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JOURNAL OF CHROMATOGRAPHY
BIOMEDICAL APPLICATIONS

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PUBLICATION SCHEDULE FOR 1977

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:
The Editor of *Journal of Chromatography*, P.O. Box 681, Amsterdam, The Netherlands
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.

Subscription Orders. Subscription orders should be sent to: Elsevier Scientific Publishing Company, P.O. Box 211, Amsterdam, The Netherlands. The *Journal of Chromatography, Biomedical Applications* can be subscribed to separately.

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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REF. NO. 332

Membrane Separation Processes

edited by PATRICK MEARES, Professor of Physical Chemistry, University of Aberdeen.

1976 xvi + 592 pages US $96.25/Dfl. 250.00 ISBN 0-444-41446-0

As standards of purity have progressively been raised in biological and chemical technology, separation procedures have become increasingly important. A whole family of such procedures is now emerging from research in which membranes, usually prepared from polymers, are used to perform the primary separation step. The applications of membranes as separation barriers are very diverse and the techniques employed vary widely. Nevertheless, the fundamental scientific principles and the problems encountered in all such processes have much in common. Thus, it is desirable and convenient to bring together, in one book, first-hand accounts of a range of membrane processes which are at or near full-scale application, so as to demonstrate their versatility as well as to describe and explain their underlying common features. The authors, all of whom have been actively engaged in research or development work, provide thorough, balanced accounts of their subjects. They outline the basic scientific principles and show how these have led to the current state of development of the process under discussion. Chapters on more advanced and widely used processes are concerned with practical technology, others deal with specification and solution of practical problems in devising the commercially viable procedure. The book will interest scientists and engineers who seek solutions to their own separation problems or who are concerned with devising and assessing new separation procedures. It will also be useful to all those directly concerned with membrane transport processes.

CONTENTS: Chapters: 1. The physical chemistry of transport and separation by membranes *(P. Meares)*. 2. Liquid permeation through polymeric membranes *(H.D. Spriggs and N.N. Li)*. 3. Principles and practice of ultrafiltration *(W.F. Blatt)*. 4. Reverse osmosis (hyperfiltration) in water desalination *(F.L. Harris, G.B. Humphreys and K.S. Spiegler)*. 5. Hollow fibres in reverse osmosis, dialysis, and ultrafiltration *(B. Baum, W. Holley Jr. and R.A. White)*. 6. Electrodialysis *(G.S. Solt)*. 7. Piezodialysis *(F.B. Leitz)*. 8. The separation of gases by selective permeation *(S.A. Stern)*. 9. Hydrocarbon separation by liquid membrane processes *(R.P. Cahn and N.N. Li)*. 10. Enzyme membranes *(D. Thomas and S.R. Caplan)*. 11. Separators and membranes in electrochemical power sources *(J.A. Lee, W.C. Maskell and F.L. Tye)*. 12. Recent developments in ion-selective membrane electrodes *(R. Bloch and E. Löbel)*. 13. The treatment of aqueous wastes and foods by membrane processes *(D.C. Sammon)*. 14. Biomedical applications of membrane processes *(C.R. Gardner)*. Author index. Subject index.

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1984 E

Analytical Pyrolysis

Proceedings of the Third International Symposium held in Amsterdam, September 7 - 9, 1976

C. E. ROLAND JONES and CARL A. CRAMERS *(Editors)*

This symposium is particularly noteworthy because of the emphasis given to the newly emergent technique of pyrolysis/mass spectrometry. The large number of papers devoted to this technique at the meeting are an indication of the impetus which this recent development has given to analytical pyrolysis.

These Proceedings provide examples of a diversity of applications of pyrolysis/ gas chromatography and pyrolysis/mass spectrometry ranging from geochemical exploration through energy resource studies to the elucidation of biopolymers and complex synthetic resins. The thirty-four papers give perspective to the current state of the fields, as well as reporting on the most recent developments in them. The introductory contributions in the sessions, provided by prominent figures in the particular fields, summarize the position to date before revealing the latest trends in the authors' own work. It could be said that each session was a miniature symposium in itself.

CONTENTS: **Automation.** Contributors: G. L. Coulter and W. C. Thompson. **Special Techniques.** Contributors: F. W. McLafferty, H.-R. Schulten and E. Stahl. **Microbiology.** Contributors: H. D. Donoghue, N. D. Fields, M. Marshall, M. Needleman, G. S. Oxborrow, J. R. Puleo, E. Reiner, M. V. Stack, P. Stuchbery and J. E. Tyler. **Forensic Science and Pharmacology.** Contributors: W. J. Irwin, J. P. Schmid, P. P. Schmid, W. Simon, J. A. Slack and B. B. Wheals. **Pyrolysis Mass Spectrometry.** Contributors: D. O. Hummel, I. Lüderwald and H. Urrutia. **Reproducibility and Specificity.** Contributors: W. Eshuis, P. G. Kistemaker and H. L. C. Meuzelaar. **Soil Chemistry and Geochemistry.** Contributors: J. M. Bracewell, J. W. de Leeuw, A. G. Douglas, B. Horsfield, S. R. Larter, F. Martin, W. L. Maters, D. v.d. Meent, H. L. C. Meuzelaar, G. W. Robertson, P. A. Schenck and P. J. W. Schuyl. **Biochemistry.** Contributors: F. L. Bayer, J. J. Hopkins, F. M. Menger and A. C. M. Weijman. **Laser Pyrolysis.** Contributors: J. C. Means, E. G. Perkins and N. E. Vanderborgh. **Reaction Mechanisms.** Contributors: D. C. De Jongh, S. Foti, I. Lüderwald, G. Montaudo, N. M. M. Nibbering, M. A. Posthumus, M. Przybylski, H. Ringsdorf and G. Schaden. **Polymers.** Contributors: M. Blazsó, J. S. Crighton, B. Dickens, J. H. Flynn, D. Gross, G. Guiochon, D. E. Henderson, C. E. R. Jones, J. Kelm, H.-J. Kretzschmar, E. J. Levy, R. J. Lloyd, W. J. Pummer, N. Sellier, T. Székely, T. Takeuchi, S. Tsuge and P. C. Uden.

1977 x + 424 pages US $39.25/Dfl. 96.00 ISBN 0-444-41558-0

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Theory and Practice of MO Calculations on Organic Molecules

by **I.G. CSIZMADIA,** Department of Chemistry, University of Toronto.

PROGRESS IN THEORETICAL ORGANIC CHEMISTRY, Vol. 1.

1976 x+378 pages US $38.50/Dfl. 100.00 ISBN 0-444-41468-1

Current chemical theory is based on approximate quantum mechanical treatments and especially molecular orbital methods. In recent years, the theory has been developed to the point where quantitative calculations can be made on many physical properties of molecules and interacting molecular systems.

This book provides an introduction to rigorous *ab initio* molecular orbital calculations for the experimental organic chemist. The necessary mathematics and quantum mechanics are reviewed as background for the experimentalist wishing to begin theoretical work. The Hartree-Fock method for both open and closed shell molecules is derived in detail. Results of many recent theoretical studies are included to illustrate the practical application of molecular orbital theory to problems in organic chemistry.

This book should be of particular value to the experimental organic chemist interested in beginning MO calculations related to his research interests. It would serve as a text for a course in Theoretical Organic Chemistry and as a useful supplementary text in courses on Physical Organic Chemistry and Molecular Quantum Mechanics. This text provides an excellent introduction to theoretical organic chemistry for the experimental organic chemist.

CONTENTS: **A. Introduction.** I. Introductory Remarks. II. Mathematical Introduction. III. Quantum Mechanical Background. **B. Theory of Closed Electronic Shells.** IV. Non-Empirical or Hartree-Fock MO Theory. V. Semi-Empirical MO Theories. VI. Excited and Ionized States in the Framework of Closed Shell MO Theories. VII. Hybrid Atomic Orbitals (HAO) and Localized Molecular Orbitals (LMO). VIII. Limitations of Molecular Orbital Theories. IX. Applications of MO Theory to Closed Shell Problems. **C. Theory of Open Electronic Shells.** X. Open Shell SCF Theories. XI. Limitations and Applications of Open Shell SCF Theories. **D. Practical Aspects of MO Computations.** XII. Basis Sets for Molecular Orbital Calculations. XIII. Information on Selected Computer Programs. XIV. Closing Remarks. **E. Appendix.** XV. Detailed Formalisms of Roothaan's SCF Theories.

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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

developing technique *(J. Blome)*. The U-chamber *(R.E. Kaiser)*. Dosage techniques in HPTLC *(R.E. Kaiser)*. High performance thin-layer chromatography: development, data and results *(H. Halpaap, J. Ripphahn)*. Consideration on the reproducibility of TLC separations *(D. Jaenchen)*. Potential and experience in quantitative HPTLC *(U.B. Hezel)*. Application of a new high-performance layer in quantitative TLC *(J. Ripphahn, H. Halpaap)*. Appendix. Index.

Volume 10

GAS CHROMATOGRAPHY OF POLYMERS

by V.G. Berezkin, V.R. Alishoyev and I.B. Nemirovskaya

1977. xiv+226 pages.
Price: US $41.95/Dfl. 103.00.
ISBN 0-444-41514-9.

At present, gas chromatography is the most widespread method for the analysis of organic compounds.

This book is devoted to the strategy of application of gas chromatography in polymer chemistry and discusses, in detail, the use of gas chromatography in research work and the polymeric compounds industry. It is the second, revised and enlarged edition of the original version published in the USSR in 1972.

The following principal applications are covered: analysis of monomers and solvents, determination of the contents of volatile substances in polymers, study of polymer formation processes, investigation into types of disintegration of high-molecular-weight compounds, polymer analysis by reaction and pyrolytic chromatography, and study of polymers and their reactivity with the aid of inverse chromatography.

This work will be of value to research institutions, industrial enterprises and senior students engaged in the fields of polymer or analytical chemistry and gas chromatography.

CONTENTS: Introduction. Basic principles of GC. GC methods for the analysis of monomers and solvents. The study of polymer formation reactions. Determination of volatile compounds in polymer systems. Study of the kinetics and mechanisms of chemical transformations of polymers at elevated temperatures. Reaction GC of polymer formation reactions. Determination Conclusion.

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CHROMBIO. 038

REVIEW

CHROMATOGRAPHY OF BIOGENIC AMINES. I. GENERALLY APPLICABLE SEPARATION AND DETECTION METHODS

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1. INTRODUCTION

Cells and extracellular fluids of all living organisms contain a complex mixture of compounds containing amino groups such as amino acids, peptides, amines and alkaloids. The study of structural, metabolic and functional relationships between these compounds frequently requires the determination of several individual compounds in biological samples. For instance, the study of the metabolism of tryptophan in the brain normally involves analytical monitoring of tryptophan, 5-hydroxytryptophan, tryptamine, N,N-dimethyltryptamine, 5-hydroxytryptamine, bufotenin (N,N-dimethyl-5-hydroxytryptamine), melatonin (N-acetyl-5-methoxytryptamine), indolylacetic acid and 5-hydroxyindolylacetic acid, and frequently also metabolites of the kynur-

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enine pathway of tryptophan, conjugates (sulphates, acetates, glucuronides, etc.) of the tryptophan metabolites and peptides. A recent trend in analytical biochemistry was therefore the development of methods that allow the determination of functionally and metabolically related compounds from the same sample. However, although in certain instances interest may be focused exclusively on a single amine, one must devise methods that allow the determination of the individual amine in the presence of many other related compounds of comparable concentration. In methods of this type chromatography is particularly useful.

The concentration of biogenic amines in tissues is, with few exceptions, low, i.e., generally lower than 10 nmole per gram of wet tissue. The necessity for the determination of low concentrations of biogenic amines in discrete areas of the brain, in small cell populations and in single cells stimulated the improvement of the sensitivity of the methods. The possibility of achieving the quantitative determination of picomole or even femtomole amounts of amines, amino acids and peptides is a major criterion for the suitability of a method in neurochemistry. Less sensitive methods may be applied successfully to urine analysis.

Many naturally occurring amines and amino acids have been identified and quantitated in tissues and body fluids in the last 20 years. The application of more advanced methods to their assay frequently revealed, however, that their actual concentrations were sometimes lower than was originally thought by several orders of magnitude. β-Hydroxy-γ-aminobutyric acid [1,2], choline and acetylcholine [3], piperidine [4] and putrescine [5,6] in the brain are examples of compounds for which revisions of their concentration in tissues were made of the basis of advanced methods. Improvements in the specificity of the methods was as important as an increase in sensitivity. The still increasing application of mass spectrometry in combination with other separation methods in analytical biochemistry is one of the latest trends aimed at improving specificity. The exploitation of the specificity of enzymic reactions, mostly combined with the use of radioactive substrates, and immunological methods are alternatives to separation methods for increasing sensitivity and specificity [7,8]. Radioenzymic assays have been established for catecholamines [9—12], serotonin [13,14], β-phenylethylamines [15,16], histamine [17—19], choline and acetylcholine [20—25] and putrescine [6], among others, and radioimmunoassays are available for a few important biogenic amines [26—29].

Further insight into the molecular events associated with metabolic aberrations enormously increased the demands for routine assays of an increasing number of amines and amino acids for diagnostic purposes, and for the monitoring of therapeutic measures. Automated methods had to be developed to meet the special requirements of clinical analysis. Automated methods, with few exceptions, do not include separation steps, but utilize specific structural features of the amines for determination. They are normally of limited specificity and applicable only under thoroughly defined circumstances.

In addition to automation and the increase in sensitivity and specificity, a further trend became apparent. After two decades of nearly exclusive consideration of a small number of physiologically and pharmacologically important amines, the catecholamines, serotonin, histamine and acetylcholine, in-

terest was extended to other amines of biological origin for which the physiological significance is less apparent or less well established. Methods for the determination of these amines are being developed. The term "biogenic amine" has gradually seemed to regain its literal sense, which was originally used, for instance, by Guggenheim in his now classic book "Die biogenen Amine" [30].

Comprehensive reviews of analytical procedures for the assay of biogenic amines do not seem to exist, if we neglect the short chapters in standard books on thin-layer chromatography (TLC) and gas—liquid chromatography (GLC), and the author's short reviews [31,32]. The reason for this is clear: most papers reviewing analytical methods for biogenic amines are devoted either to a special method or to a special approach [7,8,33—45] or a certain compound [46—57]. Furthermore, established, generally accepted and generally applicable chromatographic methods for the determination of amines are, with few exceptions, not available. Any given sample of biological origin poses different problems, depending on its complexity and the concentration of the amines in the sample. It is not possible, by using a single method, to establish a complete profile of all important amines in a given biological sample. Most methods are in principle not suitable for such an analysis: with the exception of the completely non-specific detection methods used mainly in GLC, the flame-ionization detector and the more specific nitrogen-sensitive alkali detector, and the mass spectrometer, virtually no sensitive method exists for the determination of tertiary amines. They are normally not recognized, provided that the molecule has specific features for sensitive detection, as is the case, for instance, with bufotenin. Colour and fluorescent reagents for teriary amines do not meet the requirements of the sensitivity needed for tissue analysis.

Disregarding these limitations, methods exist that are sufficiently versatile to be adapted to a given analytical problem. Existing methods have been improved successfully during recent years by the application of refined chromatographic procedures or by increasing the sensitivity of detection by suitable derivative formation. It is the purpose of this paper to review these methods.

2. GENERALLY APPLICABLE METHODS

Aliphatic mono-, di- and polyamines do not exhibit structural features that permit their sensitive or specific detection. Interference refractometry was suggested for the determination of amines after elution from thin-layer chromatograms, but its low sensitivity (μg amounts of amphetamine [58]) is one of several reasons for the restricted application of this method.

Disregarding detection with non-specific flame-ionization detectors, in all methods currently in use for the assay of primary and secondary amines the amino groups are utilized for the formation of derivatives suitable for sensitive determination and/or improvement of separation. While the sensitivity of detection depends on the derivatization reaction, the specificity is limited solely by the quality of the separation procedure. In practice, nearly all detection reactions have been combined with all separation procedures.

A. Paper and thin-layer chromatography and paper and thin-layer electrophoresis of free amines

A large number of solvent and buffer systems suitable for the separation of non-derivatized amines on paper [59—74] and thin layers by chromatography [80—106] or electrophoresis [63—65, 88,107—114] have been proposed. In addition to the usual layers (cellulose, alumina and silica gel), ion-exchange paper and thin layers [88, 115] have been considered, and also separations by ligand exchange [116]. The references cited in this paragraph mainly describe separations of aliphatic amines. Although the reference list is incomplete, it demonstrates the wide application of amine separations in analytical biochemistry. Chromatographic separations of underivatized β-phenylethylamines, catecholamines, histamine, indoleamines and acetylcholine will be discussed in a subsequent paper.

For the detection and determination of separated primary and secondary amines, ninhydrin has most commonly been used, and Dragendorff reagent for tertiary amines [117]. A number of other generally applicable detection reactions are available [105]. Colour reactions of primary amines with 2,5-dimethoxytetrahydrofuran plus *p*-dimethylaminobenzaldehyde [118], and the more generally applicable reactions with 2,6-dichloroquinone 4-chloroimide [119] or potassium permanganate [77], or the fluorimetric procedure of Segura and Gotto [120] for the detection of organic compounds on thin-layer chromatograms, may prove useful in certain amine analyses, as well as the reaction of tertiary amines with α,γ-anhydroaconitic acid to give coloured products [121]. Considerable progress in the detection of primary amines was made, however, by using fluorescamine as spray reagent [122—124], which can reveal picomole amounts. The reaction of fluorescamine with primary amines is illustrated in Fig. 1. Although there are certain difficulties, quantitative evaluation of chromatograms sprayed with or dipped in fluorescamine is possible. An alternative method is the application of the *o*-phthaldialdehyde reaction in the presence of thiol-containing compounds (2-mercaptoethanol) [125] for the fluorescence staining in TLC, thin-layer electrophoresis (TLE), paper chromatography (PC) or paper electrophoresis (PE). The sensitivity of this method [126] in our hands was not as good as that of the reaction with fluorescamine, but was useful.

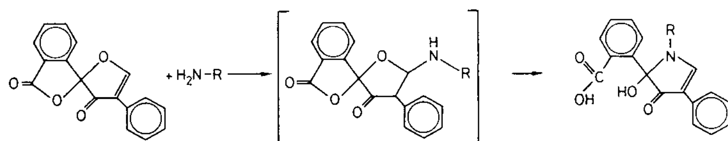


Fig. 1. Reaction of fluorescamine with a primary amine.

Despite considerable experience, there is no generally accepted approach to the analysis of the free amines in a biological sample. Disregarding the complexity of the mixture and the varying concentrations of its components, there is an additional difficulty inherent in their structural feature: the salts and the free bases of many natural aliphatic amines and β-phenylethylamines are

readily soluble in water. Homogenization with acids [0.2—0.4 *M* $HClO_4$; 10% trichloroacetic acid; acetone—0.1 *M* HCl (95:5)] is effective for their extraction from tissues, but it is difficult to concentrate large volumes of the acidic solutions without losses of trace amounts of the amines. Some of the low-molecular-weight amines are volatile. On the other hand, certain conjugates (for instance acetates) may be hydrolyzed during evaporation, and evaporation of neutralized solutions causes heavy losses. In fact, water-vapour distillation of alkaline solutions was used until recently for the separation of volatile from non-volatile amines and amino acids [62,63,127,128].

Aromatic amines and some β-phenylethylamines can be extracted from alkaline solutions with ethyl acetate, diethyl ether and similar solvents. The polyamines spermidine and spermine were mostly extracted with *n*-butanol [86,108] before chromatographic separation. A generally applicable solvent-extraction procedure for amines does not exist and, moreover, some biologically important amines are unstable in alkaline solutions. Not only catecholamines and related compounds but even simple aliphatic diamines may be partially lost due to decomposition in alkaline solutions.

A method known as ion-pair extraction may form a basis for future development. Ammonium compounds (choline, acetylcholine, etc.), primary, secondary and tertiary amines and amino acids form ion pairs with anions. The ion pairs with tetraphenylborate [129]. HgI_4^{2-} [130], anthracene-2-sulphonate [131] and di-(2-ethylhexyl)phosphate [132], among others [133—137], can be extracted with organic solvents.

Ion-pair extraction has proved useful in drug analysis [137,138] and the determination of acetylcholine [139] and of enzymes involved in acetylcholine metabolism [129, 130,140]. Ion pairs are suitable for separation by partition chromatographic methods. Column chromatographic separations utilizing this principle for the assay of some biogenic amines have recently been published [141—143].

Among the aliphatic amines, a group of di- and polyamines (Fig. 2) deserves special mention. These amines are ubiquitous in the natural world and probably play basic roles in cell biology [144,145]. Moreover, they might be useful as markers of neoplastic growth and indicators of the effectiveness of cancer chemotherapy [146—148]. These amines can serve as a typical example of the present situation in the analysis of amines in tissues and body fluids, and to

① $H_2N-(CH_2)_3-NH_2$

② $H_2N-(CH_2)_4-NH_2$

③ $H_2N-(CH_2)_5-NH_2$

④ $H_2N-(CH_2)_3-NH-(CH_2)_4-NH_2$

⑤ $H_2N-(CH_2)_3-NH-(CH_2)_4-NH-(CH_2)_3-NH_2$

Fig. 2. Structural formulae of the natural di- and polyamines. 1 = 1,3-Diaminopropane; 2 = putrescine (1,4-diaminobutane); 3 = cadaverine (1,5-diaminopentane); 4 = spermidine; 5 = spermine.

demonstrate the various strategies applied to the solution of a most important analytical problem.

The theoretical and practical importance of the polyamines has stimulated the establishment of a large number of analytical methods. Bachrach [145] summarized about 30 solvents for PC, 11 solvents for TLC and 12 different buffers for PE of the non-derivatized polyamines. Most workers used extraction of alkaline solutions with *n*-butanol in order to accumulate the polyamines and to separate them from amino acids. It should be emphasized that the chromatographic systems used for the separation of aliphatic amines are also generally suitable for amino acid separations. Ninhydrin was nearly always used for detection and determination. Some laboratories preferred, however, to determine spermidine and spermine by staining with amido black [108].

Most of these methods never gained much attention, but certain versions of PE [107,108] found wide application until recently. It turned out, however, that the urinary spermine concentrations, as measured with the PE method, were higher than those found with other procedures [148]. Putrescine concentrations in tissues were even higher by an order of magnitude than the results obtained with more advanced methods [5,6,149]. Therefore, virtually all of these methods have been abandoned as far as tissue, blood and urine analysis are concerned. For the establishment of metabolite patterns of radioactive precursors, PE and preferably TLE [150, 151] are, however, the most suitable techniques. For this purpose, it is advisable to apply trichloroacetic acid or neutralized perchloric acid extracts (neutralized with $KHCO_3$, in order to remove perchlorate) instead of *n*-butanol extracts on the chromatographic plates. Putrescine, and probably other diamines, might form degradation products in alkaline solutions, as was mentioned before.

A recent version of TLC, namely separation on silicagel sintered-glass plates combined with in situ fluorimetry after reaction with fluorescamine, was suggested as an improvement of the determination of polyamines on a micro-scale [123]. N-3-Aminopropylheptane-1,7-diamine was used as the internal standard. The sensitivity of detection with this method is of the order of 100 pmole. Its specificity and applicability in tissue and body-fluid analysis have not yet been established. However, this example shows that the TLC of free amines is still under development.

B. Ion-exchange chromatography of amines

The ion-exchange column chromatographic pre-separation of complex biological mixtures, and subsequent determination of amines in the fractions by using specific methods, has a long history. This approach is still one of the most important in the chemical analysis of biogenic amines.

The construction of automated devices for amino acid analysis following the work of Spackman et al. [152] suggested the utilization of these devices for amine analysis in the same way as for amino acid analysis. The work of Perry and co-workers [67—72,153] is exemplary in this respect. This group, and others [154—160], used almost exclusively columns packed with sulphonated polystyrene resins and either pH or salt gradients, or combined pH and salt gradients, for the successive elution of the different amines. As aromatic amines

in particular show considerable interactions with the polymer matrix, the elution patterns do not follow exactly the ion competition equilibria. The complete dissolution of a complex mixture of biogenic amines cannot be expected with this method, but starting from large tissue samples it was possible to identify a number of aliphatic amines and phenylethylamines in the brain if additional separation methods (PC, PE) were applied [72,153,154]. The same approach was successful for the detection of amines in urine [68,71,155,161] and cerebrospinal fluid [69]. On of the drawbacks of this approach is that identification of the amines is based exclusively on chromatographic criteria, which are insufficient in principle and may lead to erroneous conclusions.

Technical improvements to commercial amino acid analyzers have been considerable during the last decade. The development of spherical resins and polymer-coated glass spheres with a narrow range of diameters of 10 μm and less increased the resolution and sensitivity. Even with ninhydrin as the detection reagent, the sensitivity is now in the nanomole range. An improvement in sensitivity came from the application of continuous fluorescence monitoring, using fluorescamine [162,163] or *o*-phthaldialdehyde [164,165] as reagents. Under favourable conditions, these methods allow the measurement of picomole amounts of amines eluted from columns with diameters of 1—3 mm. Nevertheless, the routine measurement of aliphatic amines or of β-phenylethylamines in tissues and body fluids plays only a negligible role, and even the clinically important catechol- and indoleamines and their metabolites are normally not determined by automated ion-exchange methods. However, as will be discussed in a subsequent paper, some procedures meet the requirements of routine clinical analysis.

The interest in polyamines as possible markers of malignancy induced methodical developments, which are briefly summarized here. They illustrate the general trends of the current development of ion-exchange column chromatography.

Ion exchangers for the separation of di- and polyamines from amino acids and other amines have been in use for 20 years [166] and almost all types of commercial resins have been applied[145]. Elaborate separations were achieved using cellulose phosphate columns [167]. The amines were eluted by salt or pH gradients, and were determined in the collected fractions using dinitrophenylation [166,168,169], enzymatic methods [169,170] or condensation with *o*-phthaldialdehyde [167,169,171]. The tediousness of these procedures was surmounted by the automated modifications. Commercial amino acid analyzers were adapted in several laboratories for polyamine separations using, with one exception [172], reaction with ninhydrin and colorimetry at 570 nm for quantitation [173—181a]. Separations are achieved by two-or multi-step gradient elution using pH and/or sodium chloride gradients. An example is shown in Fig. 3. Recently, an attempt was made to apply ligand-exchange chromatography to the separation of polyamines [182]. Cellulose ion exchangers and different polystyrene sulphonated resins were loaded with Cu^{2+}, Zn^{2+} and Ni^{2+}, but the results were not adequate to meet current standards.

The sensitivity of detection was increased from about 500 to 0.2 nmole when fluorescamine was used [172]. The standard deviation of the procedure is of the order of ± 5% and the recovery is better than 90% [177—179].

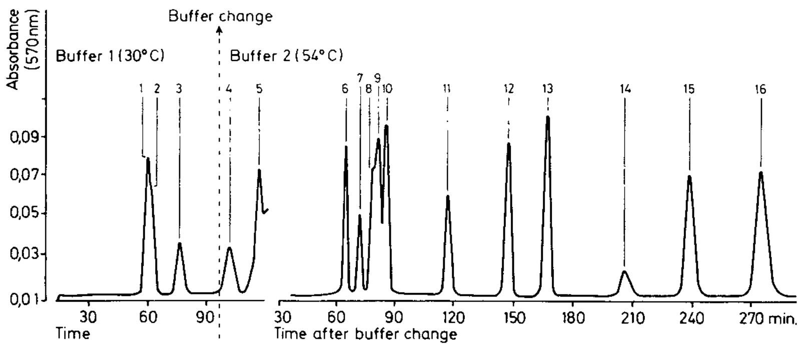


Fig. 3. Elution pattern of amines from a sulphonated polystyrene ion-exchange column using a two-step buffer system with an exponential pH and NaCl gradient in the second step. For details, see ref. 176. Buffer flow-rate, 70 ml/h. 1 = Ammonia; 2 = monoacetyl-1,3-diaminopropane; 3 = monoacetyl-1,4-diaminobutane; 4 = monocarbamyl-1,4-diaminobutane; 5 = arginine; 6 = 1,4-diamino-2-hydroxybutane; 7 = diaminopropane; 8 = N^1-monoacetylspermidine; 9 = N^8-monoacetylspermidine; 10 = 1,4-diaminobutane (putrescine); 11 = 1,5-diaminopentane (cadaverine); 12 = N-(3-aminopropyl)-1,3-diaminopropane; 13 = spermidine; 14 = agmatine; 15 = N, N'-bis-(3-aminopropyl)-1,3-diaminopropane; 16 = spermine (20 nmol of each amine). According to Tabor et al. [176].

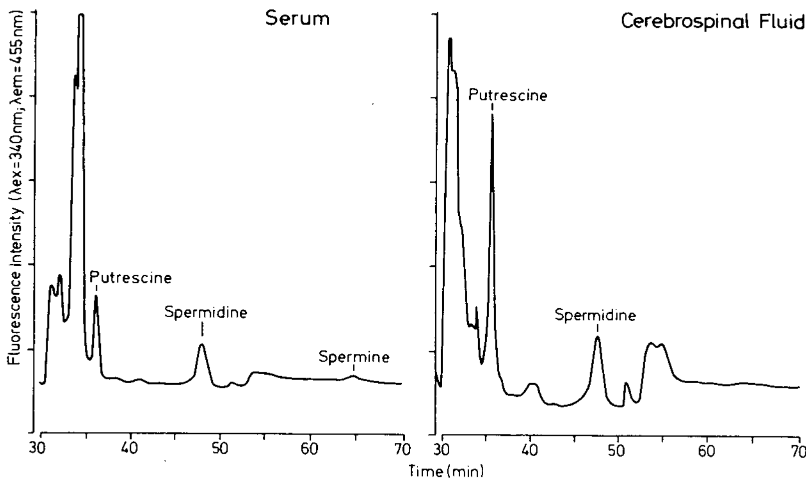


Fig. 4. Separations of serum and cerebrospinal fluid polyamines using an automated high-performance liquid chromatographic technique in combination with the reaction of amines with *o*-phthaldialdehyde. (After acid hydrolysis the equivalents of 1.25 ml of serum and cerebrospinal fluid were separated). According to Marton and Lee [183].

Recently, small high-pressure columns were utilized in combination with highly developed fully automated equipment with computerized peak-area calculation. This device allows the routine determination of 3—6 pmoles of putrescine and spermidine and 12—15 pmoles of spermine if the *o*-phthaldialdehyde method is adapted to the system [183]. Fig. 4 shows separations of polyamines from 1.25-ml serum and cerebrospinal fluid samples. Further improvements can be expected from improvements to the ion-exchange resins.

C. Coloured derivatives of amines for thin-layer chromatographic separation

A reagent suitable for the analysis of small amounts of amines (and amino acids) should fulfill the following criteria: (a) Rapid quantitative reaction under mild conditions in water or water-containing media; (b) specificity for primary or secondary amino group; (c) high sensitivity of detection; (d) favourable chromatographic properties of the derivatives; and (e) low polarity of the reaction products in order to permit the accumulation of the reaction products by solvent extraction.

Several compounds are available that react either with primary amino groups or with primary and secondary amino groups, but no reagent is known to be specific for secondary amino groups, nor is there a derivative-forming reaction known for tertiary amines that is suitable for quantitative analysis.

The reagents summarized in Fig. 5 react, with one exception, with primary and secondary amines in weak alkaline solutions to give coloured derivatives in high yields. The derivatives are stable, can be extracted from the reaction mixture with organic solvents and are suitable for chromatographic separation and spectrophotometric determination in the nanomole range. Hence they meet at least partially the above-mentioned criteria.

Solvent systems for the TLC (and PC) of these derivatives have been formulated for only a few amines. Only 2,4-dinitrofluorobenzene (Dnp-F), the well known end-group reagent of Sanger [184,185], and the more specifically reacting 2,4-dinitrobenzenesulphonic acid [186], which leads to the same derivatives, have been used extensively, both for the determination of certain urinary and tissue constituents. PC [187,188] and TLC [186, 189—194] were used for separation, and it was shown that the derivatives are also suitable for ion-exchange column and GLC separation [190]. As was mentioned before, dinitrophenylation was utilized for the determination of amines in column eluates [195]. The mass spectra of the Dnp derivatives have been studied to

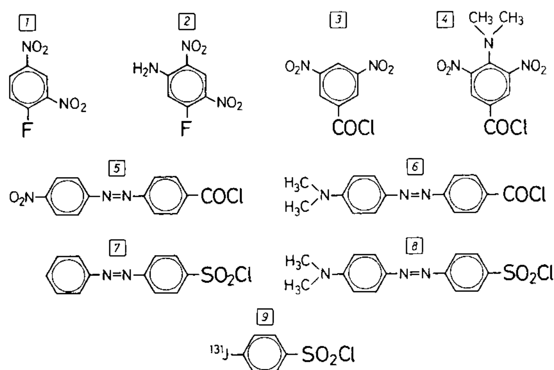


Fig. 5. Structural formulae of reagents recommended for the formation of coloured derivatives with primary and secondary amines. 1 = 2,4-Dinitrofluorobenzene [184]; 2 = 2,4-dinitro-5-fluoroaniline [197]; 3 = 3,5-dinitrobenzoyl chloride [75]; 4 = 4-dimethylamino-3,5-dinitrobenzoyl chloride [198,199]; 5 = 4-(4′-nitrophenylazo)benzenecarbonyl chloride [200]; 6 = 4-(4′-N,N-dimethylaminophenylazo)benzenecarbonyl chloride [201]; 7 = 4-(phenylazo)benzenesulphonyl chloride [202]; 8 = 4-(4′-N,N-dimethylaminophenylazo)benzenesulphonyl chloride [203,204]; 9 = [^{131}I]-4-iodobenzenesulphonyl chloride [196].

show the usefulness of this method for the unambiguous identification of amines [191].

Restriction of the sensitivity of detection to the nanomole range is the main reason for the limited use of coloured derivatives for the analysis of amines in tissues, and for the preference for fluorescent reagents of similar reactivity and separation characteristics. In order to make use of existing experience, and to increase the sensitivity of detection, tritiated Dnp-F was introduced for tissue amine analysis [128]. With this reagent, a few picomoles of a compound can be detected; however, these amounts are invisible and it is difficult, therefore, to control the quality of the separations on the thin layers. If the specificity of the separations is not adequately controlled, erroneous results might be obtained.

Increase of the sensitivity of detection by utilization of radioactive reagents is not new. [^{131}I]*p*-Iodobenzenesulphonyl chloride has been known for 30 years [196], but it was never generally applied, for the above reasons.

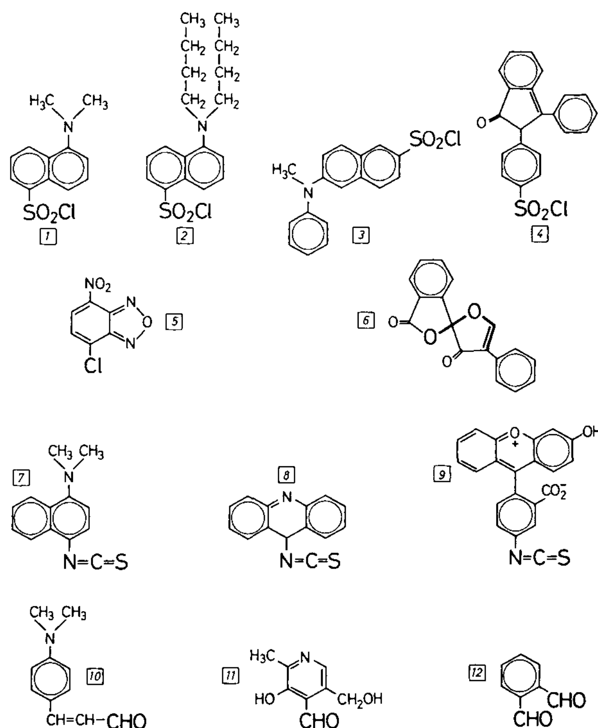


Fig. 6. Structural formulae of reagents recommended for the formation of fluorescent derivatives with primary and/or secondary amines. 1 = 5-Dimethylaminonaphthalene-1-sulphonyl chloride (Dns-Cl); 2 = 5-di-*n*-butylaminonaphthalene-1-sulphonyl chloride (Bns-Cl); 3 = 6-methylanilinonaphthalene-2-sulphonyl chloride (Mns-Cl); 4 = 2-*p*-chlorosulphophenyl-3-phenylindone (Dis-Cl); 5 = 4-Chloro-7-nitrobenzo [*c*]-1,2,5-oxadiazole (Nbd-Cl); 6 = 4-phenylspiro[furan-2(3H), 1′-phthalan]-3,3′-dione (fluorescamine); 7 = 4-dimethylaminonaphthalene-1-isothiocyanate; 8 = 9-isothiocyanatoacridine; 9 = fluorescein isthiocyanate; 10 = 4-dimethylaminocinnamaldehyde; 11 = pyridoxal; 12 = *o*-phthaldialdehyde.

D. *Fluorescent derivatives of amines for thin-layer and column chromatographic separation*

In Fig. 6, reagents currently used for the fluorescence labelling of amines (and amino acids) are summarized. The same reactive groups (activated halogen, sulphonyl chloride) are used both for fluorescence and colour labelling. Isothiocyanates and aldehydes are used only for special purposes. The specificity of the reactions and the formation of side-products during derivative formation are, in principle, the same for coloured and fluorescent derivatives. The obvious advantage of fluorescence labelling is the increased sensitivity of detection. Another advantage is the wide range of linearity between amount of substance and fluorescence intensity (i.e., photometer response), which simplifies and improves the in situ evaluation of TLC separated fluorescent compounds [205—208] and the continuous monitoring of column effluents.

The use of coloured and fluorescent reagents involves the same strategies: either the total tissue extracts or body fluids are made to react first, and separation procedures are applied exclusively to the derivatives, or a certain compound or a group of related compounds is pre-separated by solvent extraction, ion-pair extraction, column chromatography, etc., and the detection reaction is applied to the pre-separated compounds. In no case is a step involving purification of the derivatives dispensable, because normally side-products are formed during derivative formation; hydrolytic cleavage of the reagent is the most common side-reaction.

The information available does not permit a thorough comparison of the advantages of the different reagents. The amounts of information about the various reagents differ considerably and for some reagents is only fragmentary. Fluorescent reagents are briefly surveyed in the following sections; for a more detailed description, see ref. 209.

a. *4-Chloro-7-nitrobenzo[c]-1,2,5-oxadiazole*

As an aryl halogenide with activated halogen (see Fig. 6, No. 5), 4-chloro-7-nitrobenzo[*c*]-1,2,5-oxadiazole (Nbd-Cl) is closely related to Dnp-F. It reacts readily in aqueous solutions [210,211] or in organic solvents [212—214] with primary and secondary amines, and less readily with phenols and thiols at pH 8. Thiol-containing compounds, however, react rapidly at pH 7 [215]. Usually 1—20 μg of amine dissolved in 25—500 μl of solution is mixed with four volumes of a 0.05% solution of Nbd-Cl in methanol, and 50—100 μl of 0.1 *M* $NaHCO_3$ solution are added. Completion of the reaction is achieved by heating at 55° for 60 min, and the yields are mostly > 95% [210]. Separation of the reaction product from excess of reagent is usually achieved by silica-gel column chromatography.

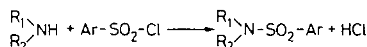
A thorough study of the chemical and physical properties of Nbd derivatives has not been published. They are stable in solution and on thin-layer plates, if protected from irradiation. In contrast with most other fluorescent labels, the absorption maxima of Nbd derivatives are in the visible region ($\lambda_{max(A)}$ = 460—470 nm), so that absorption and emission bands ($\lambda_{max(E)}$ = 510—530) overlap [210,215,216]. According to Klimisch and Stadler, about 50 pmol of Nbd-dimethylamine was measurable per millilitre of ethyl acetate. By direct (in situ) fluorescence measurement, direct proportionality between

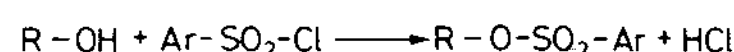
the recorded curve areas and amounts of substance was observed with 70—700 pmoles [210]. Nbd derivatives are suitable for mass spectrometric identification [217].

b. Fluorescent sulphonyl chlorides

Four sulphonyl chlorides are currently in use for the fluorescence labelling of amines and amino acids: 5-dimethylamino naphthalene-1-sulphonyl chloride (Dns-Cl), 5-di-*n*-butylaminonaphthalene-1-sulphonyl chloride (Bns-Cl), 6-methylanilinonaphthalene-2-sulphonyl chloride (Mns-Cl) and 2-*p*-chlorosulphophenyl-3-phenylindone (Dis-Cl) (see Fig. 4, Nos. 1—4). These reagents react with primary and secondary amino groups under the same conditions. Their modes of application differ only insignificantly, the differences being confined to the optical and chromatographic properties of the derivatives.

Sulphonyl chlorides react with primary and secondary amino groups even under slightly alkaline conditions, and with phenols, imidazoles [44,45] and even with some alcohols at higher pH. For instance, a method for the sensitive determination of choline has been devised [218]. Thiol compounds form the corresponding disulphides [44,45,219]. The reaction of sulphonyl chlorides with amines, phenols, alcohols and thiols is shown in Fig. 7.





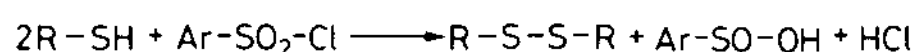


Fig. 7. Reaction of sulphonyl chlorides with amines, phenols alcohols and thiols.

In acetone--water (3:1) saturated with sodium carbonate or, in favourable circumstances, with sodium hydrogen carbonate, it is possible to obtain stoichiometric amounts of reaction products of primary and secondary amines if the reagents are applied in excess. Polyfunctional molecules, such as polyamines and aminophenols, react under these conditions with all functional groups, i.e., one obtains O,N-bis-Dns-tyramine, $N_{\alpha}N_{\pi}$-bis-Dns-histamine, tri-Dns-spermidine, tetra-Dns-spermine, tri-Dns-dopamine and tri-Dns-norepinephrine with excess of Dns-Cl.

Amino acids react first with the amino group. With excess of reagent, however, mixed anhydrides are formed, especially at elevated pH. The anhydrides of α-amino acids are partially fragmented under the usual reaction conditions to carbon monoxide, the aldehyde with one carbon atom less than the parent amino acid and the ammonia derivative [220]. Under the same conditions γ-amino acids form substituted γ-lactams, as was shown with Dns-Cl as reagent. This reaction is the basis for a sensitive and specific method for the determination of the biologically important γ-aminobutyric acid [1,57,221]. Tertiary amines may be attacked to a significant extent, especially at elevated pH and temperature, forming the derivative of a secondary amine by elimination of an alkyl or aryl group [222].

Increasing reaction velocities are normally paralleled by increased rates of hydrolysis. The formation of the corresponding sulphonic acid as a by-product

of the reaction is inevitable and methods applied for the further separation of the fluorescent derivatives have to take this into account. In the amine analyses the separation of the bulk of the sulphonic acid can usually be achieved by solvent extraction from alkaline solution.

The reaction of amines with sulphonyl chlorides in water-free organic solvents is possible [223], but has rarely been applied. This possibility of derivative formation deserves more attention.

Virtually all types of chromatographic procedures can be applied to the separation of the fluorescent sulphonamides. The most effective separations were performed on active surfaces, which have the advantage of the applicability of a wide range of separation methods, from pure adsorption systems to pure partition systems. In fact, there are few separation problems that cannot be solved on this basis by the selection of appropriate solvent mixtures, especially if the possibilities of two-dimensional separations by TLC are utilized. Numerous solvents for Dns-amide separations on thin layers have been published [44,45,209]. Bns derivatives are less polar than the corresponding Dns derivatives, but the general experience gained with Dns derivatives can be applied to their separation [224]. Less experience with other fluorescent derivatives exists. In fact, Mns and Dis derivatives, with few exceptions, have been only used for amino acid determinations [225,226]. In addition to active surfaces, polyamide sheets have been used for the separation of small amounts of amino acids and amines [40,41,43]. The high sensitivity of detection for fluorescent spots on polyamide sheets is mainly a consequence of the small diameters of the spots that can be obtained owing to the small particle size of the polyamide layers. This advantage is offset by the restriction to partition chromatographic systems, the relatively high background fluorescence of commercial sheets and especially the low capacity of the layer. This low capacity seriously limits the separation of complex mixtures with widely varying concentrations of the components, such as urine samples and tissue extracts. The recently developed high-performance thin-layer plates with silica gel layers combine the advantages of polyamide sheets with those of conventional active layers. They are especially useful for the detection of microamounts of biological amines in the form of fluorescent derivatives [227—228]. Fig. 8 shows an example of the separation of the Dns derivatives of the perchloric acid extract of mouse liver. The sensitivity of the method is shown by the fact that 5-pmole amounts of spermidine and spermine were measurable in 5 μg of liver tissue.

For the improvement of separations and especially to allow automated procedures to be introduced, column chromatographic methods have more recently been suggested for the separation of Dns derivatives of amines [229—233]. According to unpublished results in our laboratory, Dns and especially Bns derivatives are suitable for reversed-phase high-performance liquid chromatographic separation. About 3 pmoles of the polyamines spermidine and spermine in tissues were measurable within 30 min with repeated separations by using fluorescence monitoring of the column effluent.

For the quantitative assay of fluorescent sulphonamides, at least four different methods are available: absorptimetry, fluorimetry, quantitative mass spectrometry and the application of radioactive reagents.

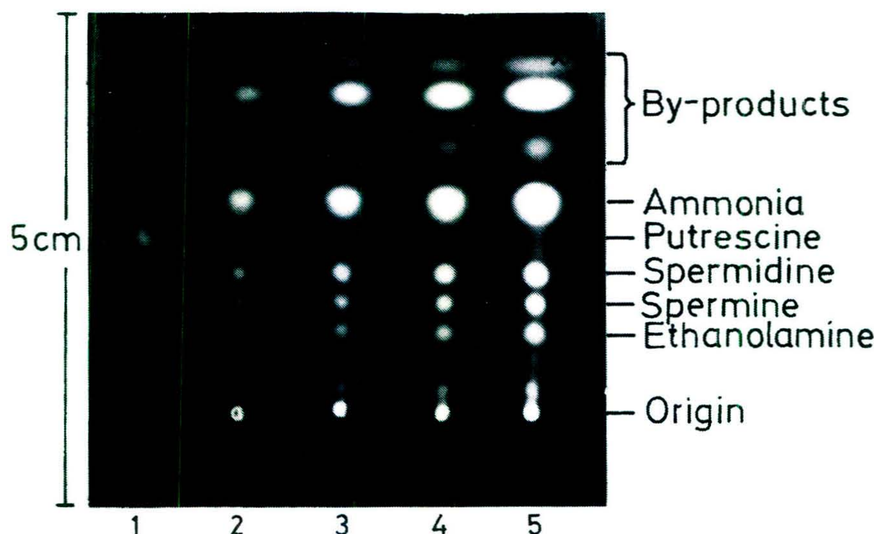


Fig. 8. One-dimensional separation of dansylated perchloric acid extracts of mouse liver on a 5 X 5 cm high-performance thin-layer plate (E. Merck, Darmstadt, G.F.R.). 1 = Bis-Dns-putrescine (reference sample); 2, 3, 4 and 5 = Dns derivatives corresponding to 5, 10, 50 and 100 μg, respectively of mouse liver tissue (about 5, 10, 50 and 100 pmoles of spermidine and spermine). Solvent, cyclohexane—ethyl acetate (1:1) (two runs). According to Seiler and Knödgen [228].

The high molar extinction of the fluorescent sulphonamides [$MnsNH_2$: $\epsilon_{255} = 4.2 \cdot 10^4$; $\epsilon_{321} = 2.3 \cdot 10^4$ (in *n*-propanol) [225]; $DnsNH_2$: $\epsilon_{252} = 1.3 \cdot 10^4$; $\epsilon_{333} = 0.43 \cdot 10^4$ (in methanol)] [44,234] allows their sensitive detection by absorptimetry, for instance in column effluents [232,235,236]. However, the most obvious method for their determination is fluorimetry. Dns derivatives are excited most effectively at 350—355 nm and the fluorescence is measured at 510—540 nm, depending on the structural features of the compound [44,45, 234]. The excitation maximum of the Bns derivatives nearly coincides with the 365-nm mercury line [224], which has considerable practical advantages as the xenon arc lamp can be replaced by the more intense and stable mercury arc lamp for fluorescence excitation. The fluorescence maxima of Bns derivatives occur at shorter wavelengths than those of the Dns derivatives (500—530 nm); their fluorescence quantum yields are comparable. Mns derivatives are excited at about 320 nm and their fluorescence maxima occur in the range 440—460 nm [225]. Dis derivatives form orange-red spots on thin-layer chromatograms, and can be detected with about the same sensitivity as Dnp derivatives, namely in the nanomole range. In strongly alkaline solutions, Dis derivatives are rearranged to strongly green fluorescing 1-phenyl-3-*p*-sulphophenyl-isobenzofuranic derivatives (Fig. 9), allowing their detection in the 0.1—1 pmole range on thin-layer plates [237]. Direct (in situ) fluorescence measurement of these spots, however, was not feasible, in contrast with all other fluorescent sulphonamides. It was therefore recommended [238] that the separated Dis derivatives be extracted with acetone, the acetone solution evaporated and the rearrangement induced by the addition of 5 ml of a solution

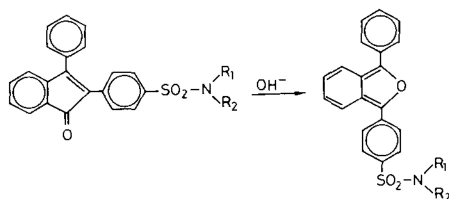


Fig. 9. Rearrangement of 2-*p*-sulphophenyl-3-phenylindone (Dis) derivatives to 1-phenyl-3-*p*-sulphophenylisobenzofuranic derivatives with sodium ethoxide [237].

of sodium ethoxide in ethanol. For the determination of pyridoxamine, the fluorescence is activated at 410 nm and emission measured at 480 nm.

Extraction of the fluorescent derivates of the adsorbent layer and in situ fluorescence scanning of the chromatograms are equally suitable procedures for the quantitative evaluation of thin-layer chromatograms [44,45,208,234]. Amounts of 100 pmoles of amines of biological origin can be determined without difficulty with a standard deviation of less than ± 5%.

In order to simplify measurements in small volumes of solvent and thus to increase the sensitivity of routine fluorescence measurements, an extraction procedure has been developed that allows the elution of TLC separated compounds with a solvent volume of about 50 μl [1,228]. In order to prevent decomposition of the Dns derivatives on the active surface, the plates are sprayed with triethanolamine—propanol-2 (1:4) immediately after chromatographic development [234]. The fluorescent spots are marked under a UV lamp (365-nm mercury line), then scraped out either using a glass capillary with a constriction [1] or a PTFE tube of about 1 mm I.D., with a cotton-wool plug fixed in the constriction (with the PTFE tube, constrictions are made with tweezers) [228]. The adsorbent is collected in one of the two compartments of the capillaries by suction with a suitable vacuum pump. For elution, the end of the tube containing the adsorbent is dipped into the solvent, and the solvent is moved through the capillary by gentle suction or, preferably, the adsorbent-filled side of the tube is connected with a motor-driven syringe, which is filled with solvent. A defined solvent volume is pumped through the capillary, the eluent being collected in the other capillary compartment, or else it is transferred directly into a small vessel, which is capped and stored at 0° until fluorescence measurements are carried out. If a spectrofluorimeter with an 8—10 μl flow-through cell or a fluorescence flow detector of a high-pressure column equipment is used for quantitative fluorimetry, an eluent volume of 50—75 μl is suitable. A few picomoles of the Dns and Bns derivatives of the polyamines spermidine and spermine can be determined routinely by using this technique, especially if high-performance thin-layer plates are used for the separations [228].

Elution of TLC separated compounds with small solvent volumes is of great importance for subsequent mass spectrometry: impurities can be kept to a minimum in the sample, and small solvent volumes simplify the transfer of the samples into the probe capillaries of the mass spectrometer. The above elution techniques, however, without prior spraying of the plates with triethanolamine, have been used in many mass spectrometric determinations of putres-

cine [149,239], piperidine [4] and serotonin [240] and for the preparation of mass spectra of TLC isolated compounds. The selection of the appropriate solvent is important. Elution should be carried out with a solvent with as low a polarity as possible, in order to minimize elution of contaminants in the adsorbent. For the elution of Dns- and Bns-amine derivatives from silica-gel plates and similar active layers, ethyl acetate is normally suitable. For subsequent radioactivity measurements, dioxan may be preferable. For derivatives of higher polarity, acetone or even methanol can be used. Dns-amino acids are extracted with methanol—25% ammonia solution (95:5) [44,234]. Silica gel G plates are preferable to plates with organic binders. If mass spectra of compounds separated on polyamide sheets are prepared, it is advisable to wash the sheets with methanol—acetic acid (3:1) before use as the spectra otherwise exhibit high backgrounds [241].

Usually molecular ions are observed in the electron-impact mass spectra of Dns and Bns derivatives and other fluorescent derivatives. The quantitative evaluation of a molecular ion or a typical fragment ion is much more specific than is fluorimetry or any other quantitative method that is currently available. Although underivatized compounds can be determined by the integrated ion current technique [242], it is advantageous to use fluorescent derivatives: (a) all separation steps in advance of the quantitative evaluation can be controlled visually; (b) owing to the high molecular weight of the derivatives, the background at the mass range of the molecular ions is normally low; and (c) erroneous peak identification is much less probable than with low-molecular-weight free amines owing to the low background.

Usually, the sample eluted from a thin-layer chromatogram is evaporated together with a suitable standard (generally the corresponding derivative of a homologue of the amine to be determined [243] or a deuterated sample [244]) from the direct probe of the mass spectrometer. The ion current of the molecular ion (or fragment ion) of the sample and standard are recorded alternately during evaporation and subsequently integrated. The ratio of the integrated ion currents of the sample and standard is a measure of the amount of sample, and is nearly independent of changes in instrumental sensitivity during the measurement. Fig. 10 shows the recorded ion currents of the molecular ions of varying amounts of bis-Dns-putrescine (*m/e* 554) and constant amounts of the internal standard bis-Dns-hexamethylenediamine (*m/e* 582), It is one of the pre-requisites of the method that the sample and standard should have similar evaporation profiles. The integrated ion current technique, using Dns or Bns derivatives, allows the precise determination of picomole or, in favourable cases, even of femtomole amounts of biogenic amines. The sensitivity of the method is dependent on the instrumental sensitivity.

Among the currently available fluorescent derivatives, Bns derivatives are especially suitable for qualitative and quantitative mass spectrometry. Dns derivatives form the ion corresponding to dimethylaminonaphthalene (*m/e* 170 or 171) with the highest abundance [244—248]. In contrast, Bns derivatives are split preferentially in the *n*-butyl side-chain, forming a fragment ion $(M—43)^+$ that still contains the complete information of the derivatized molecule [224]. Because, in addition to the $(M—43)^+$ ion, the molecular ion (M^+) is observed with about the same relative intensity as that of Dns derivatives, derivative

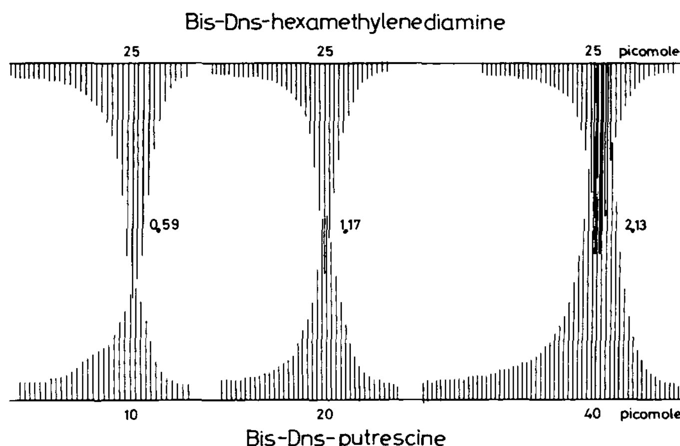


Fig. 10. Ion current curves of mixtures of 25 pmoles of bis-Dns-hexamethylenediamine (*m/e* 582) with 10—40 pmoles of bis-Dns-putrescine (*m/e* 554). The numbers are calculated peak-area ratios. Evaporation time, 30 sec. (Schematic drawing of two channel recordings from an electrostatic recorder). (For details see ref. [243]).

formation with Bns derivatives not only permits the determination of smaller amounts, but also facilitates the identification of molecular ions in the mass spectra of mixtures of compounds by means of the two characteristic ions M^+ and $(M—43)^+$, the intensities of which are observed in a fixed ratio. In addition to electron impact, field desorption may provide useful ionization methods. In combination with multiple-ion detection it may allow the quantitative analysis of complex mixtures of amines in the form of their Bns derivatives, without prior separation. A method calles metastable defocusing was recently suggested as being applicable to mixtures of Dns derivatives [249]. At a low electron beam energy (12 eV), Dns derivatives form almost exclusively the fragment ion at *m/e* 171. The instrument is focused on this fragment ion and the acceleration voltage is then varied while the analyzer voltage is kept constant. This procedure has the effect of focusing successively on the collector, during the travel from the source to the analyzer, the precursor ions that give rise to the formation of this fragment ion. Hence the method uses the determination of ions formed by metastable decomposition in the field-free region of the mass spectrometer. Applications of the method to biological samples have not yet been published.

An increase in specificity is the main characteristic of mass spectrometric methods, apart from their sensitivity. An increase in the sensitivity of detection, although not of specificity, is obtained by using radioactive reagents. N-Methyl-[^{14}C]Dns-Cl (specific radioactivity 10—30 Ci/mole) and [G-^{3}H] Dns-Cl (specific radioactivity 3—10 Ci/mmole) are commercially available. The labelled reagents can be applied in a similar manner to the unlabelled reagent, but they are normally restricted to applications with small reaction volumes (1—50 μl) because of their high cost. Their application allows the replacement of fluorescence measurements with automated liquid scintillation counting. The intense fluorescence of the derivatives is used only to reveal the separated spots on the chromatograms. If the reagent with the highest available specific

radioactivity is used, the sensitivity of the method allows the determination of a few picomoles. A further increase in the sensitivity of detection is gained by the preparation of autoradiographs from the chromatograms, which can be evaluated by microscope photometry [250]. Unfortunately, this method has disadvantages: high costs and erroneous interpretation of the autoradiographs and quantitative measurements owing to the presence of non-fluorescent but radioactive impurities and degradation products. The application of labelled reagents is improved by using double-isotope methods:

A known amount of the compound to be determined is added to the sample in the form of its ^{14}C-labelled analogue as an internal standard (in analogy with the use of deuterated standards in quantitative mass spectrometry). After derivative formation with the ^{3}H-labelled reagent, the derivative is extensively purified. Incomplete derivative formation, or losses during the purification steps do not affect the quantitative result, provided that sufficiently large amounts of the purified sample are isolated to allow precise radioactivity measurements. The amount of the non-radioactive compound present in the sample can be calculated from the $^{3}H/^{14}C$ ratio. The coefficient of variation of this method is of the order of 6% [251].

c. Isothiocyanates

Isocyanates and isothiocyanates react with primary and secondary amines to give urea and thiourea derivatives, respectively. Several isocyanates have been suggested in the past as fluorescence probes [209]. However, as they react readily with water and alcohols to give urethanes, they were replaced with the less reactive isothiocyanates.

At present, three main fluorescent labels are in use for the labelling of low-molecular-weight amino-containing compounds that bear the isothiocyanate moiety: 9-isothiocyanatoacridine [252] (Fig. 6, No. 8), fluorescein isothiocyanate [253] (Fig. 6, No. 9) and 4-dimethylaminonaphthalene-1-isothiocyanate [254] (Fig. 6, No. 7). The last two compounds seem to have been used only for end-group analysis of peptides and for the detection of free amino acids.

9-Isothiocyanatoacridine leads to the formation of several fluorescent products, and the fluorescence of one of these products could be related to the amount of amine present in the sample [252]. On chromatographic evidence, the fluorophore is a cyclization product that is formed by photo-oxidation from the primarily formed thiourea derivative (Fig. 11) [255]. A few thin-layer chromatographic separations have been carried out with isothiocyanatoacridine derivatives. A linear relationship has been observed between fluorescence intensity and amount of sample on the plates (excitation of fluorescence at 295—310 nm; emission measurement at 500—525 nm). The usefulness of 9-

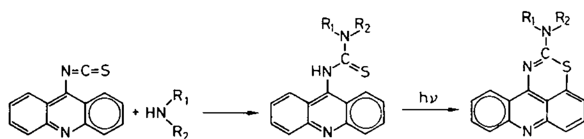


Fig. 11. Reaction of 9-isothiocyanatoacridine with an amine.

isothiocyanatoacridine as a reagent for the assay of small amounts of biogenic amines has not yet been demonstrated convincingly.

d. Fluorescamine

Fluorescamine (4-phenylspiro[furan-2(3H),1′-phthalan]-3,3′-dione) (Fig. 6, No. 6) reacts with compounds that contain nucleophilic functional groups (primary and secondary amines, alcohols, water, etc.). However, only primary amines form fluorescent products according to Fig. 1. Fluorescamine is therefore a specific reagent for compounds with primary amino groups [256]. For the reaction of aliphatic amines, a pH of 8—8.5 is adequate [257,258].

Fluorescence measurements are normally carried out in the range of maximal stability of the fluorophore between pH 4.5 and 10.5. In this range the absorption maximum is at 390 nm and the fluorescence maximum at 475 nm [257].

Fluorescamine is normally used for the assay of amines, amino acids and peptides in column effluents [162,163], or as a spray reagent for the detection of these compounds on thin-layer chromatograms [122—124]. However, in preliminary work, Imai et al. [259] ran the fluorophores of some amines (dopamine, norepinephrine and their corresponding 3-O-methylation products, polyamines) on silica-gel plates. They showed that about 250—500 pmoles of these compounds could be detected. Nakamura and Pisano [260] suggested that the compounds should be derivatized with fluorescamine at the origin of the thin-layer plates, prior to separation. As the quantitative evaluation of the fluorescent spots did not seem to be completely satisfactory, high-performance liquid chromatographic systems have been devised for the separation of these amines [261,262]. The fluorescamine derivatives were measurable at the 100-pmole level. Further experience will be necessary in order to evaluate the usefulness of this promising reagent fully.

e. Pyridoxal and pyridoxal-5-phosphate

Pyridoxal (Fig. 6, No. 11) and pyridoxal-5-phosphate form Schiff bases with primary amino-containing compounds. Complete reaction is usually achieved at pH 9.3 in phosphate buffer (yields > 90%). After 30 min, the Schiff bases are reduced to the corresponding pyridoxyl derivatives (Fig. 12). The excess of $NaBH_4$ is removed by acidification [263, 264]. Ion-exchange column chromatographic systems have been used exclusively for the separation of pyridoxyl derivatives, which were monitored in the effluent by absorptiometry (absorption maxima at 255 and 328 nm) or by fluorescene measurement (fluorescence emission maximum at 400 nm). Amounts of 10—100 pmol of an amino acid are detectable.

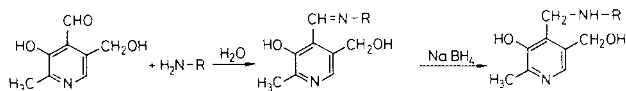


Fig. 12. Derivative-forming reaction of a primary amine with pyridoxal.

One of the advantages of the reagent is the possibility of radioactive labelling by the application of sodium borotritide ($NaBT_4$) as reducing agent. This compound is commercially available with high specific activity.

f. ω-Formyl-o-hydroxyacetophenone and benzo-γ-pyrone

ω-Formyl-*o*-hydroxyacetophenone and benzo-γ-pyrone react with primary and secondary aliphatic and aromatic amines to form β-aminovinyl *o*-hydroxyphenyl ketones (Fig. 13). Kostka [265] utilized this reaction for derivative formation with amines, and for their sensitive detection on thin-layer chromatograms. The chromatographic characteristics of 54 enamine derivatives were studied using ethyl acetate—benzene (1:5), chloroform—xylene (4:1) and acetone—xylene (1:9) and silica gel G layers. The β-aminovinyl *o*-hydroxyphenyl ketones of aliphatic amines are yellowish, while the derivatives of aromatic amines are orange; they fluoresce in the UV region. Amounts of 0.1—1 μg of the derivatives can be detected on a thin-layer plate.

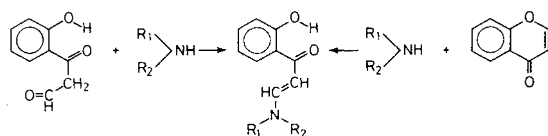


Fig. 13. Formation of β-aminovinyl *o*-hydroxyphenyl ketones from ω-formyl-*o*-hydroxyacetophenone and benzo-γ-pyrone.

As the formation of the enamine derivatives is rapid and specific for primary and secondary amines, this derivatization procedure deserves more attention than it seems to have received hitherto.

3. SUMMARY

This first part of the review on "Chromatography of Biogenic Amines" is devoted to the description of generally applicable separation and detection methods. Gas chromatographic and gas chromatographic—mass spectrometric methods, and applications of chromatographic methods to specific amines or groups of related amines and their metabolites, will be covered in Part II.

Trends in the development of separation methods (paper and thin-layer chromatography, paper and thin-layer electrophoresis and ion-exchange methods) are described, using mostly aliphatic amines as examples as they do not exhibit features that permit their specific determination. Reagents suggested for the formation of coloured and fluorescent derivatives of amines are reviewed and their applications are described. Within the limitations of the mostly inadequate information that is available, the relative usefulness of the different derivative-forming reactions are compared.

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GAS CHROMATOGRAPHY—MASS SPECTROMETRY OF METABOLITES IN HEMODIALYSIS FLUID

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SUMMARY

An analytical method has been developed for the separation and identification of several metabolites in used hemodialysis fluid obtained during the treatment of a uremic patient on the artificial kidney. The procedure involves ion exchange, evaporation, and trimethylsilylation; the derivatized components were studied by combined gas chromatography—mass spectrometry.

Twelve compounds were satisfactorily resolved; six were conclusively identified from mass spectral data. The identified components include phosphoric acid, glucopyranurono-(6→1)-lactone, citric acid, D-gluconic acid-δ-lactone, α-D-glucose, and β-D-glucose. A seventh component was tentatively identified as mannonic acid.

INTRODUCTION

Gas chromatography (GC) has been used extensively for the separation of molecular components in physiological fluids [1—3]. Identification of many of these components has been achieved by combined gas chromatography—mass

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spectrometry (GC—MS). Horning et al. [4] have demonstrated that GC—MS can be used to measure a wide variety of components of clinical interest. Thompson and Markey [5] have compared the reproducibility and efficiency of methods of isolation of organic acids from urine by means of a GC—MS—computer system.

This paper describes a GC—MS study of components found in the dialysate obtained during hemodialysis of a patient with chronic renal failure. Patients with chronic renal failure produce little or no urine, and they therefore retain large quantities of water and electrolytes, as well as those waste substances that are normally excreted. These substances, which accumulate in the blood, must be removed periodically by means of hemodialysis. In this process, the excess or waste substances are removed from the bloodstream by dialysis through a cellulose or copolymer semipermeable membrane in an artificial kidney. The aqueous receptor fluid is known as the hemodialysate (or simply dialysate); it is prepared by diluting a commercial concentrate of dissolved electrolytes (Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, and acetate) with sufficient water, so that the final volume is 120 l. In the artificial kidney, arterial blood is pumped through the membrane at ca. 200 ml/min. Dialysate flows on the outside of the membrane at ca. 0.3 l/min, its direction of flow being opposed to that of the blood, thus producing a useful concentration gradient. Typical dialysis membranes contain 40-Å pores, and are permeable to substances up to 18,000 molecular weight [6].

It has been shown in a number of studies that the syndrome of uremia is directly attributable to the presence of substances in body fluids at concentrations that are considerably higher than those normally attained in healthy subjects [6—9]. Recently, Bultitude and Newham [8] have reported a method for comparing plasma samples from chronic uremic patients before and after dialysis. They identified a number of abnormal metabolites by use of combined GC—MS of the trimethylsilyated derivatives, and showed that the concentrations of these compounds increased in uremia; however, after dialysis of the patient's blood, the concentrations became about the same as those in plasma from healthy subjects. Low-molecular-weight volatiles have also been extracted from the blood plasma of patients, before and after hemodialysis, and have been separated and identified by GC—MS—computer methods [6].

In much of the work reported thus far, attention has been devoted primarily to the analysis of blood plasma from uremic subjects. It has been generally assumed that used dialysate contains concentrations of metabolites that are too low to be detected; however, recently it has been shown that used hemodialysate can be analyzed efficiently by high-resolution liquid chromatography [10]. In the present investigation, it has been found that used dialysate sampled from the effluent of a multiple-pass artificial kidney can also be analyzed by GC—MS.

MATERIALS AND METHODS

Samples

Several 1-l samples of used hemodialysate (Travenol Laboratories, Deerfield, Ill., U.S.A.) were collected from a female patient on an artificial kidney ca. 3 h

after the start of treatment. The samples were filtered through a 115-ml, 0.20-μm Nalgene membrane filter (Sybron, Rochester, N.Y., U.S.A.). Several drops of reagent-grade chloroform were added to each sample as a bacteriostatic agent, and the samples were then frozen and stored at −15° until ready for use. Blank (or unused) hemodialysate samples were obtained from the aqueous solution of electrolytes, prior to patient treatment, and were treated and stored identically.

Sample preparation

Acidic (and some neutral) compounds were separated from hemodialysate samples by use of DEAE-Sephadex, a weakly basic anion exchanger, (Pharmacia, Piscataway, N.J., U.S.A.) [1]. The ion-exchange bed was prepared in a 25-ml buret, equipped with a small glass wool plug. A slurry of the resin suspended in 1.5 *M* pyridine acetate was poured into the buret until a 1.2 × 10 cm resin bed was produced. The column was first treated with 150 ml of 1.5 *M* pyridine acetate, followed by 200 ml of 0.5 *M* pyridine acetate. A 150-ml dialysate sample was introduced into the column by means of a constant flow device [11]; this was followed by the addition of 150 ml of doubly distilled water. The sample components were eluted with 150 ml of 1.5 *M* pyridine acetate using a flow-rate of 1 ml/min. The eluent was collected and frozen at −15° until ready for further processing.

The thawed sample was evaporated at 25° and 1 mmHg pressure. After the sample was reduced to a viscous fluid (ca. 2.5 h), crystallization occurred. This procedure was continued for an additional 2.5 h in order to remove the last traces of solvent. When drying was complete, the residue was dissolved in 2 ml of absolute methanol and quantitatively transferred to a 3-ml reaction vial (Supelco, Bellefonte, Pa., U.S.A.) for trimethylsilylation. The methanol was next evaporated from the vial under a stream of dry nitrogen at 25°.

Sample derivatization

The components in the dried residue were derivatized by adding 0.3 ml of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Pierce, Rockford, Ill., U.S.A.) from a glass syringe. The mixture was shaken and placed in a sand-bath at 60° for 5 min. The mixture was then shaken again and allowed to stand in the sand-bath for an additional 5 min, whereupon it was cooled to room temperature. Solid residue precipitated from a supernatant liquid layer that contained the dissolved trimethylsilyl (TMS) derivatives. For combined GC—MS experiments, a second portion of the sample was derivatized with N,O-bis-(perdeuterotrimethylsilyl)acetamide (BSA-d_{18}; Merck Sharp & Dohme, Montreal, Canada). This reaction produced the corresponding TMS-d_9 derivatives which facilitated mass spectral interpretation.

Gas chromatography—mass spectrometry

The TMS derivatives were separated on a Perkin-Elmer Model 900 gas chromatograph, equipped with a 12 ft. × 2 mm I.D. glass column packed with 3.3% SE-30 on 100—200 mesh Gas-Chrom Q. Helium was used as the carrier gas at a flow-rate of 71 ml/min and at a column inlet pressure of 80 p.s.i.g. The instrument was equipped with a glass-lined injection port operated at 265°,

and the eluted components were monitored by means of a flame-ionization detector operated at 270°, and fueled with hydrogen at 20 p.s.i.g. and ultrazero air at 40 p.s.i.g. Samples (1.0—5.0 μl) of the supernatant layer in the reaction vial were injected with a 10-μl syringe. The column was operated isothermally at 100° during the initial 8 min of a run, and was then programmed to 260° at 5°/min. The final temperature was maintained for 10 min. The total time of separation for a typical chromatographic elution was ca. 50 min.

For combined GC—MS studies, an LKB Model 9000 gas chromatograph—mass spectrometer was used. Aliquots (1—5 μl) of the derivatized samples were analyzed using a 10 ft. × 4 mm glass column packed with 3% OV-101 on 80—100 mesh Gas-Chrom P, which was operated as above. Helium was used as a carrier gas at a flow-rate of 30 ml/min. Mass spectrometric conditions included 70 eV ionization potential, 3.5 kV accelerating voltage, 60 μA trap current, and 270° source temperature.

RESULTS

Gas chromatograms were recorded for several blank and used hemodialysate samples for comparison purposes in order to determine the 'background', or control components, in the derivatized commercial hemodialysate concentrate and in the municipal water used for dilution. Gas chromatograms for blank and used hemodialysate samples are shown in Figs. 1 and 2, respectively. It is seen that both yield a number of components eluting between 0 and 10 min, but the patterns are very similar for the first 10 min. Gas chromatograms were also

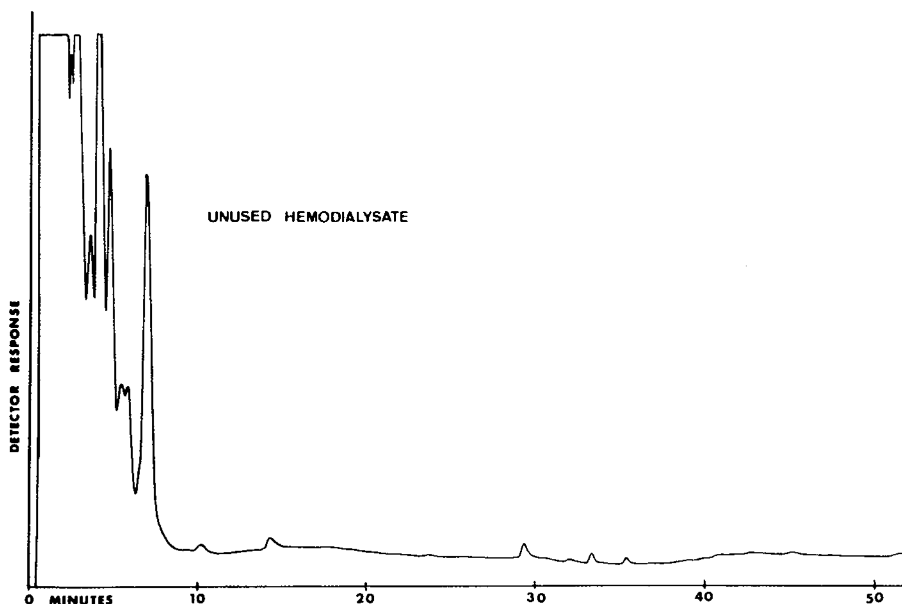


Fig. 1. Gas chromatogram for blank hemodialysate. Sample volume: 1.8 μl. Attenuation: × 80.

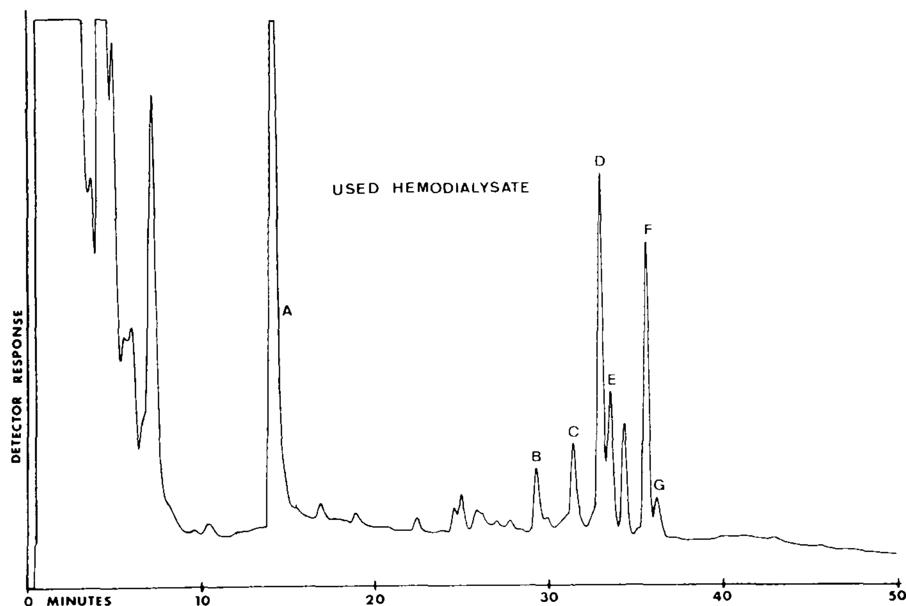


Fig. 2. Gas chromatogram for used hemodialysate derivatized with BSTFA. Sample volume: 2.5 μl. Attenuation: × 80.

recorded for reagent blanks (samples using doubly distilled water in place of hemodialysate); these were essentially identical with the blank hemodialysate samples (Fig. 1); thus no major peaks are due to the reagents used in sample preparation and derivatization. Fig. 2 shows that there are eight major components present in the used hemodialysate sample. It was found that slight variations in peak sizes did occur from run to run for individually processed aliquots of the same used hemodialysate sample; reproducibility of the retention times for the components was acceptable (±3%). The gas chromatogram resulting from a used hemodialysate sample derivatized with BSA-d_{18} is presented in Fig. 3.

Peak identification

Identification of the major components in used hemodialysate was accomplished by use of GC—MS. Mass spectral data were collected for the TMS and TMS-d_9 components, A through G (Fig. 2).

Component A was found to be the tris-TMS derivative of phosphoric acid (MW = 314). The mass spectra obtained for derivatized phosphoric acid were found to be similar to previously published data for the TMS and TMS-d_9 derivatives [12—14]. The MS data for peak A are presented in Table I; they indicate a molecular ion at *m/e* 314 and an intense M − 15 fragment ion. The *m/e* 211 ion is known [12] to be formed by the loss of a TMS group and two methyl radicals from the M^+ ion, followed by rearrangement to produce $(CH_3)_4Si_2PO_4$. Mass spectral data for the deuterium-labeled analogue are also presented in Table I. They are consistent with, and verify, the identity of peak

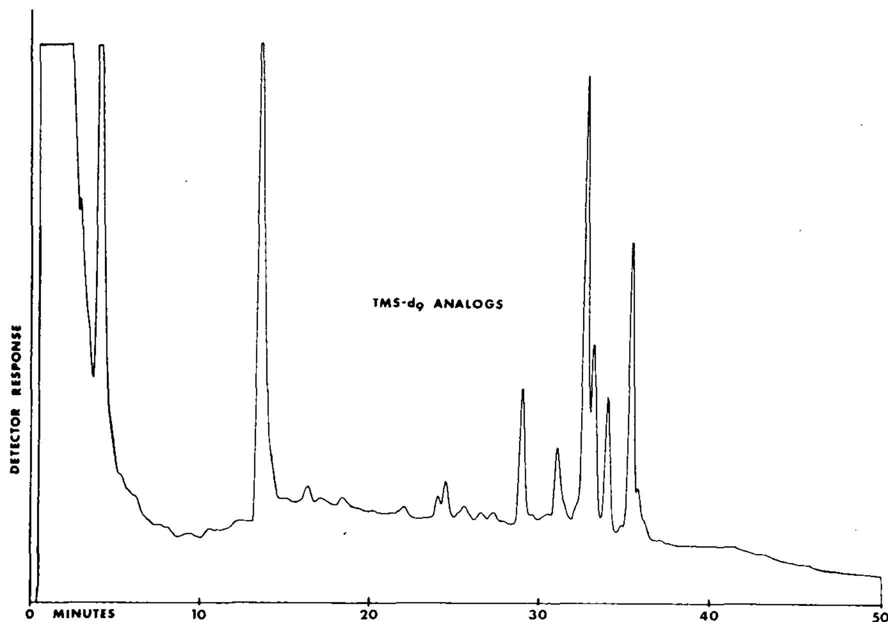


Fig. 3. Gas chromatogram for used hemodialysate derivatized with BSA-d_{18}. Sample volume: 1.4 μl. Attenuation: × 80.

TABLE I

MASS SPECTRAL DATA FOR COMPONENT A

TMS derivative		TMS-d_9 derivative	Mass shift
m/e	Rel. int. (%)	*m/e*	
314, M	23	341, M	27
299	100	323	24
283	5	303	20
211	5	223	12
207	8	222	15
147	7	162	15
133	12	142	9
73	71	82	9

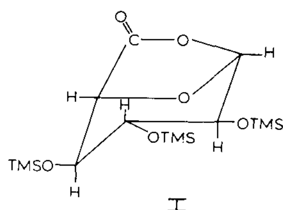
A. For example, the *m/e* TMS-d_9 molecular ion shows a mass change of 27, thus indicating the presence of three silylated positions on the phosphoric acid molecule. The *m/e* 283 ion arises from the loss of a hydrogen atom and two methyl radicals from the molecular ion (M − 31, TMS; M − 38, TMS-d_9). The retention time for derivatized authentic phosphoric acid also agreed exactly with that of component A (Fig. 2).

Component B was identified as the tris-TMS ether of glucopyranurono-(6→1)-lactone (I). The pertinent MS data are presented in Table II.

TABLE II

MASS SPECTRAL DATA FOR COMPONENT B

TMS derivative		TMS-d_9 derivative	Mass shift
m/e	Rel. int. (%)	*m/e*	
392, M	6	419, M	27
377	2	401	24
348	2	375	27
306	7	333	27
305	5	332	27
291	1	315	24
217	47	235	18
204	16	222	18
191	4	209	18
189	3	204	15
147	20	162	15
73	100	82	9



The molecular ion was observed at *m/e* 392, with an M − 15 ion at *m/e* 377. Other important signals for component B occurred at *m/e* 305, 306 and 204. The *m/e* 305 and 306 ions are known to be present in the mass spectrum of the 6→1 lactone and absent in the spectrum of the 6→3 isomer [15, 16]. Also, the intense *m/e* 230 ion found in the 6→3 lactone [16] is absent from the mass spectrum for component B. The molecular ion for the TMS-d_9 derivative appeared at *m/e* 419; this mass shift of 27 *m/e* units with respect to the TMS molecular ion provides additional evidence for the tris-TMS structure. GC of trimethylsilylated acyl glucuronides has been found to result in the elution of the trimethylsilylated 6→1 lactone [15]. Interestingly, it has been reported [16] that the 6→1 lactone is also produced during GC of a trimethylsilylated human urinary metabolite of cyproheptadine (Periactin), a drug administered to the patient who underwent hemodialysis.

Component C was identified from GC and MS data (Table III) as the tetra-TMS derivative of citric acid. Comparison of the molecular ion values for the TMS and TMS-d_9 compounds requires a molecular weight of 192 for the parent compound, suggesting C to be citric acid. The mass spectrum obtained for component C was virtually identical to that published by Dalgliesh et al. [17] for the TMS derivative of citric acid, and the GC retention time for component C was found to be identical to that of a sample of trimethylsilylated reference material.

Mass spectral data for component D are summarized in Table IV. The com-

TABLE III

MASS SPECTRAL DATA FOR COMPONENT C

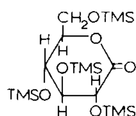
TMS derivative		TMS-d_9 derivative	Mass shift
m/e	Rel. int. (%)	*m/e*	
480, M	0.6	516, M	36
465	19	498	33
375	20	399	24
363	26	390	27
347	17	371	24
333	2	357	24
319	4	343	24
305	6	332	27
285	5	300	15
273	65	291	18
257	3	272	15
245	5	263	18
231	4	249	18
221	7	242	21
217	7	235	18
211	7	220	9
183	3	192	9
147	51	162	15
73	100	82	9

TABLE IV

MASS SPECTRAL DATA FOR COMPONENT D

TMS derivative		TMS-d_9 derivative	Mass shift
m/e	Rel. int. (%)	*m/e*	
466, M	9	502, M	36
451	7	484	33
437	–	473	36
393	2	420	27
376	2	403	27
361	6	385	24
333	11	357	24
319	58	346	27
229	17	247	18
220	18	238	18
217	17	235	18
204	14	222	18
191	7	209	18
189	10	204	15
147	33	162	15
129	19	138	9
73	100	82	9

pound is the tetra-TMS derivative of a species possessing a molecular weight of 178 (466 – 288); a likely candidate is a lactone of a hexonic acid. Petersson et al. [18] have reported characteristic intense ions at *m/e* 220 and 319 for the tetra-TMS derivative of glucono-1,5-lactone (II).



II

The spectrum of component D compares very favorably (with the exception of the ion of *m/e* 229) with that of the 1,5-lactone reported by Petersson et al. [18].

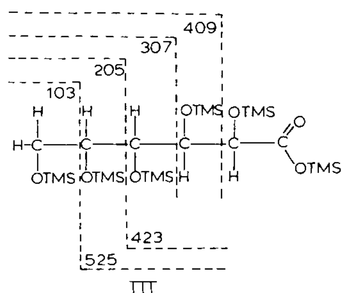
Peaks E and F yielded virtually identical mass spectral data (Table V), indicating isomeric structures. The molecular ion was not detected for either isomer; the M – 15 ion was present but not intense. Loss of trimethylsilanol from *m/e* 525 produced *m/e* 435. Subsequent loss of a second trimethylsilanol produced *m/e* 345. Injection of a sample of trimethylsilyated α- and β-D-glucose produced two major peaks with retention times that were identical to those of peaks E and F (the α-isomer is known to elute prior to the β-isomer [19]). The mass spectra of the unknown and reference standards were identical to those previously reported and described [20].

Compound G (Fig. 2) is tentatively identified as the TMS derivative of mannonic acid. The mass spectral data shown in Table VI compare favorably with those previously published [21]. Unfortunately, the ion of M – 15 (*m/e* 613) was not detected as the spectra were not scanned to >*m/e* 600. The triplet of ions of *m/e* 523, 525, and 538 (TMS) and 565, 570, and 583 (TMS-d_9), however, are strong evidence for this component being a trimethylsilylated hexonic acid. Some of the more important mass spectral peaks can be explained by the fragmentation pattern reported for this compound [21], and shown overleaf (III).

TABLE V

MASS SPECTRAL DATA FOR COMPONENTS E AND F

TMS derivative		TMS-d_9 derivative	Mass shift
m/e	Rel. int. (%)	*m/e*	
(540), M	–	(585), M	45
525	0.2	567	42
435	3	468	33
393	2	426	33
361	2	388	27
345	2	369	24
332	1	359	27
319	2	346	27
305	3	332	27
291	2	315	24
243	2	261	18
231	3	246	15
217	20	235	18
204	100	222	18
191	47	209	18
147	23	162	15
129	7	138	9
117	5	123	6
103	5	112	9
73	66	82	9



Ions such as *m/e* 217, 319, 333, 435, and 523 could be formed by loss of trimethylsilanol from *m/e* 307, 409, 423, 525, and 613, respectively. The shift data shown for the TMS-d_9 derivatives in Table VI are consistent with these observations. As Petersson et al. [21] report that the mass spectrum of the derivatized gluconic acid 'differed only slightly from that of mannonic acid', the identification of G as trimethylsilylated mannonic acid must remain tentative.

DISCUSSION

It has been demonstrated that metabolites in spent hemodialysate fluid obtained from an artificial kidney can be identified and determined by combined GC—MS. Six components have been identified or characterized by combined GC—MS. It was initially expected that these compounds would consist primarily

TABLE VI

MASS SPECTRAL DATA FOR COMPOUND G

TMS derivative			TMS-d_9 derivative *m/e*	Mass shift
m/e	Rel. int. (%)	Rel. int. (%) [21]		
613, M − 15	–	0.5	–	–
538, M − 90	0.2	0.2	583	45
525	0.5	0.5	570	45
523	0.5	0.5	565	42
435	2	3	471	36
433	2	3	466	33
423	5	5	459	36
359	3	4	386	27
333	22	24	360	27
319	12	14	346	27
307	4	5	334	27
305	11	9	332	27
292	21	20	319	27
277	3	1	304	27
217	19	14	235	18
205	18	18	223	18
204	~9	~8		
147	31	27	162	15
103	13	13	112	9
73	100	100	82	9

of acids; however, a number of neutral compounds were detected as well. One of the major components (the peak eluting between E and F, Fig. 2) has thus far not yet been identified; the mass spectral data that were obtained for this peak were inconclusive.

Although combined GC—MS analysis of spent hemodialysate is time-consuming, principally owing to the lengthy method of sample preparation, it provides at present the only way to accomplish the structural studies necessary for conclusive identification of the molecular components associated with uremia. Several of the major components known to be present in hemodialysate, such as uric acid, hippuric acid and xanthine [10] were not observed in this procedure, probably because of the apparent difficulty in derivatizing these compounds with BSTFA on the microgram scale. Efforts are presently underway in these laboratories to utilize GC—MS for the identification of components collected from liquid chromatographic separations of metabolites in used hemodialysate.

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HOCHDRUCK-FLÜSSIGKEITSCHROMATOGRAPHISCHE BESTIMMUNGSMETHODE FÜR FREIES CORTISOL IM URIN

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SUMMARY

Determination of free cortisol in urine by high-pressure liquid chromatography

The possibilities were examined for the high-pressure liquid chromatographic analysis of cortisol with methods of adsorption, distribution and reversed-phase chromatography. Free cortisol in urine can be determined by extraction with chloroform and subsequent adsorption chromatography on silica gel with a mobile phase consisting of 1.5% methanol and 0.2% water in chloroform.

The time needed for this chromatographic analysis is 10—15 min; the limit of determination is 3 ng of cortisol for one injection.

EINLEITUNG

Über die Trennung von Corticosteroiden durch die Hochdruck-Flüssigkeitschromatographie (HPLC) ist bereits eine grössere Zahl von Arbeiten veröffentlicht worden, jedoch nur in wenigen Fällen wird eine quantitative Analyse von Cortisol durchgeführt (Tabelle I).

Für arbeitsphysiologische Untersuchungen zur tagesperiodischen Schwankung der Cortisolausscheidung im Urin war es unser Ziel, eine schnelle quantitative hochdruck-flüssigkeitschromatographische Methode zu entwickeln. Dafür werden in Abständen von 2—3 Stunden Urinproben gesammelt, darin der Gehalt an freiem Cortisol bestimmt und auf die Ausscheidung pro Minute umgerechnet.

Die Abtrennung von Cortisol aus Urin erfolgt durch die Extraktion mit

TABELLE I

HOCHDRUCK-FLÜSSIGKEITSCHROMATOGRAPHIE VON CORTISOL UND ANDEREN CORTICOSTEROIDEN (LITERATURÜBERSICHT)

Trennmaterialien	Mobile Phase	Anwendung	Literatur
Kieselgel (SIL-X, Perkin-Elmer, Überlingen, B.R.D.)	Chloroform—Methanol (100:1) Chloroform—Dioxan (100:5)	Serum (qual.)	1
Kieselgel (10 µm, Varian, Darmstadt, B.R.D.)	Dichlormethan—Äthanol (95:5)	Pharmaceutica	2
Kieselgel (10 µm, Partisil 10, Reeve Angel, Clifton, N.J., U.S.A.)	*n*-Heptan—Äthanol (75:25)	-	3
Kieselgel Vydac-101 SI (30—44 µm, Macherey, Nagel & Co., Düren, B.R.D.)	*n*-Hexan—Chloroform—Methanol (60:38:2)	-	4
Kieselgel (10—15 µm)	ternäres Gemisch: 2,2,4-Trimethylpentan—Äthanol—Wasser	Serum (quant.)	5
Kieselgel (4—8 µm, Pechiney, St. Gobain, Frankreich)	ternäres Gemisch: Dichlormethan—Äthanol—Wasser	Serum (quant.)	6
ODS-Kieselgel (SIL-X(RP), Perkin Elmer)	Methanol—Wasser (40:60)	-	1
ODS-Kieselgel (CO:Pell ODS 41 µm, Reeve Angel)	Methanol—Wasser (50:50)	-	7
Amberlite LA-1 [*n*-Dodecyl-(trialkylmethyl)amin] (Rohm & Haas, Darmstadt, B.R.D.) auf Kieselgelen	Wasser	-	8
1% BOP (β,β'-Oxydipropionitril) auf Kieselgel (25—37 µm, DuPont, Bad Nauheim, B.R.D.)	Tetrahydrofuran—Heptan (20:80)	-	9
1% BOP (β,β'-Oxydipropionitril) auf Kieselgel (25—37 µm, DuPont)	5% Essigsäureäthylester + 0.2% Acetonitril in *n*-Hexan	Pharmaceutica	10
1% ANH (Cyanoäthylsilicon) auf Kieselgel (25—37 µm, DuPont)	1% Methanol in Wasser	Pharmaceutica	11

Chloroform, die organische Phase wird mit Natronlauge und Wasser gewaschen. Dieses Verfahren hat sich in der Analytik der 17-Hydroxycorticosteroide bewährt (siehe z.B. Lit. 12) und sollte zur Anreicherung für die anschliessende HPLC-Analyse eingesetzt werden.

Für die Bestimmung von Cortisol im Serum wurde von Meijers et al. [5] sowie Hesse et al. [6] ein ternäres Gemisch als System für eine Verteilungschromatographie verwendet. Wendet man dieses Verfahren für Urinextrakte an, so erscheinen nach dem Cortisol-Peak noch eine Reihe von Substanzen mit langen Retentionszeiten, die sich bei den nachfolgenden Analysen störend bemerkbar machen. Weitere Reinigungsschritte des Extraktes würden jedoch die Anwendbarkeit der Analysenmethode für die Routine einschränken.

An Kieselgel sind bisher in erster Linie Trennungen aus reinen Lösungen beschrieben worden. Die qualitative Analyse eines Plasma-Extraktes von Touchstone und Wortmann [1] ist nicht befriedigend. Als Mobile Phasen werden Gemische unpolarer Lösungsmittel wie Chloroform oder *n*-Heptan bzw.

n-Hexan mit geringen Prozentgehalten an Methanol oder Äthanol verwendet (Tabelle I).

Auch Trennmaterialien mit chemisch gebundenen Phasen sollten wegen der weniger kritischen Elutionsbedingungen für die Auftrennung von Urinextrakten zur Bestimmung von Cortisol geprüft werden.

EXPERIMENTELLES

Material

HPLC-Geräte: Hochdruckpumpe M-6000 A (Waters Assoc.), Probeninjektionssystem U 6 K (Waters Assoc.), und Zweistrahl-Mehrwellenlängen-UV/VIS Photometer M 440 (Waters Assoc.).

Sonstige Geräte: Schüttelmaschine, Fraktionenschnellverdampfer (Ed. Bühler), 50-ml Schütteltrichter, Spitzgläser (Höhe 9.5 cm, I.D. 2.3 cm, Fassungsvermögen ca. 30 ml), Faltenfilter (Durchmesser 9 cm, Selecta), und Injektionsspritze Pressure Lok, Series B-110 (Precision Sampling, Baton Rouge, La., U.S.A.).

Chemikalien: Chloroform p.a. (Merck, Darmstadt, B.R.D.) (Wassergehalt unter 0.01%), und Natriumhydroxid p.a. (Merck).

Analysenvorschrift

Vier ml Urin werden auf pH 6.0 eingestellt und mit bidestilliertem Wasser auf 10 ml verdünnt. Je 5 ml dieser Lösung werden in einem 50-ml Schütteltrichter mit je 10 ml Chloroform 3 min auf der Schüttelmaschine geschüttelt. Die wässrige Phase wird verworfen, die organische Phase dreimal mit je 2 ml 0.1 *N* Natriumhydroxid, dann dreimal mit je 2 ml bidestilliertem Wasser gewaschen. Die vollständige Trennung der Phasen muss jeweils abgewartet werden.

Nach dem Waschen wird die Chloroform-Phase über ein trockenes Faltenfilter in ein Spitzglas abgelassen, das Filter zweimal mit je 2 ml Chloroform nachgewaschen. Am Fraktionenschnellverdampfer wird das Lösungsmittel bis zur Trockene ohne Heizung verblasen. Die Innenwände des Glases wäscht man nach dem Eindampfen mit 1 ml Chloroform und verbläst nochmals bis zur Trockene. Danach werden die Spitzgläser in Eiswasser gestellt und der Rückstand in 200 μl Chloroform gelöst.

Zur HPLC-Analyse an Kieselgel (Bedingungen siehe Fig. 1) werden 25 μl in das Probeninjektionssystem gespritzt. Bereits nach maximal 15 min kann die nächste Bestimmung durchgeführt werden. Bei geringen Cortisolgehalten (z.B. Mittagsurin oder grossen Urinvolumina in kurzen Zeitintervallen) können je Analyse ohne Schwierigkeiten grössere Urinmengen unter Berücksichtigung der Volumenverhältnisse bei der Extraktion eingesetzt werden. Auch lässt sich das Einspritzvolumen für die HPLC-Analyse erhöhen. Die quantitative Auswertung der Chromatogramme erfolgt über die Peakhöhe.

ERGEBNISSE UND DISKUSSION

Die Anwendung der Adsorptionschromatographie (siehe Tabelle I) auf Urinextrakte zeigte, dass mit Materialien von 5 μm Teilchengrösse (porös) eine gute Abtrennung des Cortisols von störenden Substanzen erzielt wird (Fig. 1).

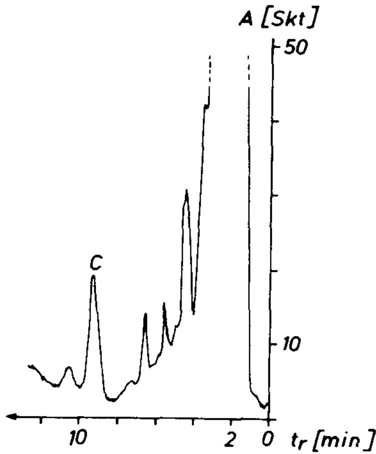

Fig. 1. Adsorptionschromatographische Analyse von Cortisol (C) in Urinextrakten. Säule: Fertigsäule Zorbax-Sil, 25 cm × 2.1 mm I.D., gefüllt mit Kieselgel Zorbax-Sil, polar (5—7 μm); Druck: 3500 p.s.i.; Temperatur: 22°; mobile Phase: Chloroform—Methanol—Wasser (98.3:1.5:0.2); Durchflussgeschwindigkeit: 0.6 ml/min; Probenmenge: 25 μl (entsprechend Extrakt aus 0.25 ml Urin); Detektor: Zweistrahl-Mehrwellenlängen-UV/VIS-Photometer M 440, 254 nm bei 0.02 a.u.f.s.

TABELLE II

VERWENDETE TRENNMATERIALIEN FÜR DIE HOCHDRUCK-FLÜSSIGKEITS-CHROMATOGRAPHIE VON CORTISOL

Trennmaterial (mittlere Korngrösse)	Mobile Phasen	Ergebnis
Kieselgele		
Nucleosil 50-5 (5 μm) (Macherey, Nagel & Co.)	1—5% Methanol in Chloroform	quantitative Analyse möglich
Zorbax-Sil (5—7 μm) (DuPont)	1—5% Methanol in Chloroform	quantitative Analyse möglich
Vydac-101 SI (30—44 μm) (Macherey, Nagel & Co.)	1—5% Methanol in Chloroform	zu geringe Bödenzahlen, keine Abtrennung des Cortisols in Urinextrakten
Perisorb A (30—40 μm) (Merck)	1—5% Methanol in Chloroform	
Chemische-gebundene Phasen		
Nucleosil 10 C_{18} (10 μm) (Macherey, Nagel & Co.)	"reversed-phase": Methanol—Wasser, bis zu 60% Wasser	Trennung in Urinextrakten möglich
Perisorb RP (30—40 μm) (Merck)	"reversed-phase": Methanol—Wasser, bis zu 60% Wasser	keine Abtrennung des Cortisols in Urinextrakten
Nucleosil 10 CN (10 μm) (Macherey, Nagel & Co.)	Methanol bzw. Iso-propanol—Chloroform	
	"reversed-phase": Methanol—Wasser, bis zu 90% Wasser	Trennung in Urinextrakten möglich

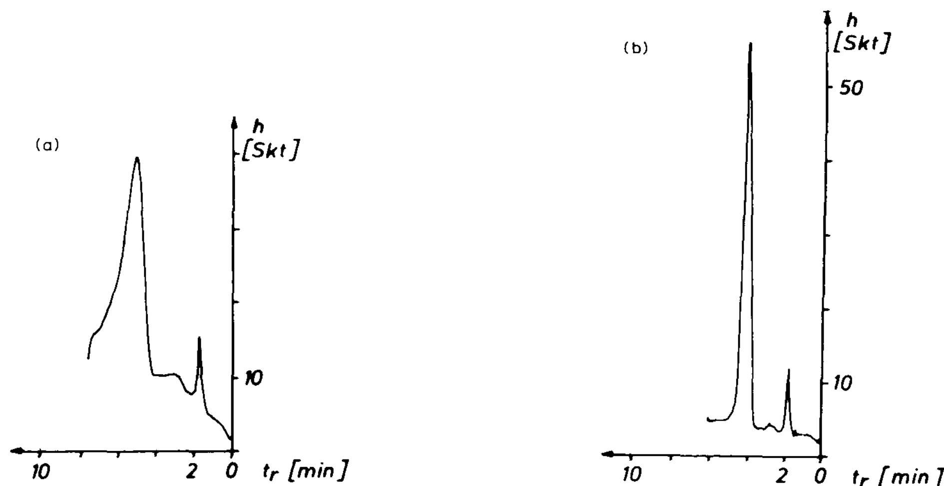

Fig. 2. Einfluss des Wassergehaltes der mobilen Phase auf die Form des Cortisol-Peaks bei der Adsorptionschromatographie, Säule: Fertigsäule Nucleosil 50-5, 20 cm × 4 mm I.D., gefüllt mit Kieselgel Nucleosil 50-5 (5 ± 1.5 μm); Druck: 1000 p.s.i.; Temperatur: 22°; mobile Phase: (a) Chloroform (über Aluminiumoxid getrocknet)—Methanol (98:2); (b) Chloroform—Methanol—Wasser (97.8:2:0.2); Durchflussgeschwindigkeit: 0.8 ml/min; Probenmenge: 25 μl (entsprechend 100 ng Cortisol); Detektor: wie Fig. 1, bei 0.02 a.u.f.s.

Kieselgele wie Vydac und Perisorb (30—44 μm, "Dünnschicht-Teilchen") lassen sich wegen der geringen Bödenzahlen nicht einsetzen (Tabelle II). Gut reproduzierbare Ergebnisse an Kieselgel sind jedoch nur dann zu erhalten, wenn der Wassergehalt in der mobilen Phase genau eingehalten wird. Werden wasserfreie Lösungsmittel (Trocknung über Aluminiumoxid) eingesetzt, so wird ausser einer geringen Bödenzahl ein Tailing des Cortisol-Peaks beobachtet (Fig. 2a). Durch den Zusatz von 0.2% Wasser zur mobilen Phase wird die Peakform für Cortisol günstig verändert (Fig. 2b) (siehe auch Lit. 13) und eine Trennung von anderen Substanzen erst möglich.

An Nucleosil-CN ist mit der Verteilungschromatographie (Methanol oder Isopropanol in Chloroform) keine Abtrennung des Cortisols von anderen Substanzen in den Urinextrakten möglich. Mit der "reversed-phase"-Chromatographie (Methanol in Wasser) gelingt dies sehr gut. Ebenso geeignet ist Nucleosil 10 C_{18} (ODS) mit Gemischen aus Methanol und Wasser als mobile Phasen (Fig. 3). Eine Übersicht über die verwendeten Trennmaterialien und Trennsäulen und deren Eignung für die Analyse von Cortisol in Urinextrakten gibt Tabelle II.

Es wurden verschiedene Urinproben mit der Adsorptions- und der "reversed-phase"-Chromatographie (Bedingungen siehe Fig. 1 und 3) quantitativ analysiert. Tabelle III zeigt die Ergebnisse dieser Bestimmungen. Bis auf eine Probe werden in allen Fällen mit der "reversed-phase"-Chromatographie niedrigere Werte erhalten. Diese unterschiedlichen Ergebnisse sind wahrscheinlich auf die schlechte Löslichkeit des Urinextraktes in Wasser—Methanol-Gemischen zurückzuführen.

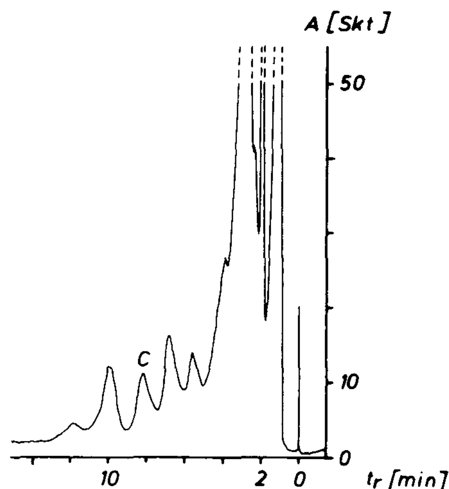

Fig. 3. "Reversed-phase"-chromatographische Analyse von Cortisol (C) in Urinextrakten. Säule: Fertigsäule Nucleosil 10 C_{18}, 20 cm × 4 mm I.D., gefüllt mit Nucleosil 10 C_{18} (= Octadecylgruppen) (10 ± 1.5 μm); Druck: 1000 p.s.i.; Temperatur: 22°; mobile Phase: Methanol—Wasser (55:45); Probenmenge: wie Fig. 1; Detektor: wie Fig. 1, bei 0.01 a.u.f.s.

TABELLE III

ERGEBNISSE DER BESTIMMUNGEN VON FREIEM CORTISOL IM URIN NACH VERSCHIEDENEN METHODEN (ng/ml)

Die HPLC-Werte sind auf eine Wiederfindung von 100% korrigiert.

No. des Urins	"Reversed-phase"-HPLC	Adsorptions-HPLC	Proteinbindung
1	8.0	8.7	30.3
2	21.5	51.3	68.3
3	23.3	42.3	42.4
4	17.4	56.3	52.6
5	37.4	44.7	43.1

TABELLE IV

ERGEBNISSE DER HPLC-ANALYSE VON FREIEM CORTISOL IM URIN

Bestimmung (N=5)	$\bar{X}$ ± S.D. (ng/ml)	Wiederfindung (%)
Ohne Zusatz	19.8 ± 1.5	—
Zusatz 100 ng/ml Urin	92.4 ± 3.9	72.6
Bestimmungsgrenze		
(6-fache Höhe des Rauschpegels)	3 ng (je Einspritzung)	

Trockene Extrakte wurden mit einem Zusatz an Cortisol zuerst in Chloroform gelöst, wieder eingedampft und anschliessend in Wasser—Methanol (45:55) gelöst. Auch hier wurde das zugesetzte Cortisol (je 100 ng) nicht vollständig wiedergefunden (Verluste zwischen 20 und 50%). Es ist daher zu vermuten, dass Urininhaltsstoffe in den Chloroformextrakten beim Lösen mit Wasser—Methanol-Gemischen als eine Art von Spurenfänger in bezug auf das Cortisol wirken. Lösungen der Extrakte in Chloroform können bei der

"reversed-phase"-Chromatographie nicht verwendet werden, da Störungen der mobilen Phase zu beobachten sind.

Der Vergleich der Ergebnisse aus der adsorptionschromatographischen Bestimmung mit den Werten, die nach der Proteinbindungsmethode durchgeführt wurden [14], zeigt eine befriedigende Übereinstimmung bis auf Urin No. 1. Diese Probe wies flockige Fällungen auf und wurde für die HPLC-Analyse filtriert. Als innerer Standard kann wie bei den Serumanalysen nach Hesse et al. [6] Prednisolon zugesetzt werden, das im Chromatogramm nach dem Cortisol auftritt. Da jedoch häufig an dieser Stelle ebenfalls ein Peak aus den Urinextrakten erscheint, wird bei Doppelbestimmungen nur einer Probe Prednisolon zugesetzt, um eine mögliche Störung bei der quantitativen Auswertung berücksichtigen zu können.

Die Ergebnisse der beschriebenen HPLC-Analyse von freiem Cortisol im Urin sind in Tabelle IV zusammengestellt. Die niedrige Wiederfindung ist auf die einmalige Extraktion bei einem Volumenverhältnis von nur 1:2 (Urin zu Chloroform) zurückzuführen. Eine Erhöhung der Wiederfindung durch mehrmalige Extraktion oder ein günstigeres Volumenverhältnis erscheint wegen des grösseren Zeit- bzw. Arbeitsaufwandes beim Extrahieren und Eindampfen nicht sinnvoll.

DANK

Wir danken Herrn Priv. Doz. Dr. Dr. H. Wisser, Leiter der Abteilung für klinische Chemie des Robert-Bosch-Krankenhauses, Stuttgart, für die Durchführung der Cortisol-Analysen nach der Proteinbindungsmethode und die Fa. Macherey, Nagel & Co., Düren, für die Fertigsäule Nucleosil 10 CN (10 μm).

ZUSAMMENFASSUNG

Es wurden die Möglichkeiten der hochdruck-flüssigkeitschromatographischen Analyse von Cortisol mit Methoden der Adsorptions-, Verteilungs- und "reversed-phase"-Chromatographie untersucht. Die quantitative Analyse von freiem Cortisol aus Urin in Chloroformextrakten ist adsorptionschromatographisch an Kieselgel mit einer mobilen Phase aus 1.5% Methanol und 0.2% Wasser in Chloroform durchführbar. Die Zeit für eine chromatographische Analyse beträgt 10—15 min; die Bestimmungsgrenze liegt bei 3 ng Cortisol je Einspritzung.

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CHROMBIO. 028

IMPROVED METHOD FOR THE ANALYSIS OF ESTROGENIC STEROIDS IN PREGNANCY URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The use of microparticulate packing materials and large injection volumes gives significant improvements in the analysis of complex samples, such as urine extracts by high-performance liquid chromatography. Lower detection limits and improved accuracy can now be attained. In addition, the combined use of adsorption and reversed-phase chromatography leads to reduced uncertainty in peak identification and gives more reliable quantification.

INTRODUCTION

Large quantities of estrogens, particularly estriol, are produced in the female body during the later months of pregnancy, and are eventually excreted in the urine mainly in the form of sulphate and glucuronide metabolites. We have previously described [1] a method for the analysis of these compounds in urine using high-performance liquid chromatography (HPLC). While demonstrating the potential of the technique, the separation was inadequate to allow accurate quantification. The availability of small-diameter porous packing materials and efficient slurry packing techniques [2] has since led to significant improvements in resolution. In addition, it has been suggested [3, 4] that use of large injection volumes can give improved sensitivity with little degradation of column performance. These factors should have important consequences, particularly in the field of body fluid analysis, where extracts are both dilute and subject to complex interferences.

Recent publications [5—7] have described the application of HPLC to the analysis of a variety of constituents in urine, and Trefz et al. [8] have analysed

human plasma for cortisol and related compounds. Synthetic mixtures of estrogens have been separated on chemically bonded stationary phases in an isocratic system by Butterfield et al. [9] and using gradient elution by Majors and Hopper [10]. However, the determination of estrogens in urine extracts has normally been performed using other techniques, such as thin-layer chromatography with spectrodensitometry [11], column chromatography followed by combined gas chromatography—mass spectrometry [12], and gel chromatography [13, 14].

This communication describes the application of improved column and sampling technology to the analysis of estrogens in pregnancy urine. Samples were analysed by both liquid—solid adsorption chromatography (LSAC) and reversed-phase chromatography (RPC) and we will demonstrate that this combination provides confirmatory information.

EXPERIMENTAL

A 40-ml sample of urine was hydrolyzed with concentrated hydrochloric acid, and the estrogens were extracted into diethyl ether as described previously [1]. The extract was reduced to 1 ml and analysed by LSAC on porous irregular chips of silica gel (Partisil-5, Whatman, Maidstone, Great Britain) of 7-μm mean particle diameter, packed in a 150 mm $\times$ 4.9 mm I.D. stainless-steel tube using a balanced density packing technique [2]. The mobile phase used with this column was 5% (v/v) ethanol in *n*-heptane.

A second extract was evaporated to dryness in a stream of dry nitrogen, the residue was redissolved in 1 ml of a 55:45 (v/v) mixture of methanol and 0.1% ammonium carbonate in water and analysed by RPC. This was performed on a column of Partisil-10 ODS (Whatman) which was purchased pre-packed in a 250 mm $\times$ 4.6 mm I.D. stainless-steel tube. This packing material consists of an octadecylsilane surface layer chemically bonded to 10-μm silica particles via Si—O—Si bonds. A 55:45 (v/v) mixture of methanol and 0.1% ammonium carbonate in water was used as mobile phase.

In both LSAC and RPC, mobile phase was delivered by a reciprocating piston pump and associated pulse damping equipment (Pye Unicam, Model 20LC chromatograph), and the components were detected using a UV detector (Cecil, Model CE212), operating at 280 nm. Samples were injected by means of a 75-μl loop valve (Valco, Model CV-6-HPA).

Synthetic mixtures were prepared using the pure estrogens purchased from BDH (Poole, Great Britain). The solvents were of 'AnalaR' grade and were variously supplied by BDH and Hopkin & Williams (Chadwell Heath, Great Britain). Deionised water was used in all cases.

RESULTS

Liquid—solid adsorption chromatography

The system was calibrated using standard solutions of the estrogens in diethyl ether and a typical chromatogram is shown in Fig. 1. The solvent peak was used for the calculation of capacity factors, which, together with the

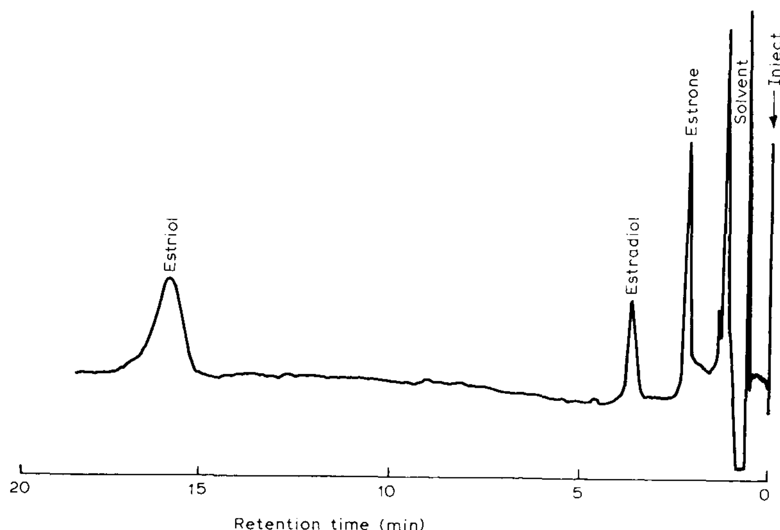


Fig. 1. Separation of estrogen mixture on Partisil-5. Mobile phase, 5% (v/v) ethanol in *n*-hexane; flow-rate, 5 ml/min; detector, 280 nm; range, 0.1 absorbance unit.

respective calibration factors, are listed in Table I. As mobile-phase flow-rate and retention times were constant, it was convenient to quantify peaks in terms of peak height, and calibration factors were defined as:

$$\text{Calibration factor} = \frac{\text{Peak height (fraction of f.s.d.)} \times \text{attenuation (absorbance units)}}{\text{Amount of solute injected (mg)}}$$

A chromatogram of urine extract is shown in Fig. 2, with the peaks having capacity factors corresponding to estrone, estradiol and estriol indicated. While those peaks corresponding to estradiol and estriol are satisfactorily resolved, it is impossible to positively identify a peak for estrone. Confirmation by another method is clearly required.

In order to quantify the peaks for estradiol and estriol in the urine extract, it was necessary to determine the efficiency of extraction. The extraction was performed in the manner described, using 1.0 mg of estriol dissolved in 50 ml of male urine, and the chromatogram (Fig. 3) was compared with a blank

TABLE I

CALIBRATION AND CAPACITY FACTORS ON PARTISIL-5 SILICA GEL

Compound	Capacity factor (k')	Calibration factor (absorbance units/mg)
Estrone	2.7	17.5
Estradiol	5.3	15.4
Estriol	26.4	2.8

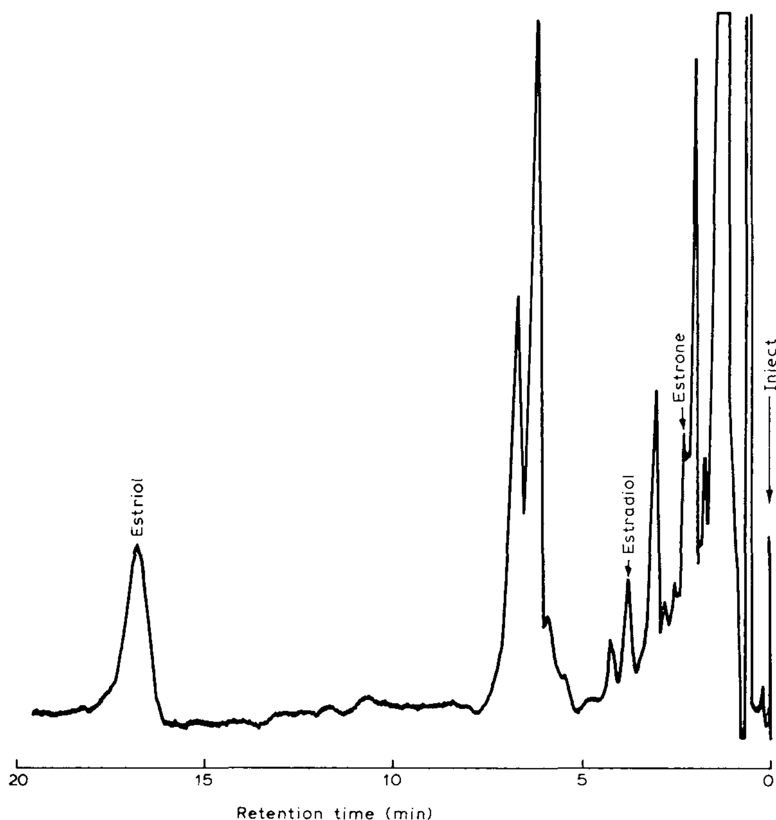


Fig. 2. Chromatogram of an extract of pregnancy urine on Partisil-5. Conditions as for Fig. 1.

obtained by extracting an 'unspiked' 50-ml sample of the same male urine. The height of the peak for estriol was measured and the extraction efficiency was determined as 27%.

The concentrations of each estrogen in the pregnancy urine were determined using the extraction efficiency, calibration factors and the volumes of extract (1 ml) and urine (40 ml). Six extractions were performed and the mean values and standard deviations are given in Table III.

Reversed-phase chromatography

The reversed-phase system was calibrated using standard solutions of estrogens in a methanol—0.1% ammonium carbonate in water (55:45, v/v) mixture. A typical chromatogram is shown in Fig. 4 and the corresponding capacity and calibration factors are given in Table II.

A chromatogram of urine extract on Partisil ODS is shown in Fig. 5, with the peaks having capacity factors corresponding to those of the estrogens indicated. In this case, all of the estrogens are adequately resolved for quantification. The extraction efficiency was not separately determined for the samples for RPC,

TABLE II

CALIBRATION AND CAPACITY FACTORS ON PARTISIL ODS

Compound	Capacity factor (k')	Calibration factor (absorbance units/mg)
Estrone	4.0	5.7
Estradiol	4.9	5.8
Estriol	1.9	20.0

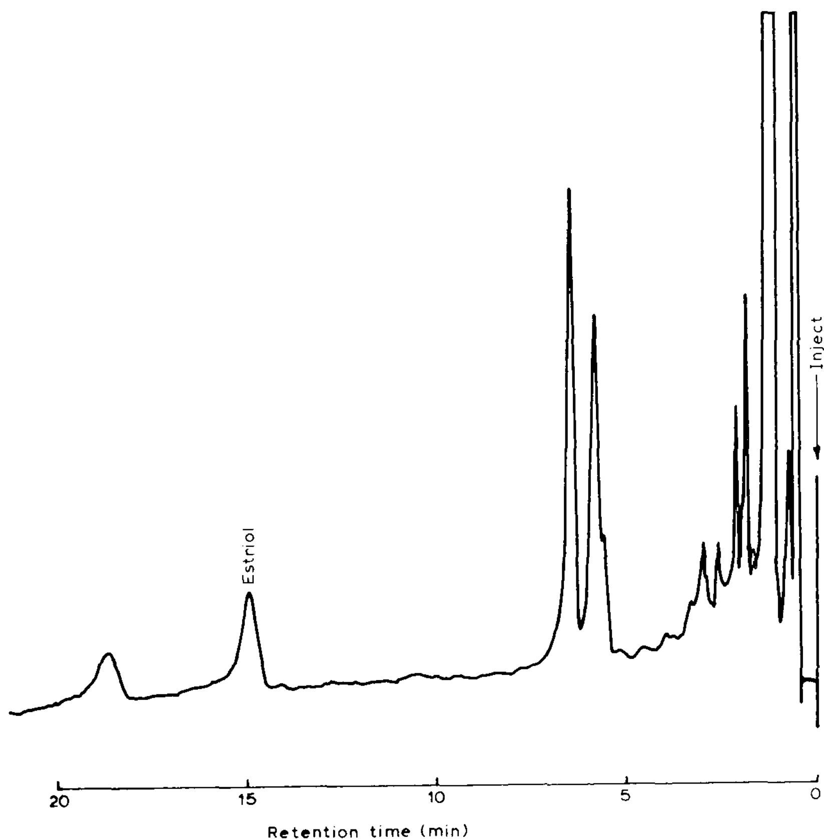
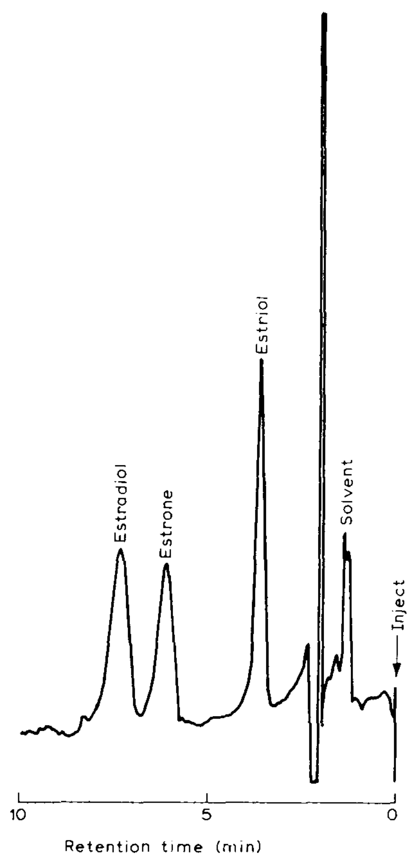


Fig. 3. Chromatogram of an extract of male urine, 'spiked' with estriol, on Partisil-5. Conditions as for Fig. 1.

but it was assumed that no loss of estrogens had been incurred in evaporating the extract to dryness and redissolving in methanol—ammonium carbonate solution. The previously measured extraction efficiency was then used to calculate the concentration of each estrogen in the pregnancy urine and the mean values obtained after analysing six extracts are given in Table III.



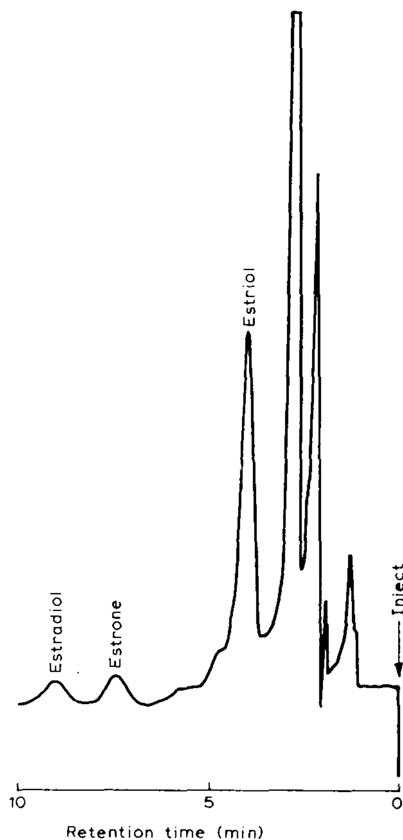


Fig. 4. Separation of estrogen mixture on Partisil-10 ODS. Mobile phase, methanol—0.1% aqueous ammonium carbonate (55:45, flow-rate 2 ml/min; detector range, 0.25 absorbance units.

Fig. 5. Chromatogram of an extract of pregnancy urine on Partisil-10 ODS. Conditions as for Fig. 4.

TABLE III

CALCULATED ESTROGEN LEVELS IN THE PREGNANCY URINE WITH RELATIVE STANDARD DEVIATIONS (RSD)

Compound	Concentration in the urine (g/l)	
	LSAC	RPC
Estrone	—	$7.3 \cdot 10^{-3}$ (RSD 18%)
Estradiol	$1.8 \cdot 10^{-3}$ (RSD 18%)	$2.5 \cdot 10^{-3}$ (RSD 18%)
Estriol	$33 \cdot 10^{-3}$ (RSD 15%)	$30 \cdot 10^{-3}$ (RSD 9%)

DISCUSSION

The results obtained from the two chromatographic systems show good agreement. Although quantification of the estrone peak on Partisil-5 was not possible, the well resolved peak obtained on Partisil ODS could readily be measured.

As would be expected, the concentration of estriol is considerably greater than that of either estrone or estradiol, and the figures are consistent with the levels normally obtained in the later months of pregnancy.

We have not attempted a rigorous determination of the extraction efficiency, but an indication of the precision is included with the results in Table III (standard deviations less than 20%).

The extraction efficiency of 27% is considerably lower than would have been anticipated from previous work [15] and merits further comment. A shorter extraction procedure was investigated, wherein a sample of 'spiked' urine was acid-hydrolysed and extracted into three 50 ml aliquots of diethyl ether which were combined and reduced in volume to 1 ml. The efficiency of this extraction was greater than 90%, indicating that considerable amounts of estriol must be removed in the usual neutralisation stages, but without neutralisation acidic co-extractants degraded the resolution and interfered in the determination of estrone and estradiol.

Huber et al. [15] emphasised the importance of reducing the pH of the neutralising sodium hydroxide solution to 10 as otherwise losses of up to 60% (w/w) of the extrogens were observed. Although care was taken over this point, our extraction efficiency was still low, and the extraction procedure could merit further investigation.

A comparison of the chromatograms in the present paper with those included in our previous publication [1] indicates the greatly enhanced resolution that can be achieved with microparticulate packing materials. This leads to important advantages in the ease and accuracy of quantification. The resolution is not significantly degraded by the use of large injection volumes (75 $\mu\ell$), which in turn lead to useful improvements in signal-to-noise ratios of the detected peaks. Such improvements would allow the quantification of much less concentrated estrogen solutions, as would be obtained when monitoring the urine in the earlier months of pregnancy.

HPLC can therefore offer the two important advantages of reduced analysis time and greater sensitivity compared with the more conventional column chromatography using Sephadex LH-20 [13, 14].

CONCLUSIONS

Advances in column packing material and techniques over the last three years lead to significant improvements in the analysis of complex samples such as urine extracts. Microparticulate adsorbents also allow the use of large sample volumes, without significant loss in efficiency, offering lower detection limits and improved accuracy.

The combined use of LSAC and RPC has led to reduced uncertainty in peak

identification and gives more reliable quantification. If only estriol is to be determined, LSAC alone would prove adequate. However, when it is necessary to quantify all three estrogens, RPC yields the more useful information. A disadvantage of RPC arises from the higher viscosity of the mobile phase, which results in a three-fold increase in pressure for a given flow-rate. This could mean that reduced flow-rates must be used in RPC because of the pressure limitations of the equipment. However, even at a lower flow-rate, the retention times are often lower with RPC than with LSAC.

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CHROMBIO. 031

ANALYSIS OF PREDNISOLONE IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A sensitive, specific high-performance liquid chromatographic procedure for the determination of prednisolone in plasma is described. The organic solvent extract from plasma is chromatographed on a silica gel column using a mobile phase of 0.2% glacial acetic acid, 6% ethanol, 30% methylene chloride in *n*-hexane on a high-performance liquid chromatograph fitted with an ultraviolet detector (254 nm). Quantitation of plasma samples containing 25 ng/ml prednisolone is reported. Metabolites and endogenous hydrocortisone do not interfere with prednisolone. The determination of prednisolone concentrations in plasma following administration of a 10-mg single oral dose to a human subject is described.

INTRODUCTION

At present, the analytical procedures for the determination of prednisolone in plasma samples (obtained during bioavailability studies) are based on either radio-immunological or competitive protein binding principles [1,2]. Qualitative [3] and quantitative [4] high-performance liquid chromatographic (HPLC) procedures for the determination of corticosteroids have been reported. The procedure reported by Trefz et al. [4] for the determination of hydrocortisone in plasma uses prednisolone as the internal standard. Although no attempt is made to do so, the method appears to be sensitive enough to allow also the quantitation of prednisolone. However, the long retention time and comparatively complex extraction procedure are not attractive for use in a bioavailability trial involving several thousand samples.

This report describes an HPLC method that is sufficiently sensitive and specific for the determination of plasma samples containing 25 ng/ml of prednisolone.

*To whom enquiries should be directed.

EXPERIMENTAL

Materials

Prednisolone (U.S.P. reference) was used for the preparation of standard solutions. 20β-Dihydrocortisone (Sigma, St. Louis, Mo., U.S.A.) was used as the internal standard. Solvents used for the mobile phase were glacial acetic acid (Mallinckrodt Canada, Montreal, Canada), methylene chloride (Spectrograde, Caledon Labs., Georgetown, Canada) and "UV"-grade *n*-hexane (Burdick & Jackson Labs., Muskegon, Mich., U.S.A.).

Anhydrous diethyl ether was freshly distilled prior to use. Prednisolone [6,7-^{3}H (nominal)] with a specific activity of 40 Ci/mmole (New England Nuclear, Montreal, Canada) was used in the extraction study.

Chromatographic procedure

A constant-volume high-performance liquid chromatograph (Waters Assoc., Milford, Mass., U.S.A.) containing a Model 6000A pump, U6K injector and Model 440 detector at 254 nm was used at an attenuation of 0.005 absorbance units full scale (a.u.f.s.). The column (250 × 3.2 mm I.D., 316 stainless steel) was packed with nominal 5-μm silica gel (LiChrosorb SI 60, Brinkman, Westbury, N.Y., U.S.A., manufactured by E. Merck, Darmstadt, G.F.R.) using a balanced density slurry packing procedure similar to that described by Majors [5]. The mobile phase, consisting of 0.2% glacial acetic acid, 6% ethanol, 30% methylene chloride in *n*-hexane (v/v), was prepared fresh daily. A flow-rate of 120 ml/h (2000 p.s.i.) was used.

Preparation of standard solutions

Prednisolone, accurately weighed, was dissolved in redistilled ethanol in a 1.0-ml volumetric flask. An aliquot of this solution was diluted with ethanol to produce a final solution of the desired concentration. Spiked plasma solutions (25—200 ng/ml) were prepared by addition of various volumes of standard prednisolone solution using a 10-μl syringe (Hamilton, Reno, Nev., U.S.A.).

The internal standard 20β-dihydrocortisone was prepared by a similar procedure.

General procedure

A 2.0-ml aliquot of plasma sample was added to a 15-ml Corex tube (Ingram & Bell, Don Mills, Canada) along with 2 μl (1 mg/ml) of internal standard solution and 6.0 ml of ether—methylene chloride (60:40). Parafilm was used to seal the tube which was then shaken for 15 min in an Evapo-Mix Constant shaker (Buchler, Fort Lee, N.J., U.S.A.) and centrifuged (10 min) at 7000 *g* (Sorvall Model RC2-B). A 5.0-ml aliquot of the organic phase was transferred to a 15-ml Corex tube containing 1.0 ml of 0.1 *N* aqueous hydrochloric acid, the tube shaken, and centrifuged as described previously. A 4.0-ml aliquot of the organic phase was transferred to a 5-ml conical tube and evaporated under nitrogen in a constant-temperature (55°) bath. The residue was immediately reconstituted with 200 μl of mobile phase and stored −20° prior to analysis. A 100-μl aliquot of the sample solution was chromatographed.

Calculations

The concentration of prednisolone in the plasma sample was determined from the following expression:

$$C_p = \frac{R}{m}$$

where C_p = concentration of the drug in plasma (ng/ml);
R = peak height ratio (drug/internal standard);
m = slope of the calibration curve.

Radioactive recovery experiment

[^{3}H]Prednisolone (62,395 dpm) was added to 2.0 ml of plasma containing either 25 ng/ml or 100 ng/ml of prednisolone and was extracted using the procedure described. The organic extract was transferred into a scintillation vial and evaporated to dryness. Ten milliliters of cocktail (BBS-3; Beckman, Fullerton, Calif., U.S.A.) were added and the radioactivity was determined by a Beckman L.S. 150 scintillation counter equipped with an automatic quench correction devise.

RESULTS AND DISCUSSION

The reproducibility and efficiency of the extraction procedure was determined using [^{3}H]prednisolone. Results, summarized in Table I, show that the extraction efficiency and reproducibility are comparable at plasma concentrations of 25 ng/ml and 100 ng/ml.

Fig. 1 shows the chromatogram obtained following the injection of a mixture of prednisolone, prednisone, dexamethasone, hydrocortisone and the internal standard. Note that prednisolone is well separated from its metabolite prednisone and endogenous hydrocortisone. A second metabolite of prednisolone, 20β-dihydroprednisolone [6—10] has a retention time greater than 20 min.

Fig. 2a shows the chromatogram obtained from the analysis of a spiked plasma sample containing 50 ng/ml of prednisolone using the system described above. This represents an injection of 16 ng of prednisolone. Note that in order to achieve this sensitivity, an attenuation of 0.005 a.u.f.s. is required. The peak to peak noise level at this attenuation was less than 1% of full-scale deflection.

Fig. 2b shows a chromatogram from pooled blank plasma obtained from 4 subjects. No interfering compounds were extracted from plasma using the described procedure.

TABLE I

EXTRACTION RECOVERIES OF [^{3}H]PREDNISOLONE FROM PLASMA

Concentration (ng/ml)	Recovery (%)	Coefficient of variation (%) (n = 4)
25	76.1	1.6
100	74.6	1.5

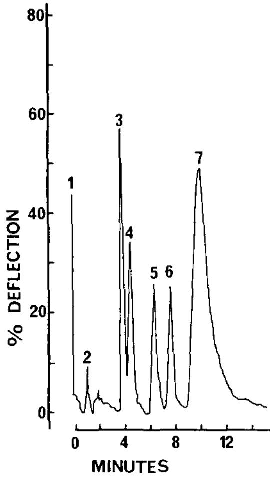

Fig. 1. High-performance liquid chromatogram showing separation of injected standards (qualitative). 1 = Injection; 2 = solvent front; 3 = prednisone; 4 = dexamethasone; 5 = hydrocortisone; 6 = prednisolone; 7 = internal standard.

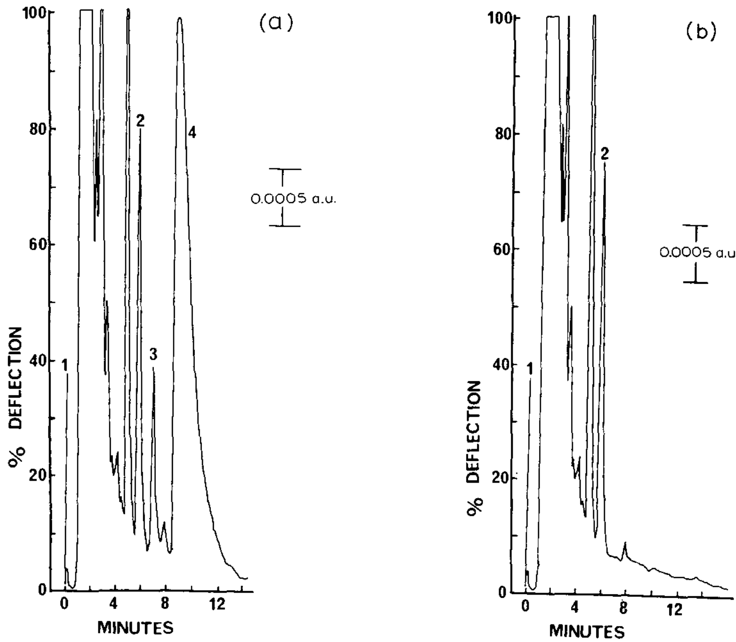

Fig. 2. High performance liquid chromatograms of (a) extracted human plasma spiked with prednisolone (50 ng/ml) and (b) human blank plasma extract. 1 = Injection; 2 = hydrocortisone; 3 = prednisolone; 4 = internal standard.

It must be emphasized that the resolution of prednisolone from endogenous substances is critically dependent on the percentage of ethanol used. The optimal percentage of ethanol necessary for a particular column was determined empirically. The mobile phase was always freshly prepared. Another problem encountered was that prednisolone extracted from plasma decomposed when stored overnight in the dried state. This problem was resolved by storing the extract in the mobile phase at −20°. Under this condition, the samples could be stored for several days without significant decomposition.

A calibration curve was obtained by plotting the peak height ratio (prednisolone/internal standard) versus the concentration of prednisolone in spiked plasma. The plot is linear and passes through the origin ($y = mx$) over the concentration range of 25 ng/ml to 200 ng/ml. The slope value is 0.0085 and its upper and lower 95% confidence limits are 0.0087 and 0.0083, respectively. The regression coefficient (R^2) is 0.999.

Three additional complete calibration curves, were constructed and the slopes were within the 95% confidence limit of the one reported. The slopes were also determined several times from the means of 4 spiked plasma standards (100 ng/ml) and these were also within the confidence limits reported.

Table II lists the results obtained from the analysis of a number of spiked plasma samples (25 ng/ml). These data reflect the accuracy and precision of the method. The mean recovery and the coefficient of variation were 98.6% and 3.9%, respectively.

The plasma prednisolone profile of a human subject (male, 90 kg) following

TABLE II

ESTIMATION OF PREDNISOLONE (25 ng/ml) IN PLASMA

Sample number	Theoretical (ng/ml)	Estimated (ng/ml)	Recovery (%)*
1	25	24.7	98.8
2	25	24.5	98.0
3	25	25.9	103.6
4	25	23.5	94.0
Mean			98.6
Coefficient of variation			± 3.9

*Recovery (%) = (estimated/theoretical) × 100%

TABLE III

PLASMA PREDNISOLONE CONCENTRATIONS IN A HUMAN SUBJECT FOLLOWING ORAL ADMINISTRATION OF A 10-mg DOSE

Time (h)	Prednisolone (ng/ml)
1	300
2	250
3	190
5	116
7	69

oral ingestion of two 5-mg prednisolone tablets (Delta Cortef; Upjohn, Toronto, Canada) is summarized in Table III. Blood was collected in heparinized 10-ml Vacutainer (Becton-Dickinson, Toronto, Canada) and the plasma, separated by centrifugation, was transferred to a 10-ml glass tube and stored at $-20°$ prior to use.

The plasma drug concentration decayed in a mono-exponential manner with time. The $t_{1/2}$ was 3 h which is in reasonable agreement with the mean $t_{1/2}$ of 2.5 h as reported by Sullivan et al. [11].

In summary, the HPLC method is a sensitive and specific procedure for the determination of prednisolone in plasma following single dose (10 mg) administration in humans. The method has been used for assessing the specificity and accuracy of a radio-immunological assay (R.I.A.) for prednisolone and can be used in laboratories which are not equipped to carry out radio-immunological assays.

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CHROMBIO. 029

THE USE OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR SAMPLE CLEAN-UP IN MASS FRAGMENTOGRAPHIC ASSAYS

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(Received June 17th, 1976)

SUMMARY

A novel high-performance liquid chromatography (HPLC) sample clean-up procedure for use in mass fragmentographic assays of (sub)-nanogram amounts of drugs in human plasma is described and compared with a conventional extraction sequence for sample purification. With the assay of the new antidepressant drug mianserin hydrochloride (Org GB 94) as an example, the HPLC procedure is discussed with respect to retention time, recovery, purification, column deterioration and convenience. It is demonstrated that HPLC sample clean-up is a useful and time-saving procedure for routine clinical analyses.

INTRODUCTION

Because of its sensitivity and specificity, mass fragmentography [1] is often used as a detection technique in assays of (sub)-nanogram amounts of drugs in biological fluids. Notwithstanding the specificity that is possible, direct low-resolution mass fragmentographic (LRMF) analyses of crude plasma extracts are impossible in many instances because of the presence of interfering substances originating from the plasma, the solvents and the reagents or the glassware used. Therefore, many ultrasensitive LRMF assays include a sample clean-up procedure to eliminate these interfering substances. For basic drugs, the clean-up of a crude extract is usually performed by extraction into an acidic solvent followed by removal of lipids by washing with non-polar solvents. These often tedious and time-consuming procedures are not amenable to automation and the limited number of samples that can be processed daily by one technician does not permit large-scale routine clinical analyses.

Most of the sample clean-up can be avoided by adjusting the mass spectrometer to a resolution of 10,000 or more and performing high-resolution mass fragmentographic (HRMF) measurements [2]. However, HRMF does not allow the simultaneous registration of internal standard ion peaks, resulting in a loss of accuracy and precision. Moreover, any sensitivity gained by elimination of

sample losses during the clean-up procedure is lost because of the reduced sensitivity of the mass spectrometer at high resolution.

The applicability of high-performance liquid chromatography (HPLC) as the sole purification step prior to LRMF has been investigated in order to simplify the time-consuming sample preparation. This paper describes the potential of HPLC for sample clean-up in comparison with a sequential extraction procedure; both methods were applied to the antidepressant drug mianserin hydrochloride (Org GB 94; 1,2,3,4,10,14b-hexahydro-2-methyldibenzo[*c*,*f*] pyrazino[1,2-*a*]-azepine monohydrochloride). Details of the assay procedure and some applications are described elsewhere [3].

MATERIALS AND METHODS

Standard and marker compounds

Amitriptyline, used as an HPLC marker compound, was kindly supplied by Merck Sharp & Dohme Nederland B.V. (Haarlem, The Netherlands). The internal standard [10,10-$^2H_2$] Org GB 94, for quantification and the HPLC marker compound [^3H] Org GB 94 were prepared by base-catalyzed isotope exchange.

Solvents

All solvents were purchased from Merck (Darmstadt, G.F.R.). *n*-Hexane and isopropanol were of Uvasol quality, ethanol and methanol were of analytical-reagent grade and the ammonia was of Suprapur grade.

Equipment

A Waters Assoc. (Milford, Mass., U.S.A.) Model ALC-202 high-performance liquid chromatograph equipped with a 2-ml loop-containing atmospheric pressure injection system (Type U6K) was used in the preparative mode. The chromatograph was equipped with a 30 cm × 4 mm I.D. stainless-steel column filled with μPorasil (10 μm; Waters Assoc.) operated at a flow-rate of ca. 2ml/min at a pressure of about 800 p.s.i. The standard UV detector was operated at 280 nm. The elution system consisted of *n*-hexane—isopropanol (80:20, v/v) to which 4% of ethanol and 0.1% of concentrated ammonia were added. A combined Varian Aerograph 2740 gas chromatograph—Varian-MAT CH7 mass spectrometer system was used. The gas chromatograph was equipped with a 4 m × 2 mm I.D. glass column filled with 1% JXR on Gas-Chrom Q, operated at 260°. The mass spectrometer was set for monitoring the total ion current (TIC) and for dual ion recording at *m/e* 264 and 266, these being the molecular ion peaks of Org GB 94 and the deuterated internal standard, respectively.

HPLC clean-up

Plasma samples of 1-ml volume, to which known amounts of deuterated Org GB 94 are added as internal standard, are extracted with two 5-ml portions *n*-hexane and the combined extracts are evaporated to dryness at 45° under a gentle stream of nitrogen. The residue is re-dissolved in 0.5 ml of the HPLC solvent system containing 1 μg of amitriptyline* and the entire solution is in-

*Once the HPLC retention time has been determined and established to be constant during an appropriate period, the marker compound can be omitted.

jected into the high-performance liquid chromatograph. The column effluent is trapped during the period from ca. 5 min after injection up to to the disappearance of the amitriptyline peak (at ca. 9 min). This solution is evaporated to dryness and the residue is re-dissolved in 8 μl of methanol prior to combined gas chromatography–mass spectrometry (GC–MS) measurements.

Sequential extraction clean-up

For comparison of the extent of purification, a sequential extraction procedure is also used. The crude *n*-hexane extract is re-extracted twince with 1.5 ml of 0.1 *N* hydrochloric acid. The combined acidic layers are washed with two 5-ml portions diethyl ether in order to remove lipids, and the aqueous phase is adjusted to pH 10 and subsequently extracted with *n*-hexane. The purified extract is evaporated to dryness and re-dissolved in 8 μl of methanol prior to GC–MS measurements.

RESULTS AND DISCUSSION

Because the HPLC procedure described here is a novel approach to the clean-up of plasma samples, some aspects are described below in more detail.

Establishment of retention times

The use of microgram amounts of the compound to be measured for establishment of retention times should be avoided as far as possible because of the risk of cross-contamination. The eluate fractions to be trapped should be selected in such a way that appropriate separation from interfering impurities is achieved and that quantitative recoveries of the compound and internal standard are guaranteed. This is achieved either by the use of radioisotope-labelled compounds and measurement of radioactivity in the eluate as a function of time, or by using appropriate marker compounds. Both procedures are illustrated in Fig. 1, which shows the HPLC 280-nm UV detector signal and the amount of radioactivity in subsequently collected 0.5-ml fractions of a plasma sample to which 7 nCi (= 0.1 μg) of titrated Org GB 94 and 1 μg of amitriptyline were added as marker compounds*.

As shown, Org GB 94 is eluted before amitriptyline. By collecting the eluate during the period from 1 min after the impurity peak up to the end of the amitriptyline peak, a quantitative recovery can be expected (see *Recovery*).

Recovery

For nanogram amounts of Org GB 94, the recovery from the HPLC was determined to be almost 100%. For microgram amounts some material is lost in the chromatograph. The carry-over to a subsequent blank injection was found to be of the order of 0.1%, which does not permit alternate processing of samples that contain microgram and nanogram amounts of Org GB 94. This

*While establishing the retention time, one should be aware of a possible separation between the compound to be determined and its deuterated analogue. In some instances, differences in retention times of more than 1 min are observed [4].

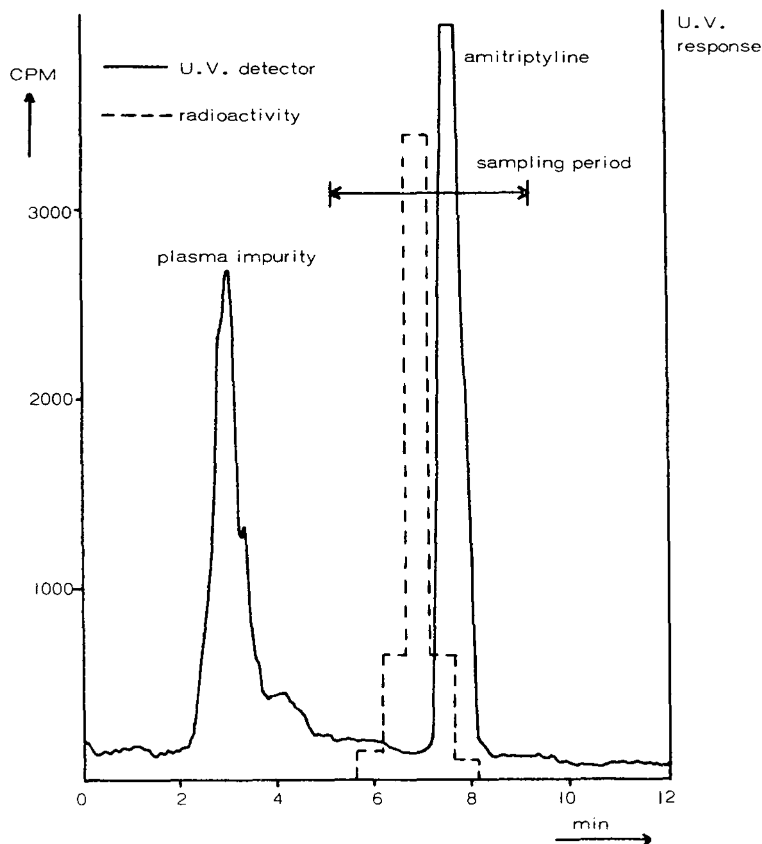

Fig. 1. High-performance liquid chromatogram (280 nm) of a 1-ml plasma extract containing 7 nCi [^{3}H]Org GB 94 (= 0.1 μg) and 2 μg of amitriptyline as marker compounds. The broken line shows the radioactivity in the 0.5-ml eluate fractions collected.

carry-over also illustrates that for the establishment of retention times, microgram amounts of the compound to be determined can not be used. Within a series of nanogram analyses, carry-over of this order of magnitude does not seriously affect the accuracy of the assay.

Extent of purification

The extent of purification is illustrated by comparison with the sequential extraction clean-up method (cf., Materials and Methods) and with the crude extract. The extracts were made from 2-ml blank plasma samples spiked with 4 ng of Org GB 94 and 4 ng of [10,10-$^2H_2$]Org GB 94. The resulting mass fragmentograms and TIC recordings are shown in Figs. 2 and 3, respectively.

The LRMF trace of the crude extract at *m/e* 264 (Fig. 2a) reveals two major impurity peaks (components 1 and 3). The sum of their peak heights exceeds the Org GB 94 peak by a factor of 1.7. In the trace at *m/e* 266, only one major impurity peak (component 2′) shows up, while some minor impurities are observed at greater retention times. The TIC trace shows two major impurities with retention times < 2 min and an abundant component with a retention time of 14.3 min.

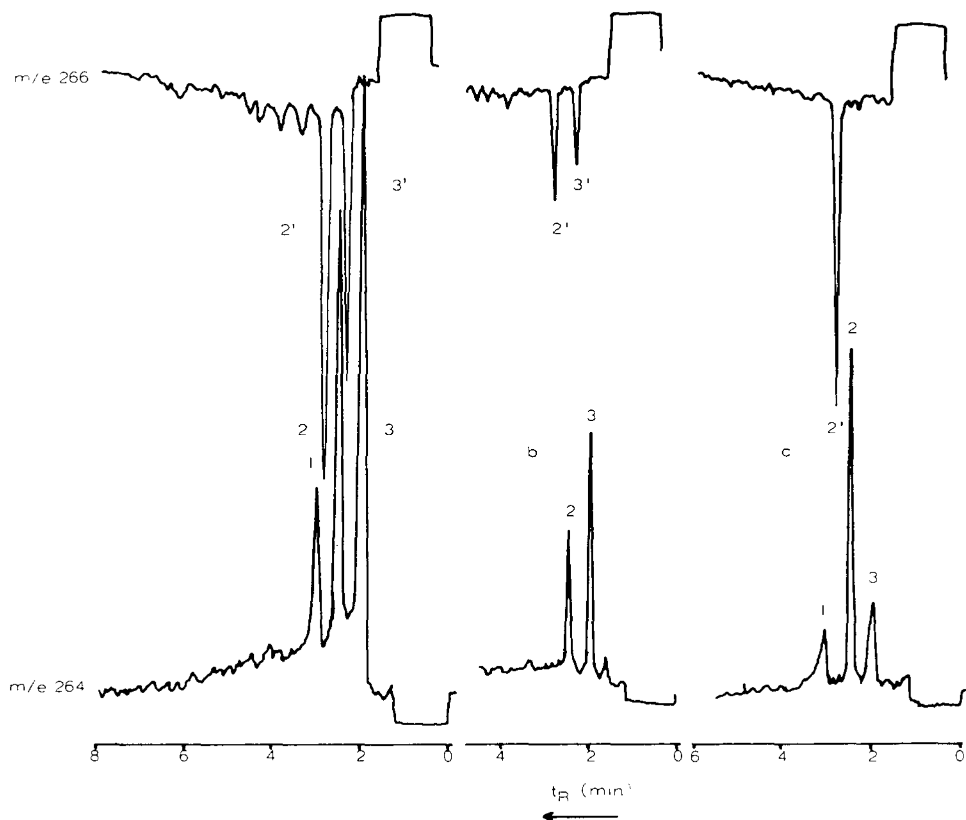

Fig. 2. Mass fragmentograms recorded at *m/e* 264 and *m/e* 266 of 2-ml blank plasma samples spiked with 4 ng of Org GB 94 and 4 ng of [10,10-$^2H_2$]Org GB 94 after (a) a single extraction, (b) a back-extraction clean-up and (c) an HPLC clean-up.

In the LRMF trace after the sequential extraction clean-up method (Fig. 2b), component 1 with *m/e* 264 disappeared, while compounds 3 and 3′ were still present with the same abundance relative to Org GB 94 as in the crude extracts. The ion intensities, however, are smaller by a factor of about 3. The corresponding TIC trace shows that the impurity peaks with short retention times are reduced whereas the component with a retention time of 14.3 min disappeared.

After HPLC purification, the trace at *m/e* 264 (Fig. 2c) shows only small residues of compounds 1 and 3, while in the trace at *m/e* 266 no other impurities show up. The Org GB 94 peak height is ca. 2.5 and 0.8 times the peak height after sequential extraction clean-up and single extraction, respectively. The corresponding TIC trace again shows further purification.

Column deterioration

With the μPorasil column currently in use, we processed over 2000 plasma samples. By flushing the column onze every 100—200 samples with appropriate polar or acidic solvents, its performance could be maintained.

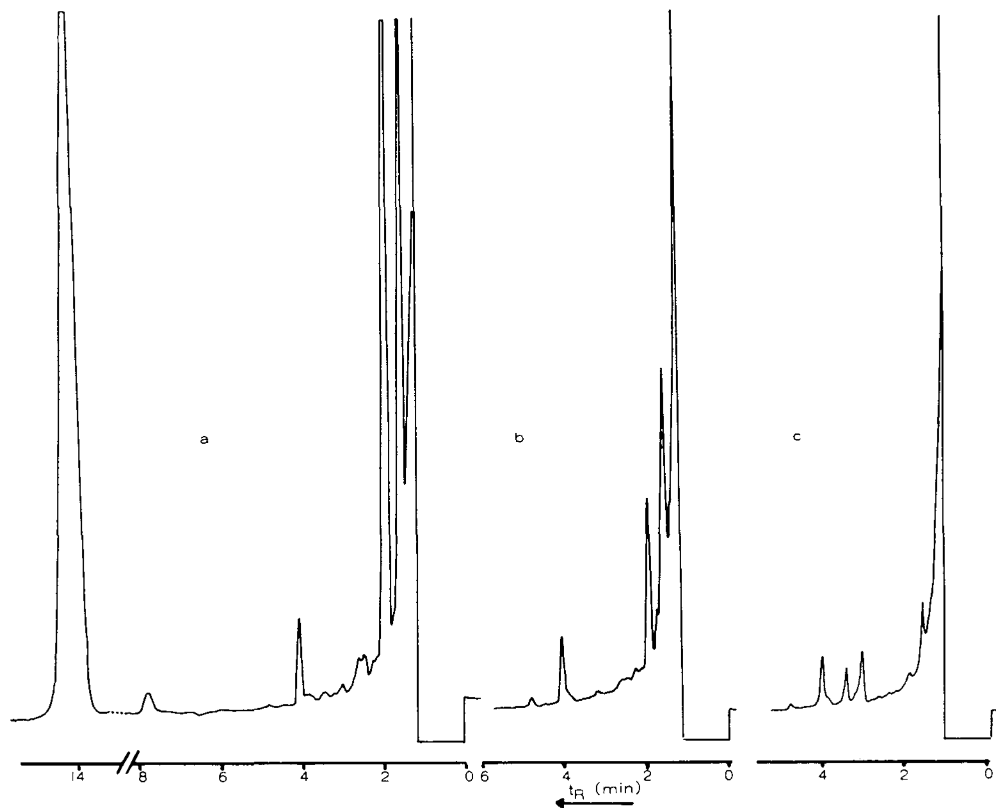

Fig. 3. TIC recordings of 2-ml blank plasma samples spiked with 4 ng of Org GB 94 and 4 ng of [10,10-$^2H_2$]Org GB 94 after (a) a single extraction, (b) a back-extraction clean-up and (c) an HPLC clean-up.

Convenience and speed

Because of its simplicity, the HPLC clean-up step can be performed by unexperienced technicians. Purification of one crude plasma extract takes 5—10 min, depending on the flow-rate used in HPLC and on the compound to be measured. An average of 60 samples can be processed per day. Because the HPLC procedure is the rate-limiting step, the total analysis capacity is also about 60 samples a day.

In contrast to other purification methods such as repeated extractions or thin-layer chromatography, the HPLC method can be automated relatively simply. This automation will improve the capacity and reliability, which are prerequisites for routine clinical analyses.

CONCLUSION

The HPLC sample clean-up proved to be a reliable, convenient and time-saving procedure for use in routine clinical mass fragmentographic quantifications.

If a small number of samples are to be assayed, a crude *n*-hexane extract can be used. For routine analyses, when 50—60 samples a day are to be processed, the crude extracts will contaminate the gas chromatographic column and mass spectrometer to an unacceptable extent and under these circumstances an HPLC clean-up provides adequate purification without significant loss of sensitivity.

ACKNOWLEDGEMENTS

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CHROMBIO. 030

MASS FRAGMENTOGRAPHIC ASSAY OF NANOGRAM AMOUNTS OF THE ANTIDEPRESSANT DRUG MIANSERIN HYDROCHLORIDE (Org GB 94) IN HUMAN PLASMA

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SUMMARY

For the assay of the antidepressant compound mianserin hydrochloride (Org GB 94) in human plasma, a mass fragmentographic method, using the deuterated analogue as internal standard and a high-performance liquid chromatographie sample clean-up procedure has been developed. The assay specifications obtained are a lower limit for reliable measurements of 1 ng/ml, an accuracy of ca. 0.01 ng/ml, a precision of 6—7% and a capacity of about 60 samples per day. The applicability of the assay method is illustrated by measurements of single-dose and steady-state plasma levels in clinical experiments, demonstrating the possibility of monitoring plasma levels during at least 24 h after a single dose of 15 mg of Org GB 94. The mean steady-state plasma levels after a daily dose of 3 × 20 mg of Org GB 94 appeared to be remarkably constant with time: 38, 36 and 34 ng/ml after 2, 4 and 6 weeks of treatment of 18 depressed patients.

INTRODUCTION

To permit a reliable pharmacokinetic and parmacological evaluation of the new antidepressant drug mianserin hydrochloride (Org GB 94; 1,2,3,4,10,-14b-hexahydro-2-methyldibenzo[*c*,*f*]pyrazino[1,2-*a*]azepine monohydrochloride), a method for its assay in human plasma has been developed. This compound, the chemical structure of which is depicted in Fig. 1, showed potent antidepressant activities in EEG profile analysis and in therapeutic studies [1].

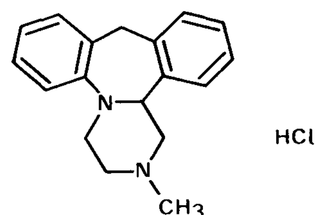

Fig. 1. Structure of Org GB 94.

In view of expected low levels after a therapeutic dose (nanogram per millilitre range) and the extensive biotransformation [2], a highly sensitive and specific assay method is required. Because of its sensitivity and inherent specificity, mass fragmentography (MF) [3] was selected as the detection method. The applicability of the assay has been illustrated by measuring plasma levels after a single oral dose and during chronic treatment.

MATERIALS AND METHODS

Internal standard

[10,10-$^2H_2$]Org GB 94, used as an internal standard, was prepared by hydrogen—deuterium exchange in the system hexamethylphosphoric triamide (HMPT) with NaO^2H and ^2H_2O at 80°. According to mass spectrometric analysis, the deuterium content was 4.2% $^2H_0$, 21.0% $^2H_2$, 73.3% $^2H_1$, 1.3% $^2H_3$ and 0.2% $^2H_4$.

Solvents

All solvents used were purchased from Merck (Darmstadt, G.F.R.). *n*-Hexane and isopropanal were of Uvasol quality, ethanol and methanol were of analytical-reagent grade and the ammonia was of Suprapur grade.

Glass-ware

Ten-millilitre glass-stoppered tubes were cleaned ultrasonically and subsequently rinsed with *n*-hexane and methanol. One-millilitre disposable glass tubes were cleaned by rinsing with methanol and subsequent heating in a gas burner until red hot.

Equipment

A Waters Assoc. (Milford, Mass., U.S.A.) high-performance liquid chromatograph equipped with a 30 cm × 4 mm I.D. µPorasil column (10 µm, Waters Assoc.) and a 280-nm UV detector was used for sample purification. The instrument was operated at a pressure of about 800 p.s.i. and a flow-rate of 2 ml/min of *n*-hexane—isopropanol (80:20, v/v) to which 4% of ethanol and 0.1% of concentrated ammonia were added.

The gas chromatograph—mass spectrometer—computer system consisted of the following:

(i) A Varian Aerograph 2740 gas chromatograph equipped with a 4 m × 2 mm I.D. glass column filled with 1% JXR on Gas-Chrom Q (80—100 mesh). The injector, column and detector oven temperatures were ca. 270, 260 and 270°, respectively. Helium was used as the carrier gas at a flow-rate of 30 ml/min. The gas chromatograph was coupled via a dual-stage Watson—Biemann separator to a mass spectrometer (see below).

(ii) A Varian-MAT CH7 mass spectrometer equipped with a peak-matching device. The spectrometer was focused at *m/e* 264 and the peak matcher was set to display alternatively the *m/e* 264 and *m/e* 266 signals, representing the molecular ion peaks of Org GB 94 and the internal standard, respectively. A home-made filter system allowed continuous, (pseudo)-simultaneous registration of the two signals on a two-pen potentiometer recorder. The mass

spectrometer was operated at electron energy 70 eV, ionizing current 300 μA, ion accelerating voltage 3 kV, electron multiplier voltage 2 kV and ion-source temperature 135°.

(iii) The computer system was a Varian-MAT SpectroSystem 100 MS with a 2.4-M-word disc backing store. Calculations were also performed with this computer system using a BASIC program.

Clinical experiments

Single-dose experiments were performed by administration of 15 mg of Org GB 94 to three healthy male volunteers aged between 21 and 35 years under the supervision of Prof. M. Fink, New York, U.S.A. Each volunteer was treated twice with a minimum time interval of 10 days between the two sessions.

Chronic therapy was performed by administration of a daily dose of 60 mg of Org GB 94 (20 mg three times a day) for 6 weeks to 18 patients suffering from depressive illness, under supervision of Dr. A. Coppen, Epsom, Great Britain. Blood samples were taken with heparinized syringes at the time intervals indicated in Tables II and III. After the addition of saturated sodium citrate solution, the blood was centrifuged and the plasma was transferred to separate sample tubes and stored at −20° until required for further analyses. Plasma was prepared within 40—100 min after withdrawal of each blood sample.

Assay methods

From the administered dose and the sampling time, the Org GB 94 concentration was estimated. For expected levels of less than 1 ng/ml in plasma, 2-ml samples were processed, while for higher levels 1-ml samples were used. An amount of [10,10-$^{2}H_2$]Org GB 94 more or less equal to the expected Org GB 94 concentration, dissolved in 0.1—2 ml of water and 100 μl of concentrated ammonia (to obtain pH 11) was added to the sample. After equilibration for at least 3 h, the plasma was extracted with tho 5-ml portions of *n*-hexane by thoroughly mixing and centrifuging for 5 min at 1200 *g*. The combined extracts were evaporated to dryness in a 10-ml conical tube at 45° under a gentle stream of nitrogen.

The residue was re-dissolved in 0.5 ml of the solvent system used for sample clean-up by high-performance liquid chromatography (HPLC). The collected fraction containing the Org GB 94 and internal standard was evaporated to dryness at 45° under nitrogen.

The residue was transferred into 1-ml glass tubes using one 200-μl portion and one 100-μl portion of methanol. The contents of each glass tube were again evaporated to dryness at 45° under nitrogen and the residue was subsequently re-dissolved in 8 μl of methanol. From this methanolic solution, the maximum possible amount (6—7 μl) was injected into the gas chromatograph—mass spectrometer for quantification. Intermittently, 2-μl portions of standard solutions containing Org GB 94 and [10,10-$^{2}H_2$]Org GB 94 in concentration ratios of 4:1, 2:1, 1:1, 1:2 and 1:4 (ng/μl) were analyzed in triplicate for establishment of the calibration graph. Peak heights (occurring at the retention time for both compounds: ca. 2.5 min) were measured manually. From these results, the amount of Org GB 94 per millilitre of plasma was calculated using a BASIC computer program. Because the amount of internal standard was adapted to each

individual sample in order to achieve optimal accuracy and precision, a relative calibration graph had to be used.

A wide-range calibration graph (concentration ratio may vary by a factor of 16) was obtained by plotting the fractional peak height $h(m/e\ 264)/[h(m/e\ 264) + h\ (m/e\ 266)]$ versus the fractional concentration [Org GB 94]/[Org GB 94] + [[10,10$^2H_2$]Org GB 94]) in the range 1:4 to 4:1. A typical example of such a calibration graph with calculated slope of 0.89 is shown in Fig. 2.

Using this calibration graph, the measured peak heights and the data on sample volume and amount of internal standard added, the program calculates the level of Org GB 94 (free base) in nanograms per millilitre of plasma. Samples in which the actual concentration ratio of Org GB 94 to internal standard exceeds the boundaries of the calibration graph have to be re-processed with different amounts of internal standard.

RESULTS AND DISCUSSION

The merits of mass fragmentographic analyses have been discussed elsewhere

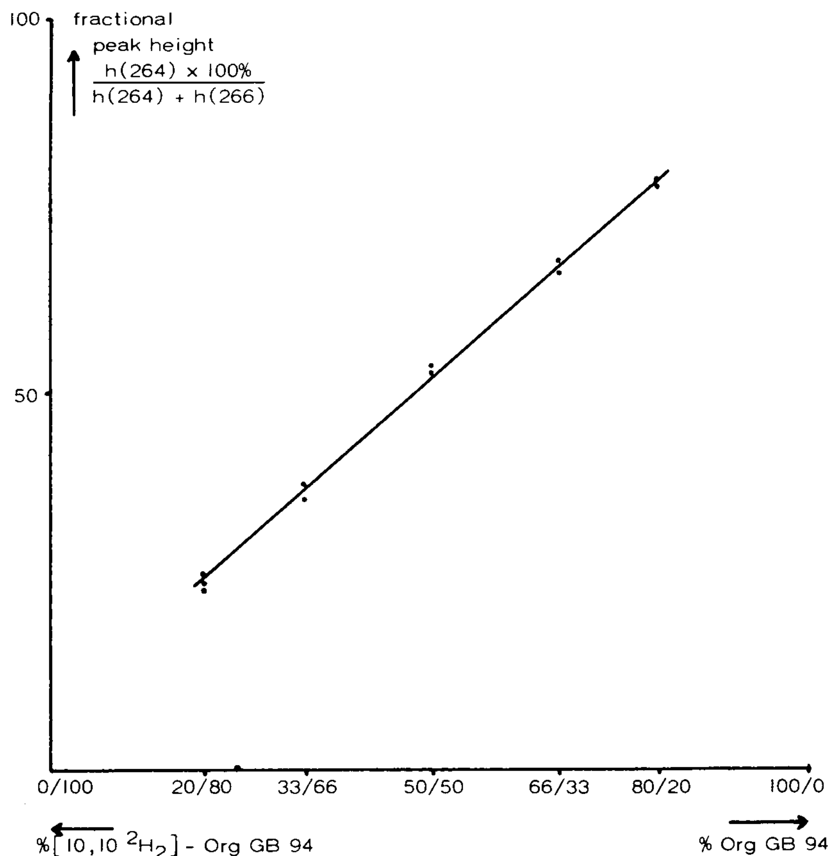

Fig. 2. Mass fragmentographic calibration graph for 1:4 to 4:1 ratios of Org GB 94 and [10,-10-$^2H_2$]Org GB 94 calibration mixtures.

[3], and the discussion of the present method is limited to the description of the assay specifications.

Assay specifications

To test the method, 1-ml samples of human plasma pools spiked with 1 or 15 ng/ml of Org GB 94 were processed. A possible disintegration of Org GB 94 during storage was investigated by analyzing a pool of plasma spiked with 15 ng/ml, after 7 months of storage at −20°. The results of these analyses are presented in Table I and are discussed in terms of accuracy, precision and sensitivity.

Accuracy and precision

The accuracy of the measurements, defined as the deviation from the true value, was found to be independent of the concentration in the range investigated. During 7 months of storage at −20° the plasma concentration of 15 ng/ml decreased by not more than 5%. The high degree of accuracy, inherent in MF assays with internal standardization, is even enhanced by the use of a sliding relative calibration graph, which allows adaptation of the amount of internal standard to each individual sample.

The precision, expressed as standard deviation, was 6—7% and was independent of the concentration and storage time. From these precision data, it can be derived that for a level of 1 ng/ml the measurements have a probability of only 0.05 of being outside the range 0.86—1.13 ng/ml. It should be borne in mind, however, that these analyses were performed with a 1:1 ratio of compound to internal standard. In separate experiments, it was demonstrated that a 1:4 or 4:1 ratio does not affect the precision significantly.

The relatively high accuracy and precision are due mainly to the method of internal standardization employed, with the stable isotope-labelled analogue added directly to the plasma sample. Because of equal recovery of drug and internal standard from plasma, any loss of drug is automatically corrected for by proportional losses of the internal standard, which makes the accuracy independent of the Org GB 94 recovery. Further, it should be realized that by using the same stock solution of [10,10-$^2H_2$]Org GB 94 for addition to the samples as for preparing the calibration graph, any inaccuracy in concentration of the stock solution is cancelled out in the calculations.

TABLE I

ACCURACY AND PRECISION OF THE ASSAY OF Org GB 94 IN HUMAN PLASMA

Org GB 94 plasma concentration (ng/ml)	Internal standard added (ng/ml)	Mean of measured concentration (ng/ml)	Number of determinations	Accuracy (%)	Precision (S.D.) (%)
1	1	1.01	10	1	6.5
15	15	15.01	20	0.1	6.0
15*	15	14.26	10	−4.9	6.8

*This pool had been prepared 7 months before analysis and had been stored at −20° in order to investigate possible disintegration of Org GB 94 during storage.

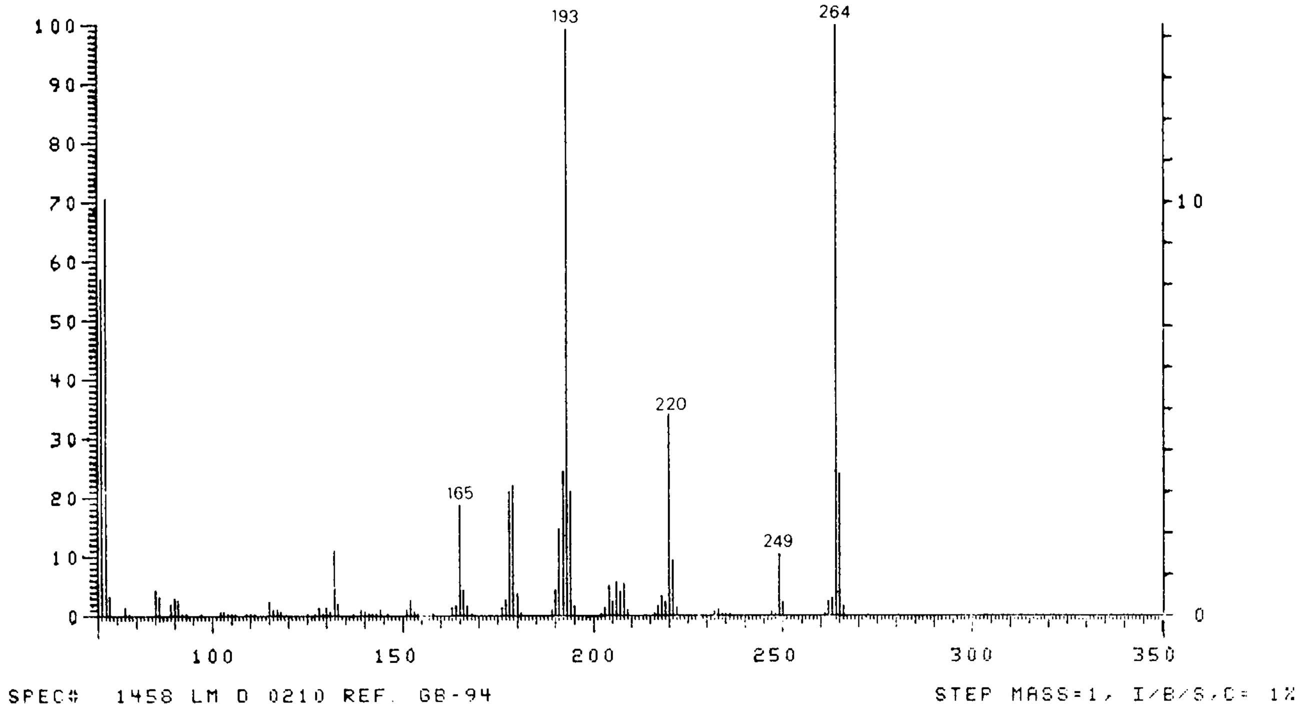

Fig. 3. Mass spectrum of Org GB 94 (Varian-MAT CH7, 70 eV).

Sensitivity

The sensitivity of the assay method is determined by (i) the detection limit of the gas chromatographic—mass spectrometric (GC—MS) system and (ii) the percentage recovery of Org GB 94 from the plasma in the extraction, purification and concentration procedure. The extreme sensitivity of the mass spectrometer in principle allows the detection of picogram amounts provided that the instrument is running under optimal conditions. In combination with a gas chromatograph, however, a decrease in sensitivity occurs owing to adsorption to the GC column packing material and disappearance of substance through the helium separator. Nevertheless, standard solutions containing 50 pg of Org GB 94 per injection yielded mass fragmentograms with a signal-to-noise ratio of better than 10:1.

The sensitivity is afforded by the relatively high abundance of the molecular ion of Org GB 94, as shown in Fig. 3. The use of the base peak at *m/e* 193 for quantitation should improve the sensitivity to only a small extent. In that event, however, the specificity is lost because ions with *m/e* 193 are also common fragments for Org GB 94 metabolites.

The percentage recovery in the sample working-up procedure was measured by processing plasma samples to which 1 ng of [^{14}C]Org GB 94 had been added. It was found that the extraction yield was almost 100% and that the HPLC purification was quantitative. Losses of 10—20% were observed during the various sample transfer, concentration and evaporation steps. Although in many instances amounts of less than 1 ng can be quantified (depending on the conditions of the equipment), for routine analyses of 1-ml samples with adequate accuracy and precision a level of 1 ng/ml in plasma is considered to be the lower limit for reliable measurements. When even lower levels are to be analyzed, decreased accuracy and precision must be accepted or larger plasma samples should be processed (permitting quantifications down to ca. 0.25 ng/ml with a 4-ml sample).

Specificity

The specificity of the method is obtained by the combination of all four successive steps in the analytical procedure: the *n*-hexane extraction, the HPLC purification, the GC separation and the MS detection. Analyses of blank plasma samples revealed vanishingly small peaks in the MF traces at *m/e* 264 and *m/e* 266 with Org GB 94 GC retention times. Moreover, of all known Org GB 94 metabolites, only the N-oxide shows a peak at *m/e* 264 in the mass spectrum and, owing to the decomposition into Org GB 94 at elevated temperatures, similar GC retention time. The N-oxide, however, cannot be extracted from plasma with *n*-hexane and consequently does not interfere in the determination. The specificity of the assay is automatically controlled during the detection by coincident onset and duration of both peaks in the mass fragmentogram*.

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*Minute differences in GC retention times of compounds and their deuterated analogues have been reported for compounds deuterated in the vicinity of a polarity centre; with Org GB 94, such differences were not observed.

Capacity

The method described permits routine measurements with a capacity of ca. 60 samples a day. The capacity can be improved by automation of HPLC and GC injection, automatic collection of the HPLC fractions and computerized digitization of the mass fragmentogram peaks.

The applicability of the assay method to the pharmacological and pharmacokinetic evaluation of the drug was illustrated by the measurement of plasma levels in patients after 2, 4 or 6 weeks of treatment with 60 mg of Org GB 94 per day and of plasma levels as a function of time after a single oral dose to male volunteers.

The steady-state plasma levels shown in Table II display a considerable inter-patient variation in the range 4—98 ng/ml, which is covered by the sensitivity of the assay. The individual levels, however, were remarkably constant during the 6 weeks of treatment, as reflected by the constant mean plasma levels over this period: 38, 36 and 34 ng/ml after 2, 4 and 6 weeks of treatment respectively.

The single-dose plasma levels in Table III demonstrate that the sensitivity of the assay is sufficient to monitor Org GB 94 in plasma from 1 h up to 24 h after a single dose of 15 mg. From these results, it can be concluded that the method permits measurements during at least 24 h following an oral dose of 15 mg, which is a prerequisite for acceptable pharmacokinetic analyses.

TABLE II

INDIVIDUAL AND MEAN EARLY MORNING Org GB 94 PLASMA LEVELS IN DEPRESSED PATIENTS AFTER 2, 4, AND 6 WEEKS OF DAILY TREATMENT WITH 3 × 20 mg OF Org GB 94

Patient	Org GB 94 plasma levels (ng/ml; free base) after treatment for		
	2 weeks	4 weeks	6 weeks
G.H.	23.6	24.3	23.9
M.Mr.	14.4	14.5	4.3
H.O.	22.1	29.9	31.4
E.T.	20.3	28.9	26.5
W.F.	98	57	52
N.L.	21.7	20.8	19.0
V.G.	35.2	—	—
R.L.	32.8	34.5	33.9
R.C.	41.3	60	45.6
M.Ms.	36.3	32.3	27.8
D.K.	15.4	16.5	22.9
M.S.	56	49.4	43.0
K.O.	28.5	23.6	20.4
J.M.	21.5	15.5	20.4
D.T.	69	64	60
S.G.	28.1	28.5	29.0
V.H.	35.8	44.2	48.7
G.S.	84	69	65
Mean ± S.D.	38 ± 24	36 ± 18	34 ± 16

TABLE III

Org GB 94 PLASMA LEVELS AFTER A SINGLE DOSE OF 15 mg OF Org GB 94 TO FOUR MALE VOLUNTEERS IN TWO SESSIONS

Subject	Session	Org GB 94 (free base) plasma levels as a function of time following administration (ng/ml)						
		25 min	1 h	2 h	3 h	5 h	7 h	24 h
J.G.	1	0.6	13.2	19.4	13.2	9.2	7.4	1.6
	2	0.4	7.6	19.5	18.4	11.7	8.8	2.3
M.P.	1	0.6	4.9	10.2	8.6	6.3	4.8	2.6
	2	1.5	6.5	11.1	10.9	8.6	5.1	1.1
M.G.	1	0.6	3.3	6.5	5.6	5.1	3.4	1.1
	2	0	0.5	8.1	8.9	7.4	6.4	1.4
S.K.	1	0.1	4.0	10.8	8.7	6.1	4.4	1.3
	2	0.3	1.0	11.8	11.4	7.5	5.2	1.8

CONCLUSIONS

The method described for the assay for Org GB 94 in human plasma, based on HPLC purification and MF quantitation, meets the requirements for routine analyses for evaluating clinical experiments and for monitoring patients' drug kinetics.

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CHROMBIO. 039

QUANTITATIVE ANALYSIS OF TERBUTALINE IN SERUM AND URINE AT THERAPEUTIC LEVELS USING GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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SUMMARY

A simple and sensitive method for the determination of terbutaline in serum and urine has been developed. A mass spectrometer in the multiple ion detection mode was used as a gas chromatographic detector. Levels were monitored after oral and subcutaneous administration of the drug. The sensitivity is 1 ng/ml using 1 ml of serum.

INTRODUCTION

Terbutaline [1-(3′,5′-dihydroxyphenyl)-2-(*tert.*-butylamino)ethanol] is a β_2-receptor stimulator and is widely used in the treatment of asthmatics to relieve bronchoconstriction [1]. Terbutaline is inactivated in humans mainly by conjugation to a sulphate and, to a minor extent, to a glucuronide. The presence of the conjugates was confirmed in tritium labelling studies by Nilsson et al. [2]. Owing to its strong hydrophilic character and its chemical instability at ele-

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vated pH, the drug requires special extraction procedures. Clean-up procedures for tritiated terbutaline with subsequent liquid scintillation counting have been performed [2]. Extraction for subsequent chromatographic analysis of this and similar drugs, as proposed by Modin and Johansson [3] and Evans et al. [4], could not be used owing to the low therapeutic levels present in serum.

Recently we developed a semiquantitative method for terbutaline using ion-pair extraction [5]. In this paper we present a fully quantitative method, which has been checked on a large number of biological samples and proved to be reliable and accurate.

EXPERIMENTAL

Materials

Pure terbutaline sulphate and deuterium-labelled d_6-terbutaline were obtained from Astra Chemicals and Pharmaceuticals (Lund, Sweden). Ethyl acetate was of analytical-reagent grade and used without further purification. Bis(2-ethylhexyl) hydrogen phosphate (DEHP) was a synthetic-grade reagent from Merck (Darmstadt, G.F.R.). The silylating reagent BSTFA was a Pierce (Rockford, Ill., U.S.A.) product. Gas chromatography (GC) was performed at 165° on a 120 × 0.3 cm I.D. glass column packed with 3% OV-1 on 80–100-mesh Gas-Chrom Q. The carrier gas was either helium or 99.95% pure methane (Matheson, Oevel, Belgium), both flow-regulated at 24 ml/min with a Brooks digital flow controller.

Mass spectrometry (MS) was performed on a Finnigan 1015 D electron impact (EI) or 3200 F chemical ionization (CI) instrument, both connected to a Finnigan 6000 computer system. In mass fragmentography at high sensitivity levels, a four-channel peak selector was used.

The temperature of the CI ion source was digitally controlled at 85 ± 1° by a CRL temperature controller/meter (Control and Readout Ltd., Worthing, Great Britain). The temperature stated was measured at the elution time of terbutaline. The mass spectrometer was tuned for optimal sensitivity at the selected ions.

Extraction

To 1 ml of a serum sample at a pH of 7.2–7.5, 20 ng of an aqueous solution of d_6-terbutaline were added. After an equilibration period of 15 min, 8 ml of a 0.015% (w/v) solution of DEHP in ethyl acetate were added. After thorough mixing on a Vortex mixer for 5 min, and subsequent centrifugation at 1500 *g* for 5 min, the organic phase was transferred into a Reacti-vial (Pierce) and evaporated to dryness under a stream of nitrogen at 55°. BSTFA (20 μl) was added and silylation was completed after 15 min at 80°. A 1-μl sample was injected into the GC–MS system.

The procedure for the determination of the free drug in urine is slightly different owing to the high levels of terbutaline involved. With 1 ml of a phosphate buffer, 0.1 ml of the urine was adjusted to a pH of 7.3, then 100 ng of the internal standard were added. The extraction was then continued as described for the serum.

RESULTS AND DISCUSSION

GC—MS of terbutaline when amounts of more than 100 ng are injected can easily be performed by EI mass spectrometry even in the presence of impurities from biological samples. The EI spectrum shows an intensive fragmentation. No molecular ion is observed.

The only intense ions are at *m/e* 86 and 356. In serum samples spiked with concentrations of 20 ng/ml or less, these ions proved to be inadequate for mass fragmentography. The ion at *m/e* 356 was always present as a background ion with varying intensity from numerous types of columns, and the ion at *m/e* 86 was present as a fragment ion from biological contaminants. CI mass spectrometry with methane as reactant gas yielded a more useful spectrum (Fig. 1). Although the ion at *m/e* 86 is the most abundant, the quasi-molecular ion $(M + H)^+$ at *m/e* 442 and the fragment ion $(M - CH_3)^+$ at *m/e* 426 are intense and proved to be very useful for quantitation at low levels in serum or urine samples. An even more intense quasi-molecular ion is obtained when isobutane is used as the reactant gas. However, the overall sensitivity for the GC—MS method is higher when methane is used as both a carrier and reactant gas. In this instance no separator is needed and the entire sample enters the mass spectrometer.

The use of the $(M + H)^+$ and the $(M - CH_3)^+$ ions in mass fragmentography placed no restrictions on the site of the deuterium label in the internal standard. The preparation of a standard with six deuterium atoms, present in the *tert.*-butyl group at a high purity level (99.8%), was performed at the Astra Laboratories. The presence of terbutaline in a sample was identified by three parameters: the retention time; the ions at *m/e* 426 and 442; and the ratio of the intensities of *m/e* 442 and 446, which should remain constant at all levels. For an accurate quantification, the ratio of the deuterium-labelled ions (*m/e* 448 and 432) should also remain constant.

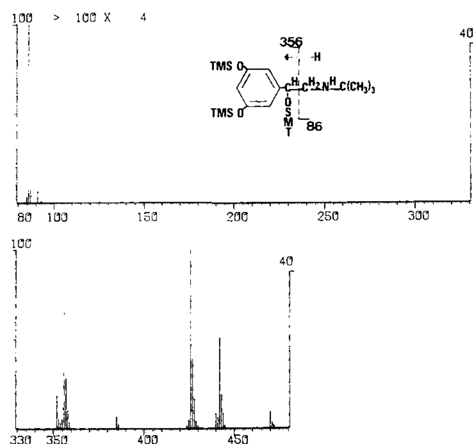


Fig. 1. The CI mass spectrum of tri-trimethylsilyl (TMS) terbutaline. The $(M - 15)^+$ ion is very abundant (25%); the abundance of the quasi-molecular ion is 12%.

TABLE I

CALIBRATION DATA FROM SPIKED SERUM SAMPLES

Correlation coefficient for the line = 0.998.

Sample	$(I_{442} + I_{426})/(I_{448} + I_{432})$				
	Concentration of terbutaline in serum (ng/ml)				
	1	2	5	10	20
1	0.045	0.095	0.27	0.55	0.96
2	0.053	0.098	0.25	0.48	1.01
3	0.055	0.095	0.24	0.52	0.99

Quantification could now be performed via a calibration equation calculated from spiked serum samples. To blank serum, 1, 2, 5, 10 and 20 ng of terbutaline were added together with a fixed amount of 20 ng of d_6-terbutaline. Each analysis was performed in triplicate and the results are given in Table I. The terbutaline concentration in an unknown serum sample is calculated from the equation

$$\text{Terbutaline concentration (ng/ml)} = 20.135 \times \frac{I_{442} + I_{426}}{I_{448} + I_{432}} - 0.066$$

where I_{442}, I_{448}, I_{426} and I_{432} represent the peak heights of the respective peaks in the fragmentogram. In urine analysis the concentration should be multiplied by 50. Frequent controls were carried through the procedure for optimal accuracy.

The overall accuracy of the extraction procedure was calculated from an actual sample divided into nine equal portions. Each portion was treated as described previously. The sample contained 2.5 ng/ml of terbutaline. The coefficient of variation was 8%.

The extraction efficiency was calculated from measurements on spiked serum samples. To each sample 2 ng of terbutaline were added and the sample was extracted without prior addition of d_6-terbutaline. Before the final step (the addition of BSTFA) 20 ng of d_6-terbutaline were added. The results from these samples were compared with those obtained from standards containing 2 ng of terbutaline and 20 ng of d_6-terbutaline in 20 μl of BSTFA. Six extractions were performed and the recovery was 80 ± 6%.

In the final part of this pilot study, we applied the method to two groups of patients. One group of seven individuals received 5 mg of Bricanyl® (equivalent to 4.1 mg of terbutaline base) on an empty stomach and the same breakfast was given to all subjects after 30 min, samples subsequently being collected after 0, 1, 2, 3 and 5 h. The serum levels are given in Table II and the mean and standard deviation are plotted in Fig. 2.

The second group of six individuals received 250 μg of Bricanyl subcutaneously and samples were collected after 0, 15, 30, 60, 120 and 180 min. The results are given in Table III and the mean and standard deviation are plotted in Fig. 3. Fig. 4 shows the recordings of the four multiple ion detection (MID)

TABLE II

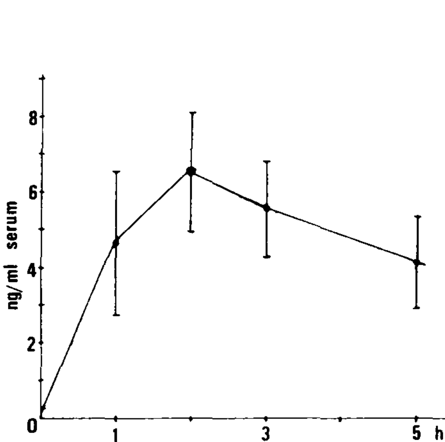
SERUM CONCENTRATIONS AFTER A SINGLE ORAL DOSE OF 5 mg OF BRICANYL

Patient	Concentration (ng/ml) after			
	1 h	2 h	3 h	5 h
B.E.	5.2	9.4	4.7	No sample
M.C.	1.0	5.1	5.3	3.9
D.C.	3.5	5.7	6.2	3.2
H.v.G.	4.4	5.3	3.9	3.9
L.R.	5.4	7.4	7.8	6.3
M.G.	6.8	5.5	4.7	2.7
M.M.	5.6	7.0	6.2	4.4

TABLE III

SERUM CONCENTRATION AFTER A SUBCUTANEOUS INJECTION OF 250 μg of BRICANYL

Patient	Concentration (ng/ml) after				
	15 min	30 min	60 min	120 min	180 min
B.R.	4.9	4.7	3.2	1.7	1.4
W.J.	5.8	4.7	4.1	2.7	1.7
S.J.	3.3	4.7	3.2	2.7	1.2
M.v.P.	3.1	4.7	4.4	2.7	1.9
M.v.S.	4.1	5.6	3.7	2.4	1.6
R.d.B.	3.4	3.7	3.6	2.7	2.5



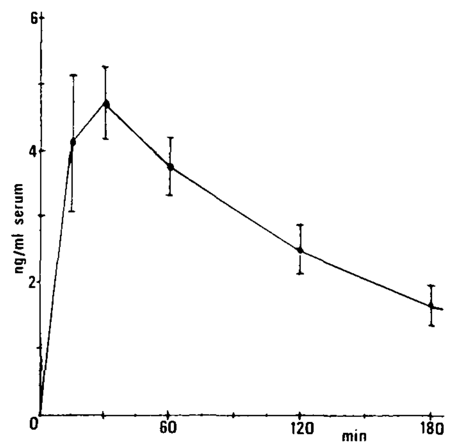


Fig. 2. Serum concentration versus time after an oral dose of 5 mg of Bricanyl. The mean and standard deviation are plotted.

Fig. 3. Serum concentration versus time after a subcutaneous dose of 250 μg of Bricanyl.

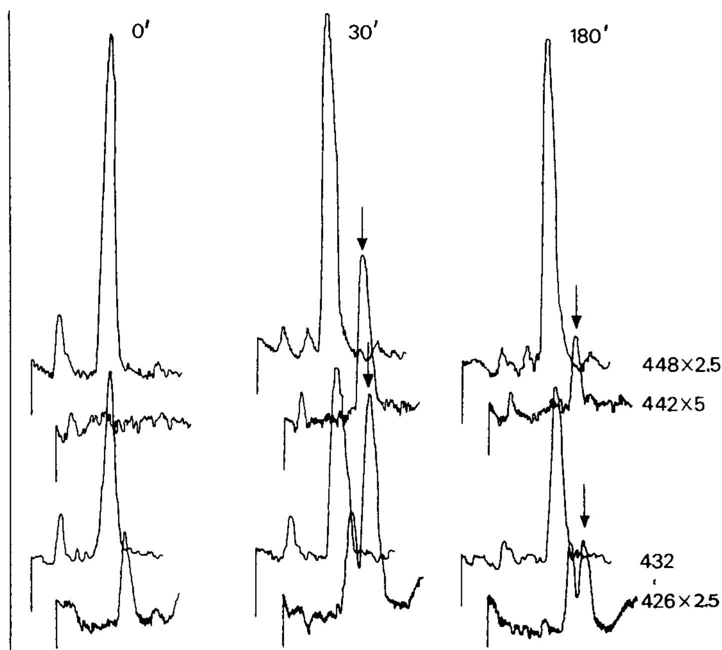


Fig. 4. Mass fragmentogram traces of serum samples from one patient (M.v.P.) at time 0, 30 and 180 min. The arrows indicate the elution time of terbutaline (5 min 11 sec). To all samples 20 ng/ml of d_6-terbutaline were added. The elution time of the internal standard was 5 min 7 sec. The traces represent the ion intensities at the *m/e* 448, 442, 432 and 426.

traces of three serum samples of a patient at zero time, at the maximum concentration after 30 min and at the minimum level after 180 min. The MID trace at *m/e* 426 contains a slightly interfering substance. Such interferences are patient related. On some occasions a clear trace is observed at all *m/e* values; sometimes the MID trace at *m/e* 448 is contaminated. If, however, the ratios *m/e* 442 : 446 and *m/e* 448 : 432 remain constant there is virtually no chance that biological compounds are contributing to the respective traces at the retention time of terbutaline.

Other β-receptor stimulating drugs, such as salbutamol, orciprenaline, isoetharine and fenoterol, do not interfere chemically, although they are extracted equally well, because of their different mass spectra and different retention times.

From the patients in the second group, urine samples were collected at the intervals 0—3 h, 3—6 h, 6—12 h and 12—14 h and analyzed. Large differences between the individuals occurred (Table IV).

All of the samples were analyzed without prior knowledge of either the patient or the time of sample collection.

From these results it can be concluded that the method described is sensitive and specific for studying serum levels in patients receiving terbutaline either parenterally or orally. Further pharmacokinetic and clinical studies are in progress.

TABLE IV

CONCENTRATION OF FREE TERBUTALINE EXCRETED IN URINE AFTER SUBCUTANEOUS INJECTION OF 250 μg OF BRICANYL

The number in parentheses are the total amounts of urine excreted in the measured intervals.

patient	Concentration (ng/ml)			
	0–3 h	3–6 h	6–12 h	12–24 h
B.R.	560 (175)	216 (25)	21 (200)	8.0 (740)
W.J.	1020 (25)	290 (75)	No sample	31 (270)
S.J.	480 (100)	190 (75)	30 (310)	8.2 (550)
M.v.P.	76 (200)	79 (240)	12 (170)	11 (550)
M.v.S.	820 (70)	218 (70)	20 (10)	24 (300)
R.d.B.	194 (80)	230 (400)	18 (700)	No sample

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CHROMBIO. 037

QUANTITATIVE THIN-LAYER CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF PROCAINAMIDE AND ITS MAJOR METABOLITE IN PLASMA

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SUMMARY

A sensitive and accurate spectrodensitometric method was developed for the determination of procainamide and its major metabolite, N-acetylprocainamide, in plasma. The method involves extraction into organic solvent at alkaline pH, separation by thin-layer chromatography and direct measurement of the absorbance of the compounds on the plate at 275 nm. Quantities as low as 10 ng could be measured and a linear relationship was obtained between peak areas and amounts of the compounds in the spots from 10 to 200 ng. The recovery of both drugs from plasma was from 95.4 to 104.8%. The method is sensitive and specific, and procainamide was well separated from N-acetylprocainamide at all investigated concentrations. The method is recommended for clinical assays and pharmacokinetics studies.

INTRODUCTION

Procainamide (PA) was studied in the dog and Rhesus monkey and 50—67% was found to be excreted unchanged in dogs and 22—49% in monkeys after intravenous administration. The main metabolite was identified as N-acetylprocainamide (NAPA) in the monkeys but not in the dogs [1]. When administered orally to patients and normal subjects, 19.2—48.8% of the drug was excreted unchanged in patients and 57.1—79.5% in normal subjects and in both cases the major metabolite was identified as NAPA [2]. Further examination of the metabolic pattern of PA showed a substantial amount of NAPA in the plasma samples of patients receiving PA and also in some experimental animals [3]. When NAPA was administered intraperitoneally to mice, it prevented coarse ventricular fibrillation, caused by deep chloroform anesthesia, and resultant hypoxia. NAPA also reduced aconitine-induced arrhythmia to atrial flutter or atrial tachycardia in dogs. NAPA was not deacetylated during these tests in animals and was assumed to be a better therapeutic agent than PA because it was less toxic. The metabolism of PA was studied in cardiac patients receiving

PA and the acetylated drug accounted for 16—63% of the administered drug and it was considered to be the chief metabolite in man (plasma level between 1 and 15 μg/ml, at times higher than that of PA) [4]. The metabolite showed weaker effect than PA on the maximal electrical driving velocity of isolated atrial strips from guinea pigs. The pharmacokinetics of the N-acetylated metabolite of PA was studied in man and the assumption, raised by previous authors, about NAPA antiarrhythmic efficacy in man [3] was strengthened, although the question of the NAPA effect in cardiac patients was left unsolved [5]. The acetylation of PA was studied in healthy volunteers and was found to be subject to the same genetic polymorphism as that of isoniazid and some sulfonamides (slow/fast acetylators) [6]. Since these results showed that NAPA may be similar in therapeutic effect to the parent drug and since the plasma concentration of NAPA may be as high as or even higher than that of the original drug in patients, it becomes important to have a reliable method to measure both compounds from one sample of biological fluid.

The first techniques used for PA determination in biological material have been spectrophotometric and fluorimetric [7, 8]. Both methods seem sensitive enough for routine measurement of PA in biological fluids but they do not provide for determination of NAPA. A gas—liquid chromatographic method was developed for PA determination in plasma [9] which gave good results at concentrations of 2 to 20 μg/ml. Another specific gas chromatographic method was divised for the determination of PA and NAPA in plasma and urine using 4-amino-N-(2-piperidinoethyl)benzamide as internal standard [10]. An attempt was made to compare the fluorimetric method [8] with the gas chromatography determination of PA [9, 10] and it was concluded that, if NAPA also has significant antiarrhythmic activity in man, the different NAPA/PA plasma ratios may require concomitant determination of both compounds to ensure adequate therapy and dose regimen adjustments [11]. The gas chromatographic methods enabled one to measure both PA and NAPA in one sample of the biological fluid, but they lack sensitivity having a lower limit of reliable reading at 2 μg/ml plasma. Measurements of lower levels are frequently needed. The spectrophotometric and fluorimetric methods require separation of PA and NAPA prior to measurement, making this procedure a tedious one.

This paper describes a thin-layer chromatographic method based on the separation of PA and its major N-acetylated metabolite on silica gel plates followed by direct determination of their absorbances. The method of quantitation on a chromatoplate has the advantage of greater precision and sensitivity because the compounds are concentrated over a small surface area. It may be used to measure very small amounts of PA and NAPA (10 ng) and therefore the determination can be made with small volumes of plasma. The procedure is fast, requiring 1.5—2 h from receipt of the samples to the output of final results for a group of samples.

PA is completely resolved from NAPA and interfering biological contaminants and both compounds appear as distinct spots on the chromatoplates. The spots are scanned at a fixed wavelength and the absorbance is recorded, while peak areas are integrated automatically.

The described method was applied to plasma samples from patients after oral doses of 250 mg of PA and typical results are presented.

EXPERIMENTAL

Materials and methods

PA (250-mg Pronestyl capsules) was obtained from Squibb & Sons, Princeton, N.J., U.S.A. and NAPA was synthesized from PA by the method of Dreyfuss et al. [1]. All other chemicals were of analytical grade. Solvents used were tested by thin-layer chromatography to assure that no traces of UV-absorbing substances were present. Thin-layer plates were 20 × 20 cm silica gel 60 on glass (E. Merck, Darmstadt, G.F.R.), which were activated at 100° for 20 min and cooled in a desiccator prior to use.

Stock solution of PA and NAPA contained 1 mg/ml in methanol. Standard solutions were prepared by dilution of the stock solution to yield concentrations of 100 μg/ml of PA and NAPA.

Application of standard solutions and extracts was achieved with a mechanical spotter which enables application of 10 spots simultaneously and automatically, leaving blank channels between spots, and yielding small spots of uniform diameter. No additional drying time was needed for the spots since gentle heat was applied to the plate during spotting.

Excellent movement and separation of PA and NAPA was achieved with a developing solvent consisting of benzene—ammonium hydroxide 28%—dioxane (10:15:80).

The plates were air-dried after development and scanned at moderate speed on a chromatogram analyzer at 275 nm recording the absorption curve and integrating the peaks simultaneously with an automatic integrator.

Apparatus

For automatic spotting of plates the Multi-Spotter AIS (Analytical Instrument Specialties) supplied with temperature and speed control was used. Ultraviolet scanning was done with a UV—VIS-2 Chromatograph Scanner (Farrand) coupled with a strip chart recorder Model 100 (Farrand), and a CDS 101 Chromatography Data System (Varian).

Extraction and development procedures

To 0.5—1.0 ml of plasma in a 20-ml glass centrifuge tube was added 0.5 ml of 0.5 *N* sodium hydroxide and the mixture was mechanically shaken for 10 min with 3—4 ml of dichloromethane as extracting solvent. The tube was then centrifuged at 1300 *g* and the organic layer was transferred to a second tube. The aqueous layer was re-extracted in the same manner with 3 ml of dichloromethane and the combined organic layers were evaporated to dryness at 40° under a stream of nitrogen. The solid residue was dissolved in 100 μl of ethyl acetate. Three spots of 1 μl each of the ethyl acetate solution were applied to a thin-layer plate along with a series of extracts from plasma spiked with measured amounts of PA and NAPA, covering the concentration range of 0.10 to 20 μg/ml and standards of PA and NAPA in methanol. The plate was developed in the solvent described above in a saturated tank allowing the solvent to migrate about 15 cm (time: about 25 min).

A calibration curve of integrated peak areas (square millimeters) against concentration (microgram per milliliter of plasma) of PA and NAPA obtained from

the respective standards was constructed and it was used for determination of the drugs in plasma specimens of patients by interpolation.

Recovery

The recovery of the added PA and NAPA, separately and when together, was determined by comparison of the absorbance peak areas obtained from spiked plasma with those of the standards scanned on the same plate.

Standard curves relating areas to drug amounts were calculated using the method of least squares.

RESULTS AND DISCUSSION

Typical recordings obtained by scanning are illustrated in Fig. 1. Calibration curves relating concentration of PA and NAPA to peak areas are shown in Fig. 2. The curves were consistently linear for spots containing 10 to 200 ng.

Recovery from spiked plasma samples was calculated by comparison with the methanol standards which had been spotted directly on the plates, and those data are included in Table I. It is seen that recovery is essentially complete, but it is still recommended that both methanol standards and extracts from spiked plasma samples be run concurrently with samples containing unknown quantities of PA and NAPA so that failure to extract completely might be detected.

Table II presents data obtained by this method on two patients at 2 and 4 h after administration of 250 mg of PA. In these cases, 1-ml samples of plasma were used, although smaller samples would be equally suitable if the extraction residue is redissolved in a smaller volume of dissolving solvent or a larger volume is applied to the plate.

The UV scanner used can be used also to measure fluorescence, and since both PA and NAPA are fluorescent, scanning in the fluorescent mode was com-

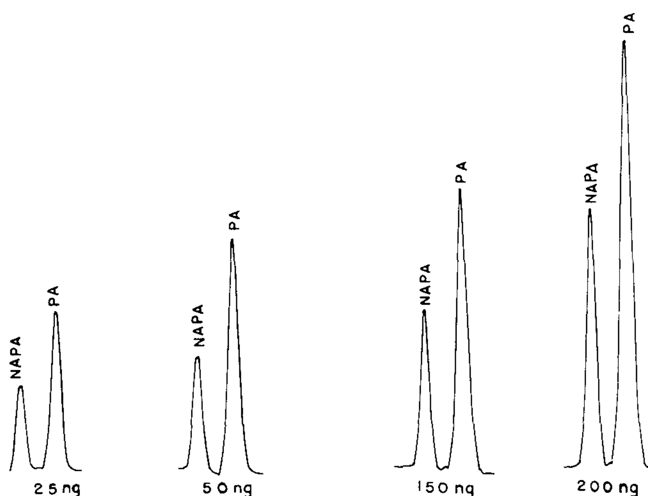

Fig. 1. Peaks observed when scanning the absorbance of procainamide and N-acetylprocainamide standards on silica gel 60.

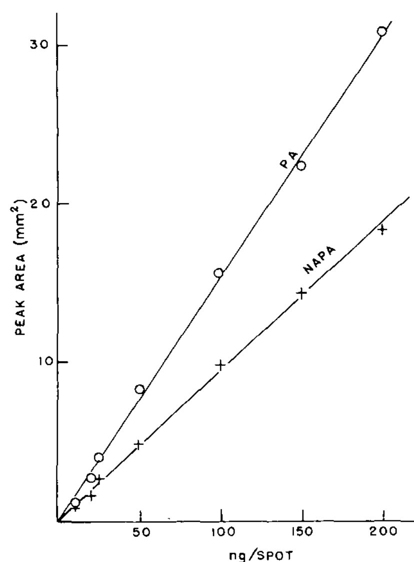

Fig. 2. Procainamide and N-acetylprocainamide standard curves in methanol.

TABLE I

RECOVERY OF PA AND NAPA FROM SPIKED HUMAN PLASMA SAMPLES

Amounts added to plasma (ng/ml)		Number of samples	Recovered (ng/ml)		Percent recovery ± coeff. variation	
PA	NAPA		PA	NAPA	PA	NAPA
25	—	6	25.1	—	100.2 ± 2.49	—
50	—	6	48.9	—	97.9 ± 3.07	—
100	—	6	98.2	—	98.2 ± 0.55	—
200	—	6	191.6	—	95.8 ± 1.68	—
—	25	6	—	22.7	—	90.6 ± 4.78
—	50	6	—	47.7	—	95.4 ± 2.40
—	100	6	—	95.9	—	96.0 ± 1.18
—	200	6	—	201.5	—	100.8 ± 1.48
25	25	3	25.6	26.2	102.5 ± 3.20	104.8 ± 5.82
50	50	3	50.2	49.4	100.4 ± 1.27	98.8 ± 3.20
100	100	3	97.1	100.1	97.1 ± 1.63	100.1 ± 0.79
200	200	3	194.4	200.0	97.2 ± 2.70	100.0 ± 0.72

pared with UV absorption. The sensitivity was found to be no greater and this method was abandoned.

This method was also compared with a published gas chromatographic method [9] on both spiked plasma and patient samples. Results are presented in Table III. It can be seen that the two methods give comparable results, though the gas—liquid chromatographic procedure is not sensitive enough and therefore is not useful below a plasma concentration of 1—2 μg/ml. The lower

TABLE II

PLASMA CONCENTRATION OF PA AND NAPA AT 2 AND 4 h AFTER AN ORAL DOSE OF 250 mg OF PA

Patient	Time after dose (h)	PA concentration (μg/ml)	NAPA concentration (μg/ml)
E.G.	2	4.8	3.0
	4	3.0	2.0
L.R.	2	6.0	3.8
	4	3.8	3.0

TABLE III

COMPARISON OF THE DETERMINATION OF PLASMA CONCENTRATIONS OF PA AND NAPA USING QUANTITATIVE THIN-LAYER AND GAS CHROMATOGRAPHY

Quantities PA and NAPA added (μg/ml)	TLC assay (μg/ml)		GLC assay (μg/ml)	
	PA	NAPA	PA	NAPA
0.5	0.45	0.51	not detected	
0.5	0.52	0.51	not detected	
1.0	1.10	0.95	not detected	
1.0	1.08	1.04	not detected	
2.0	1.92	1.95	1.84	1.90
2.0	1.98	2.03	1.98	1.90
5.0	5.08	4.89	5.00	4.91
5.0	5.0	5.04	4.92	4.95
10.0	9.90	10.05	9.89	9.90
10.0	10.04	9.95	10.06	9.96

limit of sensitivity of this thin-layer chromatography procedure is around 40 times lower than is required for the therapeutic PA plasma concentration levels.

The use of an automatic plate spotter and the thin-layer spectrophotometer equipped with a recorder and integrator makes possible the processing of up to 10 samples on a single plate. Thus, a considerable savings of time in handling a large number of samples is achievable.

This new procedure for the quantitative determination of PA and NAPA in plasma shows that thin-layer chromatography can offer an accurate, fast, sensitive and specific method for quantitative determinations.

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Note

High-performance liquid chromatographic analysis for synthetic corticosteroids in plasma

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Recently, we have developed a rapid, sensitive and specific high-performance liquid chromatographic (HPLC) procedure for the analysis of prednisolone in plasma [1]. This report describes the adaptation of the above procedure to the analysis of dexamethasone, as well as the simultaneous determination of prednisone and prednisolone in plasma.

EXPERIMENTAL

A constant volume high-performance liquid chromatograph (Waters Assoc., Milford, Mass., U.S.A.) consisting of a Model 6000A pump, U6K injector and Model 440 detector at 254 nm was used. The column (250 × 3.2 mm I.D., 316 stainless steel) was packed with LiChrosorb SI-60, 5-μm silica gel (E. Merck, Darmstadt, G.F.R.). A flow-rate of 150 ml/h (2000 p.s.i.) was used.

For the analysis of dexamethasone the internal standard was β-methasone and the mobile phase (I) was composed of glacial acetic acid—ethanol—methylene chloride—*n*-hexane (0.2 : 4 : 30 : 65.8).

For the simultaneous analysis of prednisone and prednisolone, the internal standard was dexamethasone and the mobile phase (II) was glacial acetic acid—ethanol—methylene chloride—*n*-hexane (0.2 : 3.5 : 30 : 66.3).

The procedures for extraction from plasma employed in both assays were identical to that previously reported for prednisolone [1], with the exception that the entire mobile phases consisting of the extracted samples were chromatographed.

RESULTS AND DISCUSSION

Fig. 1a illustrates the chromatogram obtained from the analysis of a plasma sample containing 75 ng/ml of dexamethasone using mobile phase I, while Fig.

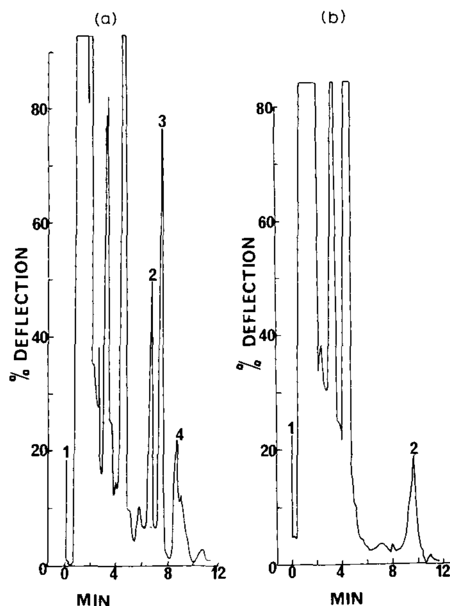

Fig. 1.(a) Chromatogram of extracted human plasma containing dexamethasone (75 ng/ml), mobile phase I. Peaks: 1 = injection, 2 = dexamethasone, 3 = β-methasone, 4 = hydrocortisone. (b) Chromatogram of human blank plasma extract, mobile phase I. Peaks: 1 = injection 2 = hydrocortisone.

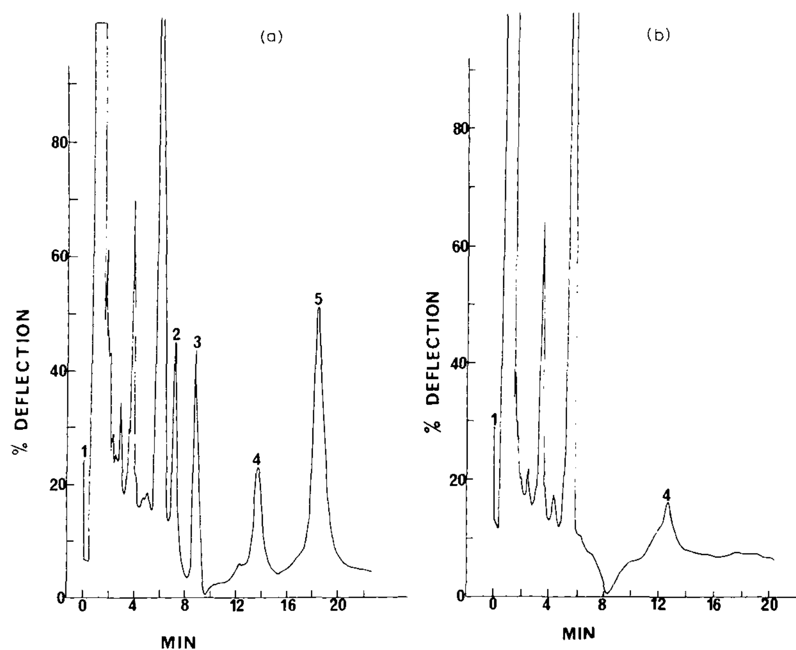

Fig. 2.(a) Chromatogram of extracted human plasma spiked with prednisone (50 ng/ml) and prednisolone (200 ng/ml), mobile phase II. Peaks: 1 = injection, 2 = prednisone, 3 = dexamethasone, 4 = hydrocortisone, 5 = prednisolone. (b) Chromatogram of human blank plasma extract, mobile phase II. Peaks: 1 = injection, 4 = hydrocortisone.

TABLE I

CALIBRATION CURVES FOR THE SYNTHETIC CORTICOSTEROIDS

Steroid	Mobile phase	Slope *	R^2 (n = 12)	Lowest level of quantification (ng/ml)	C.V. (%)
Dexamethasone	I	0.0039	0.998	25	3.2
Prednisone	II	0.0158	0.998	25	5.0
Prednisolone	II	0.0051	0.999	100	5.1

$$\star \text{ Slope} = \frac{\text{Peak height ratio (drug response/internal std. response)}}{\text{Drug Concentrations (ng/ml)}}$$

TABLE II

PLASMA CONCENTRATION FOLLOWING ORAL INGESTION OF SYNTHETIC CORTICOSTEROIDS IN HUMANS

Time (h)	Plasma concentrations (ng/ml)		
	Dexamethasone * (Subject A)	Prednisone ** (Subject B)	Prednisolone ** (Subject B)
1	207.5	27.5	345
2	171.5		
3		43.8	250
4	127.2		
5		24.5	140
6	75.2	21.2	90

* 20 mg dose of dexamethasone powder, dissolved in 2 ml ethanol.
** 20 mg of prednisolone, 4 × 5 mg tablets (Upjohn, Toronto, Canada).

1b illustrates a chromatogram of blank plasma. It is evident that no interfering compounds were extracted from plasma.

Again it is clear from Fig. 2a and b (which illustrate the chromatograms of the spiked sample and corresponding blank), that no endogenous substances interfere with the simultaneous analysis of prednisone and prednisolone.

The calibration curves for the synthetic corticosteroids and the corresponding statistics derived by using linear regression analysis ($y = mx$) are summarized in Table I. Also listed are coefficients of variation at the lowest quantifiable limits, derived from using spiked plasma samples and estimated by the use of the calibration curves. The precision of the assays is evident from the Table.

Table II summarized the plasma drug concentrations following oral administration of 20 mg of dexamethasone to a human subject and 20 mg of prednisolone to another volunteer. These results were compared to those derived by radioimmunological assays and the findings will be incorporated in a separate publication.

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Note

Steroids

V. Rapid thin-layer chromatographic assay for dehydroepiandrosterone in urine

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Dehydroepiandrosterone (DHEA, 3β-hydroxyandrost-5-en-17-one) is an important potential precursor for androgens and oestrogens, and inhibitory effects of DHEA on glucose-6-phosphate dehydrogenase, NADH-oxidase and other less important mechanisms have been found.

The DHEA is excreted in urine mainly as sulphate and glucuronide, whereas the unconjugated steroids occur only in small amounts. Studies by Lieberman et al. [1] showed that most of DHEA is excreted as sulphate. DHEA occurring as the 3β-glucuronide amounts to about 7.5% of the total [2].

Based on the fact that buffer hydrolysis described by Fotherby [3] is specific for the 3β-sulphates of 5-unsaturated steroids, and that neither sulphates nor glucuronides of androsterone and etiocholanolone hydrolyze [4], a rapid gas chromatographic assay for DHEA has been proposed [5]. The small amount of DHEA excreted as glucuronide is not determined by this method. The purpose of this paper is to present a method that can be applied in serial work in clinical laboratories in which a gas chromatograph is not available. The applicability, accuracy, precision, sensitivity, and specificity are comparable to those obtained with gas chromatographic assays for DHEA in urine [5].

REAGENTS AND EQUIPMENT

DHEA sulphate was obtained from Sigma (St. Louis, Mo., U.S.A.). The solvents, except for methanol and ethanol, were redistilled, and the other chemicals used were of reagent grade. Ready-made Silufol silica gel foils (Kavalier, Sklárny, Czechoslovakia) of dimensions 15 × 15 cm were used for thin-layer

chromatography. Absorbance readings were made with a Unicam SP 1800 spectrophotometer using glass cells of 1 cm light path.

Solutions

The following solutions were prepared: 2 *M* acetate buffer, pH 4.5 (88.5 ml of acetic acid + 136 g of sodium acetate and water to 500 ml); 2,4-dinitrophenylhydrazine reagent (15 mg of 2,4-dinitrophenylhydrazine in 50 ml of absolute benzene; stored at 4°); and 0.3% trichloroacetic acid in absolute benzene.

PROCEDURE

The procedure consists in the following steps.

(a) Hydrolysis and DHEA extraction

Conical ground-glass stoppered tubes (120 × 22 mm I.D.) were used for the hydrolysis. Urine (10 ml) is adjusted with 1 ml of 2 *M* acetate buffer to a pH of approximately 4.5 and heated for 2½ h at 100° to hydrolyze DHEA sulphate.

The well cooled mixture is shaken for 5 min with 10 ml of cyclohexane—diethyl ether (1 : 1) on a horizontal shaker at approximately 200 strokes/min. Urine samples do not form emulsions if they are cooled well before shaking.

The bottom phase is always removed by suction with the help of a capillary tube. The extract is then shaken for 1 min with 5 ml of 10% sodium hydroxide solution, then for 1 min with 10 ml of water, and centrifuged; then the aqueous phase is removed. The organic phase is evaporated to dryness.

(b) Reaction of DHEA with 2,4-dinitrophenylhydrazine

The preparation of the 2,4-dinitrophenylhydrazine was carried out by a slightly modified version of the method of Treiber and Oertel [6]. To the above residue, 0.5 ml of 2,4-dinitrophenylhydrazine reagent and 0.2 ml of trichloroacetic acid are added, then the samples are incubated at 70—80° in the dark for 10 min and subsequently evaporated slowly to dryness.

(c) Thin-layer chromatography and quantitation

The above residue is dissolved in a few drops of benzene containing 10% of ethanol, then the solution is applied on plates of silica gel divided into 2-cm strips with lines 1 mm broad. Excellent separation is achieved in chloroform—acetone (95 : 5) by the ascending technique in two runs; very often one run is sufficient for the separation. The time of development is 25 min. The R_F values of DHEA 2,4-dinitrophenylhydrazone after the first and second developments are 0.26 and 0.46, respectively. The spots on the thin layer of silica gel are cut out and placed in glass tubes.

The strips are eluted by gentle shaking on a horizontal shaker for 10 min with 3 ml of methanol. Absorbances were read at 366 nm.

RESULTS AND DISCUSSION

Evaluation of the method

Specificity. The specificity of the method is ensured by the combination of selective hydrolysis under the conditions described above, solvent extraction,

utilization of a colour reaction that is specific only for some oxosteroids and chromatographic separation. The exception in the selective hydrolysis, viz., epiandrosterone, was discussed earlier [5] and is of little practical importance.

Other sulphates of 3β-hydroxysteroids without an oxo group which might possibly be present in urine in amounts approximately comparable to that of DHEA, and which would be expected to hydrolyze, e.g., 5-pregnenediol (5-pregnene-3β, 20α-diol) and 5-pregnenolone (3β-hydroxy-5-pregnen-20-one), do not react with 2,4-dinitrophenylhydrazine. The R_F values of related oxosteroids in the system chloroform—acetone (96 : 4) have been given previously [7].

Precision. To determine the precision, 18 samples containing 1, 2 and 4 mg/l of DHEA sulphate were analyzed. The coefficient of variation was 5.8-8.0%.

Sensitivity. The sensitivity for the substance to be determined, defined as the least amount significantly differing from zero, can be calculated theoretically [8,9] from replicate analyses of the blank extracted from the plate of the non-hydrolyzed urine. This gave an arithmetic mean of absorbance 0.015 and a standard deviation of ±0.0039. Based on these values, the theoretical sensitivity of the method is about 0.1 mg of DHEA in 1000 ml of urine with a probability of 95%.

Accuracy and recovery. The standard deviations of duplicate urine assays were 0.07 and 0.08 mg/l for DHEA concentrations of 1 and 4 mg/l, respectively (24 samples in each instance). The recovery was measured by determining DHEA sulphate in urine containing 1.4 mg of conjugate per 1000 ml. For different urine samples run in triplicate the recovery was 90—97%. By assaying urine at two dilutions (urine to water ratios of 1 : 0 and 1 : 1), the recovery after dilution was 104%.

Capacity. Using this method, one technician can perform 18 determinations in one day.

Normal values. It has been reported that DHEA sulphate is secreted by the human adrenal gland in amounts comparable with cortisol [10]. The level of DHEA in the urine of an adult human undergoing constant physiological changes depends on many factors, such as age, sex and physical activity. The daily excretions of DHEA for 10 normal men and 10 normal women aged 20—40 years were 2.08 ± 1.54 (0.4—6.0) and 1.62 ± 1.09 (0.3—3.5) mg per 24 h, respectively. Comparisons of values reported in various papers for the excretion of DHEA by normal adults [5] and children [11] have been published. It is possible to conclude that the determination of urinary DHEA could be used as an index of abnormal adrenocortical function.

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Note

Improved statistical method for the calculation of protein concentration by Laurell monorocket immunoelectrophoresis

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'Monorocket' immunoelectrophoresis, first described by Laurell [1] is a simple, precise and sensitive means of assaying specific proteins. In this technique immunoprecipitates are formed in agarose gels in rocket-shaped peaks, the area of which is proportional to the amount of antigen applied. Because large numbers of area measurements are tedious and difficult to make without an automated planimeter, approximations such as peak height measurements or triangulation techniques are used to simplify the method. The use of peak height alone is satisfactory only over a limited antigen concentration range on any one plate and, although triangulation may increase this range it is still only an approximation to the true area. Therefore we report a programme suitable for use in a desk top computer to better describe the relationship between the antigen concentration and peak height, which is the simplest measurable and precise parameter.

METHOD

The calculator used was a Wang 600. Laurell 'monorocket' immunoelectrophoretic plates were run as described by Laurell [1] using antisera specific for a number of human serum proteins, a minimum of 4 standards per plate being used, to allow statistical calculation of the three coefficients.

The programme is a modification of one supplied by Wang for the calculation of (i) the least squares estimates $\hat{a}$ and $\hat{k}$ to the line $y = ax + k$ and (ii) the correlation coefficient r. In the new programme*, the variable x^b replaces x,

*Full details of the programme will be available on request.

and the value of b giving the highest correlation coefficient is determined by trial and error, together with the appropriate values of $\hat{a}$ and $\hat{k}$.

The formulae are:

$$r = \frac{n\Sigma x^b y - (\Sigma x^b)(\Sigma y)}{\sqrt{[n\Sigma x^{2b} - (\Sigma x^b)^2][n\Sigma y^2 - (\Sigma y)^2]}}$$

$$\hat{a} = \frac{n\Sigma x^b y - (\Sigma x^b)(\Sigma y)}{n\Sigma x^{2b} - (\Sigma x^b)^2}$$

$$\hat{k} = \frac{\Sigma y - \hat{a}\Sigma x^b}{n}$$

where n = number of points on standard curve; y = peak height; x = protein concentration.

To reduce computing time to a minimum, b is limited between 0.2 and 1 and the precision to within ± 0.0005, although values below 0.5 were exceptional (*i.e.* the curve was between a parabola and a straight line).

RESULTS

Table I shows the correlation coefficients obtained for different human serum proteins using the following three mathematical approaches:

(1) [height] = a [concentration] + k

(2) [Δ area] = a [concentration] + k

(3) [height] = a [concentration]^b + k

Approach No. 3 gave for most proteins better results than either of the other two, although the difference between 3 and 2 was often not significant because there is one extra coefficient in 3 and thus one less degree of freedom.

DISCUSSION

'Monorocket' immunoelectrophoresis is on theoretical grounds more accurate than the other simple method of specific protein assay, i.e. single radial diffusion [2]. The quantity measured, height, in monorocket immunoelectrophoresis, is usually greater than the diameter, in single radial diffusion, thus producing in the former a smaller relative error. In addition, the relative error in the latter is doubled, because antigen concentration is proportional to the square of the diameter, whereas using the approach outlined here for the monorocket technique the relative error in measuring peak height is multiplied by a factor between 1 and 2, ($1/b$).

Peak area approximations obtained here by triangulation give correlation coefficients similar to those using the computer; however, they are manually more tedious to measure, requiring an enlarger or projector to reduce the error in width measurement, and they also need mathematical processing.

TABLE I

CORRELATION COEFFICIENTS FOR DIFFERENT HUMAN SERUM PROTEINS

Abbreviations: RBP = retinol binding protein; TBPA = thyroxine binding prealbumin; TBG = thyroxine binding globulin; PAG = pregnancy associated α_2-glycoprotein.

Serum protein	Molecular weight $\times 10^{-3}$	Number of points	Correlation Coefficients		
			(1) height (linear)	(2) area (linear)	(3) height (power)
RBP	21	5	0.9902	0.99585	0.99899
α_1-Antitrypsin	54	5	0.9940	0.99962	0.99987
TBPA	55	6	0.9966	0.99787	0.99932
TBG	65	4	0.9940	0.99915	0.9999998
Albumin	68	5	0.9878	0.99814	0.99961
PAG	500—650	4	0.9946	0.99992	0.99994
α_2-Macroglobulin	820	5	0.9976	0.99982	0.99924
β-Lipoprotein	2400	6	0.9912	0.99883	0.99970

In conclusion, therefore, the use of this simple statistical approach increases the accuracy and reduces the number of measurements required to perform the technique.

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CHROMBIO. 032

Note

A rapid micromethod for the high-performance liquid chromatographic determination of theophylline in human serum

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Theophylline (1,3-dimethylxanthine) has been used extensively for the treatment of asthma and more recently for the management of apnea and bradycardic spells in premature infants. The absorption of theophylline varies considerably from subject to subject so that blood levels are not predictable even when the same unit dosage (mg/kg) is administered. Since adverse drug reactions have been associated with serum theophylline concentrations in excess of therapeutic levels, the rational clinical use of this agent may be facilitated by careful monitoring of serum levels.

Several methods have been described for the determination of theophylline in biological fluids. The spectrophotometric assay [1] has been largely replaced by gas chromatographic (GC) [2—9] and high-performance liquid chromatographic (HPLC) [10—15] techniques. Most of the GC procedures involve solvent extraction of the drug followed by derivatization and are often time consuming. The HPLC methods, on the other hand, do not require derivatization, but most of the reported procedures require solvent extraction.

A new HPLC procedure for the determination of theophylline in serum has been developed in which proteins are removed by perchloric acid precipitation and, after neutralization of the supernatant, an aliquot is injected directly into the chromatographic system. Serum volumes as low as 50 μl can be analyzed successfully; this makes the method particularly attractive for pediatric work.

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EXPERIMENTAL

All chemicals used were reagent grade with the exception of methanol, which was glass distilled (Burdick & Jackson Labs., Muskegon, Mich., U.S.A.).

Procedure

A 100-μl serum specimen was pipetted into a tube containing 6 μl of 70% perchloric acid. After mixing for 30 sec on a Vortex mixer, the tube was kept in ice for 20 min; this was followed by centrifugation at room temperature to remove the denatured proteins. The supernatant was transferred to another tube and 10 mg of potassium carbonate were added. The contents were mixed on a Vortex mixer and centrifuged briefly to precipitate the potassium perchlorate formed. An aliquot was then injected on the column.

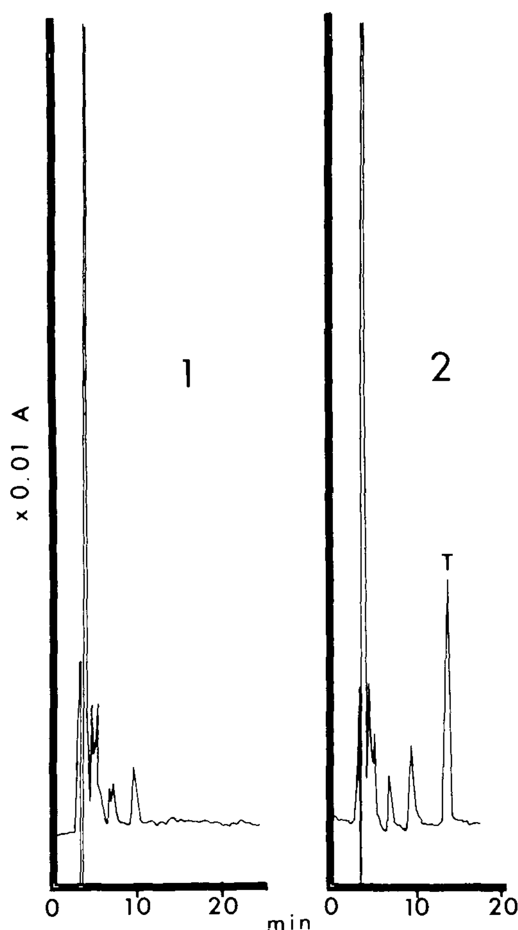

Fig. 1. HPLC of human serum samples. 1, Control serum; 2, serum containing 10 μg/ml theophylline (T). Conditions: 30 cm × 4 mm I.D. μBondapak C_{18} column, with methanol—10 m*M* sodium dihydrogen phosphate (1:4) as eluent; flow-rate 0.8 ml/min; detection at 280 nm.

Liquid chromatography

Analyses were performed on a Waters Assoc. Model 6000 pumping system coupled to a Spectroflow SF 770 multiwavelength detector (Schoeffel Instrument Corp.). A reversed-phase system consisting of a 30 cm × 4 mm μBondapak C_{18} column (Waters Assoc.) and methanol—10 m*M* sodium dihydrogen phosphate (1:4) as eluent at a flow-rate of 0.8 ml/min were used. Absorbance was monitored at 280 nm. The detector was operated at a sensitivity of 0.01 a.u.f.s. Peak heights were used for quantitation. All standard curves were linear and passed through the origin.

RESULTS AND DISCUSSION

Representative chromatograms of serum samples shown in Fig.1 demonstrate that control samples are free from contaminating peaks. Dietary xanthines, caffeine and theobromine and theophylline metabolites did not interfere with the assay. The average recovery of theophylline added to serum over the range of 1 to 25 μg/ml was 88.2 ± 4.9% (mean ± S.E., n = 16). The lower limit of detection was 1 μg/ml serum.

This technique is very simple and involves no lengthy solvent extraction or derivatization procedures. When sample size is limited, as in the case of premature infants being treated for apneic spells, the whole procedure can be successfully performed with 50 μl of serum. Most earlier chromatographic procedures require larger sample volumes. Very recently Least et al. [9] have reported a GC method that can be performed on 20-μl samples; however, their method involves extraction and derivatization. The method described in this paper is currently being used for pharmacokinetic studies of theophylline in premature and full-term infants and the results will be published at a later date.

ACKNOWLEDGEMENTS

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CHROMBIO. 048

Book Review

Separation methods in biochemistry, by C.J.O.R. Morris and P. Morris, Pitman, London, 2nd ed., 1976, VII + 1045 pp., price £ 32.50, ISBN 0-237-00716-5.

The second edition of Morris & Morris's *Separation methods in biochemistry* fills a gap in the literature that mainly affects chemists and biochemists approaching the field of separation techniques in its whole complexity. In spite of the large size of the volume, it is difficult to believe that a reader willing to devote himself to a specialized separation method would find all of the desired information in this volume, as he probably would do in a specialized monograph. The fact that the whole book has only two authors is reflected in the uniform approach to different separation techniques; on the other hand, it is obvious that the diverse separation techniques are far from equally familiar to the authors.

The twelve years that have passed since the first edition is a long period for rapidly developing separation methods, and the authors therefore had little choice but to rewrite the whole book. Numerous, today classical, techniques have been reduced in their allocation of pages (adsorption chromatography, ion-exchange chromatography, paper chromatography), while sections on other techniques that have enjoyed rapid development have been substantially enlarged (electrophoresis, gel chromatography, gas chromatography). Chapters on thin-layer chromatography and affinity chromatography have been newly included. As a result of these changes the number of pages has been increased by about 20%. New and extended tables of materials suitable for different types of chromatography will certainly be welcomed, together with the conditions suitable for some types of separations. A characteristic feature of this book is the great care with which the basic principles and theory of each method described are presented. The relatively large proportion of such chapters (about 25%) is, however, questionable in a book which is intended to offer the first acquaintance with a particular technique.

There are some features of the book that could be criticised. The title is misleading as most of the contents are devoted to chromatographic and electrophoretic techniques. The appearance of a book in its second edition places higher demands upon the authors and some discrepancies that could be pardoned in the first edition cannot be overlooked in the second. An example is the unfortunate classification of separation methods. The fact that chromatographic methods are categorized both according to the experimental arrangement (thin-layer

chromatography, paper chromatography, gas chromatography) and according to the principle of separation (adsorption, partition, ion exchange, molecular sieve) may be very misleading for a beginner, as all of these principles may occur not only in liquid column chromatography, but also in flat-bed chromatography. Another unfortunate fact is that when it appeared (1976) the book was already out of date. Most of the references are prior to 1972, and on only a few occasions is the literature quoted more recent. In the chapter devoted to gas chromatography, out of 161 references only a single one is more recent than 1971. In the third chapter, dealing with column chromatography, only two references out of a total of 96 are later than 1971. This aspect automatically makes the book imbalanced, because those techniques which have been developed mainly within the last 5 years (high-performance liquid column chromatography, affinity chromatography, etc.) receive inadequate attention. Finally, one can perhaps object that not all of the chapters are well arranged and easy to read; a few are far to long, not well laid out and have misleading titles. Sometimes it can be difficult to find a particular subject within this large volume.

Besides objections about the scope of the book and the editorial work, one can also list minor imperfections such as the fact that affinity chromatography is referred to in the chapter about applications to protein chemistry without a single mention in techniques. In chapters devoted to partition, adsorption and ion-exchange chromatography, the new types of sorbents used for high-performance liquid chromatography are hardly mentioned. In ion-exchange chromatography the reader is likely to miss silica-gel ion exchangers. In gas chromatography there is no mention of Kováts indices. A chapter about quantitative analysis (including integrators) would have been worth including. Also, the modern gas chromatography—mass spectrometry combination should have been dealt with in more detail. In general, very little attention has been paid to instrumentation, which is stressed mainly in chapters that deal with non-chromatographic procedures. The special demands put upon separations of radioactively labelled compounds also have not been included.

To summarize, although the book suffers from some drawbacks, it is generally a good text for those who wish to obtain a general insight into separation techniques in biochemistry and related fields of applied chemistry.

Prague (Czechoslovakia) K. MACEK and Z. DEYL

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Proceedings of the First International Symposium, State University of Ghent, Belgium, 16 - 18 June, 1976.

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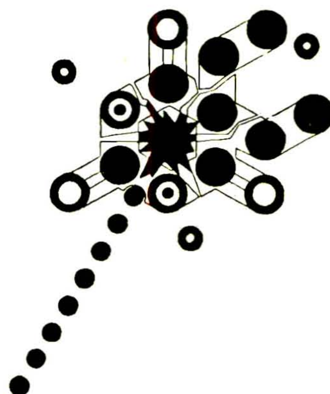

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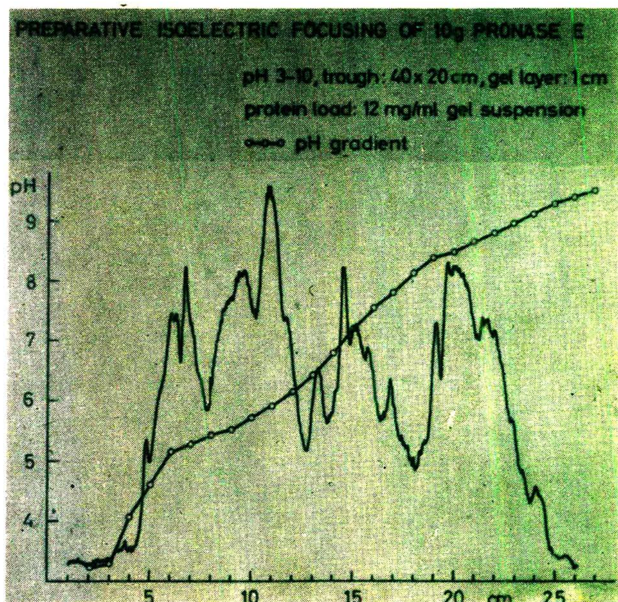

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