

Volume 3

Organometallic Chemistry Reviews

edited by **D. SEYFERTH, A.G. DAVIES, E.O. FISCHER, J.F. NORMANT** and **O.A. REUTOV.**

1977. viii+342 pages. US $41.95/Dfl. 103.00. ISBN 0-444-41538-6

This volume contains the second collection of subject reviews and presents topics in main group organometallic chemistry as well as in transition metal-organic chemistry. The organic chemistry of calcium, strontium and barium, long neglected and underdeveloped, has received more attention in recent years and these new results are surveyed in the first article. The oxidation of many types of π-alkylmetal compounds of the Group II and III elements results in formation of organoperoxy derivatives; the chemistry of this important class of compounds is reviewed here in detail. In the transition metal area, the chemistry of the novel polypyrazolyl borate-metal complexes has been updated and the organometallic chemistry of the *f*-orbital elements, reviewed. The organometallic chemistry of titanium, an important area from the industrial point of view, has been surveyed and the interesting chemistry of the π-arene-π-cyclopentadienyliron cations, described. These critical, in-depth reviews cover a broad cross-section of organomettalic chemistry and will serve to bring the reader up-to-date in a number of important current areas in this ever-growing field of research.

CONTENTS: The Organometallic Chemistry of the Alkaline Earth Metals *(B.G. Gowenlock and W.E. Lindsell).* Organic Peroxides of the Main Group II Elements *(Yu.A. Alexandrov and V.P. Maslennikov).* Organic Peroxides of the Main Group III Elements *(Yu.A. Alexandrov and V.P. Maslennikov).* Metal Complexes of Polypyrazolylborates: Recent Developments *(A. Shaver).* Recent Advances in the Organometallic Chemistry of the Lanthanides and Actinides *(S.A. Cotton).* Recent Advances in the Organometallic Chemistry of Titanium *(R.J.H. Clark, S. Moorhouse, and J.A. Stockwell).* π-Arene-π-cyclopentadienyl-iron Cations and Related Systems *(R.G. Sutherland).*

Volume 2

Organometallic Chemistry Reviews: Organosilicon Reviews

edited by D. SEYFERTH, A.G. DAVIES, E.O. FISCHER, J.F. NORMANT and O.A. REUTOV.

1976. viii+404 pages. US $41.95/Dfl. 103.00. ISBN 0-444-41488-6

The field of organosilicon chemistry has received much attention in academic, industrial and government research laboratories since the advent of the silicon polymers some thirty years ago. This volume contains four reviews which cover exciting new topics and describe, in depth, interesting new developments in this field.

Volume 1

New Applications of Organometallic Reagents in Organic Synthesis

Proceedings of a Symposium at the American Chemical Society National Meeting held in New York City, April 6-9th, 1976

edited by D. SEYFERTH

1976. x+488 pages. US $47.50/Dfl. 116.00. ISBN 0-444-41473-8

This is the first volume of a series established as a complement to the Journal of Organometallic Chemistry, and presents twelve review-type articles which are expanded versions of talks at a symposium on *"New Applications of Organometallics to Organic Chemistry"*.

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Journal of Chromatography Library

A series of books devoted to chromatographic techniques and their applications.

Although complementary to the Journal of Chromatography, each volume in the library series is an important and independent contribution in the field of chromatography. It should be stressed that the library contains no material reprinted from the journal itself.

Volume 1

CHROMATOGRAPHY OF ANTIBIOTICS

by G.H. Wagman and M.J. Weinstein.

1973. ix - 238 pages.
Price: US $28.95/Dfl. 70.00.
ISBN 0-444-41106-2

At the present time thousands of antibiotics are known, yet the systematic chromatographic classification of these substances is extremely difficult.

This book has been written to aid the identification of very similar compounds by use of specific chromatographic techniques. It contains detailed data on paper and thin-layer chromatography, electrophoresis, counter-current distribution and gas chromatographic systems for over 1,200 antibiotics and their derivatives, and provides information on chromatographic media, solvents, detection methodology and mobility of the antibiotics. Complete references are given for all methods.

CONTENTS: Chromatographic classification of antibiotics. Detection of antibiotics on chromatograms. Comments on the use of this index. Abbreviations. Index - chromatography of antibiotics. Index by compound.

Volume 2

EXTRACTION CHROMATOGRAPHY

edited by T. Braun and G. Ghersini.

1975. xviii + 566 pages.
Price: US $52.95/Dfl. 130.00.
ISBN 0-444-99878-0

This volume is the result of the collective work of many specialists, each responsible for a chapter in which a definite aspect of column extraction chromatography is thoroughly presented and discussed.

Subjects presented include the basic and technical aspects of the method, the organic stationary phases and supports, the separation of elements with particular reference to radiochemical problems, the separation of lanthanides, actinides and fission products, radiotoxicological separations and the preconcentration of trace elements in various materials prior to their determination.

Author and subject indices are included.

Volume 3

LIQUID COLUMN CHROMATOGRAPHY

A survey of modern techniques and applications.

edited by Z. Deyl, K. Macek and J. Janák.

1975. xxii + 1176 pages.
Price: US $118.50/Dfl. 290.00.
ISBN 0-444-41156-9

This book provides an up-to-date account of liquid column chromatography for the specialist and non-specialist. The main attention is focussed on techniques developed or widely used during the past 10 years. Both classical and modern techniques of chromatographic separation are treated in detail, thus providing a clear reflection of the present situation in the field.

The wide selection of applications in various fields of chemistry and biochemistry, written by specialists in the area, makes this volume a necessary reference work for those involved in chromatographic investigations.

CONTENTS: Theoretical Aspects of Liquid Chromatography. Techniques of Liquid Chromatography. Practice of Liquid Chromatography. Applications. Subject index. List of compounds chromatographed.

Volume 4

DETECTORS IN GAS CHROMATOGRAPHY

by J. Ševčík.

1976. 192 pages.
Price: US $24.50/Dfl. 60.00.
ISBN 0-444-99857-8

This publication is devoted to the function and optimal working conditions of gas chromatographic detectors.

The first systematic treatment of gas chromatographic detection techniques, it

devotes special attention to so-called specific detectors and working conditions which strongly influence results (e.g. gas flow, effect of additives in gases, working temperature, detector form and dimensions). Anomalous detector responses are explained and the form and size of response for various working conditions are indicated. The problems presented are illustrated by experimental data which are summarized in numerous tables and figures.

The book should be of interest to all who use gas chromatography in research and who would like to explore the possibilities and working conditions of different detector systems.

Volume 5

INSTRUMENTAL LIQUID CHROMATOGRAPHY

A Practical Manual on High-Performance Liquid Chromatographic Methods

by N.A. Parris.

1976. x+330 pages.
Price: US $40.95/Dfl. 100.00.
ISBN 0-444-41427-4

Available texts on liquid chromatography have tended to emphasize the developments in the theoretical understanding of the technique and methodology or to list numerous applications, complete with experimental details.

This work intends to bridge the gap between these two treatments by providing, with the minimum of theory, a practical guide to the use of technique for the development of separations. The material is based largely on practical experience and high-lights details which may have important operational value for laboratory workers. Information regarding the usefulness of available equipment and column packings is given, together with chapters devoted to the methodology of each separation method. Applications of liquid chromatography are described with reference to the potential of the technique for qualitative, quantitative and trace analysis as well as for separative applications. Numerous applications from the literature are tabulated and cross-referenced to sections concerned with the optimisation procedures of the particular methods. In addition, many of the figures have been drawn from hitherto unpublished works.

CONTENTS: Introduction and historical background. Basic principles and terminology. Chromatographic support and column. Liquid chromatographic instrumentation. Liquid chromatographic detection systems. Nature of the mobile phase. Liquid-solid (adsorption) chromatography. Liquid-liquid (partition) chromatography. Ion-exchange chromatography. Steric exclusion chromatography. Qualitative analysis. Quantitative analysis. Practical aspects of trace analysis. Practical aspects of preparative liquid chromatography. Published LC applications information. The latest trends and a glimpse into the future. Subject Index.

Volume 6

ISOTACHOPHORESIS

Theory, Instrumentation and Applications

by F.M. Everaerts, J.L. Beckers and Th.P.E.M. Verheggen.

1976. xiv+418 pages.
Price: US $65.50/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. There is rapidly growing interest in this technique which will compete with other microanalytical techniques such as liquid and gas chromatography. All kinds of ionic materials can be separated using isotachophoretic equipment. Moreover, several classes of components can be analysed in quick succession as a proper rinsing of the equipment is all that is needed between separations. Each part is detailed and comprehensive.

The various chapters can be referred to more or less independently by scientists interested in fundamental aspects, by research groups intending to construct an instrument and by workers who are mainly concerned with the analytical aspects.

CONTENTS: Historical review. **Theory.** Principles of electrophoretic techniques. Concept of mobility. Mathematical model for isotachophoresis. Choice of electrolyte systems. **Instrumentation.** Detection systems. Instrumentation. **Applications.** Introduction. Practical aspects. Quantitative aspects. Separation of cationic species in aqueous solutions. Separation of anionic species in aqueous solutions. Amino acids, peptides and proteins. Separation of nucleotides in aqueous systems. Enzymatic reactions. Separations in non-aqueous systems. Counter flow of electrolyte. Appendices. Subject Index.

Volume 7

CHEMICAL DERIVATIZATION IN LIQUID CHROMATOGRAPHY

by J.F. Lawrence and R.W. Frei

1976. viii + 214 pages.
Price: US $36.75/Dfl. 90.00.
ISBN 0-444-41429-0

This book is intended for all investigators concerned with the use of physical separation techniques for solving complex analytical problems. It is the first publication to provide a comprehensive account of modern derivatization in liquid chromatography with special emphasis on the practical aspects.

An introductory chapter familiarizes the reader with the basic philosophy of using chemical reactions and labelling procedures to enhance sensitivity, specificity and separation properties in liquid chromatographic techniques. The second chapter enables the practical worker to refresh his memory on some fundamental principles necessary to this work. The third deals with equipment and gives the analyst an idea of the choice of tools available to suit his needs. The final chapter helps the investigator to solve some concrete problems, to extend the concept of compounds and types of problems of immediate interest to him and to become familiar with the literature.

CONTENTS: Introduction. Background. Instrumentation. Applications. Subject Index.

Volume 8

CHROMATOGRAPHY OF STEROIDS

by E. Heftmann.

1976. xiv + 204 pages.
Price: US $36.75/Dfl. 90.00.
ISBN 0-444-41441-x

The qualitative and quantitative analysis of individual steroids is of great interest to pharmacologists, physicians, biochemists, plant and animal physiologists and microbiologists.

The principal chromatographic methods of analysis applicable to steroids are: liquid column chromatography (including its recent modification, high-pressure liquid chromatography), thin-layer chromatography and gas chromatography (including the recently introduced coated capillary chromatography).

Since Neher's book "Steroid Chromatography" published by Elsevier in 1964, these applications have not been surveyed in a single volume. Here, the author takes up where Neher left off and presents a detailed description of the currently used techniques. Although some theory is included, this is mainly a laboratory handbook, arranged according to the steroids analyzed as well as according to the methods used.

CONTENTS: Introduction. Liquid column chromatography. Paper and thin-layer chromatography. Gas chromatography. Relations between structure and chromatographic mobility. Sterols. Bile acids and alcohols. Estrogens. Androstane derivatives. Pregnane derivatives. Corticosteroids. Miscellaneous steroid hormones. Vitamins D. Molting hormones. Steroid sapogenins and alkaloids. Cardenolides and bufadienolides. List of Abbreviations. References. Subject Index.

Volume 9

HPTLC - HIGH PERFORMANCE THIN-LAYER CHROMATOGRAPHY

edited by A. Zlatkis and R.E. Kaiser.

1977. 240 pages.
Price: US $44.95/Dfl. 110.00.
ISBN 0-444-41525-4.

HPTLC is the advanced technology of thin-layer chromatography and is defined as the combined action of several variables which include: an optimized coating material with a separation power superior to the best high performance liquid chromatographic separation material; a new method of feeding the mobile phase; a novel procedure for layer conditioning; a considerably improved dosage method and a competent data acquisition and processing system. Thus a complete system and procedure is discussed here. This should be understood as a stepwise improvement of an analytical method, which has been a powerful tool since the pioneering work of E. Stahl.

The results achieved, as well as the promising aspects of the new method are encouraging enough to refer to the technique as the second generation of thin-layer chromatography. The final judgement however, will be left to those who use this new methodology.

CONTENTS: Simplified theory of TLC *(R.E. Kaiser).* The separation number in linear and circular TLC *(J. Blome).* Advantages, limits and disadvantages of the ring

Journal of Chromatography, 136 (1977) 361–369

CHROM. 9925

APPLICATIONS OF GAS–LIQUID CHROMATOGRAPHY IN PROTEIN CHEMISTRY

II. DETERMINATION OF AMIDE RESIDUES IN NANOMOLAR AMOUNTS OF PROTEINS

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(Received January 10th, 1977)

SUMMARY

A method for the quantitative determination of amide residues in nanomolar amounts of proteins is described, based on dilute acid hydrolysis at 100°, followed by isothermal gas–liquid chromatography of the ammonia released by on-column neutralisation of the hydrolysate and quantitation by means of a conductometric detector. Amide contents are given for twenty well characterised proteins, as well as for asparagine and glutamine.

INTRODUCTION

Up to the present time three main methods have been used for the determination of amide residues in proteins. (1) Hydrolysis by dilute mineral acids for varying periods followed by neutralisation of the hydrolysate and estimation of the ammonia liberated. On the micro-scale this has usually been carried out by the Conway micro-diffusion method combined with colorimetric assay of the ammonia. (2) Determination of free ammonia during the ion-exchange determination of amino acids in protein hydrolysates. This suffers from the disadvantage that the conditions necessary for the complete hydrolysis of all amino acids (usually 6 *M* HCl for 24–72 h) are not optimal for ammonia, and additional ammonia will be set free by the decomposition of certain amino acids such as serine and threonine. Thus incorrect results may be obtained unless the results from several hydrolyses of different duration are extrapolated to zero time. (3) Assignment of glutamine and asparagine residues during sequencing of enzymatic hydrolysates. This is probably the most precise method, but obviously is not always available.

The need for a precise amide determination applicable to minimal amounts of valuable proteins in this laboratory drew our attention to alternatives to the usual methods, and in particular to the possibility of isothermal gas–liquid chromatography (GLC) of ammonia at relatively low temperatures. On-column liberation of ammonia

from acid hydrolysates would also minimise atmospheric contamination, especially as very few nitrogenous compounds could pass through the column under low-temperature operation. An important problem was, however, the selection of a suitable quantitative detection system, since many of the commonly used GLC detectors, notably the stable and reliable flame ionisation detector, are insensitive to ammonia[1]. Our final choice was the sensitive and relatively specific conductance detector (Coulson[2]), especially since Cochrane and Wilson[3] have shown that this detector is able to detect ammonia at the nanogram level. The Coulson conductance detector is commercially available, but in this paper we describe a simple and stable conductance detector assembled from laboratory equipment which may be useful to those laboratories which have only occasional use for this detector.

Another problem encountered in the GLC of ammonia is persistent zone tailing in many chromatographic systems. In agreement with the results of Lindsay Smith and Waddington[4] with aliphatic amines, we have found that using polystyrene beads (Porapak Q), zone tailing may be virtually eliminated by pre-coating with polyethyleneimine and potassium hydroxide. Isothermal GLC of ammonia in this system could be carried out at the low operating temperature of 68°. Combination of this chromatographic system with on-column neutralisation of dilute acid hydrolysates with barium hydroxide has provided a highly specific method for the determination of amide residues on nanomolar amounts of proteins.

MATERIALS AND METHODS

Chemicals

Porapak Q (bead form, 80–100 mesh) was supplied by Waters Assoc. (Milford, Mass., U.S.A.). AnalaR-grade barium hydroxide and concentrated hydrochloric acid, Dowex 1-X8 (20–50 mesh), Amberlite Monobed Resin MB-1, analytical grade (20–50 mesh), polyethyleneimine, L-glutamine and L-asparagine were supplied by BDH, (Poole, Great Britain). N-Acetyl-D-glucosamine was obtained from Hopkins & Williams (Chadwell Heath, Great Britain).

Proteins

α-Chymotrypsin (3.4.4.5) (prepared from four times recrystallized chymotrypsinogen A), bovine insulin, crystalline bovine trypsin (3.4.4.4), soya bean trypsin inhibitor (Kunitz) and twice recrystallized porcine pancreatic elastase (3.4.4.7) were obtained from BDH; crystalline sperm whale myoglobin, horse myoglobin, chicken lysozyme (twice crystallized) (3.2.1.17), bovine β-lactoglobulin (three times recrystallized), ovalbumin (five times recrystallized), crystalline bovine serum albumin, papain (ex *Papaya latex*, twice recrystallised) (3.4.4.10), and porcine pepsin (three times recrystallized) (3.4.4.1) from Koch-Light Labs. (Colnbrook, Great Britain); crystalline ribonuclease A (2.7.7.16) from Boehringer (Mannheim, G.F.R.); ribonuclease B, horse heart cytochrome *c* and chymotrypsinogen A (six times recrystallized) from Miles-Seravac (Maidenhead, Great Britain); horse myoglobin from Serva (Heidelberg, G.F.R.); and bovine serum albumin (crystalline) and porcine pepsin (3.4.4.1) from Armour Pharmaceutical Co. (Eastbourne, Great Britain).

Proteins supplied as crystalline or lyophilized powders were used without further purification. Elastase was supplied as a suspension in water. The suspension

was centrifuged, and the solid re-suspended in water and centrifuged. The process was repeated twice, and the protein was dried over P_2O_5. Papain was also supplied as a suspension, but as it is soluble in water, it was dissolved in water and precipitated with redistilled acetone. This process was repeated three times, and the protein finally dried over P_2O_5 *in vacuo*.

Instrumentation

A Pye Series 104 gas chromatograph was used as the basic chromatographic unit, purified nitrogen being employed as carrier gas. The output from the column was led into a gas–liquid mixer–separator unit (Figs. 1 and 2), which was supplied with a constant flow of de-ionized water. The detailed construction and dimensions of the mixer–separator unit which was fabricated from a 5 × 2 × 1 cm thick block of perspex are shown in Fig. 2. The liquid output from the separator was divided into two streams, one passing through the conductance cell (Radiometer Type CDC 314, cell constant 0.316, total volume 1 ml), and thence to the reservoir (Fig. 1). The bypass stream together with nitrogen gas from the column passed directly to the reservoir. Water from the reservoir was continuously recycled by a centrifugal pump through a jacketed 40 × 1.5 cm column, the lower portion of which was packed with 50 ml of Dowex 1-X8 anion-exchange resin (20–50 mesh) in the hydroxyl form, while the upper section was packed with 25 ml of Amberlite MB-1 mixed bed resin (20–50 mesh). Water emerging from this column at a flow-rate up to 15 ml/min had an electrolytic conductance of less than 0.1 μS. The conductance cell, mixer–separator and reservoir were placed in a water-bath maintained at 25 ± 0.5°, and water from the bath was also circulated through the jacket of the ion-exchange column. Winnett and Illingsworth[5] have demonstrated that control of the water temperature is essential for reproducible operation of the Coulson conductometric detector, there being a mark-

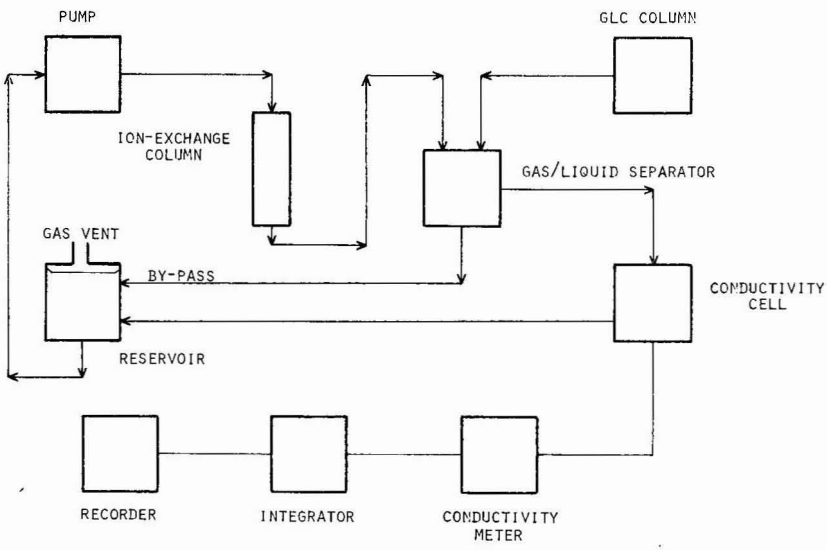

Fig. 1. Block diagram of the detector system.

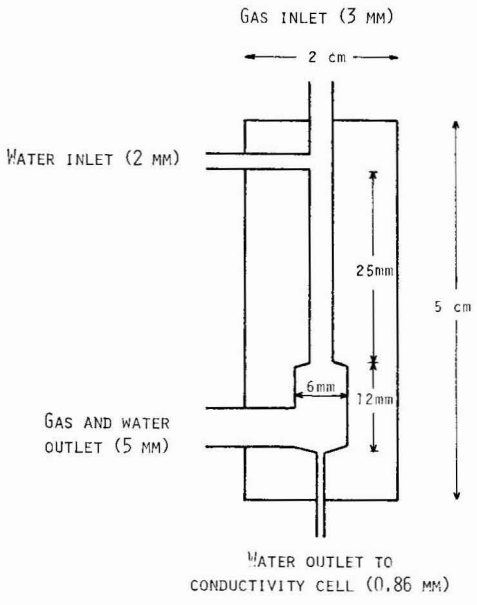

Fig. 2. Dimensions of the gas–water mixer and separator.

ed reduction of sensitivity at higher temperatures. This effect was minimal in the temperature range 22–26°.

The electrolytic conductance between the electrodes of the Radiometer CDC 314 cell was measured by a Radiometer CDM 3 conductance meter, operating at an a.c. frequency of 2 kHz on the 50 μS/full-scale deflection range. This corresponds to an output of 1.0 V, which was applied to an Autolab Model 6300 digital integrator with a printed output and peak areas were expressed in counts/sec. The output from the integrator could be varied and was usually operated at a quarter of the maximum output, which gave reasonable peak sizes when applied to a Pye Unicam Series AR 25 potentiometric recorder operating on the 10-mV input range.

Methods

Water content of the protein samples. Protein samples were not dried before hydrolysis, the water content of each protein being determined on 10–20 mg samples by drying *in vacuo* (better than 0.1 Torr) over P_2O_5. The weight of protein taken for analysis was then corrected for water content. This procedure obviated weighing problems with proteins which became very hygroscopic on intensive drying, but may be impractical when only very small samples of the protein are available.

Ammonia content of the protein samples. Chibnall *et al.*[6] showed that certain protein samples contained free ammonia (presumably as the ammonium salts of the dicarboxylic amino acids), which vitiated the results of amide analyses, and suggested a method for its estimation. The protein (10 mg) was dissolved or suspended in methanolic HCl (1.0 ml, 0.033 *M*), and at once precipitated with diethyl ether (2.0 ml). The mixture was centrifuged, the supernatant liquid removed, and the process repeated. A known amount of methylamine hydrochloride was added to the combined

supernatant liquids as internal standard, and the solvent removed. The residue was dissolved in 1 *M* HCl (25 μl), and 1-μl samples of this solution were analysed by the GLC method described below.

Protein hydrolysis for amide determinations. Although the recommended method for the determination of the amide content of proteins involves hydrolysis with 2 *M* HCl for various peroids of time (2–8 h) and extrapolation to zero time (Wilcox[7]), others, *e.g.*, Chibnall *et al.*[6], Marks *et al.*[8], and Spiro and Spiro[9] have used a single 3-h hydrolysis period.

We have investigated the rate of release of ammonia from β-lactoglobulin by 2 *M* HCl at 100°, using the GLC analytical method described in this paper. The results are given in Table I, and show that the release of ammonia is substantially complete (98%) in 2 h, and remains constant until at least 4 h. Increased amounts are obtained after 24-h hydrolyses, presumably due to degradation of serine and threonine. In agreement with the workers cited above, we have therefore adopted a standard hydrolysis time of 3 h, with 2 *M* HCl at 100°.

TABLE I

RATE OF RELEASE OF AMMONIA FROM β-LACTOGLOBULIN BY 2 *M* HCl AT 100°

Time (min)	*Ammonia released (moles)*
5	1.20
15	16.79
30	25.42
60	26.99
90	26.93
120	27.63
180	27.61
240	27.69
420	27.93
1440	31.92

The protein (0.2–1.3 mg, sufficient to give 4–14 μg of ammonia after hydrolysis) was dissolved or suspended in 2 *M* HCl (0.4 ml) containing a known weight of internal standard (methylamine hydrochloride) and hydrolysed for 3 h in a sealed glass tube. The hydrolysate was centrifuged, and the supernatant liquid evaporated to dryness using a rotary evaporator at reduced (water pump) pressure. In agreement with Wilcox[7], we have found that this concentration procedure involves no loss of amine hydrochlorides. The residue was dissolved in 1 *M* HCl (25–40 μl) and 1-μl aliquots injected directly into the column.

GLC of ammonia. (a) Preparation of the GLC column. Porapak Q (80–100 mesh, 7.44 g) was placed in a flask, just covered with methanol and a solution of polyethyleneimine (0.4 g) and KOH (0.16 g) in methanol was added. After thorough mixing the excess methanol was removed using a rotary evaporator, and the coated material dried at 110° for 18 h. The amounts given correspond to a coating of 5% polyethyleneimine and 2% KOH. The coated material was rapidly screened and the 80–100 mesh fraction packed into a 1.5 m × 4 mm all-glass helical column. The top

5 cm of the column were left unpacked. The column was conditioned by passing nitrogen at a flow-rate of 100 ml/min through the column at 150° for 24 h.

(b) Transfer and neutralisation of the acid hydrolysates. Neutralisation of the hydrolysates was achieved by injecting an aliquot into a plug of barium hydroxide formed at the top of the column (Ayres[10]). Commercial AnalaR grade barium hydroxide octahydrate was recrystallized from water and dried at 90° overnight. It was then ground sufficiently finely to pass through a 150-mesh screen, and a 2.5-cm plug of the powdered material was placed on the top of the coated Porapak Q in the column. The column was then replaced in the chromatograph oven, operating conditions established and a 1.0-μl aliquot of the hydrolysate injected directly through the septum. Barium hydroxide on-column neutralisation has many practical advantages, and if operated below 80° no pyrolysis products are released into the column. The system has, however, certain disadvantages. As previously observed by Ayres[10], no more than 10–15 μl of water could be injected into the barium hydroxide plug, either in aliquots or as a single injection, without deterioration in column performance. This necessitated changing the plug after at most fifteen 1-μl sample injections. Also H_2SO_4 could not be used for the hydrolysis of proteins, as successive injections of this acid also resulted in a progressive decrease in the response to ammonia. No such effect was observed with HCl. Neutralisation of the hydrolysate prior to injection gave low ammonia values, possibly due to loss into the partial vacuum produced during manipulation of the injection syringe.

(c) GLC operating conditions. The operating parameters (water flow-rate, cell/bypass flow ratio, carrier gas flow-rate and column and injection port temperatures were adjusted to give: (i) A good detector response without excessive peak tailing. As previously observed by Coulson[2], a decreased water flow-rate increased the sensitivity, but led to peak tailing and distortion. (ii) Rapid elution of ammonia and methylamine to give sharp peaks and a short analysis time.

The optimum conditions were found to be: nitrogen carrier gas flow-rate, 70 ml/min; oven temperature, 68°, isothermal; injection port temperature, 68°; water flow through conductance cell, 4.0 ml/min; water flow through bypass, 7.0 ml/min; detector sensitivity, 50 μS f.s.d.; paper chart speed, 0.2 cm/min.

Under these operating conditions, methylamine, dimethylamine, ethylamine, and trimethylamine could be eluted in addition to ammonia. Their retention times are given in Table II. Methylamine was chosen as internal standard as the zone pro-

TABLE II

RETENTION TIMES OF AMMONIA AND SOME ALIPHATIC AMINES UNDER EXPERIMENTAL CONDITIONS AS DESCRIBED IN THE TEXT

Amine	*Retention time (sec)*
Ammonia	66
Methylamine	222
Dimethylamine	552
Ethylamine	625
Trimethylamine	952

file and molar response were very similar to that of ammonia, but it was well separated from the latter.

A typical experimental protocol is given in Table III. The coefficient of variation both of the molar ratio ammonia/methylamine and of the amide analyses was of the order of 2%, suggesting that the precision of the determination of the molar ratio is the predominant factor in determining the overall precision of the analysis.

TABLE III

HYDROLYSIS OF LYSOZYME (DRY WEIGHT 0.216 mg) FOR 3 h AT 100° IN 2 *M* HCl (0.4 ml, CONTAINING 49.15 μg OF METHYLAMINE)

A = Zone areas of ammonia standards; B = zone areas of methylamine standards; C = relative molar responses of ammonia to methylamine; D = zone areas of ammonia from the hydrolysate; E = zone areas of methylamine internal standards; F = micrograms of ammonia released from 1 μl of the hydrolysate.

Run No.	*Standards*			*Hydrolysate*		
	A	*B*	*C*	*D*	*E*	*F*
1	148 450	345 248	1.04			
	141 180	315 005	1.11	76 572	414 706	4.57
	155 447	349 953	1.10	82 047	461 545	4.40
	174 361	405 893	1.06	75 037	434 455	4.31
	185 640	437 600	1.05	68 547	382 876	4.47
	173 425	400 805	1.07	64 035	361 030	4.43
	162 852	365 893	1.10			
2 (repacked $Ba(OH)_2$)	161 093	401 563	0.99			
	183 288	434 643	1.04	75 917	444 815	4.46
	135 578	309 055	1.08	60 504	336 942	4.61
	155 892	368 192	1.05	70 099	404 693	4.45
	165 989	388 693	1.05	64 371	372 700	4.43
	175 400	411 303	1.05	72 768	416 495	4.48
	169 970	404 421	1.04			
Mean + 1 S.D.			1.06 ± 0.022			4.46 ± 0.08
Coefficient of variation, %			2.07			1.78

RESULTS

Table IV gives the moles ammonia liberated per mole protein under our conditions of acid hydrolysis compared with the "theoretical" ammonia yields calculated from the corresponding sequence studies tabulated by Croft[11]. In the majority of cases the precision of analysis was good enough to assign the number of amide residues to ±1 residue in 20–30. Elastase was an exception and the low result must reflect the quality of our preparation, as it was not improved by further replicate analyses. The amide analyses of asparagine and glutamine were carried out on 100-μg samples, and serve as checks on the overall precision of the method. N-Acetyl-D-glucosamine was included because of its occurrence in glycoproteins. It will be seen that its contribution to amide nitrogen would be negligeable. There has been disagreement in the literature regarding the amide content of myoglobins[12–14]. Our results support the lower estimates in both whale and horse myoglobin. Only our prep-

TABLE IV

AMIDE AND FREE AMMONIA CONTENTS OF SOME PROTEINS AND OF RELATED COMPOUNDS

	μg free ammonia/mg of protein	*Moles NH_3 found/mole protein*	*Moles NH_3/mole protein calculated from sequence studies*
N-Acetyl-D-glucosamine	—	0.02	0
L-Asparagine	—	1.002	1
L-Glutamine	—	0.981	1
Bovine insulin	Negligeable	6.23	6
Sperm whale myoglobin	0.135	6.19	6 or 7
Seravac horse myoglobin	Negligeable	8.17	8 or 9
Koch-Light horse myoglobin	0.125	7.54	8 or 9
Horse heart cytochrome *c*	0.295	8.11	8
Soya bean trypsin inhibitor	0.798	13.71	14
Lysozyme	0.429	16.99	16
Ribonuclease A	Negligeable	16.76	17
Ribonuclease B	Negligeable	18.25	17
α-Chymotrypsin	3.112	22.87	23
Chymotrypsinogen A	0.077	23.87	24
Armour porcine pepsin	0.140	26.16	25
Koch-Light porcine pepsin	0.180	26.10	25
Papain	Negligeable	24.51	26
β-Lactoglobulin	0.1052	28.01	28
Trypsin	Negligeable	27.17	29
Ovalbumin	Negligeable	33.70	30–32
Elastase	Negligeable	26.92	33
Armour bovine serum albumin	Negligeable	33.72	34–37
Light bovine serum albumin	Negligeable	32.04	34–37

aration of α-chymotrypsin gave a significant free ammonia content by the method of Chibnall *et al.*[6].

DISCUSSION

It must be emphasized that although only 2–20 μg of the protein hydrolysate are actually used for a single analysis, the method as described does not approach the limits of sensitivity possible, but is rather chosen to avoid the difficulties of weighing low microgram amounts of possibly hygroscopic proteins. A tenfold higher sensitivity is available in the Radiometer CDM 3 conductance meter, although its use would involve modification to the zero setting arrangements of the actual recorder used. The volume of the conductance cell could also be reduced with advantage. Hall[15] has described a number of improvements to the original Coulson[2] detector, claimed to increase its sensitivity 20–50 times, the most important being a smaller conductance cell constructed of PTFE, and the replacement of water by 50% aqueous propan-2-ol as circulating liquid. These claims were only partially confirmed by Wilson and Cochrane[3]. We have not investigated them further, but it would appear that a picogram-scale ammonia determination with a precision ($\pm 2\sigma$) of $\pm 5\%$ or better is quite feasible.

The specificity of the method described here is very high, as not only must any contaminant pass through the GLC column with a retention time similar to that of ammonia, but it must also dissolve in water to give a conducting solution. An important advantage of the present method (in contrast to the micro-Conway diffusion method) is that it does not require rigorous cleaning and careful storage of glassware, as the only glassware used are the syringe and the sealed ampoules which are made from disposable Pasteur pipettes and used once only.

Although described here for amide determinations in proteins the method is obviously applicable for many similar ammonia estimations, *e.g.*, asparaginase or glutaminase determinations. The rapidity of the GLC technique makes it very suitable for multiple analyses, as in the investigation of enzyme kinetics.

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SELECTIVE CONCENTRATION OF AMINES FROM AQUEOUS SOLUTIONS BY A GAS PURGING TECHNIQUE

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SUMMARY

Amines are removed from heated, basic solutions saturated with salt by inert gas stripping and selectively trapped as coordination complexes on columns containing copper(II) salts coated on an inert support. An estimate of the amine concentrations at ppm levels can be made by measuring the length of the trapping column colored by the complexes. Concentrations of amines in ppb* can be determined by eluting this column with potassium hydroxide and determining the amines in the effluent directly by gas chromatography. The procedure allows amines to be concentrated by a factor of 200 and removes potentially interfering substances from the sample.

INTRODUCTION

Simple methods for determining amines at ppb* concentrations in aqueous samples such as waste water and biological fluids are not presently available. Direct gas chromatographic (GC) methods[1,2] are not applicable to amine concentrations much below the ppm level, and other organic contaminants in water can overlap the chromatographic peaks of amines. Pre-concentration of amines by solvent extraction or other techniques prior to their GC determination will allow more sensitive determinations, but interfering substances will also be concentrated. If available, nitrogen-specific GC detectors may be used to reduce or eliminate non-amine interferences. In this work techniques for selectively concentrating amines from aqueous solutions as a prelude to their GC determination were investigated.

Cation-exchange resins have been used for retaining amines from aqueous solutions[3]. We have found, however, that amine recoveries are not quantitative using this technique at concentrations below the ppm level. Walton and co-workers[4,5] have successfully separated compounds containing the amine functional group by ligand exchange chromatography. Investigations of the feasibility of using this technique for the pre-concentration of amines revealed that amines can be quantitatively retained on the copper, nickel and zinc forms of cation-exchange resins. Unfortunately no

* Throughout this article the American billion (10^9) is meant.

sufficiently simple technique was discovered whereby amines could be quantitatively eluted from the resin in a form amenable to their GC determination.

Several workers[6,7] have reported the isolation of different organic compounds from aqueous solutions by gas purging techniques (also referred to as headspace techniques), coupled with trapping of the volatilized substances on materials such as Tenax GC. In previous work we have shown that amines can be selectively removed from gas streams using a support phase coated with copper(II) salts as an abstractor material[8]. By combining the gas stripping techniques with selective trapping of amines on columns containing copper(II) salts, an extremely simple and effective method has been developed for concentrating amines by a factor of up to 200-fold and simultaneously eliminating interfering substances. Amines are purged from a heated, basic solution saturated with salt and trapped on a column packed with Chromosorb W AW DMCS coated with copper(II) chloride. The amines are subsequently eluted with potassium hydroxide and determined directly in the potassium hydroxide solution by GC.

In addition to its utility as a selective method for concentrating ppb levels of amines prior to their GC detection, the gas purging method also provides an extremely simple method for determining ppm levels of amines. By merely measuring the lengths of trapping columns colored by the copper–amine complexes, estimates of amine concentrations as low as 0.5 ppm can be made.

EXPERIMENTAL

Chemicals and apparatus

A Hewlett-Packard Model 5711A gas chromatograph equipped with dual flame ionization detectors was used for determining amines. Amines were separated on 6- or 10-ft. × 1/8 in. O.D. stainless-steel columns packed with 28% Pennwalt 223 and 4% potassium hydroxide on 80–100 mesh Gas-Chrom R. Amines were purged from water using a modified 500-ml gas wash bottle. A coarse mesh gas diffusion tip was attached to the immersion tube, the exit tube was bent upward to prevent water condensation in the trapping tube, and the exit arm was drawn out to 1/4 in. O.D. to accommodate the use of Swagelok unions. The amine trapping material was prepared by dispersing (9 g of Chromosorb W AW DMCS in a minimal amount of methanol containing 1 g of dissolved copper chloride dihydrate (0.37 g Cu(II)). The mixture is heated to evaporate most of the methanol leaving a paste-like material that is then air dried until free flowing. The coated support is packed into glass tubes (1/8 or 1/4 in. O.D.) to give 2–5-in. bed volumes, and is retained with glass wool plugs.

Determination of amines in water by GC

Add a sample of water containing amines to a gas wash bottle and bring the total volume to 100–200 ml with distilled water. Add 75 g potassium chloride and 1 g potassium hydroxide to the solution. Attach a 1/8-in. trapping column containing a 2-in. bed of coated Chromosorb to the exit arm of the gas wash bottle using a Swagelok union with PTFE ferrules. Seal the system and start purge gas flow at a rate of *ca.* 300 ml/min. Heat the water to a temperature of 60°. After stripping is completed ($1\frac{1}{2}$ h) remove the trapping tube and attach to an unused inlet of a gas chromatograph and allow carrier gas to flow through the tube at a temperature of 100°

for 5 min to volatilize any interfering organic substances that may have condensed on the trapping material. Remove the tube and attach to a syringe and slowly force 1 ml of 1 *M* potassium hydroxide through the column. Collect the effluent, allow any copper hydroxide precipitate to settle (*ca.* 5 min), and chromatograph a 2-μl aliquot of the solution, using a 6-ft. Pennwalt column and a flow-rate of 25 ml/min. Acceptable chromatograms have been obtained at an isothermal oven temperature, 30° below the boiling point of the least volatile amine in a sample.

Method for estimating amine concentrations

Estimate ppm concentrations of amines using the same stripping procedure described above. Use 1/8-in. trapping columns containing 5 in. of sorbent for amine concentrations up to 10 ppm in 200 ml samples; use 1/4-in. trapping columns for higher concentrations. Mark the top of the colored amine band after 30 min and then at 15 min intervals until lack of movement indicates that all amines have been purged from the sample. After stripping is completed, measure the length of the colored amine band and determine the amine concentration from a calibration curve. In some cases the distinction between the copper–amine complex (generally blue) and the copper–aqua complex (light blue) covering the rest of the column may be difficult. In such cases heat the column with gas flowing through it. This will destroy the copper–aqua complex and leave a brown color without affecting the blue copper–amine complex.

RESULTS AND DISCUSSION

Recovery of amines added to water

The GC method gives nearly quantitative recovery of most amines added to water at 100 ppb (μg/l) concentrations (Table I). Of the amines tested only morpholine gave unacceptable results. Based on 40 determinations all other amines tested gave an average recovery of 98.2% with a relative standard deviation of 7.6%. Morpholine cannot be efficiently purged from water because it is both very water soluble and relatively non-volatile. More volatile water-soluble amines, such as diethylamine, can be readily stripped from water. Less volatile amines that are sparingly soluble in water,

TABLE I

RECOVERY OF AMINES BY THE GAS CHROMATOGRAPHIC METHOD

Amines tested in mixtures containing 3 to 6 components each at a concentration of 100 ppb, *N* = number of determinations.

Amine	*N*	*Average recovery (%)*	*Amine*	*N*	*Average recovery (%)*
Triethylamine	3	95	Octylamine	3	95
Tributylamine	3	102	Hexylamine	3	98
Dibutylamine	6	104	Piperidine	4	97
Butylamine	3	90	Pyridine	6	100
Cyclohexylamine	3	91	Aniline	3	100
Diethylamine	3	101	Morpholine	3	8

Average recovery (excluding morpholine), 98.2%. R.S.D., 7.6%.

such as tributylamine, can also be stripped from water. The gas stripping method fails however, when applied to amines that are both non-volatile and extremely water soluble.

Gas stripping conditions

The type of purge gas used, its flow-rate, and the method of dispersing it in water all affect the removal rate of amines from water. All preliminary work on this method was performed using air as a purge gas because it appeared as effective as other gases evaluated (helium, nitrogen, and methane) and less expensive. However, when the method was applied to piperidine, recoveries of only 50% were attainable and an unidentified peak appeared in the chromatograms. When helium was used as a purge gas the recoveries improved and the unidentified peak disappeared. It is reasonable to assume that piperidine and presumably other amines can be air oxidized and thus use of an inert gas is recommended. Stripping time decreases as gas flow-rate is increased. A flow-rate of 300 ml/min is used because amines can be stripped in a reasonable length of time and higher flow-rates lead to a pressure build-up in the system. Use of a gas dispersion tip on the immersion tube is essential for the rapid stripping of amines. Using an open tube di(*n*-butyl)amine, for example, cannot be stripped in 4 h. However, when a coarse mesh sparger tip is used purging is complete in less than 30 min.

The temperature and salt content of the solution affect the time required for complete removal of amines. At room temperature most amines cannot be purged to any appreciable extent. At 60° all amines tested can be stripped in less than 1 h. Saturating the solution with salt decreases the purging time significantly. As an example of its effect, diethyl amine requires 22 h for complete stripping from a solution containing no added salt but less than 30 min from a solution saturated with potassium chloride. Potassium hydroxide prevents ionization of amines and thus facilitates the sparging process.

Amine trapping materials

Prior work indicated that copper(II)chloride coated on Chromosorb W AW DMCS would be effective and selective for removing amines from gas streams[8]. No serious effort was made to discover if other combinations are as effective for this application. When zinc salts are used in place of copper salts, amine peaks do not appear in the chromatograms at their expected retention times. Instead, broad humps occur at longer retention times. Macroreticular resins were found to be effective as support phases, but other organic compounds are strongly retained on them. In previous work when copper-coated Chromosorb was used as an amine abstractor material for use in GC, a coating of 25% copper(II) chloride dihydrate was used[8]. For the present application a 10% coating has as high a capacity for removing amines and a more uniform coating is obtainable. Presumably at higher coating levels a large fraction of the copper is inaccessible to the amines.

Elution and GC determination of amines

Amines can be eluted from the sorbing material with alkaline alcohol solutions, with cyanide solutions, or with aqueous basic solutions. Alcohols are undesirable as eluting solvents because the alcohol peaks or their tailing edges obliterate the peaks

of early eluting amines. The use of cyanide to displace amines from copper complexes is effective, but offers no advantages that outweigh the hazards involved in its use. Since the Pennwalt columns used for separating amines can tolerate water and contain potassium hydroxide as a supplementary stationary phase, it was believed that aqueous potassium hydroxide would not adversely affect the column. Aqueous potassium hydroxide effectively elutes amines from the trapping column and offers the additional benefit that any copper washed off is precipitated as its hydroxide. Several hundred injections of potassium hydroxide solutions of amines into the Pennwalt column did not produce any significant changes in the chromatographic separations; thus the assumption that this eluting solvent would not adversely affect the column was borne out. No serious efforts were made to modify or improve previously reported GC methods for separating amines because the goal of this work was to develop techniques for applying existing methods for determining lower concentrations of amines after removal of interferences.

Interference studies

Of the organic compounds studied, none was retained to a significant extent on the amine sorbent used (Table II). However, nearly all of these substances condense on the trapping column and the heating step is required to remove them. Only acetone and pentanone are retained on the column after the heating step and these only to an extent of about 1% of the amount added to the sample as an interference. It was anticipated that metal ions present in samples might complex amines and interfere with their removal from water. However, the potassium hydroxide added to adjust sample pH also serves to precipitate most transition metals and no interferences were encountered. One devious interference was encountered when the semi-quantitative method was applied to the determination of ammonia in urine. The standard stripping procedure hydrolyzes urea in urine to release ammonia. This problem is eliminated by omitting the heating step, although this lengthens the required stripping time considerably.

TABLE II

SUBSTANCES NOT AFFECTING THE DETERMINATION OF AMINES

Mixture of all organic compounds each at a concentration of 10 ppm added to a mixture containing 100 ppb each of diethylamine, butylamine, pyridine, cyclohexylamine, and dibutylamine. Inorganic ions added singly to same amine mixture at concentrations, in ppm, indicated in parentheses.

Organic		*Inorganic*
Methanol	Benzene	Calcium (450)
Ethanol	Benzaldehyde	Magnesium (1000)
Propanol	Ethylene dichloride	Copper (1000)
Acetone	Carbon tetrachloride	Nickel (1000)
2-Pentanone	Phenol	Iron (1000)
Isopropyl ether	Benzyl alcohol	Chloride (1000)
Ethyl acetate	Dichlorobenzene	Sulfate (1000)
Octane	Cumene	Carbonate (1000)
Acetonitrile	Acetic acid	Zinc (1000)

Semi-quantitative determination of amines

The semi-quantitative method was applied to a variety of amines and was found to be reasonably sensitive and reproducible (Table III). The detection limits for amines established for this procedure are based on an arbitrarily chosen band length of 1 mm when 1/8 in. O.D. columns are used. Lower detection limits would be obtainable with smaller diameter columns, but such columns create excessive back pressure. Lower concentrations of copper coating on the Chromosorb also lead to greater sensitivities, but the use of significantly lower amounts of copper coatings usually makes the bands difficult to detect. Pyridine, aniline, and some other amines form extremely intensely colored copper(II) complexes and in such cases the coating could be reduced.

TABLE III

SEMI-QUANTITATIVE METHOD PARAMETERS

Amine	*Amine/Cu(II)* *	*Detection limit*** *(ppm)*	*Calibration curve**** *slope (μM/mm)*
Trimethylamine	1.0	1.0	2.2
Dimethylamine	1.0	0.5	2.2
Butylamine	1.1	1.0	2.4
Cyclohexylamine	0.9	1.0	1.9
Aniline	0.9	1.0	1.9
Dibutylamine	1.5	2.0	3.2
Tributylamine	1.5	3.0	3.2
Pyridine	2.0	1.5	4.3
Ammonia	4.0	0.5	8.6

* Known amount of each amine stripped from 200 ml of water and trapped on 1/8 in. O.D. column containing 2.16 μmoles Cu(II)/mm of length.

** Calculated concentration of amine giving a 1-mm band length when purged from a 200-ml sample.

*** Slope obtained using 1/8 in. O.D. columns coated with 10% $CuCl_2 \cdot 2H_2O$.

The reproducibility of the method was tested on six replicates of 1.4 mg of di(*n*-butyl)amine purged from volumes of water ranging from 100 to 200 ml and trapped on different batches of the sorbent. Bands ranging from 14.5 to 16.0 mm were measured, with an average of 14.8 and an average deviation from the mean of 0.5 mm. The variability is primarily due to the non-planarity of the leading and tailing edges of the amine band which leads to an uncertainty in the measurement of length. This uncertainty is relatively independent of the length of the amine band; thus the relative uncertainty will increase as the amount of amine decreases.

Linear calibration curves were obtained when the semi-quantitative method was applied to a representative group of amines. The slopes of calibration curves (Table III) are, of course, influenced by the amount of copper coated on the support and the column diameter. Both of these variables, however, are readily controlled. The slope obtained for an individual amine is determined by the stoichiometry of the complex formed. Metal to ligand ratios for the copper–amine complexes were calculated by stripping a known amount of amine and trapping it on columns containing a known amount of copper(II) per unit length. The molar ratios obtained (Table III)

were quite reasonable in most cases which also indicates that practically all the copper is accessible to the amines. Because many amines form 1:1 complexes with the copper(II) under stripping conditions, it is possible in some cases to estimate the total amine concentration in a sample even if the identity of the amines is not known. Such an estimate may, however, be in error if a sample contains significant amounts of amines, such as ammonia or pyridine, that form higher order complexes.

Tubes containing 5 in. of sorbing material are used in the semi-quantitative method. In general this length of column is adequate for trapping at least 0.25 mmole of amines when 1/8 in. O.D. columns are used and 1.0 mmole when 1/4 in. O.D. columns are used. The larger columns were rarely used in developing the method, but the reproducibility is similar on both column sizes.

Applications

The semi-quantitative method was used to determine ammonia in urine and in the effluent from a municipal sewage plant. With no heating the urine sample required 2 h for complete stripping and 28 ppm ammonia were found. A second sample was heated to 95° during the purging and gave 1300 ppm ammonia, due primarily to the hydrolysis of urea. The sewage plant effluent sample contained 45 ppm of ammonia. A sample of condensed steam from a power plant containing a proprietory mixture of amines as a corrosion inhibitor was analyzed using both the GC technique and the semi-quantitative procedure. The gas chromatographic method indicated the sample contained 1.3 ppm of cyclohexylamine plus four other unidentified amines having retention times less than that of diethylamine. The semi-quantitative method indicated a total amine concentration of 1.6 ppm based on a cyclohexylamine calibration curve.

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THIN-LAYER CHROMATOGRAPHY OF CHLORINATED CRESOLS

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SUMMARY

The thin-layer chromatography of four chlorinated cresols was studied on five layer materials using eleven solvent systems. Sharp spots were obtained except on Kieselguhr G layers. The best separation of the individual compounds occurred on silica gel-containing layers with dichloromethane as the solvent. Each solvent system was found to cause a different separation on different layers.

INTRODUCTION

Thin-layer chromatography (TLC) is one of the most widely used analytical techniques[1] and is applied extensively for the separation and identification of pesticides and their residues[2–4] and for their multiple detection[5–8]. TLC is an inexpensive and sensitive technique for the rapid screening and multiple detection of pesticide residues even at the 0.5-μg level[9,10]. TLC procedures devised for organophosphorus and organochlorine pesticides and developed for the detection of common pesticides on a single plate using one or more developing reagents can be adopted for routine toxicological analysis[11]. TLC is reported[12] to be more suitable for organochlorine than for organophosphorus pesticides. From the different layer materials available for TLC, silica gel, alumina, Kieselguhr and cellulose are the most widely used[13]. In a TLC study of seven pesticides, Narayanaswami *et al.*[11] obtained good results on silica gel and silica gel–alumina (7:3) layers with three different solvent systems.

Chlorinated cresols are persistent environmental residues and 4-chloro-*o*-cresol is important in Finland as the first metabolite and impurity of the most used pesticide MCPA[14]. No systematic TLC study on chlorinated cresols has been reported, although the TLC separation of some chlorinated cresols was considered together with other chlorinated phenolic compounds on silica gel using both polar and non-polar solvents[15]. Our results on the TLC of chlorinated catechols[16] prompted us to undertake the present study for the separation, identification and determination of chlorinated cresols with different layer materials using several solvent systems.

EXPERIMENTAL

Apparatus and methods

The sizes of the standard TLC plates were 20 × 20 cm and five plates were

prepared in a single operation with a Desaga/Brinkmann Model S-11 applicator (Brinkmann Instruments, Westbury, N.Y., U.S.A.). Ascending elution in a closed glass chamber (Desaga, Heidelberg, G.F.R.) was applied. The samples were spotted with a 10-μl syringe (Hamilton, Whittier, Calif., U.S.A.) to a starting line 1 cm from the bottom of the layer, the first spot 1 cm from the side of the layer and the following three spots at 4-cm intervals. A Desaga scale plate was used to measure the R_F values of the spots.

Layers

The following materials were used: (i) silica gel G ("nach Stahl", Typ 60; Merck, Darmstadt, G.F.R.); (ii) alumina ("150 Sauer", Typ T, Merck); (iii) Kieselguhr G (Merck); (iv) silica gel–alumina (7:3, w/w); and (v) silica gel–Kieselguhr (3:2, w/w).

Samples

The compounds studied were 2-chloro-*p*-cresol (I), 3-chloro-*o*-cresol (II), 4-chloro-*o*-cresol (III) and 4-chloro-*m*-cresol (IV). III was a commercial sample (Fluka, Buchs, Switzerland), which was purified by vacuum distillation, and I, II and IV were synthesized in our laboratory. Proof of their structures obtained by infrared, mass and ^1H and ^{13}C nuclear magnetic resonance spectrometry will be reported elsewhere. The analytical purity of the samples was verified by gas chromatography.

Solvent systems

A preliminary screening of 45 different solvents and solvent mixtures was carried out in order to select those which gave good spots and reasonable R_F values for all of the compounds studied. The 11 most suitable solvents were as follows: 1, light petroleum (b.p. 40–60°)–acetone (80:20, v/v); 2, light petroleum–ethyl acetate (70:30); 3, light petroleum–methanol (75:25); 4, dichloromethane; 5, dichloromethane–benzene–methanol (60:30:10); 6, benzene–ethanol–acetic acid (85:10:15); 7, benzene–chloroform–acetic acid (60:30:10); 8, *n*-hexane–ethyl acetate–acetic acid (80:15:5); 9, light petroleum–ethyl acetate–acetic acid (80:15:5); 10, light petroleum–acetone–acetic acid (80:15:5); and 11, benzene–acetone–acetic acid (80:15:5).

Chromogenic reagent[17]

A 2% solution of 3,5-dichloro-*p*-benzoquinonechlorimine in benzene was used for spot detection.

Development of chromatograms

A slurry of the absorbent was applied to the glass plates to form a 1-mm thick layer. The plates were activated at 110°C for 12 h and spotted with 10 μl of 0.1% (w/v) solutions in diethyl ether of each cresol studied. The elution was continued up to a height of the solvent front of 15 cm. The plates were then dried in air and sprayed with the chromogenic reagent.

RESULTS AND DISCUSSION

The R_F values and colours of the spots obtained using the 11 solvent systems are given in Tables I–V.

(i) Silica gel G layer (Table I)

All four chlorinated cresols formed sharp spots with all solvent systems. After elution with acetic acid-containing solvent mixtures (6–11) all spots gave yellow colour reactions, whereas variable colours were obtained when the eluent was a neutral organic solvent (1–5). The elution times at room temperature (20°) varied from 30 to 70 min.

TABLE I

R_F VALUES OF CHLORINATED CRESOLS (I–IV) ON A SILICA GEL G LAYER (i) WITH DIFFERENT SOLVENT SYSTEMS (1–11)

Solvent	*I*	*II*	*III*	*IV*	*Elution time (min)*
1	0.33	0.36	0.33	0.30	30
2	0.56	0.56	0.56	0.56	40
3	0.23	0.26	0.26	0.26	70
4	0.56*	0.46	0.43	0.36	30
5	0.73	0.66	0.66	0.63	30
Colour	Yellow	Dark blue	Brown	Brown	
6	0.63	0.60	0.60	0.60	50
7	0.53	0.50	0.50	0.53	50
8	0.50	0.40	0.36	0.33	40
9	0.52	0.46	0.43	0.43	35
10	0.26	0.26	0.23	0.23	35
11	0.66	0.63	0.63	0.63	40
Colour	Yellow	Yellow	Yellow	Yellow	

* Brown spot.

The fastest ascending solvent (5) had the greatest eluting power, giving R_F values of 0.63–0.73. The best separation of the individual compounds from each other was obtained with eluents 4 and 8. These solvents could therefore be used for the identification of chlorinated cresols on silica gel. Solvent 2 eluted all four compounds at the same speed, giving R_F values of 0.56, and seems to be very suitable for the group separation of chlorinated cresols from other components in a clean-up process in residue analysis.

(ii) Alumina layer (Table II)

All spots with all 11 solvent systems were sharp and yellow, except for the dark blue colour produced with II and brown with III when a neutral eluent (1–5) was used. The elution times were 50–65 min.

The eluting powers of the different solvents varied considerably, being greatest with solvent 5 and least with solvent 3.

None of the eluents was suitable for the separation of the compounds I–IV from each other. However, all compounds had identical R_F values with eluents 2 and 11 (0.63 and 0.73, respectively), and hence elution on an alumina layer using solvent 2 or 11 seems to be applicable for group separation purposes.

(iii) Kieselguhr G layer (Table III)

The ascending elution was rapid with all solvents, being completed in 25–60

TABLE II

R_F VALUES OF CHLORINATED CRESOLS (I–IV) ON AN ALUMINA LAYER (ii) WITH DIFFERENT SOLVENT SYSTEMS (1–11)

Solvent	*I*	*II*	*III*	*IV*	*Elution time (min)*
1	0.73	0.70	0.70	0.66	50
2	0.63	0.63	0.63	0.63	60
3	0.36	0.16	0.16	0.13	65
4	0.73	0.60	0.60	0.50	55
5	0.86	0.83	0.83	0.80	60
Colour	Yellow	Dark blue	Brown	Yellow	
6	0.76	0.73	0.73	0.73	50
7	0.76	0.70	0.70	0.70	55
8	0.63	0.60	0.60	0.60	50
9	0.60	0.56	0.56	0.56	50
10	0.51	0.43	0.43	0.43	55
11	0.73	0.73	0.73	0.73	55
Colour	Yellow	Yellow	Yellow	Yellow	

TABLE III

R_F VALUES OF CHLORINATED CRESOLS (I–IV) ON A KIESELGUHR G LAYER (iii) WITH DIFFERENT SOLVENT SYSTEMS (1–11)

Solvent	*I*	*II*	*III*	*IV*	*Elution time (min)*
1	—	0.93	0.93	—	25
2	—	0.96	0.96	—	30
3	0.96	0.96	—	—	60
4	—	0.96	0.96	0.96	25
5	—	0.96	0.96	0.96	25
Colour	Yellow	Blue	Brown	Yellow	
6	—	0.96	—	—	40
7	—	0.96	—	—	25
8	—	0.98	0.98	—	40
9	—	0.96	0.96	—	25
10	—	0.96	0.96	—	30
11	—	0.96*	0.98	—	25
Colour	Yellow	Brown	Yellow	Yellow	

* Yellow spot.

min. The chlorinated cresols were eluted very rapidly, giving high or unmeasurable R_F values. Hence one could conclude that Kieselguhr alone is not a useful layer material for the TLC of chlorinated cresols.

(iv) Silica gel–alumina (7:3) layer (Table IV)

The sharpness of the spots and their colour reactions were the same as on the alumina layer but the R_F values were significantly different. Dichloromethane (4) appeared to be a suitable eluent for identification purposes, all four R_F values being different. For group separations, solvents 3, 6 and 10 seem to be the best, the R_F values of the individual chlorinated cresols being identical.

TABLE IV

R_F VALUES OF CHLORINATED CRESOLS (I–IV) ON A SILICA GEL–ALUMINA (7:3) LAYER (iv) WITH DIFFERENT SOLVENT SYSTEMS (1–11)

Solvent	*I*	*II*	*III*	*IV*	*Elution time (min)*
1	0.50	0.46	0.43	0.43	60
2	0.40	0.40	0.43	0.50	30
3	0.23	0.23	0.23	0.23	50
4	0.56	0.43	0.40	0.33	40
5	0.66	0.66	0.60	0.60	50
Colour	Yellow	Dark blue	Brown	Yellow	
6	0.60	0.60	0.60	0.60	50
7	0.60	0.53	0.53	0.50	50
8	0.43	0.33	0.33	0.30	40
9	0.60	0.53	0.53	0.50	35
10	0.26	0.26	0.26	0.26	30
11	0.70	0.66	0.66	0.63	35
Colour	Yellow	Yellow	Yellow	Yellow	

(v) Silica gel–Kieselguhr G (3:2) layer (Table V)

Sharp spots were obtained with solvents 1–8, 10 and 11. With solvent 9 the compounds were eluted with the solvent front. The colours of the spots were same as on the alumina (ii) and silica gel–alumina (iv) layers, but the R_F values were different. Dichloromethane (4) proved to be a suitable solvent for identification purposes, giving a different R_F value for each chlorinated cresol. With the other solvents, the differences in the R_F values were too small for the separation of the individual chlorinated cresols from each other, but these solvents could be applied for group separations in some instances. The elution times varied from 30 to 60 min.

TABLE V

R_F VALUES OF CHLORINATED CRESOLS (I–IV) ON A SILICA GEL–KIESELGUHR G (3:2) LAYER (v) WITH DIFFERENT SOLVENT SYSTEMS (1–11)

Solvent	*I*	*II*	*III*	*IV*	*Elution time (min)*
1	0.46	0.43	0.43	0.40	35
2	0.70	0.70	0.63	0.60	40
3	0.33	0.33	0.30	0.26	60
4	0.66	0.56	0.53	0.46	35
5	0.73	0.66	0.66	0.60	35
Colour	Yellow	Dark blue	Brown	Yellow	
6	0.76	0.73	0.73	0.73	60
7	0.66	0.60	0.63	0.63	60
8	—	0.56	0.53	0.53	30
9	—	—	—	—	30
10	0.40	0.36	0.36	0.36	30
11	0.66	0.76	0.76	0.76	30
Colour	Yellow	Yellow	Yellow	Yellow	

CONCLUSIONS

Chlorinated cresols can be detected and analyzed by TLC. Separation of the individual compounds can be achieved by elution with dichloromethane on silica gel or on mixtures of silica gel with alumina or Kieselguhr G. Elution with light petroleum–ethyl acetate (70:30) on silica gel or alumina is applicable for the group separation (clean-up) of the chlorinated cresols from other compounds.

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DETECTION OF SYMPATHOMIMETIC CENTRAL NERVOUS STIMULANTS WITH SPECIAL REFERENCE TO DOPING

II. COMPARATIVE STUDY OF TWO ADSORPTION CHROMATOGRAPHY METHODS USING DIFFERENT XAD RESINS

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SUMMARY

Recoveries of a series of sympathomimetic central nervous stimulants in human urine are measured using either adsorption chromatography on self-filled columns (method A) or with a special resin method suitable for racehorse urine (method B). The Amberlite resins used are XAD-2, XAD-4, XAD-7 and XAD-8 and elution is performed using chloroform.

The reported comparative drug extractabilities indicate that in most instances the recoveries follow the sequence XAD-4 $>$ XAD-2 $\approx$ XAD-8 $\gg$ XAD-7 using method A. Based on the recovery and purity of the extracts obtained, XAD-8 is preferred for gas chromatographic analysis while XAD-4 is very suitable for thin-layer chromatographic screening work.

Comparing the two methods, equally good or better results were obtained with method A for all of the resins studied except XAD-7. Finally, it was found that the effect of refrigerated storage of the resins on the drug extractabilities for central nervous stimulants could be neglected.

INTRODUCTION

In a previous paper[1], the recoveries of a series of sympathomimetic central nervous stimulants (CNS) in human urine were measured using either conventional liquid–liquid extraction with chloroform or resin adsorption chromatography on prepacked columns filled with XAD-2. The comparative drug extractabilities found between chloroform extraction and adsorption chromatography indicated that in most instances the drugs were extracted almost equally well by the rapid XAD-2 technique using chloroform as elution solvent.

As a result of this work, a comparative study was undertaken in order to optimize the recoveries using different XAD resins and the method already described[1] (method A).

On the other hand, owing to the frequently high viscosity of alkalinized horse urine, the direct passage of such samples through a column is not recommended. Therefore, large volumes of both diluted and undiluted racehorse urine adjusted to an appropriate pH were extracted by shaking with XAD-2 resin[2-5], and the resin washed and transferred into a column for the elution step. The drug recoveries from human urine using this method (method B) were also determined in this work, using XAD-2, XAD-4, XAD-7 and XAD-8 resins.

Further, the effect of refrigerated storage of the different XAD resins on the drug extractabilities of some CNS compounds using both methods was investigated.

EXPERIMENTAL

Apparatus

All gas chromatography (GC) experiments were performed with a Varian 1400 FID gas chromatograph connected to a Varian CDS 101 integrator. The glass column (3 m × 1/8 in. I.D.) was packed with Apiezon L (15%) and potassium hydroxide (5%) on 80–100-mesh Chromosorb W. The operating conditions were: column oven temperature, 160°; injection port temperature, 255°; detector block temperature, 230°; and carrier gas (nitrogen) flow-rate, 25 ml/min.

Sample reservoirs and empty chromatography columns were purchased from Brinkmann (Westbury, N.Y., U.S.A.).

Amberlite XAD resins (300–1000 μm) were purchased from Serva Feinbiochemica (Heidelberg, G.F.R.). The pore sizes and surface areas of the resins were: XAD-2, 90 Å and 330 m^2/g; XAD-4, 50 Å and 750 m^2/g; XAD-7, 80 Å and 150 m^2/g; and XAD-8, 250 Å and 140 m^2/g.

A polyester screen (80 mesh) was kindly supplied by Mr. G. H. Johnston, Lynn & Johnston Labs. (Lachine, Canada).

Compounds

The following compounds were investigated: *d,l*-amphetamine sulphate, chlorphentermine hydrochloride, cyclopentamine hydrochloride, dimethylamphetamine hydrochloride, *d,l*-N-ethylamphetamine hydrochloride, fenfluramine, mephentermine sulphate, methoxyphenamine hydrochloride; *d,l*-methylamphetamine hydrochloride, phendimetrazine bitartrate, phenmetrazine, phentermine hydrochloride and *d,l*-propylhexedrine hydrochloride. Stock solutions (250 μg/ml) of these drugs were freshly prepared with double-distilled water. All analytical work was carried out at 20°

Column preparation and conditioning of Amberlite resins

The chromatographic columns used in method A were filled with 2.0 ± 0.1 g of resin. The bottom of the column contained a piece of 80-mesh polyester screen while the top of the resin bed was covered with a small plug of cotton-wool. The resin was washed with the following solvents: 10 ml of chloroform, 10 ml of methanol and 2 × 10 ml of double-distilled water. Immediately before use, the columns were treated with 10 ml of 0.01 *N* sodium hydroxide solution.

Using method B, 2.0 ± 0.1 g of resin were rinsed in an erlenmeyer flask (50 ml) with the same solvents, except 0.01 N sodium hydroxide solution, and the sequence described in method A.

RESULTS AND DISCUSSION

Recovery of method A

The method developed by Kullberg *et al.*[6] and modified as mentioned in a previous paper[1] was used (urinary pH, 11–12; elution solvent, chloroform). The urinary drug concentration and standard solutions were as described earlier[1]. All experiments were replicated six times for each drug. The adsorption and elution of the compounds were performed under conditions of free gravitational flow. The recoveries of this procedure for XAD-2, XAD-4, XAD-7 and XAD-8 are given in Table I.

TABLE I

COMPARATIVE DRUG EXTRACTABILITIES (%) USING DIFFERENT XAD RESINS (METHOD A)

The figures in parentheses are standard deviations.

Drug	*XAD-2*	*XAD-4*	*XAD-7*	*XAD-8*
Amphetamine	72.8 (3.70)	79.6 (3.15)	63.4 (5.28)	78.6 (3.72)
Chlorphentermine	78.6 (8.10)	92.4 (1.98)	76.2 (6.19)	86.3 (2.04)
Cyclopentamine	68.9 (5.66)	70.2 (4.61)	11.6 (2.02)	43.1 (4.02)
Dimethylamphetamine	60.3 (6.01)	81.4 (7.16)	36.5 (3.85)	53.4 (2.89)
Ethylamphetamine	80.3 (5.03)	92.7 (2.10)	53.8 (6.18)	76.9 (2.76)
Fenfluramine	75.7 (2.41)	67.5 (1.31)	31.7 (3.56)	77.9 (5.55)
Mephentermine	51.1 (7.68)	69.3 (1.96)	34.5 (2.03)	77.1 (4.40)
Methoxyphenamine	68.4 (2.52)	70.4 (4.61)	12.5 (1.79)	62.7 (5.79)
Methylamphetamine	75.1 (5.47)	82.3 (2.86)	47.3 (2.01)	90.2 (7.33)
Phendimetrazine	91.8 (9.32)	93.6 (5.56)	99.8 (8.11)	92.5 (8.16)
Phenmetrazine	85.8 (8.00)	89.4 (2.10)	88.9 (3.77)	70.5 (6.97)
Phentermine	81.7 (2.71)	96.1 (8.32)	69.3 (5.52)	81.4 (2.78)
Propylhexedrine	68.9 (0.76)	42.7 (6.45)	16.1 (2.98)	46.2 (6.93)

The results in Table I indicate that the drug extractabilities on XAD resins follow the sequence XAD-4 $>$ XAD-2 $\approx$ XAD-8 $\gg$ XAD-7. Nevertheless, as a result of the great adsorption of urinary impurities on XAD-4 and/or the incomplete removal of the styrene monomers in the column cleaning procedure, this resin is not recommended for use in the GC of very concentrated urinary extracts. Moreover, the use of the purer Servachrom XAD-4 resin did not improve the results.

As mentioned by Machata *et al.*[7], the pore size of XAD-4 seems to be optimal for the extraction of drugs. Nevertheless, we believe that in addition to the lower pore size, the large surface area also plays an important role in the very good results obtained with XAD-4.

Although the recoveries of cyclopentamine, dimethylamphetamine and propylhexedrine on XAD-8 are poor, this resin is to be preferred to XAD-2 for the analysis of drugs in concentrated urinary extracts by GC owing to the very pure chromatograms obtained. For screening purposes using thin-layer chromatography, however, adsorption chromatography on XAD-4 could be used without difficulty.

Further, it is noteworthy that in most instances the extractabilities using XAD-2 in this work were lower than the corresponding recoveries obtained with the prepacked XAD-2 resin cartridges[1]. It was demonstrated by Kullberg and co-workers[6,8]

that in contrast to morphine and phenobarbital, the extractability of amphetamine on XAD-2 resin was independent of the urinary flow-rate. Nevertheless, the lower recoveries with the procedure used compared with the pre-packed column method[1] could be due to the greater urinary and elution solvent flow-rates resulting from the replacement of the cotton-wool plug at the bottom of the column with an 80-mesh screen. Moreover, it should be noted that the dependence of amphetamine recovery on urinary flow-rate seems to be rather controversial[6,8,9].

Recovery of method B

As already mentioned, the passage of undiluted horse urine through XAD-2 columns causes some difficulties owing to the high viscosity. This problem was overcome by shaking 100 ml of buffered urine (pH 9.5) with 5 g of XAD-2 resin, pouring the resin through a glass column and eluting with 25 ml of ethyl acetate–dichloromethane (60:40)[2]. Other workers[5] used four 5-ml fractions (aqueous drug solutions), which were shaken with the same amount of resin.

To compare the two methods (A and B), 2.0 ± 0.1 g of rinsed resin were shaken with 20 ml of spiked human urine (pH 12–13) for 15 min. After decanting the urine, the resin was poured through the column with small volumes of 0.001 *N* sodium hydroxide solution. The columns were sucked dry and eluted with 20 ml of chloroform Subsequent stages, urinary drug concentration and standard solutions were as described earlier[1].

The recoveries of this method for the resins used are given in Table II, and are the mean values of six determinations.

The recoveries in Table II do not obey the general sequence found with method A. For use in doping analysis with method B, XAD-8 and XAD-2 are to be preferred to the other resins.

On comparing the drug extractabilities for the two methods, it should be mentioned that the lower recoveries with XAD-7 in method A are substantially higher

TABLE II

COMPARATIVE DRUG EXTRACTABILITIES (%) USING DIFFERENT XAD RESINS (METHOD B)

The figures in parentheses are standard deviations.

Drug	*XAD-2*	*XAD-4*	*XAD-7*	*XAD-8*
Amphetamine	71.2 (4.20)	51.1 (2.96)	72.1 (4.17)	45.3 (2.72)
Chlorphentermine	84.8 (1.58)	70.7 (3.94)	85.6 (2.96)	87.0 (3.53)
Cyclopentamine	57.5 (3.36)	39.8 (3.80)	13.2 (1.41)	44.1 (6.09)
Dimethylamphetamine	65.8 (5.90)	*	29.1 (5.95)	49.8 (5.90)
Ethylamphetamine	86.1 (5.21)	80.7 (5.35)	65.6 (3.30)	84.4 (3.20)
Fenfluramine	66.6 (4.12)	69.9 (3.56)	71.2 (8.72)	80.9 (6.69)
Mephentermine	51.5 (4.99)	74.7 (6.46)	34.0 (4.08)	57.9 (4.82)
Methoxyphenamine	57.5 (5.47)	56.4 (3.39)	21.3 (3.73)	53.8 (5.76)
Methylamphetamine	76.2 (6.31)	68.4 (3.22)	52.5 (5.00)	86.3 (5.64)
Phendimetrazine	82.9 (8.48)	97.0 (4.37)	99.0 (6.37)	90.2 (5.39)
Phenmetrazine	76.2 (4.75)	96.0 (3.80)	63.5 (4.32)	54.6 (2.36)
Phentermine	59.4 (6.17)	75.4 (3.89)	60.4 (4.12)	68.9 (1.63)
Propylhexedrine	28.9 (4.46)	43.0 (7.22)	11.0 (0.74)	55.9 (2.30)

* Not measured owing to interfering peak.

TABLE III

EFFECT OF REFRIGERATED STORAGE OF XAD RESINS ON THE DRUG EXTRACTABILITY (%) USING METHOD A

The figures in parentheses are the recoveries (%) without refrigerated storage of the resin.

Resin	*Amphetamine*	*Cyclopentamine*	*Fenfluramine*	*Methoxyphenamine*	*Methylamphetamine*	*Phendimetrazine*	*Phenmetrazine*
XAD-2	77.9 ± 4.63	70.8 ± 3.62	73.9 ± 4.46	91.0 ± 3.60	78.9 ± 0.34	97.8 ± 3.02	96.6 ± 3.20
	(72.8 ± 3.70)	(68.9 ± 5.66)	(75.7 ± 2.41)	(68.4 ± 2.52)	(75.1 ± 5.47)	(91.8 ± 9.32)	(85.8 ± 8.00)
XAD-4	78.1 ± 2 12	65 2 ± 2.85	66.0 ± 3.72	66.4 ± 2.93	80.2 ± 4.47	87.3 ± 5.06	93.3 ± 2.09
	(79.6 ± 3.15)	(70.2 ± 4.61)	(67.5 ± 1.31)	(70.4 ± 4.61)	(82.3 ± 2.86)	(93.6 ± 5.56)	(89.4 ± 2.10)
XAD-7	72.8 ± 5.02	17.9 ± 2.03	72.9 ± 6.34	36.3 ± 3.83	69.1 ± 5.44	— *	90.3 ± 5.08
	(63.4 ± 5.28)	(11.6 ± 2.02)	(31.7 ± 3.56)	(12.5 ± 1.79)	(47.3 ± 2.01)		(88.9 ± 3.77)
XAD-8	65.1 ± 2.82	55.5 ± 5.96	77.8 ± 3.53	74.4 ± 3.57	100.1 ± 5.86	91.5 ± 3.38	59.6 ± 2.30
	(78.6 ± 3.72)	(43.1 ± 4.02)	(77.9 ± 5.55)	(62.7 ± 5.79)	(90.2 ± 7.33)	(92.5 ± 8.16)	(70.5 ± 6.97)

* Not measured.

TABLE IV

EFFECT OF REFRIGERATED STORAGE OF XAD RESINS ON THE DRUG EXTRACTABILITY (%) USING METHOD B

The figures in parentheses are the recoveries (%) without refrigerated storage of the resin.

Resin	*Amphetamine*	*Cyclopentamine*	*Fenfluramine*	*Methoxyphenamine*	*Methylamphetamine*	*Phendimetrazine*	*Phenmetrazine*
XAD-2	80.8 ± 1.82	53.6 ± 3.70	61.5 ± 4.81	55.1 ± 4.72	63.7 ± 4.91	81.8 ± 1.76	76.3 ± 3.14
	(71.2 ± 4.20)	(57.5 ± 3.36)	(66.6 ± 4.12)	(57.5 ± 5.47)	(76.2 ± 6.31)	(82.9 ± 8.48)	(76.2 ± 4.75)
XAD-2	50.6 ± 3.09	43.2 ± 4.41	65.3 ± 1.31	54.9 ± 3.38	63.4 ± 4.50	99.6 ± 2.79	83.6 ± 0.77
	(51.1 ± 2.96)	(39.8 ± 3.80)	(69.9 ± 3.56)	(56.4 ± 3.39)	(68.4 ± 3.22)	(97.0 ± 4.37)	(96.0 ± 3.80)
XAD-7	58.0 ± 4.70	16.4 ± 3.10	71.4 ± 1.70	27.5 ± 3.99	54.9 ± 2.06	101.1 ± 5.00	63.7 ± 4.86
	(72.1 ± 4.17)	(13.2 ± 1.41)	(71.2 ± 8.72)	(21.3 ± 3.73)	(52.5 ± 5.00)	(99.0 ± 6.37)	(63.5 ± 4.32)
XAD-8	50.1 ± 5.15	47.4 ± 3.96	72.6 ± 2.25	53.9 ± 4.23	98.1 ± 3.46	76.1 ± 3.34	42.8 ± 1.67
	(45.3 ± 2.72)	(44.1 ± 6.09)	(80.9 ± 6.69)	(53.8 ± 5.76)	(86.3 ± 5.64)	(90.2 ± 5.39)	(54.6 ± 2.36)

in some instances when method B is used. In a liquid chromatographic separation study of phenols using XAD-7 resin, it was noticed by Fritz and Willis[10] that this resin had been chemically altered under alkaline conditions. Hence the low recoveries using XAD-7 (method A) could be attributed to the lower absorptive capacity of the resin owing to partial hydrolysis of the ester groups during the washing step with 0.01 *N* sodium hydroxide solution. Indeed, the results in Table II demonstrate the better recoveries with XAD-7 in method B without preliminary washing with sodium hydroxide solution.

On the other hand, generally similar (XAD-2) or even better results (XAD-4, XAD-8) are obtained with method A. The low values obtained in method B could not be improved by increasing the shaking time; in an additional experiment with propylhexedrine using XAD-8 resin, the recoveries were 35.3 ± 4.62, 51.2 ± 4.72, 55.9 ± 2.48, 47.9 ± 8.04 and 51.2 ± 8.05% for shaking times of 5, 10, 15, 30 and 60 min, respectively. Nevertheless, it is possible that an enhancement of the resin to urine ratio could increase the recoveries for method B.

Effect of refrigerated storage of XAD resin on the drug extractability

Bastos *et al.*[11] mentioned that the refrigerated storage of XAD-2 under distilled water for 7–14 days increased the recoveries of morphine and phenobarbital by 20% and 12.6%, respectively.

The effect of refrigerated storage on the recovery of some CNS stimulants was studied here using different XAD resins and methods A and B, 2.0 ± 0.1-g portions of the XAD resins being washed and stored for 7 days under distilled water at 4°. The results of these experiments (mean values of four determinations) compared with those found with the normal procedure are given in Table III (method A) and Table IV (method B).

Taking into account the standard deviations, the drug extractabilities in Tables III and IV clearly show, with the exception of XAD-7 (method A), that in contrast to morphine and phenobarbital the effect of refrigerated storage of the resins on the recovery of CNS stimulants is negligible for both methods.

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CHROM. 9943

DETERMINATION OF THE ANTI-INFLAMMATORY AGENT CARPROFEN, (D,L)-6-CHLORO-α-METHYLCARBAZOLE-2-ACETIC ACID, IN BLOOD BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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SUMMARY

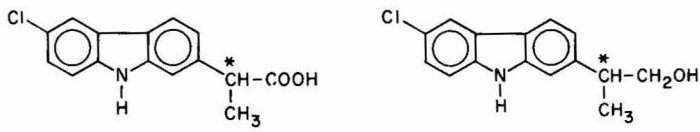
A rapid, sensitive, and specific high-pressure liquid chromatographic (HPLC) assay was developed for the determination of (D,L)-6-chloro-α-methylcarbazole-2-acetic acid (carprofen) in blood. The assay involves extraction into diethyl ether from blood buffered to pH 6. The overall recovery of carprofen from blood is 97.3 ± 5.3% (S.D.), and the sensitivity limit of detection is 100–200 ng/ml of blood using a UV detector at 254 nm or 3 ng/ml of blood using a fluorescence detector with excitation at 240 nm and emission at wavelengths greater than 350 nm. The HPLC assay is amenable to rapid routine analysis of clinical specimens, and the data obtained using this assay showed an excellent correlation coefficient (0.99) compared with a previously published spectrofluorometric assay. The method was used to monitor the blood level–time fall-off profiles in four subjects following single and multiple dose administration of carprofen.

INTRODUCTION

The compound (D,L)-6-chloro-α-methylcarbazole-2-acetic acid (carprofen, compound I in Fig. 1) was synthesized by Berger[1] and is a member of a series of carbazoles undergoing pharmacological testing as anti-inflammatory agents[2].

Previously published luminescence and electron-capture gas–liquid chromatographic (EC–GLC) procedures for compound I in blood and urine[3,4] were time-consuming for routine analysis of the large number of specimens usually obtained from clinical studies. Consequently, a sensitive and specific high-pressure liquid chromatographic (HPLC) assay was developed for the determination of compound I in blood. The HPLC assay was equivalent to the luminescence and EC–GLC procedures in sensitivity and specificity but was much simpler to use in routine analysis.

The analogous compound 2-[(D,L)-6-chloro-2-carbazolyl]-propanol (compound II in Fig. 1) was used as the reference standard in the assay.



COMPOUND [I] COMPOUND [II]

Fig. 1. Chemical structures of compounds I and II. The asterisk indicates the asymmetric carbon atom.

EXPERIMENTAL

HPLC analysis of compound I in blood

Column. The column used was a 0.25 m × 4.6 mm I.D. stainless-steel column containing Partisil silica gel 10 μm (Whatman, Clifton, N.J., U.S.A.).

Instrumental parameters. A DuPont Model 830 high-pressure liquid chromatograph equipped with a Model 835 multiwavelength UV detector operated at 254 nm and a Waters Assoc. loop injector Model No. U6K was used. A Schoeffel Model FS-970 fluorescence detector operated at 240 nm for excitation and at wavelengths greater than 350 nm for emission (Corning No. 0-52 filter) was used for fluorimetric detection. The isocratic mobile phase used was a mixture of methylene chloride–methanol–acetic acid (98:1:1) at a head pressure of 750 p.s.i. and a flow-rate of 1.5 ml/min. Under these conditions, the retention time of compound I was 3.8 min and that of compound II 5.2 min. The UV detector sensitivity was 1×10^{-2} a.u.f.s., and the fluorescence detector sensitivity was 0.1 μA.f.s. The chart speed on the 1.0-mV Honeywell recorder (Model No. 194) was 30 in./h. Under these conditions 200 ng of compound I and 150 ng of compound II per 10 μl injected give nearly full-scale pen response when operated in the UV mode, whereas 3 ng of compounds I and II per 10 μl injected give nearly full-scale pen response when operated in the fluorescence mode. The minimum detectable amounts of compounds I and II are 100 and 150 ng/ml of blood, respectively, using the UV detector and 3 ng/ml of blood using the fluorescence detector.

Analytical standards. Compound I ($C_{15}H_{12}ClNO_2$, MW = 273.72, m.p. = 192–194°) and compound II ($C_{15}H_{14}ClNO$, MW = 259.73, m.p. = 170–171.5°) of pharmaceutical grade purity (>99%) are used as analytical standards.

Prepare stock solutions of compounds I and II in separate 10-ml volumetric flasks by dissolving 10 mg of each compound into 1 ml of methanol. Dilute to volume with methylene chloride–acetic acid (99:1). These stock solutions (containing 1 mg/ml) are used to prepare the following mixed standard solutions (Table I) by suitable dilutions in methylene chloride–methanol–acetic acid (98:1:1), 100 μl of which are added to blood as internal standards.

Ten-microliter aliquots of solutions A to D or E to H are injected as external standards for establishing the HPLC parameters using either the UV or the fluorescence detector, respectively. Aliquots (100 μl) of the same solutions are added to blood as the internal standard calibration curve for the determination of the concentration in the unknowns and for the determination of percent recovery.

Calibration of compounds I and II by HPLC. A calibration (external standard) curve of the peak area ratio of compound I to compound II *vs.* the concentration of

TABLE I

STANDARD SOLUTIONS TO BE USED WITH AN UV AND A FLUORESCENCE DETECTOR

Standard	*Compound I*	*Compound II (ref. std.)*
UV detector (μg/100 μl)		
A	0.5	1.5
B	1.0	1.5
C	1.5	1.5
D	2.0	1.5
Fluorescence detector (ng/100 μl)		
E	7.5	30
F	15	30
G	22.5	30
H	30	30

compound I per 100 μl of methylene chloride–methanol–acetic acid (98:1:1) is constructed. A fresh calibration curve of the external standards and of the recovered internal standards are prepared for each day of analysis to establish the reproducibility of the HPLC system.

Reagents. All reagents must be of analytical reagent grade (>99% purity). Potassium phosphate buffer (1.0 *M*, pH 6) is prepared by mixing equal volumes of 1 *M* $K_2HPO_4 \cdot 3H_2O$ (228.23 g/l) and 1 *M* KH_2PO_4 (136.09 g/l). Mix well by inversion and check final pH with a pH meter. Absolute diethyl ether (Mallinckrodt, St. Louis, Mo., U.S.A.) is the extraction solvent, and a mixture of methylene chloride–methanol–acetic acid (98:1:1) is used for both the mobile phase for the HPLC system and to make standard solutions of compounds I and II.

Analysis of blood. The flow diagram of the extraction procedure is shown in Fig. 2.

Into a 15-ml conical centrifuge tube (PTFE No. 13 stoppered), add 0.5 ml oxalated whole blood, 2 ml of 1 *M* phosphate buffer (pH 6), mix well, and extract with 8 ml of diethyl ether by shaking for 10 min on a reciprocating shaker (Eberbach) at 80–100 strokes/min. Along with the samples, run a specimen of control blood and four 0.5-ml control blood specimens containing 0.1 ml of either standard solution A, B, C, or D (equivalent to 0.5, 1.0, 1.5, 2.0 μg of compound I and 1.5 μg of compound II per 0.5 ml blood) when using the UV detector or solutions E, F, G, or H (equivalent to 7.5, 15, 22.5, 30 ng of compound I and 30 ng of compound II per 0.5 ml blood) when using the fluorescence detector. Centrifuge the samples at 2500 rpm (1500 *g*) in a refrigerated centrifuge (Model PR-J, rotor No. 253, Damon/IEC Corp.) at 5°. Repeat the extraction with another 8-ml portion of diethyl ether, centrifuge, and combine the ether extracts in a 15-ml conical centrifuge tube. Evaporate the ether extracts to dryness at 60° in a N-EVAP evaporator (Organomation Assoc.) under a stream of clean, dry nitrogen. Dissolve the residues in 100 μl of methylene chloride–methanol–acetic acid (98:1:1) and inject a 10-μl aliquot into the liquid chromatograph. Typical chromatograms of blood extracts are shown in Figs. 3 and 4.

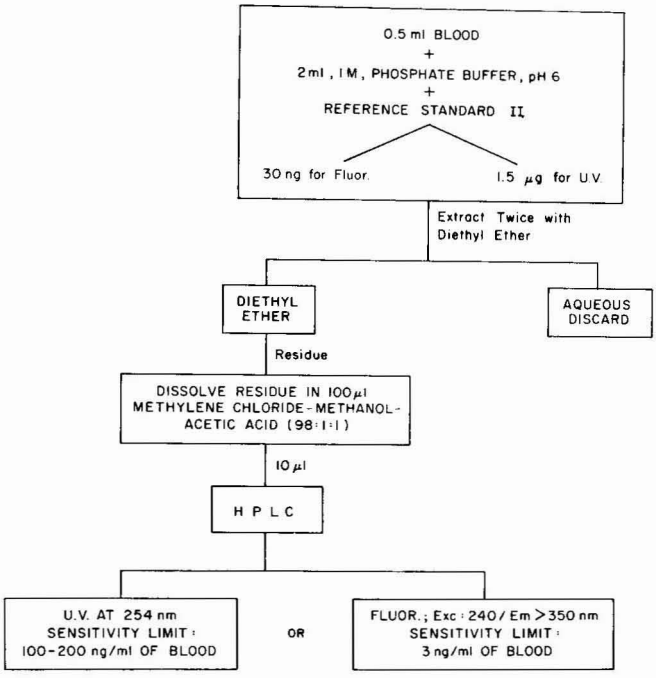


Fig. 2. Flow diagram of the extraction procedure for compounds I and II.

Calculations. The peak area ratio of compound I to compound II in the respective recovered internal standards is determined and plotted graphically *vs.* total concentration to establish the blood recovery curve. Similarly, the peak area ratio of compound I to compound II in the aliquots of the respective unknowns injected is also determined. The concentration of compound I in the unknowns represented by its peak area ratio is interpolated from the blood recovered internal standard curve. Since the peak area ratio of compound I to compound II is constant irrespective of the actual volume of sample injected or the total volume of the solvent, no dilution or aliquot factor is needed in the quantitation of the unknowns, even with further dilutions (*i.e.*, $>100\ \mu l$), provided the peak due to the reference standard (compound II) is still measurable. The recovery factor for both internal and reference standards also remains constant throughout and is not needed for the calculation of the unknowns. Thus, concentration (ng) in the unknowns interpolated from the internal standard curve = ng of compound I per 0.5 ml of blood.

If, however, the peak due to the reference standard is diluted out, a direct calibration technique must be employed whereby a calibration curve of peak area of the recovered internal standard of compound I *vs.* concentration is plotted and used for the quantitation of the unknowns. Furthermore, the amount of compound I per aliquot of the unknown sample injected has to be corrected for the dilution of the total sample.

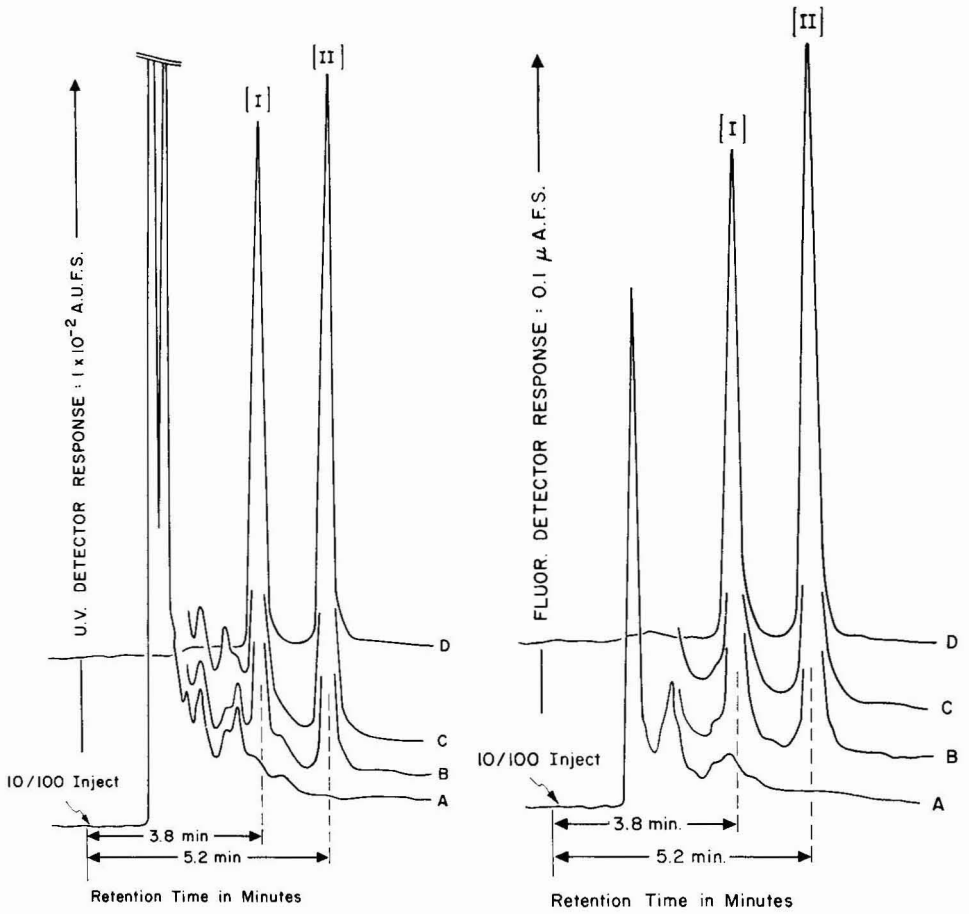


Fig. 3. Chromatograms of HPLC analysis, using a UV detector, of diethyl ether extracts of (A) control blood, (B) control blood containing added authentic standard, (C) subject blood post oral dose, and (D) authentic standard.

Fig. 4. Chromatograms of HPLC analysis, using a fluorescence detector, of diethyl ether extracts of (A) control blood, (B) control blood containing added authentic standard, (C) subject blood post oral dose, and (D) authentic standard.

RESULTS AND DISCUSSION

The intense UV absorption and luminescence properties of the carbazole class of compounds is well documented[5,6]. A sensitive and specific HPLC assay was developed for the determination of compound I from 1 ml or less of blood, employing either a UV or a fluorescence detector. This method provides for rapid and simple quantitation of compound I for routine analysis of the large number of samples obtained from clinical studies.

The major UV absorption bands of compounds I and II occur at 240–242 nm and are shown in Fig. 5. The DuPont Model 835 multi-wavelength UV detector was

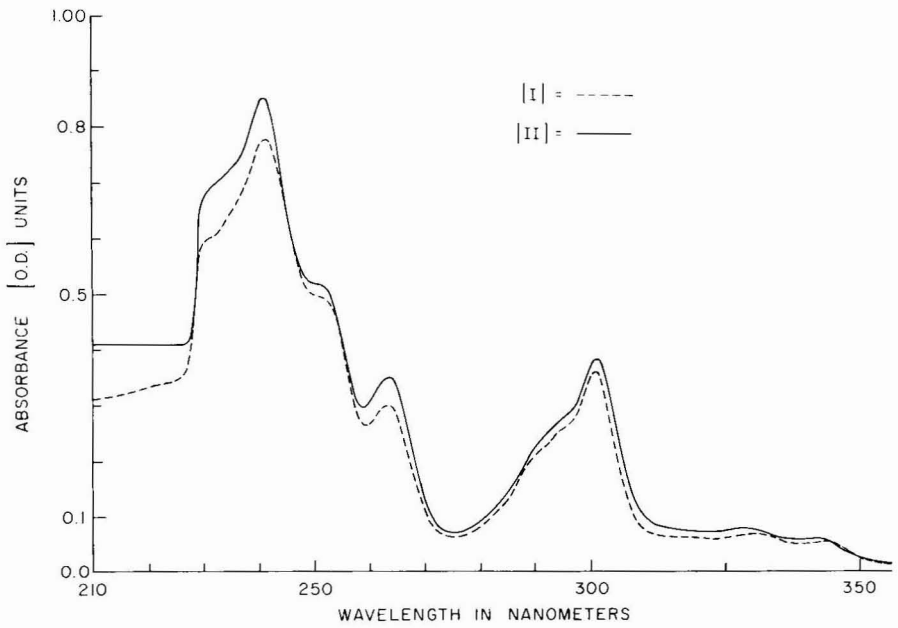


Fig. 5. UV absorption spectra of 4 μg/ml solutions of compounds I and II in methylene chloride–methanol–glacial acetic acid (98:1:1).

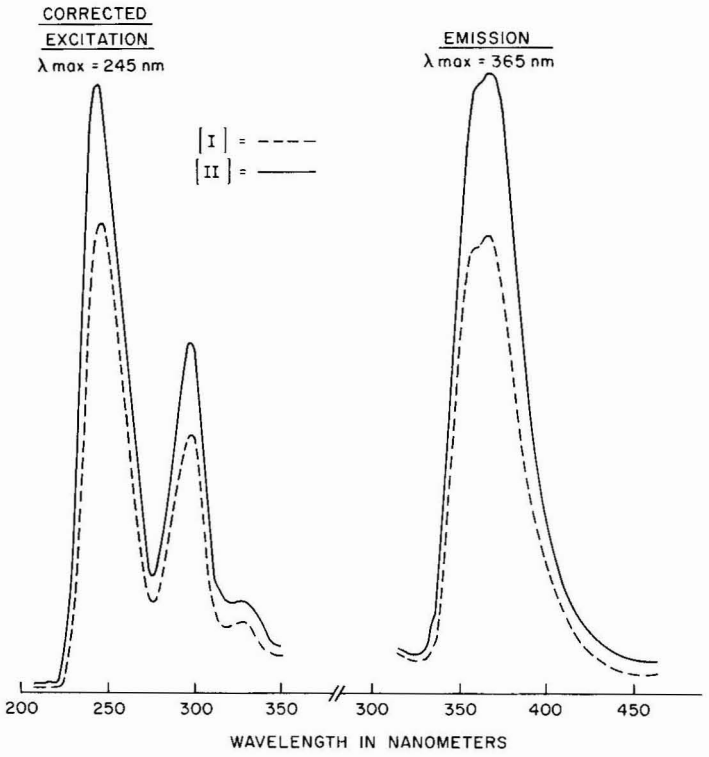


Fig. 6. Corrected excitation and emission spectra of 100 ng/ml solutions of compounds I and II in methylene chloride–methanol–glacial acetic acid (98:1:1).

used at 254 nm in conjunction with a low-pressure mercury lamp. Although measuring the UV absorption of compounds I and II at 254 nm is not at the maxima for both compounds (Fig. 5), the sensitivity at this wavelength was sufficient for the comparison of this method to the previously published luminescence methods[3,4]. If more sensitivity is required (approximately fifty fold), then the fluorescence detector should be used in tandem with the UV detector. The corrected excitation and emission spectra of compounds I and II using a Farrand spectrofluorometer equipped with a xenon lamp are shown in Fig. 6. The corrected excitation maximum is at 245 nm and coincides with the absorption maxima at 240–242 nm. The emission maximum is at 365 nm. The excitation monochromator of the Schoeffel Model FS970 fluorescence detector is set at 240 nm owing to the higher energy output of the deuterium lamp used as its energy source. A Corning No. 0-52 filter (greater than 350 nm band-pass) is used for measuring the fluorescence emission of both compounds.

The HPLC assay is the method of choice because it is a simple three-step operation that involves selective extraction, sample concentration, and direct analysis by HPLC. An earlier luminescence method[3] employs double extraction, a two step thin-layer chromatographic (TLC) separation and elution prior to fluorometric determination. The EC–GLC method[3], in addition to the above steps, also requires esterification prior to analysis.

TABLE II

COMPARISON OF THE HPLC METHOD USING A UV DETECTOR AND THE TLC–FLUORESCENCE METHOD

n.d. = Not detectable. Limit of sensitivity = 0.1–0.2 μg/ml blood. Correlation coefficient = 0.99.

Subject	*Day of dose*	*Time after a 100-mg dose t.i.d. (h)*	*Concentration of compound I in blood (μg/ml)*	
			HPLC	*TLC–fluorescence*
A	Day 14	0	1.63	1.93
		0.5	10.1	8.92
		1	6.42	6.09
		2	4.30	4.26
		5	2.00	2.10
		8	1.29	1.48
		12	0.86	1.13
		24	0.64	0.85
		48	0.32	0.48
		168	n.d.	n.d.
B	Day 1	0	n.d.	n.d.
		0.5	0.40	0.68
		1	2.99	3.81
		2	5.60	6.24
		5	2.70	3.04
	Day 3	0	3.01	3.63
		0.5	4.20	5.06
		1	7.19	7.67
		2	6.70	7.37
		5	4.83	5.11

Recovery and sensitivity limits of the HPLC assay

The overall recovery of compounds I and II from blood is of the order of 97.3 ± 5.3% (S.D.). The sensitivity limit of detection is 100–200 ng/ml of blood, using a UV detector at 254 nm, or 3 ng/ml of blood using a fluorescence detector with excitation at 240 nm and emission at wavelengths greater than 350 nm.

Application of the method to biological specimens

In order to evaluate the clinical utility of the HPLC method, it was necessary to analyze blood samples obtained from clinical studies on compound I that were previously analyzed by the TLC–fluorescence method[3].

The HPLC method was used to monitor the blood level–time profiles in two subjects following 14 consecutive days of oral dosing at 100 mg three times a day. One subject was monitored for 168 h, following the last dose on day 14. A second subject was monitored only on days 1 and 3 following the first daily dose of compound I The blood levels obtained by the HPLC method were statistically compared with those obtained by the TLC–fluorescence method using linear regression analysis. The

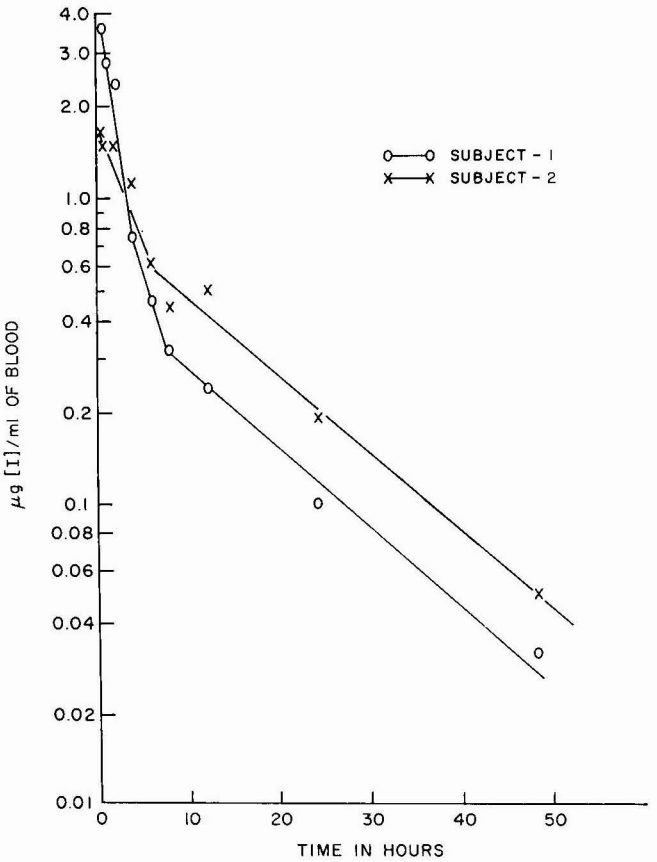


Fig. 7. Blood level fall-off curves in man following the oral administration of a single 100-mg dose of compound I.

resulting least squares line ($r = 0.99$) indicated that a slope of 1 and an intercept of 0 fell within the 95% confidence limits. The results and comparison of the two methods are shown in Table II. The HPLC method was also applied to the analysis of blood samples following a single 100-mg oral dose of compound I in two subjects (Fig. 7). The blood level–time curves from these two subjects show peak levels at 0.5 h post administration of 3.6 and 1.6 μg/ml of blood, respectively, indicating rapid absorption of the drug. The blood levels at 24 and 48 h in both subjects were below the limit of quantitation of the UV detector, hence required the sensitivity of the fluorescence detector for their quantitation.

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CHROM. 9942

GAS–LIQUID CHROMATOGRAPHIC DETERMINATION OF 1,3-DIHYDRO-3-PHENYLSPIRO[ISOBENZOFURAN-1,4-PIPERIDINE], HP 505, IN BIOLOGICAL FLUIDS USING A NITROGEN-SPECIFIC DETECTOR

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SUMMARY

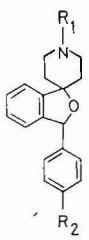
A gas chromatographic method for the determination of 1,3-dihydro-3-phenylspiro[isobenzo-1,4-piperidine], HP 505, in plasma, red blood cells and urine has been developed. HP 505 and internal standard are extracted from basified fluid with hexane and then back extracted into acetic acid. After re-extraction into hexane, HP 505 and internal standard are analysed by gas–liquid chromatography as the N-propionyl derivatives using a nitrogen-specific detector. Concentrations of HP 505 can be measured over the range 2–100 ng/ml plasma.

The method has been applied to the analysis of biological fluids from volunteers receiving oral doses of HP 505.

INTRODUCTION

The compound 1,3-dihydro-3-phenylspiro[isobenzofuran-1,4-piperidine], HP 505 (I in Fig. 1), is currently being developed as a new drug acting on the central nervous system.

In order to examine its bioavailability and pharmacokinetics in man, it was necessary to have an analytical method to determine its concentration in biological



	R_1	R_2
I	H	H
II	$COCH_2CH_3$	H
III	H	CH_3
IV	$COCH_2CH_3$	CH_3

Fig. 1. Structural formulae of HP 505 (I), HP 1197 (III) and their N-propionyl derivatives (II and IV, respectively).

fluids. It was anticipated, from the known metabolism of HP 505 in animals[1], that the levels of HP 505 in plasma resulting from the projected oral dose of 5–50 mg would be very low (*ca.* 10–50 ng/ml). Preliminary investigations indicated that neither fluorimetry nor spectrophotometry would be sufficiently sensitive, but that gas–liquid chromatography using a nitrogen-specific detector would be sufficiently sensitive and specific after conversion of HP 505 to the corresponding N-propionyl derivative (II in Fig. 1).

MATERIALS AND METHODS

Reagents

All chemicals were of analytical grade and were used without further purification, unless otherwise indicated.

Hexane ("Distol" grade, Fisons, Loughborough, Great Britain) was allowed to stand over concentrated sulphuric acid for 24 h, and then over an acidic solution of potassium permanganate (0.5% in 1 *N* sulphuric acid) for a further 24 h; it was then washed with water, dried over sodium sulphate and distilled. A reagent mixture in hexane containing 0.5% (v/v) propionic anhydride (G.P.R. grade; Hopkins and Williams, Chadwick Heath, Great Britain) and 1% (v/v) pyridine was freshly prepared for each batch of samples. Acetic acid (1 *M*) was prepared by diluting glacial acetic acid with distilled water. Cyclohexane was "Distol" grade (Fisons).

Standard solutions

A solution of 1 mg/ml HP 505 was prepared by dissolving HP 505 in the minimum amount of 1 *M* acetic acid and making up to the required volume with distilled water. This solution was then diluted with distilled water to provide the stock solution containing 1 μg/ml HP 505.

A stock solution containing 1 μg/ml of the internal standard HP 1197 (III in Fig. 1) was prepared in exactly the same way. The stock solutions were stable for at least a month if stored below 5°.

Extraction and derivatization from plasma and urine

Hexane (10 ml), 1 *M* sodium hydroxide (0.5 ml) and 100 ng of the internal standard, HP 1197, (0.1 ml of the 1 μg/ml stock solution) are added to plasma (2 ml) [or urine (1 ml)] in a screw-capped test tube. The plasma is extracted for 15 min using a mechanical rotary inversion mixer at 20 rpm (Heto Rotamix, V.A. Howe) and the layers are separated by centrifugation at 2000 *g* for 5 min. The hexane phase is transferred to a clean test tube containing 1 *M* acetic acid (1 ml) and is then extracted for 15 min using the inversion mixer. After centrifugation at 800 *g* for 2 min to separate the layers, the upper hexane phase is aspirated and discarded. After the aqueous phase has been washed with hexane (1 ml), it is made alkaline by the addition of 1 *M* sodium hydroxide (1.5 ml), and extracted with hexane (5 ml). The layers are separated by centrifugation at 800 *g* for 2 min and the hexane phase is transferred to a clean test tube. A freshly prepared hexane solution (0.5 ml) of 0.5% propionic anhydride and 1% pyridine is added to the test tube; the contents are mixed and reacted for 1 h at 60° in a water-bath.

One *M* acetic acid (1 ml) is added to the cooled reaction mixture, and the contents are extracted for 15 min using the inversion mixer. After centrifugation at 800 *g* for 2 min, the hexane phase is transferred to a tapered test tube. The tubes are immersed in a water-bath at 40°, and the solvent is removed by a gentle stream of nitrogen. Cyclohexane containing 5% of ethyl acetate (100 μl) is used to wash the walls of the tube and concentrate the residue in the tapered tip of the test tube. The samples can then be stored below 5° until needed for analysis, at which time the solvent is removed by a nitrogen stream at room temperature. The dry residue is then taken up in the mixture of cyclohexane and ethyl acetate (25 μl) and aliquots (5 μl) are analysed by gas–liquid chromatography.

Extraction and derivatization from packed red blood cells

For determinations in packed red blood cells, about 1 g of cells is accurately weighed into a test tube and diluted with water (1 ml) before proceeding in exactly the same way as for plasma.

Gas–liquid chromatography

Analyses were performed on a Perkin-Elmer F17 gas chromatograph equipped with a Perkin-Elmer nitrogen–phosphorus detector which has a nitrogen:carbon selectivity of at least 5000:1 (ref. 2). The coiled glass column (2 m × 1.75 mm I.D.) was packed with 3% OV-25 on Chromosorb W-HP (100–120 mesh). The carrier gas flow-rate was 20 ml/min of helium and the oven temperature was 275°. The injector and detector were maintained at 300°. The hydrogen and air flow-rates to the detector were 3 ml/min and 60 ml/min, respectively.

Because of the high temperatures required to achieve short analysis times, only the thermally stable silicone phases were considered suitable. Of the phases investigated, the relatively polar OV-25 was chosen, rather than OV-1 or OV-17, as this gave better separation of N-propionyl HP 505 from other peaks in the chromatogram arising from endogenous plasma constituents. Under these conditions the retention times of N-propionyl HP 505 and N-propionyl HP 1197 were 5.5 and 6.5 min, respectively. Typical chromatograms obtained from plasma are shown in Fig. 2.

Gas chromatography–mass spectrometry

Mass spectra were determined on an AEI MS 30 mass spectrometer coupled to a Pye 104 gas chromatograph (Pye Unicam) via a membrane separator. The mass spectometer was operated at 45 eV, and 300 μA ionizing current; the source temperature was 250° and the separator temperature was 230°. Chromatography was performed at 280° using a glass column (1.5 m × 4 mm) packed with 3% OV-17 on Chromosorb W-HP (100–120 mesh). Helium flowing at 45 ml/min was the carrier gas.

Quantification of HP 505 levels

HP 505 levels in biological fluids were calculated using a response factor obtained by analysing blank samples of the fluid to which 100 ng of HP 505 (0.1 ml of the 1 μg/ml stock solution) had been added. These calibration samples were analysed in parallel with the unknown samples, and the response factor was calculated for each

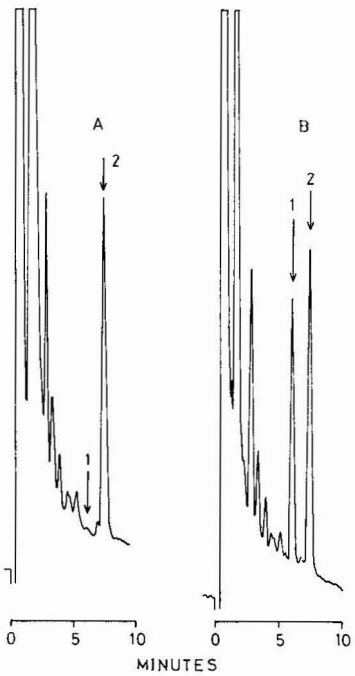


Fig. 2. Examples of chromatograms: A, extract of plasma (2 ml) taken from a volunteer prior to dosing; B, extract of plasma (2 ml) taken from the same volunteer 8 h after a 50-mg oral dose of HP 505. The arrows 1 and 2 indicate the retention times of the N-propionyl derivatives of HP 505 and HP 1197, respectively. The calculated amount of HP 505 in B was 35 ng/ml.

batch of samples. The concentration of HP 505 was calculated from the following formula

$$\text{concentration of HP 505} = \text{response factor} \times \frac{\text{peak height of N-propionyl HP 505}}{\text{peak height of N-propionyl HP 1197}} \times \times \text{concentration of added HP 1197}$$

$$\text{where: response factor} = \frac{\text{peak height of N-propionyl HP 1197}}{\text{peak height of N-propionyl HP 505}} \times \frac{\text{concentration of added HP 505}}{\text{concentration of HP 1197}}$$

Over a number of months during which the method was applied to the analysis of HP 505 in plasma, the average value of the response factor was 1.18 (S.D. $\pm$ 0.04, 32 observations).

RESULTS AND DISCUSSION

Low levels of primary and secondary amines cannot usually be analysed quantitatively by gas–liquid chromatography because of high adsorptive losses on the column and poor peak shape. However, the gas chromatographic properties of amines can be considerably improved by formation of N-acyl derivatives which can be prepared in good yield by reacting the amine with anhydrides, often in the presence

of pyridine as a catalyst. Four derivatives of HP 505 were prepared, namely, N-heptafluorobutyryl, N-acetyl, N-trifluoroacetyl and N-propionyl. Of these, the N-propionyl was found to have the longest retention time on OV-25 and this resulted in better separation of the drug and internal standard from other compounds present in the final extract.

As it was anticipated that a very sensitive assay would be required for this drug, a preliminary study was carried out using the N-heptafluorobutyryl derivative and electron capture detection (Hewlett-Packard ^{63}Ni). Although the electron capture detector was extremely sensitive to N-heptafluorobutyryl derivative of HP 505, this approach was abandoned because the detector response was found to be non-linear over a wide concentration range and because there was considerable interference from contaminants in the final extract arising from either solvent residues or from compounds co-extracted from plasma. These problems were overcome by using a nitrogen–phosphorus detector; backgrounds were considerably lower and the detector response was found to be linear over at least two orders of magnitude. In addition, this nitrogen–phosphorus detector has been shown to have high stability and reliability approaching that of a conventional flame-ionization detector[2].

EVALUATION OF THE METHOD

Optimization of the extraction

The procedure for isolating HP 505 from plasma was optimized by using [^{14}C]-HP 505. The maximum yield of HP 505 in the first hexane extract, obtained at pH 14 and after 20 min extraction, was found to be about 70%; lower pH resulted in lower recovery, and longer extraction times did not significantly increase the amount of HP 505 extracted. About 50% of the HP 505 remained after the subsequent extraction into 1 *M* acetic acid and back extraction into hexane. Further losses in the remaining steps of the method were found to be negligible and thus the overall recovery for the entire method is about 50%.

Accuracy and precision

The accuracy of the method was established by analysing blank plasma to which had been added HP 505. The results of four separate determinations are summarised in Table I. They show that the accuracy is satisfactory over the range 2–100 ng/ml. In all control samples, a small peak equivalent to about 1 ng/ml of HP 505 was found at the retention time of N-propionyl HP 505. The presence of this peak adversely affects the accuracy of the method for concentrations of less than 2 ng/ml of HP 505.

The precision of the method was determined from duplicate analyses of 2-ml portions of plasma from volunteers who had taken HP 505 orally. The method of Snedecor[3] was used to analyse the data and the results are shown in Table II.

Specificity

The small endogenous peak at the retention time of the N-propionyl derivative of HP 505 could not be eliminated completely by solvent purification or glassware cleaning. However, in the analysis of predose plasma samples from thirty volunteers, this peak never amounted to more than about 1 ng/ml of HP 505.

TABLE I

DETERMINATION OF HP 505 ADDED TO BLANK PLASMA

Each result is the mean of 4 determinations.

HP 505 added (ng/ml)	*HP 505 found (ng/ml)*	*Standard deviation (ng/ml)*	*Coefficient of variation (%)*
0	1.1	0.1	9
0.6	1.5	0.4	27
1.1	1.9	0.6	32
2.6	3.1	0.5	16
5.1	5.4	0.4	7
10.0	10.3	0.5	5
26.7	27.7	0.7	3
51.4	54.1	0.3	0.5
76.2	79.3	2.3	3
100.2	104.7	3.0	3

TABLE II

ESTIMATE OF THE PRECISION OF THE METHOD FROM DUPLICATE DETERMINATIONS

Concentration range of HP 505 (ng/ml)	*Number of samples analysed in duplicate*	*Mean concentration (ng/ml)*	*Estimated standard deviation (ng/ml)*	*Coefficient of variation (%)*
1–10	17	4.8	0.80	16.7
10–20	27	14.8	0.77	5.2
20–30	17	25.8	0.80	3.1
30–50	14	35.2	0.53	1.5

Combined gas chromatography–mass spectrometry was carried out on a urine extract. The spectra obtained from the compounds at the retention times of the N-propionyl derivatives of HP 505 and HP 1197 were identical to the spectra of the authentic standards.

Application of the method

The method has been applied to the analysis of plasma, urine and red blood cells from volunteers who had taken single or multiple oral doses of HP 505. In a typical experiment, a volunteer was given 50 mg of HP 505 orally in capsule form and at various times during the next 24 h blood samples were collected. Immediately after withdrawal, the blood was added to a heparinized tube and the plasma and red blood cells separated by centrifugation. The plasma and red blood cell profiles from one of these experiments are shown in Fig. 3.

In a similar study, a volunteer was given 25 mg of HP 505 orally in capsule form. Urine was collected at various intervals during the next six days and analysed for HP 505. The results are given in Table III.

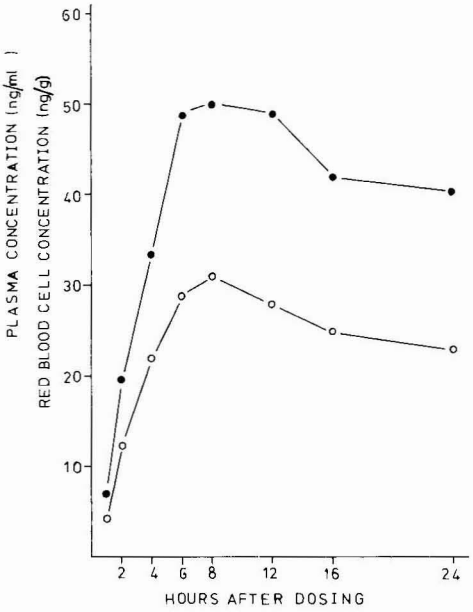


Fig. 3. Plasma (○) and red blood cell (●) levels of HP 505 in a volunteer after a 50-mg oral dose of HP 505.

TABLE III

URINE LEVELS OF HP 505 IN A VOLUNTEER AFTER A SINGLE ORAL DOSE OF HP 505 (25 mg)

Total amount of HP 505 recovered = 4.85 mg.

Time after dosing (h)	*Volume of urine collected (ml)*	*Concentration of HP 505 in urine (μg/ml)*	*Amount of HP 505 recovered (mg)*
0– 4	1145	0.09	0.10
4– 12	2010	0.28	0.56
12– 24	515	0.86	0.44
24– 48	1600	0.76	1.22
48– 72	1400	0.68	0.95
72– 96	1555	0.42	0.65
96–144	5450	0.17	0.93

ACKNOWLEDGEMENT

We wish to thank Mrs. M. A. Hill for her excellent technical assistance in the analysis of the plasma and urine samples.

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APPLICATION OF THE EXTRACTIVE ALKYLATION TECHNIQUE TO THE PENTAFLUOROBENZYLATION OF MORPHINE (A HEROIN METABOLITE) AND SURROGATES, WITH SPECIAL REFERENCE TO THE QUANTITATIVE DETERMINATION OF PLASMA MORPHINE LEVELS USING MASS FRAGMENTOGRAPHY

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SUMMARY

The pentafluorobenzylation of morphine and related phenolic alkaloids by extractive alkylation is described. The alkylation is performed using tetrabutylammonium as counter ion and ethyl acetate as solvent. Optimum reaction conditions are presented together with the gas chromatographic properties of the derivatives formed.

The technique is applied to the quantitation of plasma morphine levels. Using morphine-d_3 as internal standard mass fragmentographic analysis of morphine as its pentafluorobenzyl- and pentafluorobenzyl, mono-trifluoroacetyl derivatives is demonstrated, and a case report is presented. Quantitation to a plasma morphine level of 5 ng/ml is readily attainable.

INTRODUCTION

Biological and forensic samples frequently contain low concentrations of drugs and related metabolites. Drugs of the morphine alkaloid type are highly polar. Their hydrophilic nature necessitates rigorous extraction conditions and often derivatization prior to analysis as exemplified by the submicrogram quantitation of morphine by gas chromatography (GC) using flame ionization detection[1], electron capture detection[2,3], and mass spectrometry (MS)[4]. Extractive alkylation affords a method of isolating polar compounds with simultaneous derivatization; alkylation of phenolic compounds using pentafluorobenzyl (PFB) bromide has been reported[5,6].

This investigation evaluates the use of PFB bromide as a reagent for the extractive alkylation of morphine and related phenolic alkaloids. The conditions for derivatization and their GC properties are presented together with the application of the technique to the quantitation of plasma morphine concentrations using mass fragmentography.

EXPERIMENTAL

Reagents and chemicals

PFB bromide and trifluoroacetic (TFA) anhydride were supplied by Pierce-Warriner (Chester, Great Britain). All solvents (AnalaR grade and redistilled prior to use) and tetrabutylammonium (TBA) hydroxide were obtained from BDH (Poole, Great Britain). Levallorphan and levorphanal tartrate were supplied by Roche Products (Welwyn Garden City, Great Britain), pentazocine by Winthrop Labs. (Newcastle-upon-Tyne, Great Britain), nalorphine hydrobromide by Burroughs Wellcome & Co., (London, Great Britain), and morphine base by MacFarlan Smith (Edinburgh, Great Britain). Morphine-d_3 was synthesized from morphine as previously described[7]. Monoacetylmorphine (MAM) was obtained as a gift from Dr. S. J. Mulé (Narcotic Addiction Control Commission, New York, N.Y., U.S.A.).

Glass equipment

All test-tubes, pipettes, flasks and reactivials (Pierce-Warriner) were washed with concentrated hydrochloric acid distilled water and dried. The glassware was subsequently silanized by treatment with a 4% (v/v) solution of dimethyldichlorosilane in toluene, washed with methanol and dried at 110°.

Gas chromatography

A Pye Unicam GVC gas chromatograph equipped with a pulse modulated electron capture detector (ECD) of the ^{63}Ni type and a flame ionization detector (FID) was used. The detectors were maintained at 300°. Borosilicate glass columns (213 × 0.4 cm I.D.) were packed with 2% OV-17 coated on 100–120 mesh Diatomite C (Pye Unicam, Cambridge, Great Britain) and conditioned for 24 h prior to use. The columns and support material were deactivated by silanization as previously described[8]. The nitrogen carrier gas was freed from contaminants by molecular sieve 13X and used at a flow-rate of 50 ml /min. The FID was operated with hydrogen and air flow-rates of 40 and 500 ml/min, respectively.

Preparation of derivatives

Pentafluorobenzyl derivatives. An aqueous solution (1 ml) containing 0.4 *M* TBA hydroxide, 0.2 *M* sodium hydroxide and 2 mg of alkaloid was added to ethyl acetate (1 ml) containing PFB bromide (20 μl). The reaction tube was stoppered and shaken at 22° until the amount of derivative formed was constant, and minimal or no underivatized alkaloid could be detected by GC–FID.

Pentafluorobenzyl,mono-trifluoroacetyl (PFB,TFA) derivatives. Ethyl acetate solutions of morphine, morphine-d_3, and nalorphine pentafluorobenzyl derivatives prepared similarly to those above were transferred to a reactivial (1 ml) heated at 75° and evaporated to dryness with a stream of nitrogen. Benzene–methanol (1:4, v/v) (50 μl) was added to the vial, and again taken to dryness to remove last traces of water. After addition of benzenes (50 μl) and TFA anhydride (25 μl) the vial was capped and heated for 15 min at 75°.

Plasma extraction

To plasma (1 ml) containing morphine as standards or unknown concentra-

tions were added morphine-d_3 (60 ng from a stock solution), 4 *M* sodium hydroxide (50 μl), TBA hydroxide (250 μl), ethyl acetate (1 μl) and PFB bromide (20 μl). The capped reaction tube was then shaken for 30 min at 22°. After centrifugation (3000 *g* for 5 min) the ethyl acetate layer was aspirated into a clean tube, to which was added 0.05 *M* sulphuric acid (1 ml). The mixture was shaken for 10 min and following centrifugation (3000 *g* for 5 min) the acid layer was removed, brought to pH 14 with 4 *M* sodium hydroxide, and the solution re-extracted with ethyl acetate (1 ml) for 15 min. The organic phase was washed with a little water and evaporated to dryness in a reactivial heated at 75° using a stream of nitrogen.

Mass spectrometry

An MS30 gas chromatograph–mass spectrometer (AEI, Manchester, Great Britain) was used with a coiled glass column (91.4 × 0.4 cm I.D.) packed with 2% OV-17 coated on 100–120 mesh Diatomite "C". The column was maintained at a temperature of 265° and perfused with helium at 40 ml/min. The silicone membrane separator was maintained at 200°. The mass spectrometer was operated with an ion-source temperature of 250°, a trap current of 300 μA, an ionizing voltage of 20 eV and an accelerating voltage of 4 kV. Spectra were recorded with an ultraviolet oscillograph (Bryans Southern Inst., Croyden, Great Britain) using a chart speed of 3 cm/min. Molecular ion and principal fragment ions of the alkaloid derivatives are listed in Table I.

TABLE I

MOLECULAR IONS AND PRINCIPAL FRAGMENT IONS OF THE ALKALOID DERIVATIVES STUDIED

The values in parentheses are relative abundancies.

Derivative	M^+	*M – PFB*	*M – OTFA*
Morphine PFB	465 (16)	284 (100)	—
Morphine-d_3 PFB	468 (14)	287 (100)	—
Nalorphine PFB	491 (13)	310 (100)	—
Pentazocine PFB	465 (23)	284 (100)	—
Levorphanol PFB	437 (61)	256 (100)	—
Levallorphan PFB	463 (87)	282 (100)	—
Morphine PFB,TFA	561 (24)	380 (100)	448 (30)
Morphine-d_3 PFB,TFA	564 (25)	383 (100)	451 (29)
Nalorphine PFB,TFA	587 (23)	406 (100)	474 (19)

Mass fragmentography

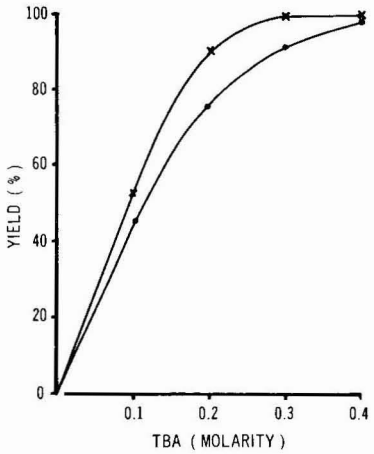
Mass fragmentography studies were performed using the gas chromatograph–mass spectrometer described above equipped with a six-channel multiple peak monitor with sample hold unit. Separations were made using a glass column (45 cm × 0.4 cm I.D.) packed with 2% of OV-17 perfused with helium at 40 ml/min. The column temperature was maintained at 265° for elution of PFB derivatives of morphine and morphine-d_3 with the peak monitor continuously recording the generation of ions at *m/e* 284 and *m/e* 287. The corresponding PFB,TFA derivatives were eluted with a column temperature of 245° and the ions at *m/e* 380 and *m/e* 383 being continuously monitored.

RESULTS AND DISCUSSION

Reaction conditions

Pentafluorobenzylation of the alkaloids was performed by an adaptation of the extractive alkylation technique used by Ehrsson[9] to prepare PFB derivatives of phenols and carboxylic acids. The method has been successfully employed to study chlorthalidone[10] and sulphonamides[11]. The PFB derivatives are normally prepared using methylene dichloride as the organic phase. However, when used with plasma as the aqueous phase, separation of the two components proved impossible owing to protein precipitation. Ethyl acetate was selected as an acceptable alternative, however, when after separation of the two phases by centrifugation, emulsions were either absent or of acceptable minimal proportions.

Quantitative PFB derivatization was dependent on the molarity of the TBA hydroxide used (Fig. 1). Morphine proved to be the most difficult alkaloid to extract from aqueous solution, 0.4 *M* TBA being required to effect a 98% yield as determined by GC–FID. The reaction was also time dependent (Fig. 2). Most of the alkaloids were fully derivatized within 20 min; morphine, however, required a reaction time of 30 min, in the presence of 0.4 *M* TBA, before the GC–FID peak heights of the PFB derivative became constant.



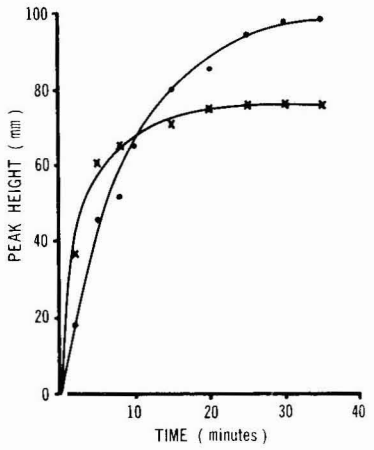


Fig. 1. Influence of TBA on the pentafluorobenzylation of morphine (●) and nalorphine (×). Temperature: 22°. The yields were determined by GC–FID.

Fig. 2. Influence of time on the PFB alkylation of morphine (●) and nalorphine (×). Aqueous phase: 0.4 *M* TBA in 0.2 *M* sodium hydroxide; organic phase: ethyl acetate containing PFB bromide (25 µl); temperature: 22°. Peaks heights determined by GC–FID.

The PFB derivatives of morphine and nalorphine were found to be stable in solution for at least 48 h at 4°. The stability of PFB compounds has previously been demonstrated[5].

All derivatives when examined by GC–MS demonstrated molecular and fragment ions consistent with the addition of one PFB group to the phenolic hydroxyl

group present. Only morphine and nalorphine possess an additional alcoholic function capable of incorporating a TFA group.

Gas chromatographic properties

The derivatives exhibited good peak symmetry. Retention data for the alkaloids studied are given in Table II. Incorporation into the molecule of one PFB group causes less than a three fold increase in retention time; the large increase in molecular weight being partly overcome by the degree of volatility imparted by the PFB group. Reaction of morphine and nalorphine to form the PFB,TFA derivatives further increased the volatility, such that their retention times were intermediate between that of the free bases and their corresponding PFB derivatives. Morphine-d_3 derivatives possessed the same retention data to the corresponding morphine compounds.

TABLE II

RETENTION TIMES RELATIVE TO CODEINE – 1 OF THE ALKALOIDS AND THEIR DERIVATIVES

Column: 2% OV-17 operated at 265° and 245° for PFB and PFB,TFA derivatives, respectively.

Compound	t_{ret}		
	Underivatized	*PFB*	*PFB,TFA*
Pentazocine	0.56	1.16	
Levorphanol	0.56	1.11	
Levallorphan	0.73	1.59	
Morphine	1.19	2.78	1.95
MAM	1.44	2.75	
Nalorphine	1.56	3.70	2.43

The ECD response for morphine and nalorphine PFB derivatives were measured; the minimum detectable quantities[12] were estimated to be $1.2 \cdot 10^{-17}$ and $2.3 \cdot 10^{-17}$ moles/sec, respectively. The high ECD response is in accord with that of other PFB compounds[11,13].

Mass fragmentography

Quantitative determination of plasma morphine levels was studied initially by monitoring the ions at *m/e* 284 and *m/e* 287 generated by the loss of the PFB group from morphine PFB and morphine-d_3 PFB, respectively. Pentazocine is the only narcotic likely to cause ion interference (*m/e* 284), it is, however, easily separated from morphine as indicated by the GC data.

Using a column temperature of 265° and a helium flow-rate of 40 ml/min, morphine eluted with a retention time of 2.25 min. The slight degree of tailing observed (similar to Fig. 3) in the ion intensity peaks recorded was probably due to the short length of column (45 cm) used and/or the relatively low temperature (200°) of the silicone membrane separator. Nevertheless a good linearity graph (analogous to Fig. 4) of the ratio of ions *m/e* 284 to *m/e* 287 *versus* morphine concentration was obtained, and proved applicable to the determination of unknown plasma morphine levels.

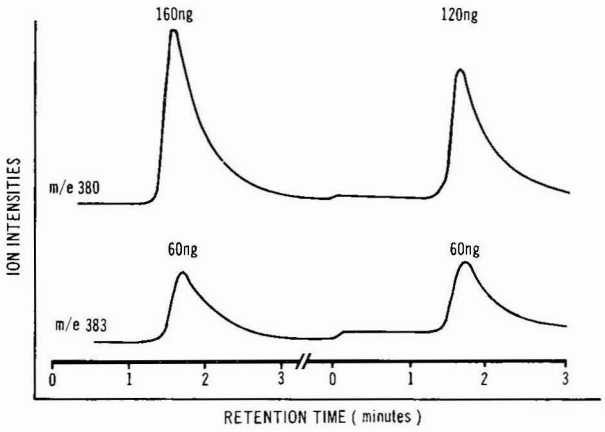


Fig. 3. Mass fragmentogram obtained by continuously monitoring the generation of ions at *m/e* 380 and *m/e* 383. The morphine standards (160 ng and 120 ng) were isolated by the extractive alkylation technique from plasma, containing morphine-d_3 (60 ng) as internal standard, and converted to their corresponding PFB,TFA derivatives. Column: 2% OV-17; column temperature: 245°; helium flow-rate: 40 ml/min.

The method has a number of advantages over that reported for plasma morphine quantitation employing mass fragmentography of the di-TFA derivatives[4]. In our hands, although the di-TFA derivatives gave excellent peak symmetry, long retention time impurities always interfered with subsequent analyses unless given sufficient time to clear the instrument. Also for low plasma morphine level detection the spectrometer multiplier had to be used with the highest setting possible that allowed a permissible signal to noise ratio. Using the PFB derivatives no impurities with long retention times were encountered; this allowed the continuous analysis of samples. Also a lower multiplier setting could be used, presumably owing to the presence of increased morphine levels resulting from its more efficient isolation using the extractive alkylation technique; it was estimated that the method gave a five fold in-

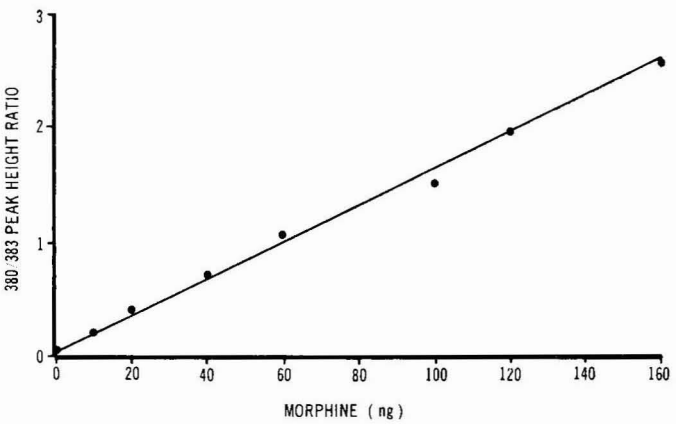


Fig. 4. Standard curve for plasma morphine concentrations analysed as the PFB,TFA derivatives by mass fragmentography (*M* − PFB ions). Internal standard: morphine-d_3 (60 ng); column: 2% OV-17; column temperature: 245°; helium flow-rate: 40 ml/min.

crease in sensitivity over that experienced with the di-TFA derivatives. The method should be generally applicable to the other phenolic alkaloids mentioned in the GC section with the exception of MAM, which undergoes deacetylation with subsequent conversion to morphine PFB.

The main disadvantage of the method was that the *m/e* 284 and *m/e* 287 ion intensity traces did contain ill-defined short retention time (1–2 min) impurities which produced an initial base line drift. Thus the corresponding PFB,TFA derivatives were examined by monitoring the ions at *m/e* 380 and *m/e* 383 generated by the loss of the PFB group from morphine PFB,TFA and morphine-d_3 PFB,TFA, respectively. Using a column temperature of 245° and a helium flow-rate of 40 ml/min morphine eluted with a retention time of 1.25 min. Although the peak shape still exhibited some tailing (Fig. 3), no short or long retention time impurities were discernable, thus allowing continuous sample injection. From a duplicate series of morphine standards extracted from plasma incorporating morphine-d_3 (60 ng) as internal standard, the calibration curve (Fig. 4) was constructed. The curve was used to determine the decay of plasma morphine levels (Fig. 5) resulting from the intramuscular administration of morphine to a 69-kg man. The curves are typical for the dose administered (0.15 mg/kg) and the morphine is readily detectable at the 5 ng/ml level. It is estimated that, if the area of the ion intensity traces were determined by computer linked integration, the overall sensitivity for the total method could be an order of magnitude greater, *i.e.*, quantitation at the picogram level, than those of similar GC–MS methods. Work relating analgesia to plasma morphine levels in patients is currently in preparation.

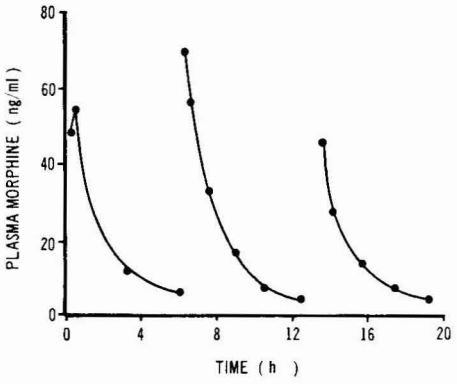


Fig. 5. Time-concentration curves illustrating the decay of plasma morphine concentrations. The morphine (0.15 mg/kg) was administered to a 69-kg man, once preoperatively and twice postoperatively by intramuscular injection.

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CHROM. 9997

Note

Amino acid analysis. A novel reaction chamber

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The heating bath supplied with the Technicon AutoAnalyzer (Model AAA-1) has many undesirable features.

These features include: thermostat malfunction, periodic replacement of a special oil, removal of the oil's decomposition products which coat the glass coil with an insulating layer, manipulations with the glass coil can lead to fractures that are difficult to repair, continuous operation of a stirrer motor and heating element over long periods resulting in energy wastage, and replacement parts for the oil bath being sometimes difficult to obtain. Hence with these problems in mind an alternative method for heating was sought.

MATERIALS AND METHODS

Fig. 1 is a sketch of the new reaction chamber. To the stainless steel base plate ($7\frac{1}{2} \times 7\frac{1}{2} \times \frac{7}{8}$ in.) has been spot welded 4 brackets. The brackets support the glass coil and the metal cylinder cover. The cover is 8 in. long and has a 7 in. diameter.

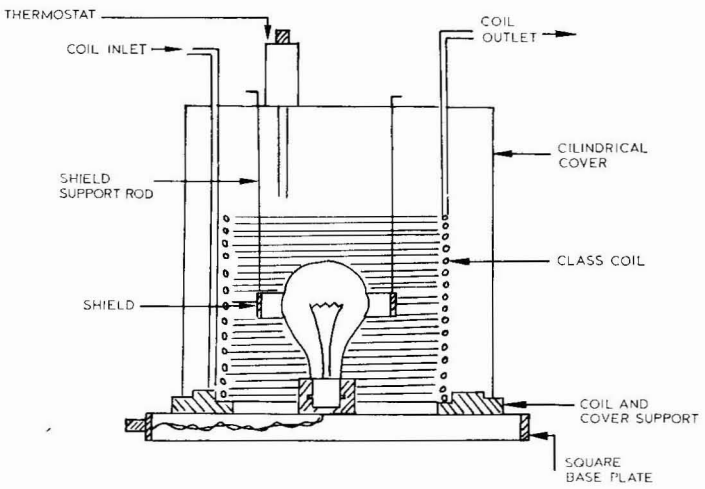

Fig. 1. Sectional view of the reaction chamber.

The inner surface of the cover is reflecting and the outer is painted black. There is a gap of $\frac{1}{2}$ in. between the cover and the base plate. The top of the cover has 5 holes drilled into it. Two holes (5/8 in. diameter) at $6\frac{1}{2}$ in. centres through which approximately 3 in. of coil glass tubing projects; a 5/16-in. hole to allow insertion of the thermostat probe and two holes (1/8 in. diameter) at $3\frac{3}{4}$ in. centres for the positioning of a circular shield around the light globe filament. The width of the shield is 3/8 in. and the thickness 1/32 in. There is a space of 3/4 in. between the shield and the globe. A 100-W clear light globe was located centrally on the base plate and push-in type electrical connections, through which power was supplied to the thermostat, were also fitted to the base plate. A type T.S. 2 N.C. thermostat from Associate Electrical was used in the construction of the reaction chamber. The thermostat can be adjusted by rotation of a cam to obtain the desired temperature of 80°.

The Technicon AutoAnalyzer had previously been converted from a single to a dual column instrument with increased sensitivity[1]. Colour development was obtained by the reaction of amino acids with ninhydrin reduced with titanous chloride[2].

RESULTS

Table I contains the results of the analysis of a standard mixture of amino acids with the new reaction chamber or the commercial oil bath installed in the Technicon. 100 nmoles of each amino acid were present in the standard mixture. Under the conditions of AutoAnalyzer operation given previously, the use of a double

TABLE I

COMPARISON OF CONSTANTS OBTAINED WHEN SYNTHETIC MIXTURES OF AMINO ACIDS (100 nmoles) WERE ANALYSED UNDER VARIED CONDITIONS

Constants shown are average of 3 determinations and the variation for most amino acids in all the analyses was within ±2%.

Amino acid	*Double glass coil*		*Single glass coil*	
	in oil bath at 96°	*with reaction chamber*	*in oil bath at 96°*	*with reaction chamber*
Lys	60	63	62	50
His	57	61	56	50
Arg	54	60	52	50
Asp	55	54	51	47
Thr	58	59	60	50
Ser	58	61	60	50
Glu	63	60	60	51
Pro	16	12	12	10
Gly	61	60	58	52
Ala	63	58	58	50
Hcy	32	29	29	29
Val	61	53	60	50
Met	57	61	55	56
Ile	60	61	56	50
Leu	62	64	56	55
Tyr	60	60	58	55
Phe	60	58	56	50

glass coil allows the reactants to be heated for a period of 17 min with the given flow-rate[1]. As can be seen from Table I there is not much variation in the value for a constant when using the double glass coil in oil bath or reaction chamber but with the single glass coil in the reaction chamber there is a consistent decrease in the value comparative with that obtained using the oil bath. However, this decrease in sensitivity comes about partly from the particular coil used in the reaction chamber. In order to speed the analysis only the inner glass coil was used. The inner coil diameter is approximately 1 in. smaller than that of the outer coil and is consequently about 3 ft. shorter in length. Another contributing factor is that for these analyses the thermostat was set at 80°.

Thus, samples containing as little as 40 nmoles of most amino acids can be analysed satisfactorily with the single coil installed in the reaction chamber without resorting to electronic amplification of the recorder print-out. Although obviously the ninhydrin reaction with amino acids has not gone to completion when using the single coil and reaction chamber, duplication of analyses have shown that the results obtained are accurate and reproducible.

Finally, the desirable features of installing the reaction chamber are: (1) a considerable saving in time and fuel consumption is achieved, as it is only necessary to switch on the light globe at commencement of an analysis; (2) it is speedier to flush out the coil at the termination of an analysis, especially if the proportioning pump is equipped with a two-speed motor, a 14 min flush at high speed is sufficient (Beckman analyzers require 60 min for this operation); (3) no longer will it be necessary to replace oil in the heating bath and descale the glass coil to obtain maximum efficiency of heat transfer; (4) back pressure from the shorter coil length does not overtax the peristaltic operation of the proportioning pump, thus, a smoother liquid flow through the colorimeter cuvettes is achieved without the necessity of installing in-line pulse suppressors and (5) there is a slight improvement in the resolution of threonine and serine, again possibly due to the smoother liquid flow and the shorter coil preventing prolonged mixing of the column effluent containing these two amino acids.

ACKNOWLEDGEMENT

I wish to thank Mr. G. W. McLennan for assembly of the reaction chamber.

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CHROM. 9992

Note

Short-time pyrolysis and spectroscopy of unstable compounds

V*. Improvement in Curie-point pyrolysis gas chromatography

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The method of Curie-point pyrolysis[2,3], in combination with gas chromatography and mass spectrometry offers a fast and reproducible way of studying pyrolysis reactions. The substance, coated on a ferromagnetic wire, is heated by a high-frequency pulse to the Curie temperature of the wire. To increase the thermal strain on the substance, spirals or thin tubes of ferromagnetic materials have been recommended[4] instead of wires.

The slight variation of this method described here has been used in our laboratory for some years[5–8]; the pyrolysis unit is shown in Fig. 1. When using materials having high Curie temperatures (up to 900°), the usual soft-glass tubes (1) are unsuitable because of the resulting thermal strain, and the use of quartz tubes (1) has the disadvantages that a metallic needle (2) cannot be fused on to the tip of the tube, and a quartz needle-tip is very fragile. To overcome these problems, we use a thin-walled quartz tube (4), resembling a melting-point capillary with an I.D. slightly larger than the ferromagnetic wire (3). This tube is heated easily by radiation from the glowing wire to a temperature only slightly lower than the Curie temperature of the wire, especially when long pyrolysis times (10 sec) are used. The molecules of substances evaporated from the wire are reflected from the hot capillary (4) back on to

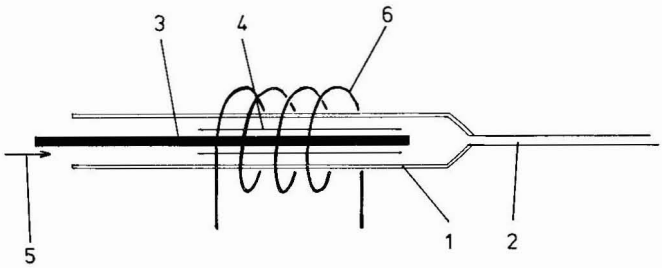

Fig. 1. Modified chamber for Curie-point pyrolysis. 1 = Glass tube; 2 = needle; 3 = ferromagnetic wire; 4 = thin-walled quartz tube; 5 = carrier-gas supply; 6 = high-frequency coil.

* For Part IV, see ref. 1.

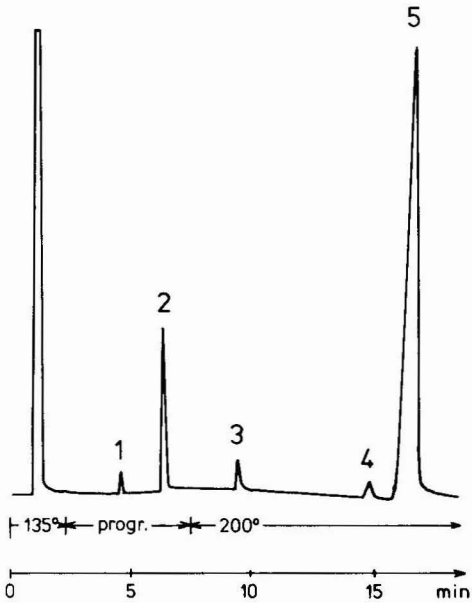

Fig. 2. Pyrolysis gas chromatogram of phenanthrenequinone: pyrolysis temperature 900°, pyrolysis time 10 sec. Column: 5 m × 0.125 in., packed with 2.5% of XE-60 on Chromosorb G AW DMCS (80–100 mesh). Peaks: 1 = naphthalene; 2 = biphenyl; 3 = fluorene; 4 = phenanthrene; 5 = fluorenone.

the wire, so that the substance receives more "impacts" on the hot surfaces and the pyrolysis rate is increased.

The advantages of this arrangement are as follows. The use of expensive and fragile quartz tubes is avoided, the period of thermal contact between the substance and the hot surfaces is increased, the ferromagnetic wire and the capillary can be cleaned easily, and ferromagnetic wires are available for more temperatures than are metallic tubes.

An example of the application of this method is the thermolysis of phenanthrenequinone (Fig. 2), which gives results similar to those of gas-phase thermolysis[9,10]; the substances formed were identified by coupled mass spectrometry.

ACKNOWLEDGEMENTS

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CHROM. 10,020

Note

Gas chromatographic determination of nitrilotriacetic acid using a nitrogen-selective detector

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(Received January 21st, 1977)

In preparation for a survey to determine levels of nitrilotriacetic acid (NTA) in drinking water, previously published methods of analysis for NTA[1–7] were evaluated. The method of Aue *et al.*[1] was considered to be the most appropriate but quantitation of NTA was difficult at the very low levels (*ca.* 1 ppb*), expected in drinking water. We now report the use of a nitrogen-selective detector which allows gas chromatographic (GC) quantitation of NTA as its tri-*n*-butyl ester, at sub-ppb levels in raw water and drinking water.

EXPERIMENTAL

General procedure

The method of Aue *et al.*[1] was followed. The formic acid was re-distilled in glass before use and the ion-exchange resin was washed well with this formic acid before use. All glassware was soaked for at least 24 h in concentrated hydrochloric acid, rinsed with distilled water and dried before use.

Gas chromatographic analysis

A Perkin-Elmer Model 910 gas chromatograph, equipped with a single column, a two-way effluent splitter, a flame ionization detector and a nitrogen–phosphorus detector operating in the nitrogen mode was used for this study. The column was 6 ft. × 1/4 in. O.D. glass, packed with either 5% OV-101 or 3% OV-210 on 80–100 mesh Chromosorb W HP. The carrier gas was helium at a flow-rate of 60 ml/min and the effluent splitter diverted 60% to the flame ionization detector and 40% to the nitrogen detector. Hydrogen and air flows were optimized for each detector. The injector and detector temperatures were 240° and 280°, respectively, and the column temperature as indicated in the text.

Gas chromatographic–mass spectrometric analysis

Qualitative and quantitative analysis were performed on a Finnigan Model 4000 GC–mass spectrometry (MS)–data system operating in the electron-impact mode.

* Throughout this article, the American billion (10^9) is meant.

The GC conditions were: injector temperature, 220°; column temperature, 200°; interface temperature, 250°. The column was glass, 6 ft. × 2 mm I.D., packed with 3% OV-1 on 80–100 mesh Chromosorb W HP and the carrier gas helium at a flow-rate of 40 ml/min.

The MS conditions were: source temperature, 270°; electron energy, 70 eV; resolution $M/\Delta M = 1200$ (10% valley).

RESULTS AND DISCUSSION

Evaluation of methods of analysis for NTA indicated that the method of Aue *et al.*[1] was the most suitable for low levels of NTA and was applicable to the wide variety of waters likely to be sampled during a survey to determine levels of NTA in drinking water. Essentially this procedure[1] consists of passing the water sample through an ion-exchange column, washing off interferences and then eluting the NTA. The NTA is then converted to its tri-*n*-butyl ester which is analysed by GC using a flame ionization detector. Aue *et al.*[1] claimed a limit of detection of 1 ppb NTA for a 50-ml water sample, but our preliminary investigations with standard solu-

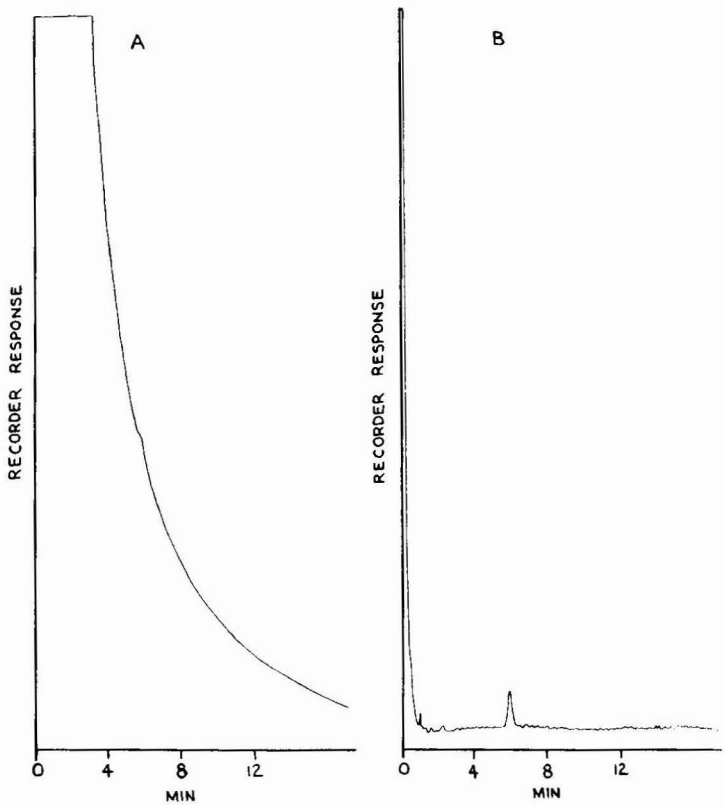

Fig. 1. Gas chromatograms of tri-*n*-butyl ester of NTA, retention time 6 min. Column, 5% OV-101 at 220°; 2.6 ng injected. (A) Flame ionization detector, 60% of effluent, attenuation 10 × 4; (B) nitrogen-selective detector, 40% of effluent, attenuation 10 × 1.

tions of the tri-*n*-butyl ester showed that quantitation at this level was difficult due to interference from the solvent peak (Fig. 1A) when using acetone as the injection solvent as specified by Aue *et al.*[1]. The use of alternate injection solvents gave some improvement but quantitation was still difficult.

Somewhat surprisingly no one has previously reported the use of a nitrogen-selective detector for GC detection of esters of NTA. Analysis of standard solutions of the tri-*n*-butyl ester, equivalent to 1 ppb NTA in a 50-ml water sample, showed that the sensitivity of this detector was adequate, quantitation was straight forward and there was minimal interference from the injection solvent, acetone (Fig. 1B). The nitrogen-selective detector gave a linear response over the range 1–1000 ng injected of the tri-*n*-butyl ester of NTA.

The isolation procedure of Aue *et al.*[1] gave a satisfactory chromatogram (Fig. 2A) for a control blank water sample provided that all solvents were re-distilled in glass and the ion-exchange resin and glassware were thoroughly washed before use. The

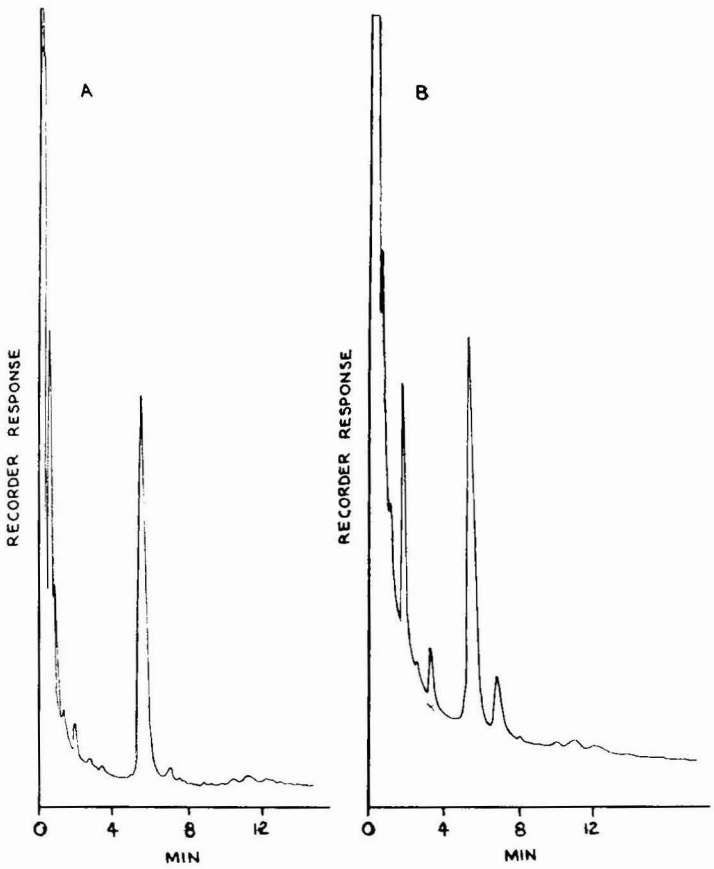

Fig. 2. Gas chromatograms on 5% OV-101 column at 235°, nitrogen-selective detector, attenuation 10 × 1. (A) Control water blank, butylated residue dissolved in 100 μl acetone and 4.8 μl injected; (B) raw water sample containing 0.4 ppb NTA butylated residue dissolved in 100 μl acetone, 4.9 μl injected, retention time 3.4 min.

lower detection limit was considered to be four times the level of the blank which would give a detection limit of *ca.* 0.2 ppb NTA for a 50-ml water sample. A typical chromatogram obtained from a 50-ml raw water sample analysed as containing 0.4 ppb NTA is shown in Fig. 2B. Recoveries of NTA from water samples spiked with 1–1000 ppb NTA were greater than 90%.

Confirmation of the *n*-butyl ester at the ppb level by GC–MS was possible using multiple ion monitoring of the major fragments, *m/e* 88, 158, 258, (Fig. 3) obtained in the electron impact mass spectrum of the tri-*n*-butyl ester of NTA (Fig. 4).

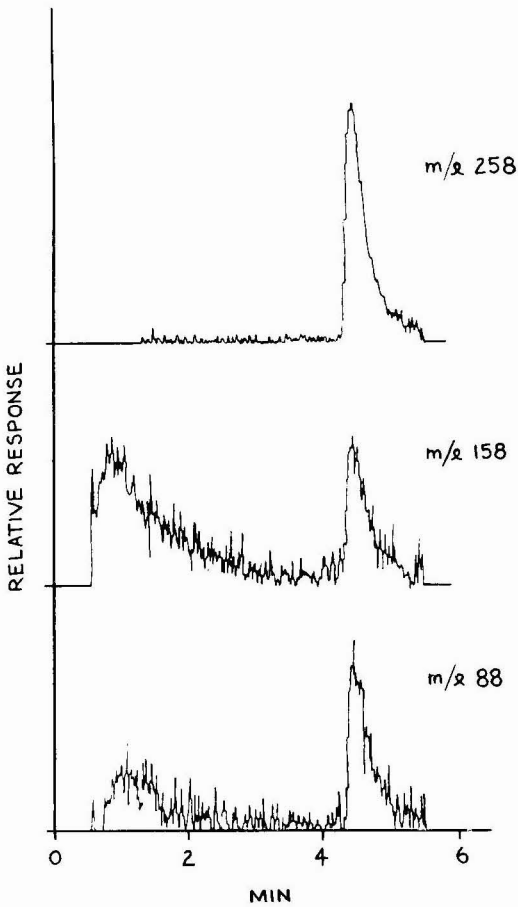

Fig. 3. Gas chromatography–mass fragmentography of tri-*n*-butyl ester of NTA, 3% OV-1 column at 200°, 15 ng injected, retention time 4.4 min.

Analysis of some typical water samples for NTA using the nitrogen-selective detector (Table I) showed that the method was applicable to both raw water and drinking water and that using this method NTA could be detected and quantitated at the sub-ppb level.

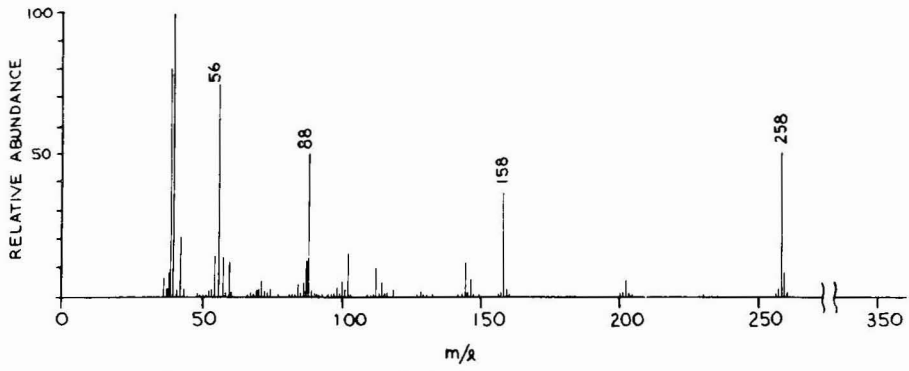

Fig. 4. Electron impact mass spectrum of tri-*n*-butyl ester of NTA. The molecular ion, *m/e* 359, can be detected if the spectrum is magnified *ca.* 10×.

TABLE I

LEVELS OF NTA IN RAW WATERS AND DRINKING WATERS

The letters A–H refer to local municipalities from where the samples were obtained.

Sample	*Concentration of NTA (ppb)*
Raw water A	1.03
Drinking water A	0.84
Raw water B	Trace*
Drinking water B	Trace*
Raw water C	1.75
Drinking water C	1.37
Raw water D	0.42
Drinking water E	Trace*
Drinking water F	0.87
Drinking water G	1.60
Drinking water H	0.84

* Indicates detectable levels ~0.2 ppb.

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CHROM. 9971

Note

Separation of L- and D-amino acids as diastereomeric derivatives by high-performance liquid chromatography

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(First received November 9th, 1976; revised manuscript received January 25th, 1977)

In a preliminary communication[1], we reported an effective chromatographic separation of four racemic amino acids as the diastereomeric mixture of N-*d*-10-camphorsulphonyl *p*-nitrobenzoate by high-performance liquid chromatography (HPLC) using a silica gel (MicroPak Si-5) column packing and 1.5% isopropanol in isooctane as the eluting solvent. The N-*d*-10-camphorsulphonyl moiety served to introduce an additional asymmetric centre and the *p*-nitrobenzyl group as a chromophore for detection.

We now report the application of the method to the amino acids methionine, glutamic acid, tryptophan, tyrosine, isoleucine, leucine, phenylalanine and alanine.

EXPERIMENTAL

Apparatus and conditions

An FLC 350 high-performance liquid chromatograph (JASCO) with gradient capability and a UV-254 detector monitoring at 253.7 nm were used. The column employed was a stainless-steel tube, 25 cm × 2.2 mm I.D., slurry-packed with microporous chemically bonded silica gel (Varian MicroPak-NH_2, average particle size 10 μm) and operated at ambient temperature. The flow-rate of the mobile phase was adjusted using pressures of 20–50 kg/cm^2.

Reagents and chemicals

All solvents were of reagent grade and were distilled prior to use. Amino acids were obtained from Katayama (Osaka, Japan), while *d*-10-camphorsulphonyl chloride was prepared from the corresponding acid[2].

Preparation of amino acid derivatives

A 30-ml volume of a solution of 2.0 mmole of *d*-10-camphorsulphonyl chloride in anhydrous diethyl ether was added dropwise to a solution of 1.0 mmole of amino acid in 10 ml of diethyl ether plus 20 ml of 1 *N* sodium hydroxide solution with vigorous stirring at 0°. Stirring was subsequently continued at room temperature for 3 h. The aqueous layer was separated from the ethereal layer, washed with twice diethyl ether, acidified with concentrated hydrochloric acid and then extracted with diethyl ether. The ethereal solution was dried over anhydrous sodium sulphate and evapo-

rated to dryness. The residue was dissolved in 10 ml of N,N-dimethylformamide, then one drop of trimethylamine and 1.1 mmole of *p*-nitrobenzyl bromide were added. The reaction mixture was heated at 55° for 2 h, diluted with 40 ml of chloroform, washed with water, dried over anhydrous sodium sulphate and then evaporated to dryness to obtain the N-*d*-10-camphorsulphonyl *p*-nitrobenzoate of the amino acid.

Unless otherwise stated, a chloroform solution of the diastereomeric mixture of the derivatives of DL-amino acids was used for HPLC.

RESULTS AND DISCUSSION

We investigated the separation of DL-amino acid derivatives with dichloromethane as the eluting solvent. The purification has to be carried out carefully, in order to obtain constant retention times of the amino acid derivatives. The purification procedure was as follows: washed with 5% hydrochloric acid, 5% potassium carbonate solution and then water (five times each), dried over anhydrous sodium sulphate, distilled to collect the fraction of b.p. 39°, and used immediately. The derivatives of D- and L-alanine, -glutamic acid, -methionine and -phenylalanine were separated completely, as shown in Fig. 1. However, the long retention times of each of the amino acid derivatives were not convenient for our purpose.

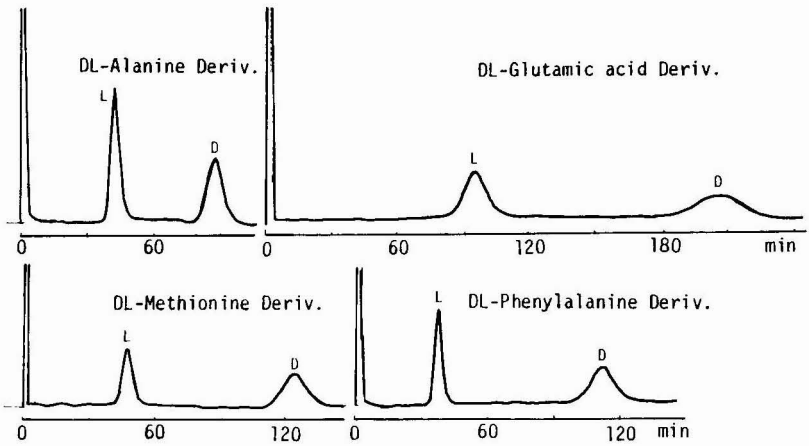

Fig. 1. Chromatograms of the diastereomers of N-*d*-10-camphorsulphonyl *p*-nitrobenzyl amino acids. Flow-rate, 0.4 ml/min; column, MicroPak-NH_2; eluent, dichloromethane.

To shorten the analysis time, a variety of solvents and gradient systems were investigated and chromatograms of mixtures of some DL-amino acid derivatives and the corresponding gradient diagrams are illustrated in Figs. 2 and 3. Excellent separations of all amino acid derivatives were observed. In order to identify the peaks, optically enriched amino acid derivatives were prepared under the same reaction condition as described above. No racemization was observed during the preparation of the derivatives, because each derivative showed a single peak in the chromatogram.

Table I shows typical retention times obtained with two solvent systems consisting of isooctane plus dichloromethane in different proportions, each containing 5%

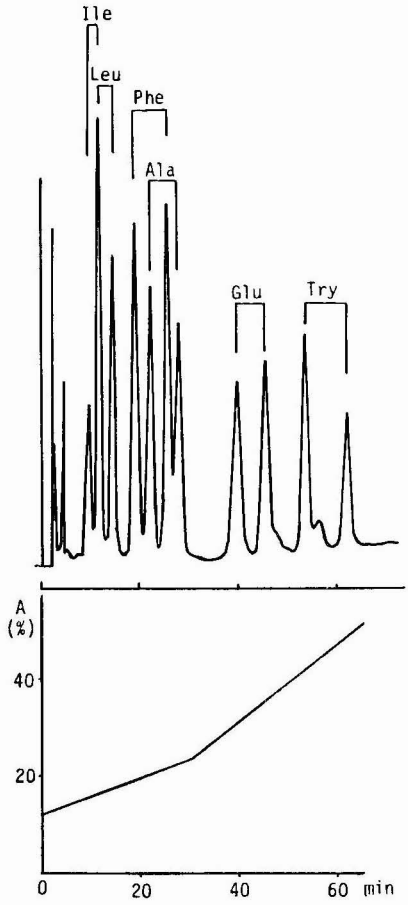

Fig. 2. Chromatogram and gradient diagram of the diastereomers of N-*d*-10-camphorsulphonyl *p*-nitrobenzyl amino acids. Flow-rate, 0.5 ml/min; column, MicroPak-NH_2. Eluent: A, isooctane–dichloromethane–isopropanol (70:15:15); B, isooctane–dichloromethane (90:10).

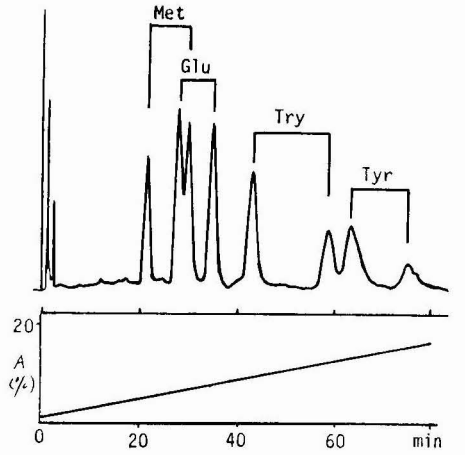

Fig. 3. Chromatogram and gradient diagram of N-*d*-10-camphorsulphonyl *p*-nitrobenzyl amino acids. Flow-rate, 0.7 ml/min; column, MicroPak-NH_2. Eluent: A, isooctane–dichloromethane–isopropanol (35:50:15); B, isooctane–dichloromethane (50:50).

TABLE I

RETENTION TIMES (min) OF D- AND L-AMINO ACID DERIVATIVES

Eluent: A, isooctane–dichloromethane–isopropanol (79:16:5); B, isooctane–dichloromethane–isopropanol (63:32:5). Flow-rate, 0.4 ml/min. Column, MicroPak-NH_2.

Amino acid	*Eluent A*			*Eluent B*		
	L	D	D/L	L	D	D/L
Leucine	3.9	4.4	1.1	2.7	2.8	1.0
Isoleucine	4.4	5.0	1.1	2.9	3.1	1.1
Phenylalanine	6.2	8.5	1.4	3.3	4.1	1.2
Methionine	7.4	10.0	1.4	3.6	4.6	1.3
Alanine	7.2	9.3	1.3	3.7	4.4	1.2
Glutamic acid	12.8	16.8	1.3	4.2	5.2	1.2
Tryptophan	29.2	49.6	1.7	9.0	14.9	1.7
Tyrosine	33.2	47.2	1.4	11.6	16.2	1.4

of isopropanol. The results show that the variation of the ratio of isooctane to dichloromethane affects the absolute retention time of each amino acid derivative but not the relative retention times of corresponding D- and L-amino acids.

Of the amino acids tested, tryptophan and phenylalanine were detectable with the UV-254 detector without introducing the *p*-nitrobenzyl moiety as a chromophore. The chromatogram of the methyl ester of N-*d*-10-camphorsulphonyl phenylalanine is shown in Fig. 4. Compared with the chromatogram of the *p*-nitrobenzoate of the corresponding derivative, the methyl ester seems to be more efficient from the point of view of the separation of enantiomers, the retention times and the preparation of the derivative.

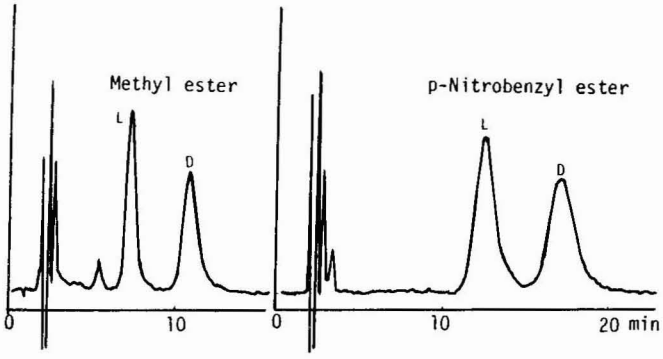

Fig. 4. Chromatograms of the methyl ester and *p*-nitrobenzyl ester of the diastereomers of N-*d*-10-camphorsulphonyl phenylalanine. Flow-rate, 0.4 ml/min; column, MicroPak-NH_2; eluent, isooctane–dichloromethane–isopropanol (87:8:5).

CONCLUSIONS

For all of the amino acid derivatives tested ,the retention times of the L-amino acid derivatives were consistently shorter than those of the corresponding D-amino acid derivatives. Consequently, it could possibly be assumed tentatively that there is a correlation between retention time and absolute configuration, which might be useful for the assignment of the absolute configuration of new amino acids.

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CHROM. 9993

Note

Chromatography of the reduction products of spectinomycin

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Spectinomycin* (I) (Fig. 1) and its reaction products[1] are generally basic, highly water soluble compounds, many of which are stable only within a narrow pH range. The polyfunctional nature of these molecules makes it difficult to prepare homogeneous UV-absorbing derivatives in a quantitative manner. Because of these factors, thin-layer chromatography and liquid chromatography are not readily applicable and it has always been difficult to establish the purity of modified spectinomycins and degradation products.

Fortunately, the various reduction products which no longer contain the α-ketol system are stable to strongly basic ion-exchange resins. They are therefore amenable to separation by ion-exclusion chromatography[2], a process that has been widely used in the analysis of other antibiotics such as neomycin[3–7], kanamycin[4,8], and butirosin[9]. In these instances the amines under study were chromatographed on a quaternary ammonium resin (OH^-) with a low degree of cross-linking, *e.g.*, Dowex 1-X2. The same procedure is equally applicable, however, to the separation of acids on an acidic resin such as Dowex 50W-X8 (refs. 10–12).

EXPERIMENTAL

Chromatronix columns of various internal diameters were used in conjunction with either a Milton Roy Minipump (Model 196-89) or a Chromatronix Cheminert CMP-2 metering pump. The column effluent was monitored with a differential refractometer (Waters Assoc., Model R4) and in some cases a Bendix photoelectric polarimeter (Model 143A) as well. Columns were packed with AG 1-X2 ion-exchange resin (200–400 mesh) obtained from Bio-Rad Labs. (Richmond, Calif., U.S.A.), and converted to the hydroxyl form before use. The eluant in all cases was degassed water, and the reservoir was fitted with a sodium-hydroxide containing trap to prevent entry of carbon dioxide which deactivated the column by conversion of the resin to the carbonate form. The compounds referred to were prepared as described by Knight and Hoeksema[13].

* Formerly known as actinospectacin. Trobicin is the registered US trademark of The Upjohn Company for spectinomycin hydrochloride. Additional trademarks include Togamycin and Stanilo.

DISCUSSION

Reduction of spectinomycin with either sodium borohydride in methanol, or by catalytic hydrogenation in ethanol, leads to the epimeric dihydro derivatives IIa and IIb, one of which (IIb) is identical with the naturally occurring dihydrospectinomycin[14]. Both may be further reduced to tetrahydro compounds (IIIa and b, IVa and b) by sodium borohydride in aqueous solvents[13].

Whereas the tetrahydro epimers were readily eluted, and best resolution was obtained using long, narrow bore columns at low flow-rates (Fig. 2), the dihydro epimers were much more strongly retained. To obtain a separation in a reasonable length of time for analytical purposes a much shorter column and higher flow-rate

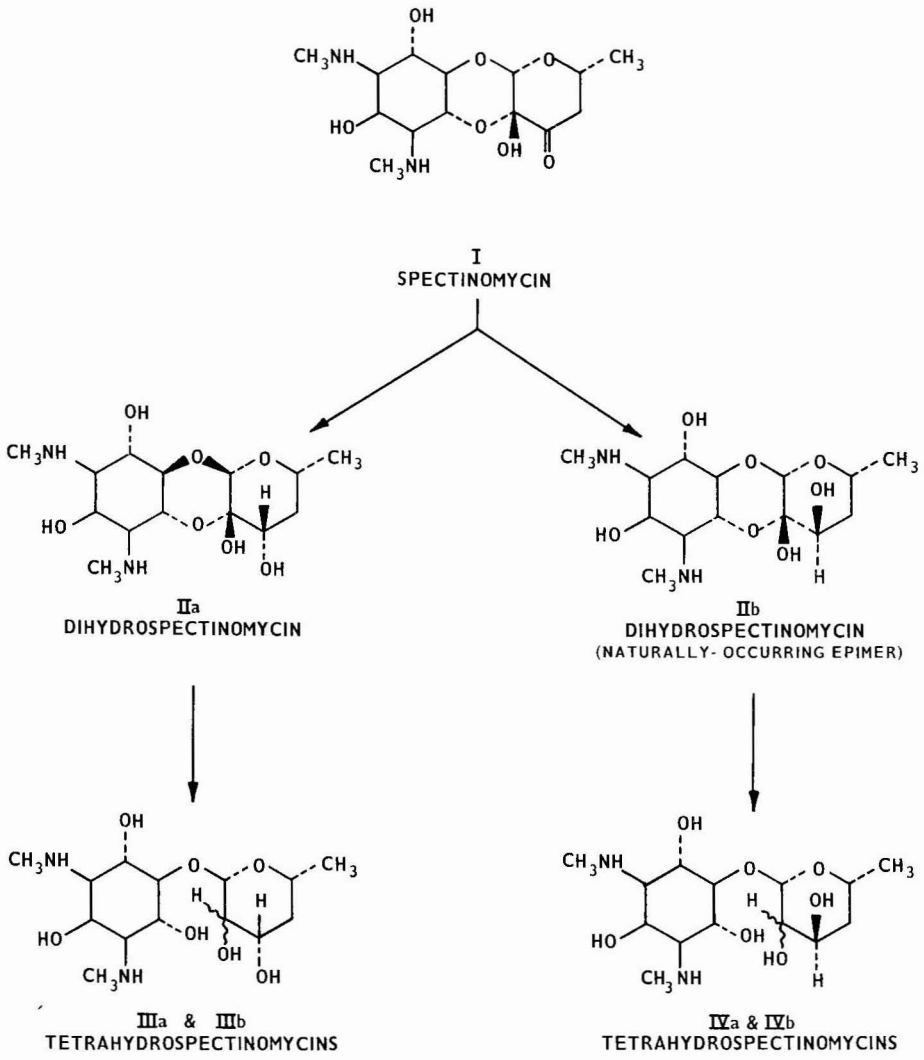

Fig. 1. Spectinomycin and its reaction products.

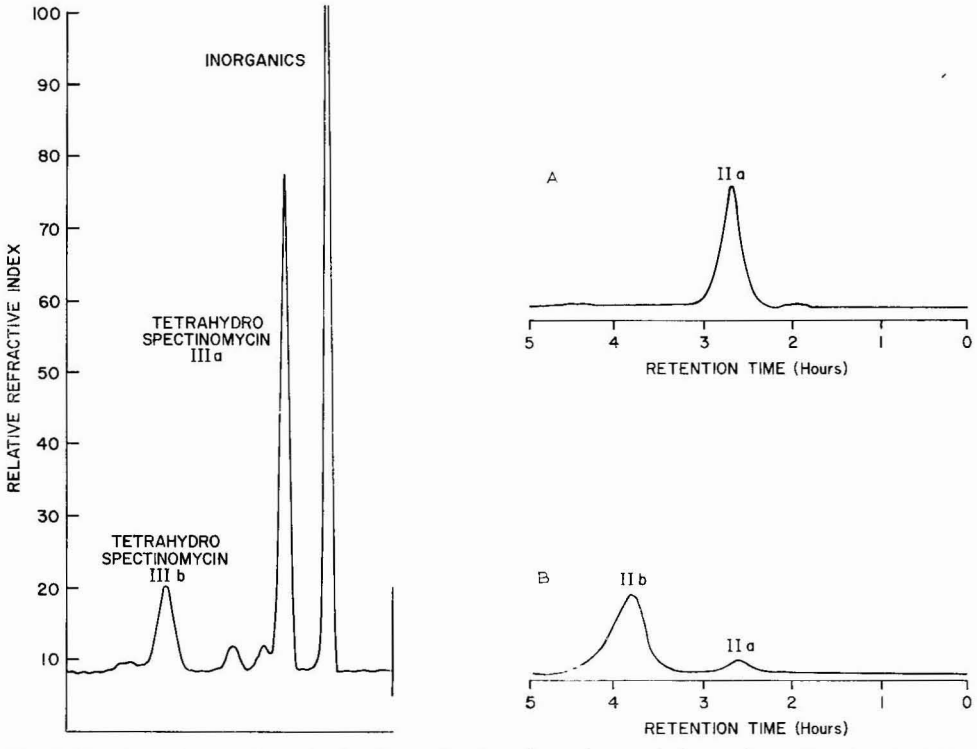

Fig. 2. Total reaction product obtained by reduction of spectinomycin base with sodium borohydride in 50% aqueous methanol. Column, 7 ft. × 2.8 mm I.D.; flow-rate 25 ml/h.

Fig. 3. Analytical separation of dihydrospectinomycin epimers. Products obtained by hydrogenation in 95% ethanol (A) and in water (B). Column, 210 mm × 9 mm I.D., flow-rate 75 ml/h.

were needed. Using this technique it was possible to demonstrate that hydrogenation in ethanol gave a product opposite in configuration to that obtained by hydrogenation in water (Fig. 3). Preparative scale separation of the dihydrospectinomycins was possible on a 1 in. I.D. column in a run time of 18 h (Fig. 4).

Each of the dihydro epimers gave two tetrahydro compounds on further reduction with sodium borohydride in aqueous solvents, and the epimer pairs were readily

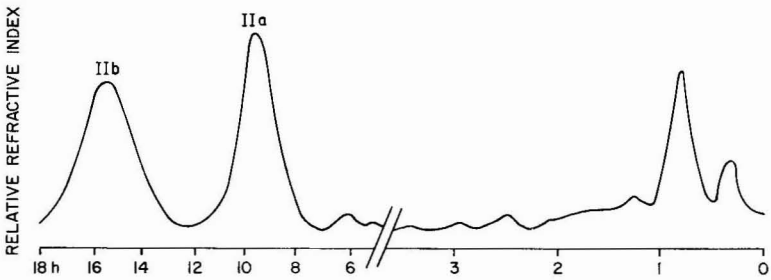

Fig. 4. Preparative separation of dihydrospectinomycin epimers. Column, 11 × 1 in. I.D., flow-rate 120 ml/h.

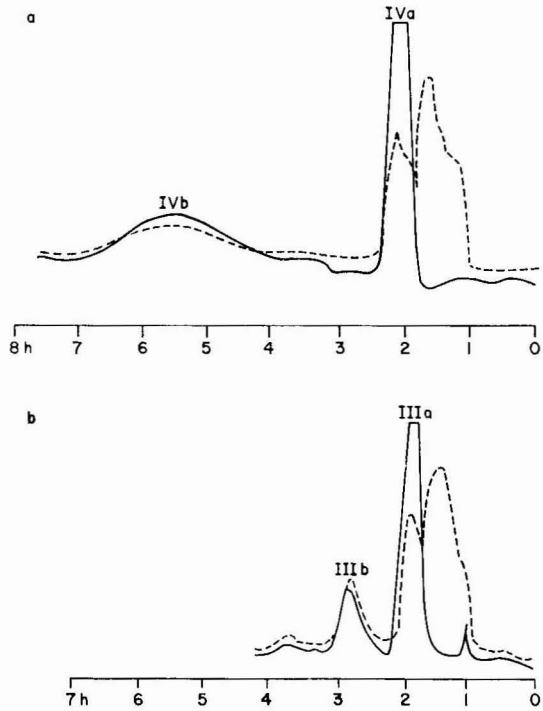

Fig. 5. Sodium borohydride reduction of dihydrospectinomycin epimers. (a) Reduction of naturally occurring epimer IIb; (b) Reduction of epimer IIa. Column, 16 × ½ in. I.D., flow-rate 92 ml/h. Solid trace, optical rotation (negative); Dotted trace, relative refractive index.

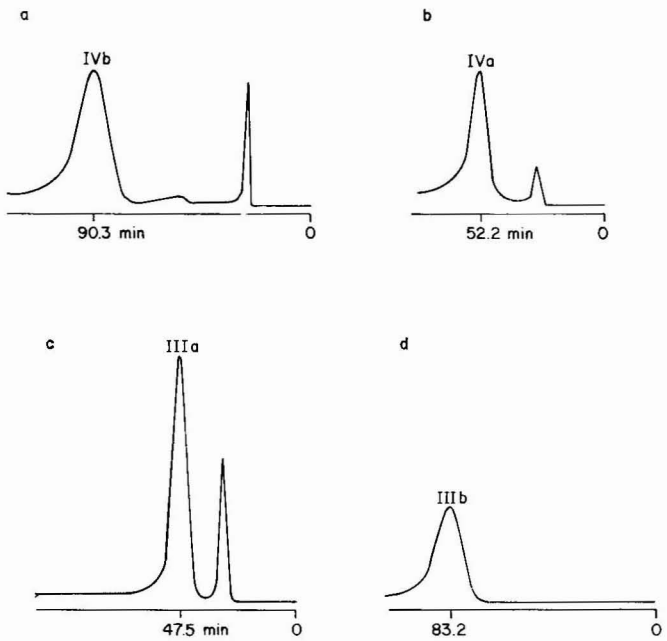

Fig. 6. Individual compounds from sodium borohydride reduction of dihydrospectinomycin epimers, isolated from the chromatograms shown in Fig. 5. a and b, from naturally-occurring isomer IIb. c and d, from isomer IIa. Column, 1 m × 2.8 mm I.D., flow-rate, 11.8 ml/h.

resolved in each case (Fig. 5), which allowed isolation of all four epimers for further characterization. The purity of each one was checked by re-chromatography (Fig. 6). The first eluting peak in each case, which occurs at one column volume, is due to small amounts of inorganic materials, solvent of crystallization, etc. The method described here has definite advantages over ion exchange, thin-layer, and gas–liquid chromatography for the analysis of this type of compound, even where such other methods are applicable. The free base can be analysed on a micro or a preparative scale without the need to form derivatives, and since the eluant is simply water, freeze-drying of the collected fractions is all that is required for isolation, and the question of separation from buffer salts does not arise.

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20a. Proteins of plant origin including bacteria

See 1589.

21. PURINES, PYRIMIDINES, NUCLEIC ACIDS AND THEIR CONSTITUENTS

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See 1614, 1679.

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See 1446.

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Liquid Column Chromatography

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8. SUBSTANCES CONTAINING HETEROCYCLIC OXYGEN

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10. CARBOHYDRATES

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19. PEPTIDES; CHEMICAL STRUCTURE OF PROTEINS

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See also 1769, 1804, 1805, 1807, 1819.

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21. PURINES, PYRIMIDINES, NUCLEIC ACIDS AND THEIR CONSTITUENTS

21a. Purines, pyrimidines, nucleosides, nucleotides

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Thin-Layer Chromatography

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See also 2401, 2664.

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32d. Biomedical applications

See 2482, 2511, 2517, 2604, 2693, 2694.

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21. PURINES, PYRIMIDINES, NUCLEIC ACIDS AND THEIR CONSTITUENTS

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28. ANTIBIOTICS

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35. SOME TECHNICAL PRODUCTS AND COMPLEX MIXTURES

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2 L. R. Snyder, *Principles of Adsorption Chromatography*, Marcel Dekker, New York, 1968, p. 201.
3 H. C. S. Wood and R. Wrigglesworth, in S. Coffey (Editor), *Rodd's Chemistry of Carbon Compounds, Vol. IV, Heterocyclic Compounds*, Part B, Elsevier, Amsterdam, Oxford, New York, 2nd ed., 1977, Ch. 11, p. 201.
4 E. C. Horning, J.-P. Thenot and M. G. Horning, in A. P. De Leenheer and R. R. Roncucci (Editors), *Proc. 1st Int. Symp. Quantitative Mass Spectrometry in Life Sciences, Ghent, June 16–18, 1976*, Elsevier, Amsterdam, Oxford, New York, 1977, p. 1.

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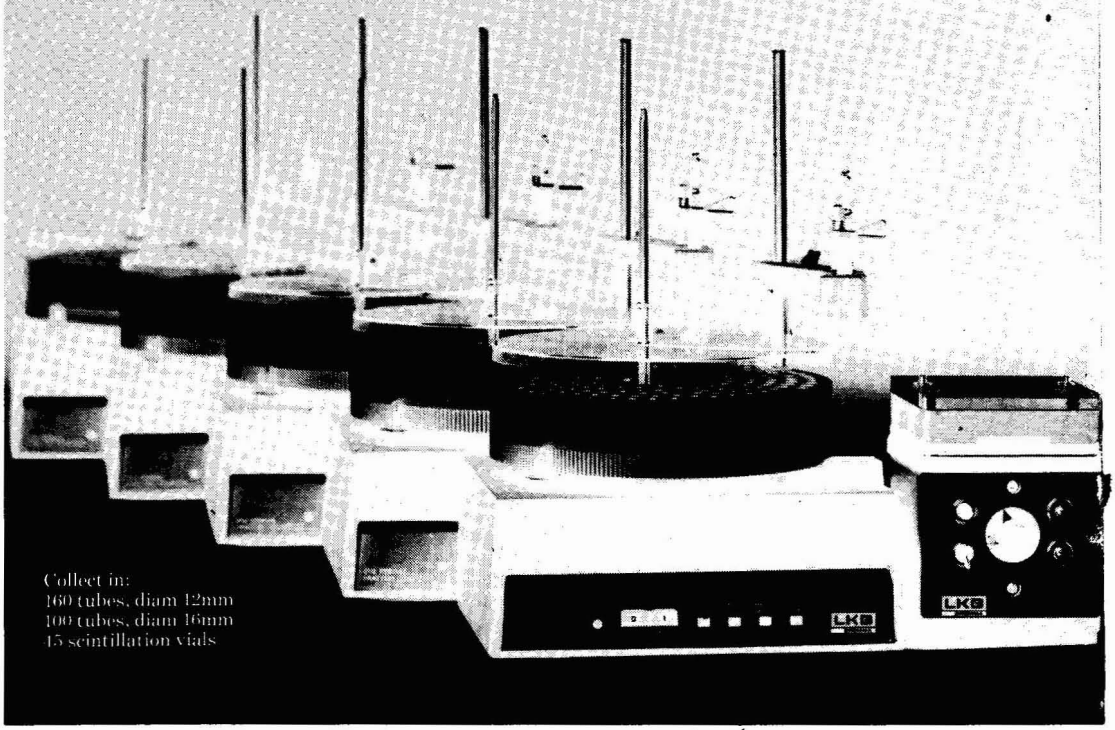

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