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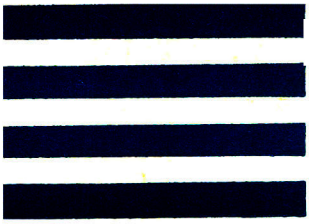
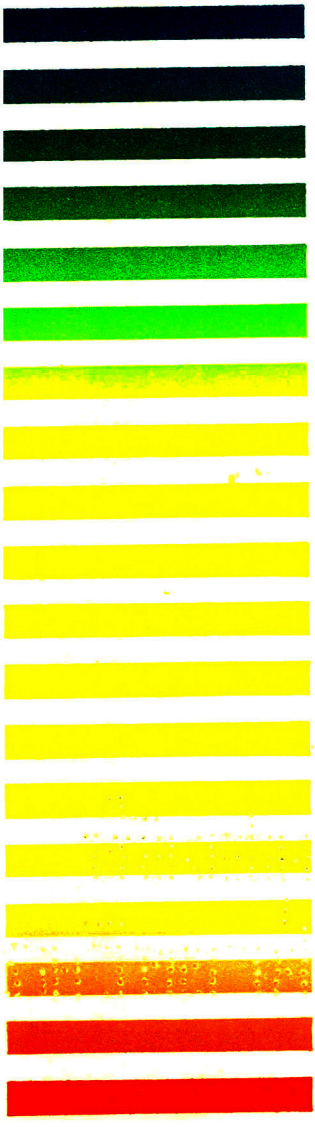
(Biomedical Applications, Vol. 3, No. 2)



JOURNAL OF CHROMATOGRAPHY BIOMEDICAL APPLICATIONS



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PUBLICATION SCHEDULE FOR 1978

Journal of Chromatography (incorporating *Chromatographic Reviews*) and *Journal of Chromatography, Biomedical Applications*

MONTH	J	F	M	A	M	J	J	A	S	O	N	D
Journal of Chromatography	147 148/1	148/2 149	150/1 150/2	151/1 151/2 151/3	152/1 152/2	153/1 153/2	154/1 154/2	155/1 155/2 156/1	156/2 157	158 160/1	160/2* 161	
Chromatographic Reviews		159/1				159/2				159/3		
Biomedical Applications	145/1		145/2		145/3		146/1		146/2		146/3	

* Cumulative indexes Vols. 141–160.

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, 1000 AR Amsterdam, The Netherlands
or to:
The Editor of *Journal of Chromatography, Biomedical Applications*, P.O. Box 681, 1000 AR 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, 1000 AE Amsterdam, The Netherlands. The *Journal of Chromatography*, *Biomedical Applications* can be subscribed to separately.

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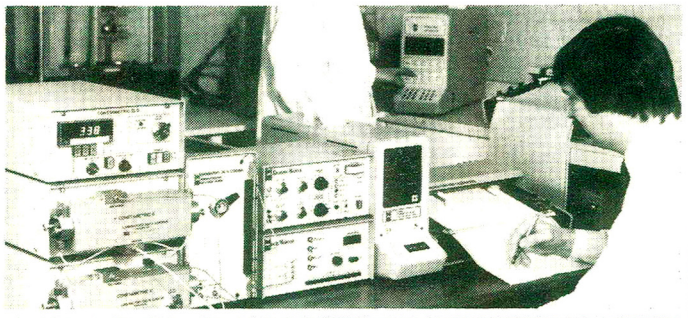

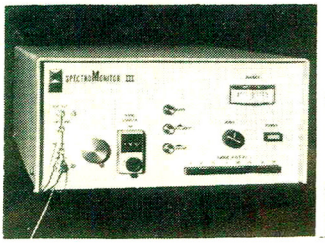

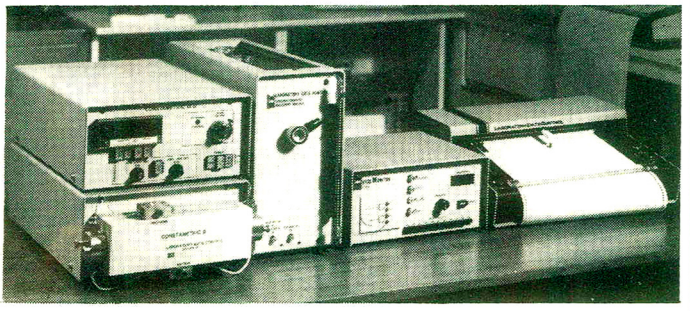

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Volume 16

POROUS SILICA

Its Properties and Use as Support in Column Liquid Chromatography

by K. K. Unger

1979. about 300 pages
Price: US $52.25 / Dfl. 120.00
ISBN 0-444-41683-8

Although an enormous amount of literature exists about basic surface chemistry of silica on the one hand and about its use as packing in high-performance liquid chromatography on the other, no comprehensive and systematic survey has previously been published to cover both subjects.

This book bridges the gap between these two treatments and provides the chromatographer with full information on the properties of silica and its chemically bonded derivatives in context with its chromatographic behaviour. The book is divided into two parts: the first deals with the physical and chemical properties of silica including pore structure, surface chemistry, particle preparation and characterization, while the second surveys the wide-spread application of untreated and chemically modified silica as adsorbent, support and ion exchanger in the four modes of HPLC, i.e. adsorption, partition, ion-exchange and size-exclusion chromatography. A separate chapter is devoted to packing procedures and performance of silica columns.

The text is illustrated throughout by rich experimental data presented in the form of numerous tables and figures, and the book will be of undoubted value to all who use silica in HPLC and who seek to choose the optimum silica packing for a given separation problem.

CONTENTS: General chemistry of silica. Pore structure of silica. Surface chemistry of porous silica. Particle characteristics. Silica columns – packing procedures and performance characteristics. Silica and its chemically bonded derivatives as adsorbents in liquid–solid chromatography. Silica as support in liquid–liquid chromatography. Chemically modified silica as packings in ion-exchange chromatography. Silica as a packing in size-exclusion chromatography. Subject index.

Volume 15

ANTIBIOTICS

Isolation, Separation and Purification

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

Aug. 1978. x + 772 pages
Price: US $84.75 / Dfl. 195.00
ISBN 0-444-41727-3

This book has been written in response to the great interest currently being shown in modification of some of the older, and many newer antibiotics to improve upon existing, naturally produced compounds.

Twenty-four eminent scientists in the field of antibiotic isolation have contributed chapters on key chemical families of antibiotics, with emphasis placed on isolation, separation and purification of these substances, many of which can be used as starting materials for further modification. In addition to the detailed descriptions of these procedures (in many instances giving assay and detection techniques), the authors have also provided brief summaries of the chemical, physical and biological properties, usage and structural formulae of many of the compounds. Isolation methods include solvent and resin extractions, counter-current distribution, gas–liquid and high-pressure chromatography and electrophoresis. Many of the recently discovered naturally produced antibiotics are also discussed.

The strong emphasis on isolation methodology is a particularly valuable feature of the book, as those seeking information on this aspect of antibiotic production have previously had to consult a myriad of journal papers. It will however appeal to all involved in the field of antibiotics and will provide useful background material for those not directly involved with isolation technology.

CONTENTS: Actinomycins *(A. Mauger and E. Katz)*. Ansamycins *(A. Ganguly)*. Cephalosporin Antibiotics *(R. L. Hamill and L. W. Crandall)*. Coumarin-Glycoside Antibiotics *(J. Berger and A. D. Batcho)*. 2-Deoxystreptamine-Containing Antibiotics *(J. A. Marquez and A. Kershner)*. Griseofulvins *(G. H. Wagman and M. J. Weinstein)*. Lincomycin Related Antibiotics *(T. E. Eble)*.

Volume 8

CHROMATOGRAPHY OF STEROIDS

by E. Heftmann

1976. xiv + 204 pages
Price: US $ 39.25 / Dfl. 90.00
ISBN 0-444-41441-x

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. Currently used techniques are described in detail.

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 7

CHEMICAL DERIVATIZATION IN LIQUID CHROMATOGRAPHY

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

1976. viii + 214 pages.
Price: US $ 39.25 / 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 provides a comprehensive account of modern derivatization in liquid chromatography with special emphasis on the practical aspects.

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 $ 69.75 / 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. 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 concerned mainly with the analytical aspects.

Volume 5

INSTRUMENTAL LIQUID CHROMATOGRAPHY

A Practical Manual on High-Performance Liquid Chromatographic Methods

by N. A. Parris

1976. x + 330 pages
Price: US $ 43.50 / 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.

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 apects 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 4

DETECTORS IN GAS CHROMATOGRAPHY

by J. Ševčík

1976. 192 pages
Price: US $ 30.50 / Dfl. 70.00
ISBN 0-444-99857-8

The first systematic treatment of gas chromatographic techniques, this publication 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).

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 $ 125.95 / Dfl. 290.00
ISBN 0-444-41156-9

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.

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.

Volume 2

EXTRACTION CHROMATOGRAPHY

edited by T. Braun and G. Ghersini

1975. xviii + 566 pages
Price: US $ 60.95 / Dfl. 140.00
ISBN 0-444-99878-0

Volume 1

CHROMATOGRAPHY OF ANTIBIOTICS

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

1973. ix + 238 pages
Price: US $ 41.50 / Dfl. 95.00
ISBN 0-444-41106-2

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.

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Volume 14
RADIOCHROMATOGRAPHY
The Chromatography and Electrophoresis of Radiolabelled Compounds

by T. R. Roberts

May 1978. x + 174 pages
Price: US $39.95 / Dfl. 90.00
ISBN 0-444-41656-0

The aim of this book is to describe and discuss all of the various radiochromatography and radioelectrophoresis methods in a single volume. For each technique, the historical development is outlined and the relative merits of the radiochemical detection methods currently available are assessed. This is followed by a discussion of the method of choice for any particular application. Each chapter also describes in detail the practical aspects of the various techniques and provides examples of applications taken from the recent literature.

This work will be of great value to workers with only limited experience of radiochromatography. It will be particularly valuable in enabling the inexperienced worker to select the optimum method for his situation.

Radiochromatography will be of special interest to industrial workers and academics in the fields of pesticide metabolism, drug metabolism and biochemical analysis.

CONTENTS: Introduction. Radioactivity detectors used in chromatography. Radio-paper chromatography. Radio-thin-layer chromatography. Radioelectrophoresis. Radio-column chromatography. Radio-gas-liquid chromatography. Miscellaneous applications related to radiochromatography. Appendix. Subject index.

Volume 13
INSTRUMENTATION FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

by J. F. K. Huber

Aug. 1978 xii + 204 pages
Price: US $34.75 / Dfl. 80.00
ISBN 0-444-41648-X

A practical guide for all those involved in the application of column liquid chromatography, this book provides a valuable, up-to-date review of the large selection of instrumentation currently available, describing the general design features and the specific technical solutions in the instrumentation for high-performance liquid chromatography. For the purposes of this survey, the chromatographic system has been divided into a number of interacting sub-systems and these are described in detail by experts in the field. Special emphasis is given to discussion of the general principles of design which will remain relevant even if new technical solutions are found in the future.

The final chapter comprises a useful compilation of commercially available chromato graphs together with their specifications.

CONTENTS: The chromatographic apparatus from the viewpoint of system theory *(J. F. K. Huber)*. Pump systems *(M. Martin and G. Guiochon)*. Solvent gradient systems *(M. Martin and G. Guiochon)*. Sample introduction systems *(J. C. Kraak)*. Column design selection *(J. C. Kraak)*. Components and accessories for preparative high-performance liquid chromatography *(A. Wehrli)*. Detectors based on the measurement of optical and electrical properties of the mobile phase *(H. Poppe)*. Electrochemical detectors *(H. Poppe)*. Radiometric detectors *(P. Markl)*. Combination of liquid chromatography and mass spectrometry *(E. Kenndler and E. R. Schmid)*. Specifications of commercial liquid chromatographs *(R. R. Becker)*. Subject index. Manufacturer index.

Volume 12
AFFINITY CHROMATOGRAPHY

by J. Turková

April 1978. x + 406 pages
Price: US $ 69.75 / Dfl. 167.00
ISBN 0-444-41605-6

This book reviews the application of affinity chromatography for the isolation of various biologically active substances. The bibliographic review comprises almost 1,400 references and is completed by data on use of solid supports and spacers. Great attention is given to the review of the most commonly used solid supports and to the method of attachment, together with the methods of characterization of both the carriers and the immobilized affinity ligands.

This extensive and up-to-date review is intended mainly for biochemists and biologists. It will be particularly useful to clinicians engaged in human or veterinary medicine, as well as to those dealing with chromatography and industrial chemistry.

CONTENTS: Introduction. The principle, history and use of affinity chromatography. Theory of affinity chromatography. Application of affinity chromatography to the quantitative evaluation of specific complexes. General considerations on affinant–sorbent bonding. Choice of affinity ligands for attachment. Hydrophobic chromatography, covalent affinity chromatography, affinity elution and related methods. Solid matrix supports and the most used methods of binding. Characterization of supports and immobilized affinity ligands. General considerations on sorption, elution and non-specific binding. Examples of the use of affinity chromatography. Immobilized enzymes. Subject index. List of compounds chromatographed.

Volume 11
LIQUID CHROMATOGRAPHY DETECTORS

by R. P. W. Scott

1977. x + 248 pages
Price: US $ 36.75 / Dfl. 84.00
ISBN 0-444-41580-7

The rapid development of liquid chromatography over the past decade has been due to the introduction of highly sensitive linear liquid chromatography detectors. This book provides a comprehensive treatment of the function and optimal working conditions of liquid chromatography detectors. It is divided into four parts.

Part 1 includes a detailed discussion of properties of the detecting system that can impair column performance and how these effects can be minimized. In Parts 2 and 3, the various types of detectors that have been developed are described and a detailed treatment given of commercially available detectors. Part 4 discusses the practical operation of liquid chromatography, including methods for quantitative analysis as well as practical hints on detector operation and special detector techniques. The final chapter deals with spectroscopic detectors and provides a detailed description of LC/UV and LC/MS systems.

This work is particularly useful because of the presentation of the necessary detector specifications which enables readers to make a rational comparison of the performance of one detector with that of another.

CONTENTS: Introduction. General characteristics of liquid chromatography detectors. Bulk property detectors. Solute property detectors. The use of detectors in liquid chromatography. Subject index.

Volume 10
GAS CHROMATOGRAPHY OF POLYMERS

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

1977. xiv + 226 pages
Price: US $ 44.95 / Dfl. 103.00
ISBN 0-444-41514-9.

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.

Volume 9
HPTLC – HIGH PERFORMANCE THIN-LAYER CHROMATOGRAPHY

edited by A. Zlatkis and R. E. Kaiser

1977. 240 pages
Price: US $ 47.95 / 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.

Journal of Chromatography, 146 (1978) 185—196
Biomedical Applications

CHROMBIO. 177

REVIEW

THE PLACE OF GAS CHROMATOGRAPHY — MASS SPECTROMETRY IN CLINICAL CHEMISTRY

H.M. LIEBICH

Medizinische Universitätsklinik, Otfried-Müller-Strasse 10, 7400 Tübingen, (G.F.R.)

(Received February 22nd, 1978)

CONTENTS

1. INTRODUCTION

Mass spectrometry (MS) in its modern and advanced form has been used for approximately ten years in clinical and clinically related laboratories. However, it should not remain unmentioned that MS techniques have been applied in clinical research for more than twenty years in the analysis of respiratory and blood gases. It was employed in lung function tests to determine nitrogen, oxygen and carbon dioxide in expired air, sampled over a mouth piece, and to perform regional analyses within the bronchial tree in diagnostic bronchoscopy [1]. The technique is suitable for instantaneous and continuous gas measurements. For continuous in vivo determinations of O_2- and CO_2-pressures in animals the gases were sampled from circulating blood through a permeable membrane at the tip of an intravascular cannula which was connected to the mass spectrometer [2]. In general, the instruments used for respiration gases are built for small mass ranges, e.g. *m/e* 18—80 [2] and have low resolution. In spite of the potentials of the technique for gas analyses MS has not been widely

accepted in this area. The reasons for this were summarized by the following comment in The Lancet in 1960: "Unfortunately a mass spectrometer is a formidable instrument, only likely to be found in specialized laboratories" [3]. Even today many clinical chemists are reluctant to use MS.

The object of this paper is to attempt a brief survey of the potentials and the current situation of MS in clinical chemistry and clinical biochemistry. To present a complete review of the subject would exceed the scope of the paper. Only the main aspects shall be outlined, and technical questions cannot be discussed. A recent and detailed review has been written by Lawson [4]. Since the majority of the problems in clinical chemistry using MS apply to its coupling with gas chromatography (GC), GC—MS combination will be emphasized.

GC is a method used for separating volatile compounds and all substances which can be made volatile by derivatization. Because of its flexibility and broad applicability it has been, until now the most frequently used analytical separation method. No other method equals GC in separating power and efficiency. Using calibration curves and internal standards, quantitative analyses with high precision are possible.

MS is an analytical technique for: (i) identification of unknown substances; (ii) sensitive and highly specific detection of known substances.

For the clinical chemist the combination of GC and MS can be an almost ideal analytical system for the following problems:

(i) Identification of unknown substances of low concentration in complex mixtures of biological origin. Depending on the chemical properties of the substances 10—100 ng of injected material are sufficient for identification.

(ii) Specific qualitative detection of known substances. The detection limit is 1—100 pg.

(iii) Specific quantitative determination of known substances. Depending on the chemical nature of the substance, the detection limit is approximately 5—100 pg.

(ii) and (iii) are achieved by mass fragmentography (MF), also called selective ion monitoring (SIM), either by single ion detection (SID) or multiple ion detection (MID).

Substances which cannot be made volatile are not amenable to analysis by GC—MS. For such substances the combination of liquid chromatography with MS would be a most valuable addition to GC—MS.

Considering the efficiency of the GC—MS system it appears understandable that until now the following attributes are still not completely eliminated from GC—MS: high costs, complexity, need for skilled personnel, frequent maintenance requirements.

By attaching a computer to the GC—MS system (GC—MS—COM), the potentials are enhanced considerably, and the following operations become feasible:

(i) Automatic repetitive scanning, enabling a more complete and less tedious analysis of complex profiles.

(ii) Storage and documentation of the MS data, with the possibility of presentation on display or plotter.

(iii) Spectrum transformations, background subtraction and further manipulations to facilitate spectrum identification.

(iv) Elemental composition analysis using high MS resolving power, to give additional information on unknown substances.

(v) Computer matching of recorded spectra against files of reference spectra.

(vi) Computer MF, enabling selective detection of various, not pre-selected compounds and classes of compounds after a single GC—MS run. Spotting of known, and searching for predicted, substances.

In Fig. 1 the GC—MS—COM system of our laboratory is schematically represented. Similar configurations are used elsewhere. An example of a GC—MS—COM analysis of volatile metabolites in urine using automatic repetitive scanning is given in Fig. 2.

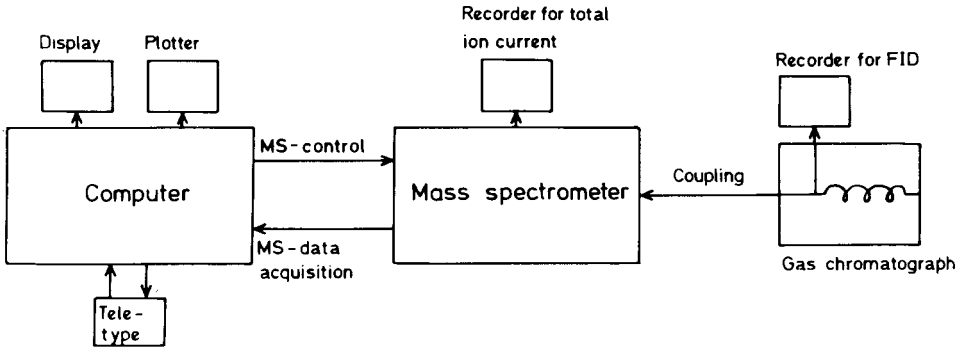

Fig. 1. Configuration of a GC—MS—COM system.

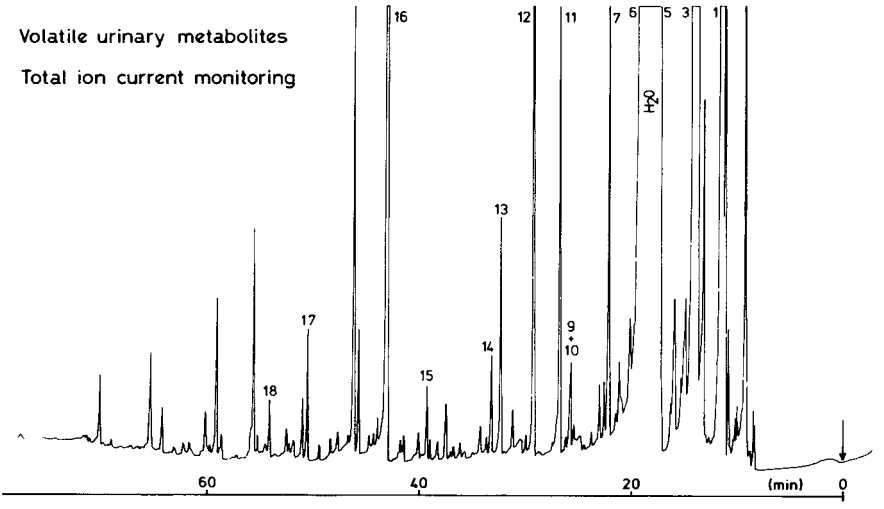

Fig. 2. GC—MS analysis of volatile metabolites in urine. 1 = Acetone, 3 = ethanol, 5 = 2-pentanone, 6 = *n*-propanol, 7 = dimethyl disulfide, 9 = 3-penten-2-one, 10 = N-methylpyrrole, 11 = *n*-butanol, 12 = 4-heptanone, 13 = isopentanol, 14 = 2-heptanone, 15 = cyclohexanone, 16 = allyl isothiocyanate, 17 = pyrrole, 18 = benzaldehyde. From J. Chromatogr., 112 (1975) 539.

The enormous number of applications of GC—MS and GC—MS—COM shall be classified in four areas:

I. Structural identification of substances.

II. Profile analyses and pattern recognition.

III. MF determinations and reference methods.

IV. In vivo experiments with substances labeled with stable isotopes.

2. STRUCTURAL IDENTIFICATION OF SUBSTANCES

More than any other analytical technique MS is suited to the identification of substances. In simple compounds the molecular ion and fragmentation pattern enable the analyst to identify the substance. As an example (Fig. 3), the molecular ion *m/e* 114, the fragment ions *m/e* 43 and *m/e* 71, and the McLafferty rearrangement ion *m/e* 86, together with the GC retention time suggest peak number 12 to be 4-heptanone. Computer matching against files of reference spectra and elemental composition analysis are aids in identifying more complicated molecules.

To a large extent structural identifications by GC—MS or GC—MS—COM have been performed on urine, plasma and tissue steroids in normal newborns, infants and adults [5—13] and in patients e.g. with adrenal malfunctions and enzyme defects [14—16]. The steroids are analyzed as derivatives such as trimethylsilyl ethers or methoximes—trimethylsilyl ethers. Packed glass columns originally used for steroid analyses, have been almost completely replaced by glass capillary columns, introduced for instance by Grob [17], as having much higher separation efficiency [11, 18, 19]. Not only for steroids but for practically all other complex biological mixtures, capillary columns should be used instead of packed columns.

A great multitude of organic acids and amino acids have been identified in body fluids and tissues [20—24]. Many of these studies are connected with investigations of metabolic abnormalities and inborn errors of metabolism,

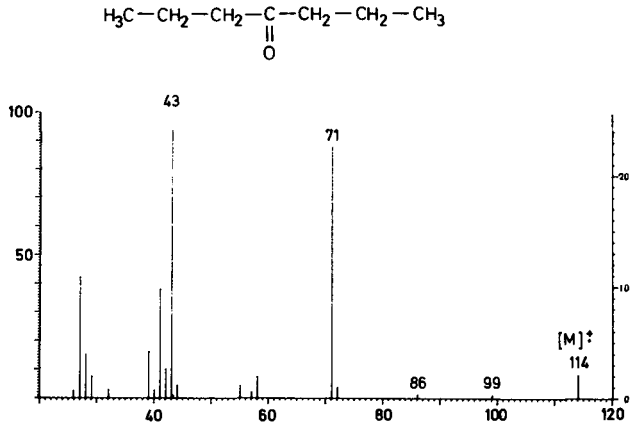

Fig. 3. Mass spectrum of peak number 12 in Fig. 2, identified as 4-heptanone.

such as congenital lactic acidosis [25, 26], ketoacidosis [27], propionic and methylmalonic acidemia [28], β-methylcrotonylglycinuria [29], dicarboxylic aciduria [30] and the corresponding enzyme deficiencies. Due to the great amount of GC and MS data on acids and other metabolites, it has been suggested by Jellum [107] and by Macek [31], that the data may be coordinated on an international basis.

Metabolites which are volatile without derivatization, such as ketones, alcohols, sulphur compounds and furans are especially amenable to GC—MS analysis, and were identified in urine and blood [33—36].

Considerable achievements were made in the structural identification of the different types of prostaglandins and their metabolites. Methyl ester—trimethylsilyl ether—methoxine derivatives enabled the determination of the hydroxyl- and keto- groups and of the double bond positions [37].

In the analysis of very complex molecules, such as free ceramides in human platelets [38], ceramides from plasma sphingomyelin [39], oligosaccharides in urine [40, 41] and glycosphingolipids with blood group specificity [42—44] MS is indispensable. Sequence and bond positions in oligosaccharides and glycosphingolipids could be established. Mass spectrometric sequencing was also used in peptides [45] by analyzing di-, tri- and tetrapeptides obtained by partial hydrolysis. The primary structure of ovine hypothalamic luteinizing hormone-releasing factor (LRF), a decapeptide, was established using GC—MS [46].

3. PROFILE ANALYSES AND PATTERN RECOGNITION

GC—MS is most powerful for profile analyses and recognition of normal and abnormal patterns of biological substances in body fluids and tissues. Whereas conventional clinical chemical tests determine individual substrates, hormones or groups of hormones which are known to be present, profile analyses offer the opportunity to:

(a) detect and characterize a large number of substances simultaneously;

(b) find new and unexpected substances;

(c) determine changes in the ratios of different constituents;

(d) give much more precise information, e.g. on steroid hormones, than a group test.

Abnormalities in urinary or blood steroids may be overlooked by a group test or by radioimmunological determination of one or a few steroids, but are more likely to be detected by profiling. Profile analyses must be expected to add to the understanding of metabolic abnormalities and diseases.

Analysis of metabolic profiles was introduced approximately seven years ago for urinary steroids, sugars, sugar alcohols, aromatic acids, plasma sugars and serum acids of the Krebs cycle [47]. Especially after the introduction of glass capillary columns, steroid profiles were studied with respect to establishing normal profiles and aberrations due to disease or stress [6, 7, 11, 18, 19, 48, 49].

Multicomponent profile analysis with special emphasis on organic urinary acids and the detection of inborn errors of metabolism, has been developed to a very advanced state by workers in Oslo [50—54]. Together with other

workers investigating abnormalities in urinary acids [25, 55], more than twenty new inborn errors of metabolism were discovered by GC or GC—MS, many of which were caused by enzyme deficiencies in amino acid metabolism. In addition to new disorders, substantial new information was obtained about metabolism in known defects.

A broad study on abnormal organic acidurias in mentally retarded patients was described by Watts et al. [56]. From 1778 mentally retarded patients, 5% had an abnormal organic aciduria. As expected, the most frequently observed abnormalities were those of phenylalanine metabolites in cases of phenylketonuria. An excellent review on profiles of organic acids has been given by Jellum [57].

Low-molecular-weight and volatile metabolites in blood [35, 36, 58—60] and urine [32—34, 61—65], and to a lesser extent also in cerebrospinal fluid [61], breath, saliva and tissue, have been profiled with the object of establishing normal patterns, on the basis of which pathological abnormalities can be recognized. The topic has been reviewed by Politzer et al. [66].

Detailed studies have been made in our laboratory on the volatile compounds in urine of patients with diabetes mellitus. In comparison with normal individuals, diabetic patients develop distinct changes in the ketone and alcohol excretion [63]. High total 4-heptanone (4-heptanone plus its precursor, a β-ketocarboxylic acid) was found in urine of approximately 75% of the diabetic patients with balanced glucose levels [65]. During hypoglycemic periods the total 4-heptanone excretion increases, in severe hyperglycemic periods it decreases. Increased ethanol excretion is found in 80% of the urines, whereas higher-molecular-weight alcohols were detected mainly in the urine of patients with diabetic complications.

4. MASS FRAGMENTOGRAPHIC DETERMINATIONS AND REFERENCE METHODS

4.1. Direct mass fragmentography

Selective ion monitoring was first introduced by Sweeley et al. [67] and by Hammar et al. [68], who applied the technique to the identification of chlorpromazine and its metabolites in human blood and called it MF. Since then the potentials of this highly sensitive and specific method have been used for qualitative detection and quantitative assays. The detection limits are in the low picogram range (e.g. 4-heptanone, 2—5 pg). In order to interfere in an assay, a compound must have the same GC retention behaviour and the same specific ions, which is not very likely to occur. In quantitative analyses, calculations are based on calibration curves. To correct for losses in the sample preparation procedure as well as for uncontrolled variation of the instrumental conditions, internal standards should be used from the beginning of the procedure. They are either compounds with similar chemical properties, isomers or homologous substances, or substances labeled with stable or radioactive isotopes (isotope dilution method).

We have chosen the isomer 3-heptanone for MF determination of total 4-heptanone by SID using molecular ion *m/e* 114 [36, 69]. Very low background noise is observed for this ion, and the determination can be performed in a

non-concentrated extract obtained by a single-step extraction of urine with cyclohexane. The calibration curve is linear over more than three orders of magnitude [69]. The excretion of total 4-heptanone in normals ranges between 50 and 450 μg/24 h, in diabetics it is increased up to several mg/24 h. Using overlapping injections, the analysis time is approximately 8 min.

One of the obstacles for a wide use of GC—MS in clinical chemical routine laboratories is the fact that sample preparation prior to GC—MS analysis is often laborious. In general, aqueous samples should not be introduced into the mass spectrometer. For the MF determination of ethanol in urine and serum of diabetic patients we have overcome this problem by by-passing the water between the outlet of the GC-column and the interface to the mass spectrometer. In this way we analyze ethanol in a true micromethod by directly injecting 1 μl of urine or serum (Fig. 4).

A large number of publications has appeared on MF determination of biogenic amines and their metabolites in urine, plasma, cerebrospinal fluid or brain tissue. Some of the authors use internal standards of type 1 [70—73], others deuterium-labeled species [74—77].

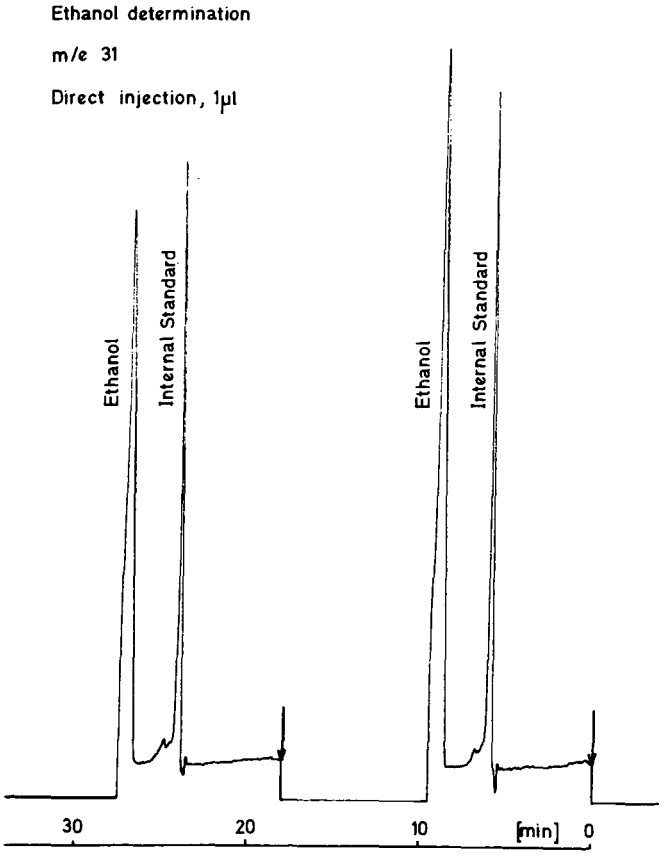

Fig. 4. MF determination of ethanol in urine. SID with *m/e* 31, $H_2C = \overset{+}{O}H$, direct injection of 1 μl of urine. Internal standard: diethyl ether.

Direct MF for steroids was used very early by Siekmann et al [78] and by Adlercreutz and Hunneman [79].

Using MF together with isotope dilution, reference and definitive methods were established for clinical chemistry. The MS determination of calcium with ^{44}Ca as the added isotope [80] gives results within a few tenths of one per cent of the true or absolute value.

For substrates and steroids, MF methods with isotope dilution have been developed mainly by workers led by Breuer and Björkhem, respectively. Methods are available for steroid hormones such as oestrogens [81, 82, 90], testosterone [81, 82, 84—87], 5α-dihydrotestosterone [84], progesterone [89], aldosterone [81—83] and cortisol [81, 82, 88], for cholesterol [91, 92], triglycerides [93], urea [94] and for glucose [95]. Deuterium-, tritium- and ^{14}C-labeled internal standards were used for the steroids, deuterated glycerol trioleate and glucose for the triglyceride and glucose determinations, respectively, and ^{15}N-labelling for urea. Mass differences between unlabeled and labeled compound should be small, otherwise partial chromatographic separation may occur. Variation coefficients were mostly between 1.3 and 4% for the various parameters, whereby pipetting errors are perhaps the most important source of variation. Special attention was also directed toward the type of derivative, heptafluorobutyric esters giving higher sensitivity than trimethylsilyl ethers.

Correlations between MF and radioimmunological or chemical methods showed either good agreement or lower values measured by MF (progesterone, cortisol), most probably as a result of the higher specificity of MF. The use of MF with isotope dilution has been reviewed by Björkhem et al. [96].

Using the advantage of a quadrupole mass spectrometer to simultaneously detect many specific ions over a broad *m/e* range, twelve amino acids from 50 μl of plasma or urine were determined with deuterated standard amino acids [97].

4.2. Computerized mass fragmentography

Whereas in direct MF one specific ion, or a small number of specific ions, has to be pre-selected prior to analysis, computerized MF allows the use of any recorded ion after the GC—MS run for specific detection or determination of a substance. This is especially suited to profile analyses. However, the advantage of the greater flexibility of this method must be weighed against the disadvantage of considerably lower sensitivity, because a larger mass range is scanned and fewer ions of one type reach the multiplier. If not prohibited by too low sample concentration, the method has a very wide use. From complex profiles, selective patterns can be obtained by computerized MF, giving information on the presence of a single compound or groups of compounds [34, 36]. Computer MF has proved very valuable for steroid [9, 12, 13, 98], amino acid [99] and bile acid [100, 101] analyses.

5. IN VIVO EXPERIMENTS WITH SUBSTANCES LABELED WITH STABLE ISOTOPES

With the increased use of MS in clinical laboratories it became possible to

use stable isotope labeling for in vivo experiments and diagnosis, and to complement or replace experiments with radioactive tracers, thus eliminating possible radiation hazards connected with radioactive substances. Compounds labeled with stable isotopes can be easily detected and quantitated in blood, urine or tissue by MS and MF.

By administering deuterium-labeled homovanillic acid (HVA), the turnover of HVA in man was determined [102]. Very detailed studies on phenylalanine and tyrosine metabolism were published by Curtius and coworkers. Patients with phenylketonuria and hyperphenylalaninemia were loaded with deuterated phenylalanine and the aromatic acids were determined in urine. No tyrosine metabolites were found [103], expressing a deficiency in the enzyme phenylalanine-hydroxylase. Administration of deuterated L-tyrosine to patients with phenylketonuria and determination of the excretion of Dopa metabolites, suggested that the tyrosine-3-hydroxylase activity and the formation of catecholamines depend on the phenylalanine concentrated in plasma [104].

Deuterated ethanol has been extensively used by Cronholm et al. in studies of biosynthetic pathways, e.g. the biosynthesis of cholesterol and bile acids [105] or Krebs cycle acids [106].

6. CONCLUSION

Since MS, especially in the form of GC—MS, is the most flexible and most powerful analytical technique available today for organic substances that are volatile or can be made volatile, and that are present in complex mixtures of biological origin, it is indispensable for biochemical and clinical research. Its potentials would be enhanced even more by combination with other techniques, particularly high-pressure liquid chromatography, thus eliminating to a large extent the requirement of volatility.

A mass spectrometer is no longer the formidable instrument, if it is operated by well trained personnel. However, because of its great potential a GC—MS system should not be hindered by analyses that can be performed by simpler and more economical methods. Only when the separating power, the sensitivity and the specificity of GC—MS are needed or when no other method is available for the analysis, GC—MS should be used. Often GC alone will suffice.

Instrument manufacturers are beginning, and should be encouraged to continue, to develop smaller, more economical, less flexible instrumentation, which still offers the optimal efficiency for a given type of analysis. In clinical chemistry, today's mass spectrometers are used in the first place in research and in diagnostic centers specializing in detailed metabolic investigation for diagnostic purposes.

7. SUMMARY

In clinical chemistry and clinical biochemistry mass spectrometry is used mainly in combination with gas chromatography, in some cases supported by a computer. The combination is distinguished by its separating efficiency for complex mixtures of substances and by its high sensitivity together with very

high specificity. An amount of 10—100 ng is sufficient for the identification of an unknown substance, and even 1—100 pg can be adequate for qualitative detection or quantitative determination by mass fragmentography. The principal areas of application are: (a) structural identification of substances; (b) profile analyses and pattern recognition; (c) mass fragmentographic determinations and reference methods; (d) in vivo experiments with substances labeled with stable isotopes.

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GAS CHROMATOGRAPHIC—MASS SPECTROMETRIC ANALYSIS OF OPTICALLY ACTIVE METABOLITES AND DRUGS ON A NOVEL CHIRAL STATIONARY PHASE

HARTMUT FRANK, GRAEME J. NICHOLSON and ERNST BAYER

Institut für Toxikologie, Wilhelmstrasse 56 and Institut für Organische Chemie, Auf der Morgenstelle 18, 74 Tübingen (G.F.R.)

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SUMMARY

Chirasil-Val, a novel chiral polysiloxane-type stationary phase is capable of separating the enantiomers of optically active drugs and metabolites of several compound classes; α-amino acids, α-amino alcohols, glycols, aromatic and aliphatic α-hydroxy carboxylic acids and amines. Due to their high thermal stability, columns coated with Chirasil-Val may be coupled to a mass spectrometer. Potential applications of the new stationary phase include analysis of the optical purity of enantiomeric drugs, determination of the configuration of metabolites, and quantitation of optically active drugs and metabolites using the unnatural enantiometer as internal standard. Direct separation of enantiomers on Chirasil-Val is especially useful if only minute amounts of the optically active compounds are available for analysis.

INTRODUCTION

Stereoselectivity is an inherent phenomenon of biological systems. In most cases optical enantiomers exhibit different biological activities due to differences in strength of interaction with the corresponding receptor, different transport mechanisms and metabolism along different pathways [1]. Well known examples are the sympathomimetic α-phenyl-β-aminoalcohols, e.g. ephedrine, epinephrine, phenylephrine and others [2]. L-DOPA is rapidly absorbed and extensively metabolized in intestinal tissues, whereas D-DOPA is absorbed more slowly and not metabolized at all [3]. The D-enantiomer of penicillamine is applied in cases of cystinuria and mercury poisoning, the L-enantiomer is toxic [4]. L-Lactic acid is a normal constituent of human urinary samples, whereas presence of D-lactic acid signals metabolic disorder [5]. Only the L-enantiomer of N-phthaloyl glutamic acid, a metabolite of thalidomide, is teratogenic [6].

Basically, gas chromatographic (GC) analysis of optically active compounds

from biological samples can be performed in two ways: by derivatization with a pure enantiomer of an optically active reagent and separation of the resulting diastereomers on a nonchiral stationary phase [7, 8], or by direct separation of the enantiomers on a chiral stationary phase [9, 10].

Analysis via the detour of diastereomers has four principal shortcomings: (a) choice of derivatization agents is limited by the requirement of optical activity, (b) the derivatization reagent must be optically pure, (c) differences in the reaction kinetics of derivatization between two enantiomers leads to systematic errors in quantitative analysis and (d) enhanced probability of racemization at one of the asymmetric carbons during derivatization increases the error of quantitative determinations.

Direct separation of enantiomers on a chiral stationary phase offers the advantage that reagents commonly used for derivatization in GC may be applied. The two enantiomers to be separated interact with the chiral stationary phase via hydrogen bonds to form diastereomeric association complexes with differences in their solvation enthalpy of several hundred calories [11].

The low temperature range over which the hitherto known chiral compounds are suitable as GC stationary phases prevented the general use of the direct separation of enantiomers. Most of the phases have melting points of 80—100° and only a few are of sufficiently low volatility at temperatures above 130°. At elevated temperatures such columns show increased bleeding and deteriorate rapidly also combination with a mass spectrometer is unsatisfactory due to a high background in the mass spectra.

We recently synthesized a chiral stationary phase, referred to below as Chirasil-Val [12, 13], possessing low volatility and high thermal stability by coupling L-valine *tert.*-butyl-amide to a copolymer of dimethylsiloxane and carboxy-alkyl-methyl-siloxane units of appropriate viscosity and molecular weight. Chirasil-Val opens up a new temperature range for the analysis of optical isomers and consequently additional compounds of low volatility are now amenable to this method. Also, Chirasil-Val offers, for the first time, the possibility of coupling a mass spectrometer to a GC column capable of separating optical enantiomers.

EXPERIMENTAL

Preparation of capillaries

Capillaries of 0.3 mm I.D. made from borosilicate glass are dynamically coated with 0.5% colloidal silicic acid in acetone [14] and dried thoroughly at 200—250° with carrier gas flowing. They are coated with Chirasil-Val in methylene chloride by the static method [15], conditioned by programming to 210° with a slow hydrogen stream and kept there overnight.

In order to ensure that deactivation is always complete and to elute high boiling compounds, the capillaries are routinely kept at 200° overnight. Under these conditions the average life span of a column exceeds 6 months.

Esterification of carboxyl groups

A 500-μg amount of carboxylic acid is placed in a screw-cap vial, dissolved in 200 μl 2 *N* hydrogen chloride in anhydrous isopropanol and heated to

90° for 30 min. If the carboxyl group is sterically hindered (e.g. valine) 1 h is preferable. Excess reagent is removed under a gentle stream of nitrogen with moderate heating.

N, O, S-Acylation with pentafluoropropionic anhydride

A 250-μl volume of ethyl acetate and 50 μl pentafluoropropionic anhydride are added to the sample in a heavy-walled screw-cap vial. Air is displaced with nitrogen and the mixture is heated to 110° for 10 min. Excess reagent and solvent are removed with a gentle stream of nitrogen. The dry residue is dissolved in an appropriate volume of methylene chloride.

O-Pentafluoropropionyl lactic acid cyclohexylamide

A 1-mg amount of lactic acid (D or L) is dissolved in 100 μl methanol and 100 μl methylene chloride. An ethereal solution of diazomethane (or diazomethane gas) is added until the solution stays yellow for 2 min. Most of the solvent is evaporated, the concentrated solution is diluted with 100 μl methanol and 5 μl of cyclohexyl amine are added. After 30 min at 50° the solvent is removed under a gentle stream of nitrogen and the residue is O-acylated as described above.

Analysis of penicillamine

Penicillamine is desulfurized with Raney nickel according to ref. 16 and derivatized as described above, resulting in N-pentafluoropropionyl valine isopropylester.

GC is performed on a Carlo Erba Model 2101 gas chromatograph with glass capillaries 20 m × 0.3 mm coated with Chirasil-Val, film thickness ca. 0.1 μm. Hydrogen is used as carrier gas, inlet pressure 0.35 bar. Other conditions as given in the corresponding legend.

Peak areas are measured with the electronic integrator Spectra Physics System I.

GC—mass spectrometry is performed on a Varian Model 2740-1 gas chromatograph interfaced to a mass spectrometer Varian MAT 711 by an open split. The mass spectrometer is set to a resolution of approximately 1000; ionizing voltage 70 eV; ionizing current 0.8 mA; multiplier voltage as given in the mass spectra; interface temperature 220°; ion source temperature 220°.

RESULTS AND DISCUSSION

Chirasil-Val was synthesized primarily for the purpose of analyzing the optical purity of amino acids from synthetic peptides, but it is well suited to the separation of enantiomers of a large number of optically active compounds occurring in biological systems.

The general structure of the phase is given in Fig. 1. L-Valine *tert.*-butylamide is linked to the polysiloxane via the highly stable carboxamide group. Each chiral moiety is separated from the next by approximately 7 dimethylsiloxane units, thus avoiding interaction between neighbouring valine residues by hydrogen bonding. This seems to be an important factor for resolution and thermal stability. Phases with a smaller number of "diluting" dimethyl-

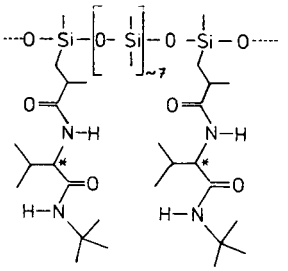

Fig. 1. Structure of the chiral stationary phase Chirasil-Val.

siloxane groupings were thermally less stable and, due to high melting temperatures, unsuitable as stationary phases. The phases synthesized so far can be used over a temperature range from 70° to 230°, for a short period even to 250°. Racemization of the chiral moiety is tolerable at 230°; after 24 h at this temperature approximately 3% of L-valine is racemized.

For derivatization we prefer perfluoroacyl derivates [17] whenever feasible, i.e. for amines, alcoholic and phenolic hydroxyl groups and thiols. These are among the most volatile derivatives used in GC. This is of importance, since the free enthalpy-differences decrease with increasing temperature.

In Fig. 2, the separation of the N, O-pentafluoro-propionyl derivatives of a number of sympathomimetic drugs and epinephrine metabolites is shown. Baseline resolution for most of the compounds is achieved but resolution factors vary considerably. They are mainly dependent upon the functional

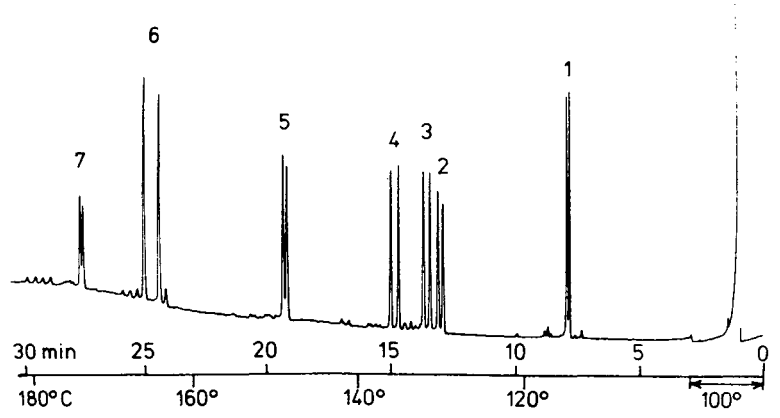

Fig. 2. GC separation of the enantiomers of several sympathomimetic drugs and epinephrine metabolites as N, O-pentafluoropropionyl-derivatives. Chromatographic conditions: 20 m × 0.3 mm Chirasil-Val, injector and detector 220°, carrier gas hydrogen 0.38 bar. Peaks: 1 = phenyl-2-methylamino-propanol (ephedrine), 2 = 1-(4′-hydroxyphenyl)-2-methylamino-propanol (suprifen), 3 = 3′-methoxy-4′-hydroxyphenylglycol, 4 = 1-phenyl-2-aminoethanol, 5 = 1-(3′-methoxy-4′-hydroxyphenyl0-2-methylaminoethanol (metanephrine), 6 = 3,4-dihydroxyphenylalanine isopropyl ester (DOPA), 7 = 1-(3′,4′-dihydroxyphenyl)-2-amino-ethanol (norepinephrine). In all cases investigated the D-enantiomer is eluted prior to the L-enantiomer.

groups and their relative location within the molecule. Amino acids, as N-pentafluoro-propionyl amino acid isopropylesters, exhibit the highest resolution factors. Hydrogen-donating amino groups and hydrogen-accepting carbonyl groups of the N-acyl and ester moiety are in optimal sequence and similar spatial relationship for "fitting" onto the "hydrogen-bonding-matrix" of the stationary phase. The amino acid DOPA therefore exhibits one of the largest resolution factors. Enantiomers of the 1-phenyl-2-amino alcohols are not as well separated due to the dissimilar spatial relationship of the hydrogen-donating and accepting moieties of solute and solvent. If the amino group carries an additional substituent, no hydrogen bond can be formed at this site after acylation, and one point of an optimal "three-point-fit" is lost, with consequent reduction in the resolution factors. The same applies to the phenylglycols.

As already indicated, the enantiomers of optically active drugs and metabolites often differ greatly in their efficacy and activity. D-Ephedrine for instance is the isomer with the highest pressor-activity. If D-ephedrine racemizes at the asymmetric carbon adjacent to the amino group the depressor-active D-pseudoephedrine is produced. Clearly, an ephedrine preparation which contains significant amounts of D-pseudoephedrine may falsify the results of a study of its adrenergic activity. In such cases Chirasil-Val offers the possibility of analyzing the optical purity in a simple, time-saving manner with a high degree of sensitivity and accuracy.

A further example investigated was D-penicillamine; since this drug is administered often in doses of up to 1 g per day, an impurity even of a fraction of a percent of the toxic L-enantiomer may be hazardous. We therefore developed a method for analysis of the optical purity of this compound.

In order to test the linearity and accuracy of the method, samples of D-penicillamine are spiked with amounts of 0—10% of L-penicillamine and converted to valine with Raney-nickel [16]. The enantiomers are separated isothermally at 110° with a resolution factor $\alpha_{L/D}$ of 1.134. In Fig. 3 the percentage of L-penicillamine found is plotted against the amount of L-penicillamine added. Each value represents the mean of five determinations within the indicated standard deviations. The line obtained by the least square method has a slope of 0.9945 indicating a nearly perfect correlation of values-found with amount-spiked. The intercept of 0.55% is in very good agreement with the value obtained for the sample not spiked: 0.56 ± 0.04%. Percentages of L-penicillamine present in different preparations of D-penicillamine are listed in Table I.

In principle, the determination of the enantiomer-ratio by GC alone may be falsified by a compound with the same retention time as one of the enantiomers. This may occur especially in the analysis of a component in a drug formulation containing other additives. In such a case the reliability of the analysis is greatly improved by mass fragmentography, monitoring an ion typical for the compound to be quantitated. Due to its low volatility, Chirasil-Val is well suited for combination with a mass spectrometer. Fig. 4a shows the electron impact mass spectrum of 2.4 ng of L-DOPA, esterified, N, O-acylated and chromatographed isothermally at 180°. Fig. 4b shows the background mass spectrum of Chirasil-Val shortly after emergence of the DOPA

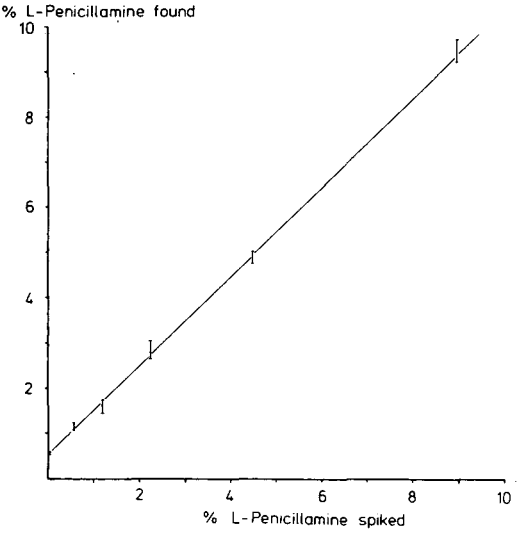

Fig. 3. Quantitative determination of L-penicillamine impurities in D-penicillamine spiked with differing amounts of L-penicillamine (n = 5).

TABLE I

DETERMINATION OF L-PENICILLAMINE IN D-PENICILLAMINE

Sample	L-Enantiomer (%)	Standard deviation (n = 6)
1	0.57	0.05%
2	0.56	0.04%
3	0.54	0.06%
4	0.35	0.08%
5	0.52	0.05%

peak. The background ions are mainly those expected for a poly-dimethylsiloxane.

For analysis of L-DOPA, the base peak at m/e 472 is chosen. In Fig. 5 an actual quantitation of the enantiomer ratio of a commercial L-DOPA is presented. For quantitative analysis by mass fragmentography especially in the picogram range, absence of disturbing background ions is a prerequisite. Chirasil-Val possesses only a few background ions of a very low intensity above m/e 360. Quantitation of minute amounts of L-DOPA by mass fragmentography therefore is feasible, as demonstrated in Fig. 6, depicting the mass fragmentogram of 30 pg of L-DOPA with a signal-to-noise ratio of 10.

Other compounds amenable to separation on Chirasil-Val are asymmetric amines, glycols and α-hydroxy carboxylic acids. For the latter, conversion to the corresponding ester often leads to insufficient separation of the enantiomers. This is due to the absence of a nitrogen-attached hydrogen which is

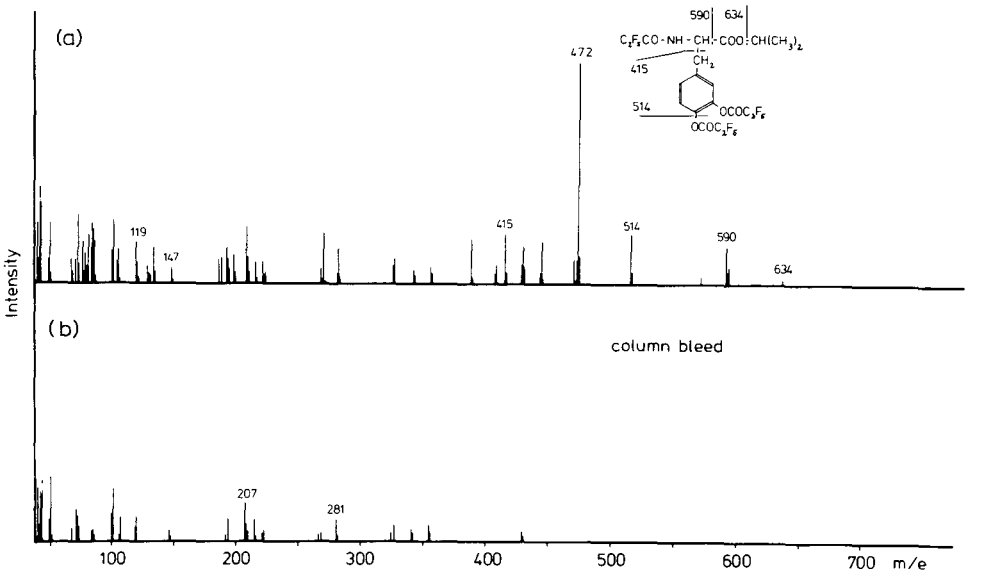

Fig. 4. (a) Electron impact mass spectrum of 2.4 ng commercial L-DOPA, converted to the isopropylester and N, O-pentafluoropropionyl derivative injected on a capillary 20 m × 0.3 mm Chirasil-Val, 180° isothermal, carrier gas Helium, 0.32 bar, mass spectrometer Varian-Mat 711, interface 220°, ion source 220°, 70 eV, multipl. volt 2.4 kV. (b) Background mass spectrum of Chirasil-Val, 2 min after emergence of L-DOPA. Conditions as in (a).

shown to be important for generation of a strong diastereomeric association complex between solvent and solute. If, however, the carboxylic acids are converted to the corresponding O-pentafluoropropionyl carboxylic amides, astonishingly high resolution factors are achieved. In Fig. 7 the separation of the enantiomers of the O-pentafluoropropionyl lactic acid cyclohexylamide is shown. The resolution factor for this simplest α-hydroxy carboxylic acid is in the same range as those achieved for α-amino acids on Chirasil-Val. As is the case for amino acids, the D-form of lactic acid is eluted prior to the L-enantiomer.

Another powerful application of such optically active stationary phases is the quantitation of optically active metabolites in biological samples by GC. Often the choice of a proper internal standard presents difficulties, and incomplete derivatization or decomposition of the derivative lower the accuracy of determination. Metabolites are usually present as only one enantiomer. By adding the optical enantiomer as internal standard [18], all the problems arising from incomplete recovery, incomplete derivatization, hydrolysis, thermal decomposition and shifting response factors are eliminated. The internal standard has the same solubility, reactivity and chemical stability as the compound to be quantitated. The mass spectra of both internal standard and compound to be analyzed are identical, which is of importance for selected ion recording.

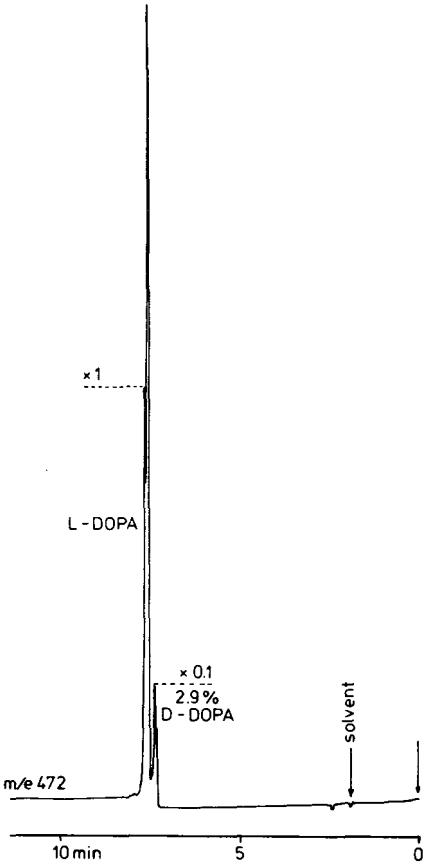

Fig. 5. Selected ion recording of 10 ng L-DOPA, chromatographed on a capillary 16.5 m × 0.3 mm Chirasil-Val at 190°. Other conditions as in Fig. 4.

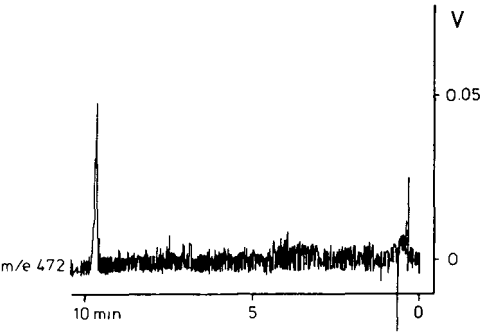

Fig. 6. Selected ion recording of 30 pg L-DOPA, chromatographed on a capillary 16.5 m × 0.3 mm Chirasil-Val. Mass spectrometer Varian MAT 711, sensitivity 0.01 V/cm, filter 5, multipl. volt. 2.6 kV. Other conditions as in Fig. 4.

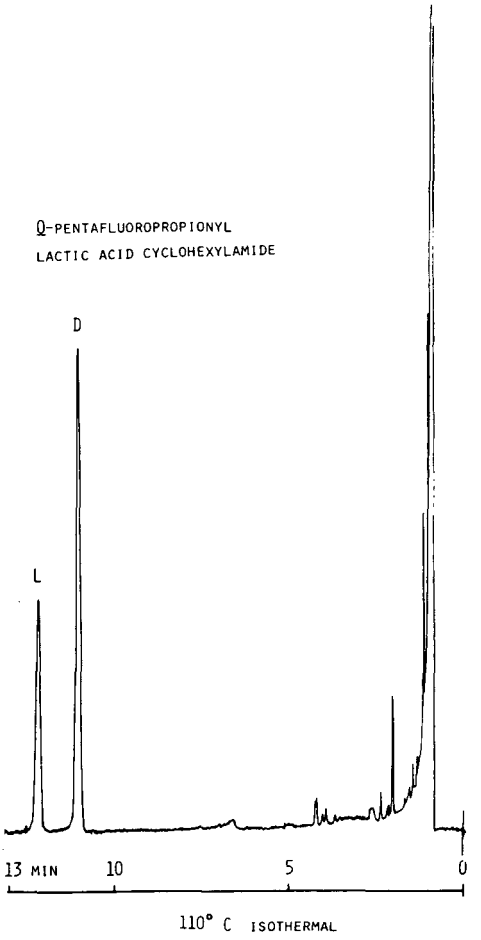

Fig. 7. Gas chromatographic separation of the enantiomers of O-pentafluoropropionyl lactic acid cyclohexyl amide. Conditions: 20 m × 0.3 mm Chirasil-Val, injector and detector 220°, carrier gas hydrogen 0.3 bar, 110° isothermal.

CONCLUSIONS

Chirasil-Val proves to be a valuable stationary phase for the separation of optical isomers and quantitative determination of the optical purity of various classes of enantiomeric drugs and metabolites by GC. The stationary phase is well suited for combination with mass spectrometry. Further, Chirasil-Val may be applied to the assignment of configurations to optically active metabolites and their quantitation using the unnatural enantiomer or the racemic mixture as an internal standard.

ACKNOWLEDGEMENT

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Biomedical Applications

CHROMBIO. 174

NEW TYROSINE METABOLITES IN HUMANS: HAWKINSIN AND *CIS*- AND *TRANS*-4-HYDROXYCYCLOHEXYLACETIC ACIDS

UNUSUAL ADSORPTION OF DEUTERATED AND NON-DEUTERATED HAWKINSIN DURING GAS CHROMATOGRAPHY

A. NIEDERWIESER, A. MATASOVIĆ, F. NEUHEISER and E. WETZEL

Universitäts-Kinderklinik, Steinwiesstr. 75, CH-8032 Zürich (Switzerland)

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SUMMARY

In a new inborn error of metabolism, where obviously a defect of 4-hydroxyphenylpyruvate dioxygenase (EC 1.13.11.27) exists, hawkinsin [(2-cystein-S-yl-1,4-dihydroxycyclohex-5-en-1-yl) acetic acid] and *cis*- and *trans*-hydroxycyclohexylacetic acids were found in the urine. A partially reversible adsorption of deuterated and non-deuterated hawkinsin (as the penta-trimethylsilyl derivative) in gas chromatography—mass spectrometry has inhibited a mass fragmentographic quantitation of this compound to date. However, quantitation seems to be possible using mass fragmentography of 1,4-dihydroxycyclohexylacetic acid, formed by desulfuration of the sample with active nickel.

INTRODUCTION

We have recently been studying the unusual urinary excretion products of an Australian woman, and her daughter who suffered from transient tyrosinemia during her first year of life. One of these compounds revealed a new sulfur amino acid, namely (2-cystein-S-yl-1,4-dihydroxycyclohex-5-en-1-yl) acetic acid, which we named hawkinsin (Haw) [1]. The others were *cis*- and *trans*-4-hydroxycyclohexylacetic acids [2]. All of these unusual compounds were proved to be tyrosine metabolites by a loading test with 50 mg/kg of 3,5-bis-deutero-L-tyrosine, orally, and selected ion-monitoring of the non-deuterated and bis-deuterated molecule species in urine [2].

In this paper we report on the unusual adsorption effect of Haw on the columns used for gas chromatography—mass spectrometry (GC—MS), an effect

which has inhibited quantitation of this compound by mass fragmentography to date.

MATERIALS AND METHODS

Haw and 1,4-dihydroxycyclohexylacetic acid were prepared as previously described [1]. Hexa-deutero-Haw was prepared similarly. 4-Hydroxyphenylacetic acid was deuterated in D_2O-H_2SO_4 to give 2,3,5,6,α,α hexa-deutero-4-hydroxyphenylacetic acid (contaminated with the penta- and tetra-deutero derivatives) and the product was transformed into the hexa-deutero-4-quinolacetic acid by photo-oxidation using Rose—Bengal as catalyst. The quinolacetic acid was then reacted with cysteine to give a mixture of the mono- and bis-addition products. These were separated by ion-exchange chromatography and precipitated from the methanolic solution in diethyl ether.

Active nickel for desulfuration was prepared from nickel chloride and sodium borohydride [3]. Trimethylsilylation was performed using bis-trimethylsilyltrifluoracetamide (BSTFA)—acetonitrile (1:1, v/v) for 1—4 h at 135° (hawkinsin) or 60° (1,4-dihydroxycyclohexylacetic acid). GC of penta-trimethylsilyl (TMS)-Haw was performed on 15—60 cm × 2 mm columns of 1% SE 30 or 1% Dexsil 300 on Chromosorb W AW DCS at 210—220° and on 1.8-m long OV-17 Pyrex glass capillary columns at 180—200°.

GC—MS was performed on a Micromass F-16 mass spectrometer (Vacuum Generators Micromass, Winsford, Great Britain) combined with a Carlo Erba Model 2101 AC gas chromatograph over a jet separator (for packed columns) and a further Model 2101 AC gas chromatograph equipped with a glass capillary column coupled directly with the ion source.

RESULTS AND DISCUSSION

In order to be able to quantitate, and also to detect traces of Haw with selected ion monitoring, we prepared a hexa-deutero-Haw (Haw-d_6) in a similar manner to which we prepared non-deuterated Haw (Haw-d_0) [1]. The mass spectrum of the corresponding penta-TMS derivative is shown in Fig. 1. As can be seen from the fragment at 540, the product was a mixture of hexa-, penta- and tetra-deutero-Haw. The base peak at *m/e* 218 is a typical α-amino acid fragment. The molecule ions are below 1% and just visible. The fragments at *m/e* 444 and 450 (non-deuterated and hexa-deuterated, respectively) result from the loss of COOTMS and TMSOH. The fragments at *m/e* 360 and 364, respectively, result from a retro-Diels—Alder reaction and characterize the position of the double bond. Also of note is the presence of the tropylium ion at *m/e* 179 and 184; this is an ion which intrigued us during our efforts to identify this compound. Two residues, namely trimethylsilanol and cysteine, must be split off in order to obatin a phenolic intermediate from this aliphatic compound. At least one or even two deuterium ions are lost during the tropylium ion formation (Fig. 1).

GC of Haw was possible only as the penta-TMS derivative. The best results were obtained using very short columns of 15—60 cm in length. It was necessary to treat the columns repeatedly with BSTFA reagent at a high temperature

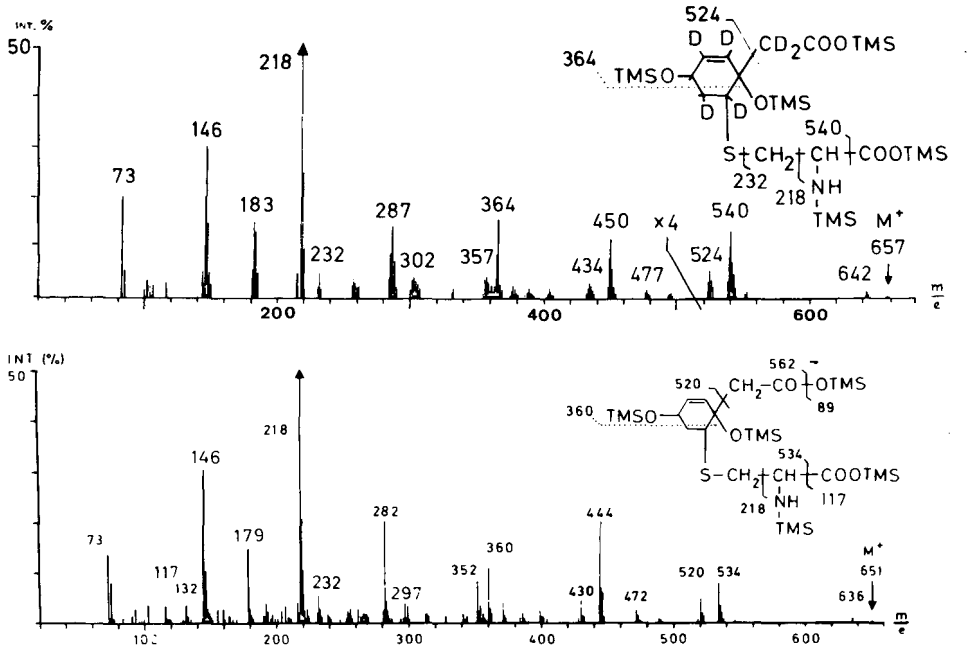


Fig. 1. Electron impact (EI) mass spectrum of deuterated Haw (above) and non-deuterated (below) as the penta-TMS derivative at 20 eV. GC—MS on a 60 cm × 2 mm column of 1% SE-30 on Chromosorb W AW DCS at 210°. VG Micromass F-16 mass spectrometer.

before use. The possible temperature range of the column was narrow: no peak at all was obtained if the compound eluted slowly because of lower temperature, lower carrier gas flow-rate or longer columns. Experiments with selected ion monitoring of non-deuterated and deuterated derivatives gave quite unexpected results; these are summarized in Fig. 2. The fragments at *m/e* 444 and 450 were monitored for Haw-d_0 and -d_6, respectively. At first non-deuterated Haw was injected several times. Then, pure deuterated Haw was injected. Unexpectedly, a high signal from the previously-injected Haw-d_0 was observed at *m/e* 444, simultaneously with the expected signal at *m/e* 450. The signal of the unlabeled compound decreased only slowly during further injections of pure Haw-d_6. This phenomenon can only be explained by a partially reversible adsorption of Haw on the column; a newly injected sample equilibrates partially with the adsorbed one. As can be seen in Fig. 2, the amount of Haw-d_0 which was desorbed by the first injection of Haw-d_6 even increased in the second and third series. Under such conditions, no quantitative analysis and no trace analysis was possible.

Attempts to diminish the adsorption effects by using glass capillary columns have not been successful to date. No peak at all could be obtained on 20-m OV-17 glass capillaries. Only on very short capillary pieces of less than 2 m was a flame-ionization detector signal obtained. In order to demonstrate the adsorption effect, a dilution series of the same mixture of Haw · $(TMS)_5$ and an alkane as an internal standard was analyzed. Because the same mixture was

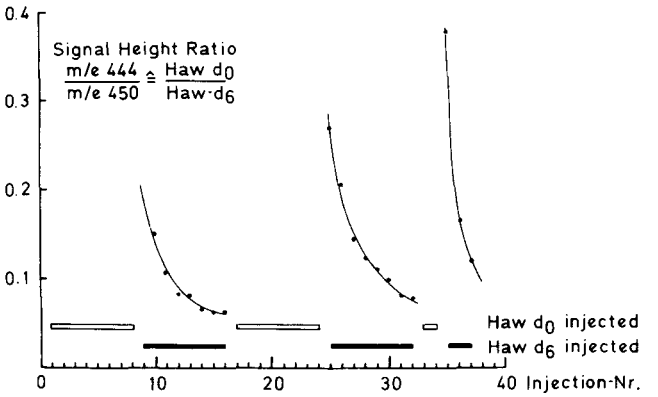


Fig. 2. Desorption of Haw-d_0 ◦ $(TMS)_5$ from a packed GC column by repeated injections of hawkinsin-d_6—$(TMS)_5$. A 300 × 2 mm column of 1% SE-30 on Chromosorb G AW DMCS was operated at 215°. Selected ion monitoring of Haw-d_0 and Haw-d_6 at *m/e* 444 and 450, respectively.

analyzed, a constant peak height ratio could be expected if no adsorption ocurred. However, a dramatic decrease of this ratio with decreasing amounts of sample injected was observed (Fig. 3). Adsorption was even higher on a Pyrex capillary which had been pretreated several times with Carbowax 20M before it was coated with OV-17 (Fig. 3).

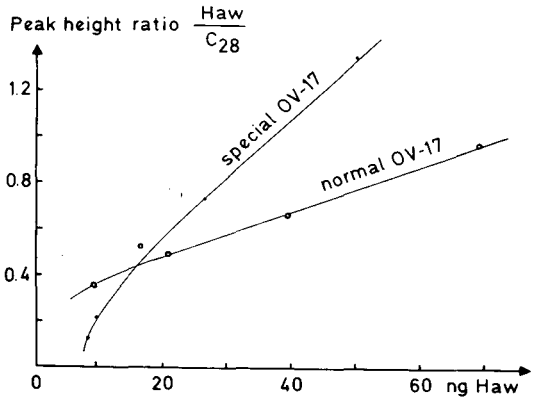
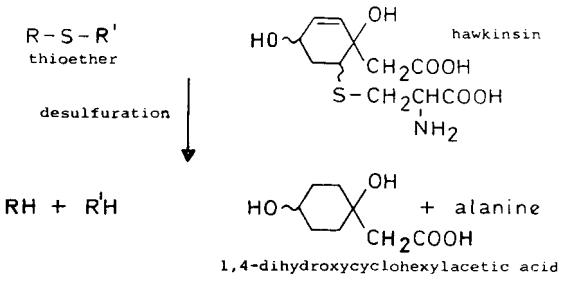


Fig. 3. Adsorption of Haw on 1.8-m long OV-17 glass capillary columns. The sample consisted of a mixture of 500 ng Haw and 200 ng of alkane C_{28}. The mixture was reacted with BSTFA—acetonitrile (1:1, v/v) at 135° for 1 h and then diluted with the reagent. The actual amount separated on the capillary column was estimated, assuming an inlet split ratio of 1:10. A constant peak height ratio would be expected if no adsorption occurred. Temperatures: 265°, injector, 195°, normal OV-17 and 188°, special OV-17 column. The special column was treated 4 times with Carbowax before it was coated.

Hence, we had to look for another possibility for quantitation. Haw is a thio-ether which can be split by desulfuration with active nickel into 1,4-dihydroxycyclohexylacetic acid and alanine under reduction of the double bond [1]:



It has been shown recently [3, 4] that desulfuration can be used as a general method for the quantitation of N-acetylcysteine conjugates (mercapturic acids), which are products of the glutathione detoxification pathway of many drugs and other xenobiotics. In the case of the mercapturic acids, N-acetyl-alanine is split off and measured with selected ion monitoring, and a trideuterated N-acetylcysteine derivative is used as an internal standard [3, 4].

Hence, we tried to apply the desulfuration technique to the quantitation of Haw. Measurement of the resulting 1,4-dihydroxycyclohexylacetic acid could be a specific means for quantitation. After several trials we found conditions were such that the 1,4-dihydroxyhexylacetic acid could be gas chromatographed practically without adsorption as the per-TMS derivative on 1 m × 2 mm columns of 1% Dexsil on Chromosorb W AW DCS. (Methylation with diazomethane followed by silylation gave incomplete derivatisation of the hydroxyl group at C-1). On the other hand, Haw-d_6 could be desulfurated practically without loss of the isotope labels. This is demonstrated by the mass spectrum in Fig. 4. The intensity at *m/e* 381 is a little lower than one would expect from the Haw-d_6 spectrum, but this is certainly no contra-indication to the use of the desulfuration technique. Thus, even our imperfect hexadeuterated hawkinsin preparation can be used as an internal standard for mass fragmentography of Haw-d_0 and Haw-d_2. Nevertheless, we hope to be able to synthesize an isotopically pure Haw-d_6 in the near future and we intend to search for the presence of traces of Haw in the urine of patients with liver diseases and tyrosinemia.

However, it could well be that the intermediate, from which Haw and *cis*- and *trans*-4-hydroxycyclohexylacetic acids are derived, is not normally in a free state but is immediately rearranged by the enzyme into homogentisic acid. From our observations we postulate a defective 4-hydroxypyruvate dioxygenase which is still able to oxidize and decarboxylate the substrate, but is unable to rearrange the intermediate into homogentisic acid. The occurrence of the fully reduced 4-hydroxycyclohexylacetic acid and of Haw strongly indicates an epoxide intermediate, namely (1,2-epoxy-4-hydroxycyclohexa-3,5-dien-1-yl) acetic acid, which can lose its potentially aromatic character by

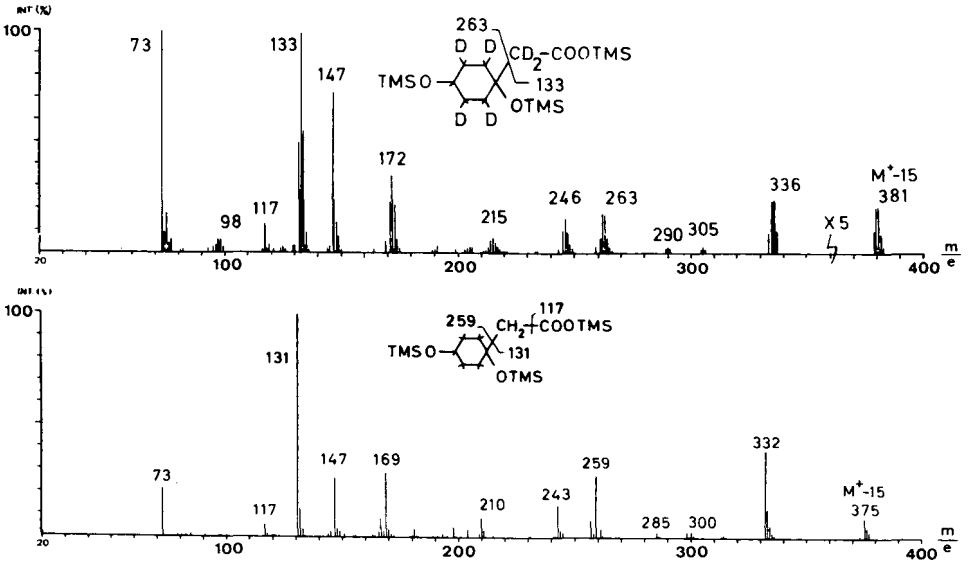


Fig. 4. EI mass spectra of deuterated and non-deuterated 1,4-dihydroxycyclohexylacetic acid—$(TMS)_3$ at 20 eV. The acids were obtained by desulfuration of Haw-d_6 and Haw-d_0 with active nickel, and analyzed on a Vacuum Generators Micromass F-16 mass spectrometer.

a simple tautomerization into (1,2-epoxycyclohex-5-en-4-on-1-yl) acetic acid. An attack at C-2 by a hydride ion followed by further reductions will lead to 4-hydroxycyclohexylacetic acid (predominately *trans*), and an attack at C-2 by cysteine (or glutathione) followed by reduction will lead to Haw.

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QUANTITATIVE ANALYSIS OF β-PHENYLPYRUVIC ACID BY SINGLE ION MONITORING

EVALUATION OF ISOMERIC INTERNAL STANDARDS*

U. LANGENBECK, A. MENCH-HOINOWSKI and I. RØD-URBAN**

Institute of Human Genetics, University of Göttingen, D-3400 Göttingen (G.F.R.)

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SUMMARY

Quantitative single ion monitoring of β-phenylpyruvic acid at high sensitivity is possible after derivatization first with *o*-phenylenediamine and then with a silylating reagent. The resulting O-trimethyl-silyl-quinoxalinol (O-TMS-Q) has previously been shown to be highly stable during storage and on chromatography. As an internal standard the isomeric *o*-methyl-phenylglyoxylic (*o*-toluylformic) acid is introduced. The mass spectra of both O-TMS-Q's are characterized by abundant $[M]^+$ at *m/e* 308. The concept of "class specific metabolic profiling" is discussed in relation to quantitative gas chromatography—mass spectrometry detection of aliphatic and aromatic α-ketoacids.

INTRODUCTION

Gas chromatographic (GC) and mass spectrometric (MS) methods in combination with electronic data acquisition and handling (Comp) allow very efficient detection of primary and secondary metabolic anomalies in patients with inherited disease. Such "profiling" of human body fluids [1] has greatly contributed to our understanding of a group of diseases known as the organic acidurias [2, 3].

By definition, profiling techniques are qualitative or semiquantitative in nature, i.e., deviations of metabolite levels in the positive or negative direction are indicators of possible metabolic disease [4]. If general profile data are also analyzed quantitatively [5], erroneous conclusions may be reached.

However, GC—MS—Comp profiling techniques can yield reliable quantitative

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**Part of the results was obtained during medical thesis work of I.R.-U.

data if only a single class of metabolites is detected, e.g. steroids [6, 7] or ketoacids [8, 9]. In such cases, chromatographic properties and quantitative behavior are more uniform and better understood, provided also that the internal standards belong to the same chemical class. Such "class specific metabolic profiling" as we would like to call it, can be made possible either through specific purification [6, 7] or through specific detection [8, 9].

Aliphatic α-ketoacids can be specifically detected as O-TMS-Q derivatives [8, 9, 10] either with nitrogen selective detectors [11] or even more specifically with multiple ion detection [9, 12]. In this case *o*-phenylenediamine serves as a label: it imposes a characteristic fragmentation pattern on the small molecular weight keto acids by converting them to hetero-aromates [9]. With the resulting high mass fragments (*m/e* 217, 232, 245) relatively little interference from other compounds is observed.

Recently, we have extended the O-TMS-Q procedure to the quantitative GC analysis of aromatic α-ketoacids in urine [13]. In the following lines we introduce a suitable internal standard which allows β-phenylpyruvic acid (PPA) to be included in a ketoacid specific profiling of body fluids using GC—MS—Comp techniques.

MATERIALS AND METHODS

The gas chromatograph and other equipment, the source of chemicals, the derivatization of aliphatic and aromatic ketoacids and the quantitative evaluation, have been fully described in previous communications [8, 13]. An O-TMS-Q procedure is also available for ketoacids in blood [14]. In short, the procedure consists of reacting unextracted ketoacids in 2 *N* HCl for 30 min at 70° with *o*-phenylenediamine, extraction of the quinoxalinols with chloroform (the aqueous phase being saturated with $(NH_4)_2SO_4$), evaporation of the organic phase, and final derivatization for 30 min at 70° with 50 μl of pyridine and 50 μl of bis-(TMS)-trifluoro acetamide. Care is taken to condense the chloroform fully by feeding the rotary evaporator with an ethylene glycol—water (1:1, v/v) mixture at −10° [14].

The deutero-TMS-Q's were prepared as described in ref. 10.

Low resolution 70-eV mass spectra were obtained on a Varian Model CH7 magnetic mass spectrometer. Samples were introduced via a Varian Model 1700 gas chromatograph with a 150-cm 3% SE-30 column and a Biemann—Watson separator. Parameters of operation have been given previously [13].

Single ion monitoring with α-ketocaprylic acid as internal standard was performed on a Finnigan Model 3000 quadrupole mass spectrometer coupled to a Varian Model 1400 gas chromatograph. The samples were introduced via a 180 cm × 1/8 in. O.D. spiral glass column filled with Dexsil 300 on Supelcoport. The temperature program was from 180 to 220° at 2°/min. The injection port temperature was 200°, the glass-jet separator and metal transfer line were kept at 250°. Electron energy was 70 eV and ion energy 5 V.

Preparation of isomeric methylphenyl glyoxylic (toluylformic) acids

In very low yields the acids could be obtained from the corresponding methylacetophenones (EGA Chemie, Steinheim, G.F.R.) by reacting them for

6 h in icecold 1% KOH with $K_3[Fe(CN)_6]$ [15].

For preparation of larger amounts with optimal purity we started from the isomeric methylbenzoic acids. These are converted: (i) to the chloride [16]; (ii) to the cyanide [17]; and (iii) by acid hydrolysis to the free ketoacid.

All chemicals were from Riedel de Haën (Seelze, G.F.R.).

(i) Dimethylformamide (in traces) was dropped into a stirred suspension of methylbenzoic acid in thionylchloride (molar ratio 1.0:1.5) and the suspension was refluxed for 1 h. After stirring at room temperature overnight, the excess thionylchloride was removed under vacuum, using a waterpump and a 15-cm Vigreux column to facilitate separation. By distillation of the residue the acid chloride was obtained. Yield 95%. $B.p._{10}$: 2-methylbenzoylchloride, 100—103°; 3-methylbenzoylchloride, 99—101°; 4-methylbenzoylchloride, 95°.

(ii) Anhydrous cuprous cyanide and methylbenzoylchloride in a molar ratio of 1.2:1 are thoroughly mixed and heated. The temperature is raised to 220—230° and maintained between these limits for 1.5 h. At the end of this time the crude toluylcyanide is purified by fractional distillation through a column. The distillate of the *meta* and *para* derivatives solidifies to colorless crystals. The *ortho* derivative is obtained as an oil. Yield 60—65%. $B.p._{10}$: 2-toluylcyanide, 109—111°; m.p.: 3-toluylcyanide, 24°; 4-toluylcyanide, 46°.

(iii) Toluylformic acid is prepared by the hydrolysis of toluylcyanide with concentrated hydrochloric acid. The mixture is shaken occasionally until the cyanide is dissolved completely and is then allowed to stand at room temperature for 5 days. The clear solution is poured into water and extracted with ether. The ether is removed by distillation. The residual oil is placed in a vacuum desiccator containing phosphorus pentoxide and solid sodium hydroxide and allowed to remain there until dry. The crude acid is dissolved in hot carbon tetrachloride and cooled until crystallization is complete.

The free *ortho* acid is a high-boiling oil. Equimolar amounts of the *ortho* acid and concentrated ethanolic sodium hydroxide were heated. The resulting sodium salt was recrystallized from 70% ethanol. Yield 73—77%. M.p.: sodium salt of 2-toluylformic acid, 297. 3—297.9°; 3-toluylformic acid, 71°; 4-toluylformic acid, 95—97°. All melting points are uncorrected.

RESULTS

Mass spectra

In Fig. 1 the mass spectra of the β-phenylpyruvic acid (PPA) derivative, O-TMS-benzylquinoxalinol (BQ) (data from ref. 13), and of O-TMS-*p*-methylphenylquinoxalinol (*p*MPQ) are shown. A high similarity with regard to high abundance of the molecular ion was expected and was indeed found. The MS of *m*MPQ is almost identical to the one of *p*MPQ. The peculiarities of *o*MPQ are described below.

The spectrum of BQ has been interpreted in ref. 13. Major ions of *p*MPQ and *m*MPQ are (*m/e* of the deuterated derivative in brackets): *m/e* 235 (235), 219 (219), and 217 (226). The ion *m/e* 217 is formed by the loss of a methylphenyl radical from the molecular ion (metastable ion at *m/e* 152.8). The ion *m/e* 219 is formed by loss of $(CH_3)_2SiO$ from the [M—15] ion (metastable ion

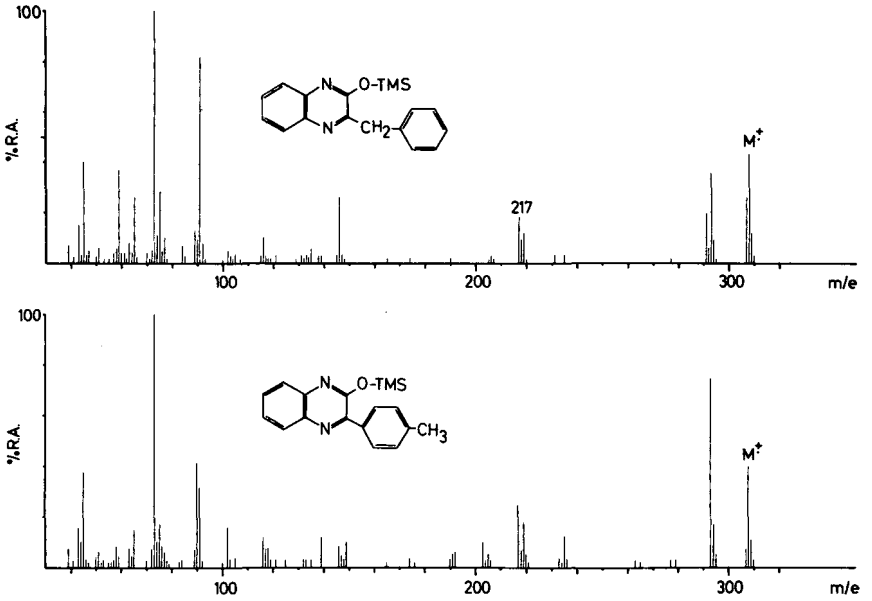

Fig. 1. 70-eV Mass spectra of O-TMS-Q's from phenylpyruvic acid (upper panel; data from ref. 13) and *p*-methylphenylglyoxylic (*p*-toluylformic) acid (lower panel). See text for interpretation.

at *m/e* 163.7). Ion *m/e* 235 is probably the result of loss of a TMS radical from the molecular ion. As evidenced by very low abundance of *m/e* 226 in the deuterated derivative, there is almost no cleavage of a methylphenyl radical from the molecular ion of *o*MPQ.

The ion *m/e* 135 (141) of BQ has been interpreted as a rearrangement product with the composition $[C_6H_5\text{-}Si(CH_3)_2]$ (ref. 13). All three isomers have a conspicious ion *m/e* 149 (155) which may be due to a similar rearrangement.

Evidence from isotope peaks shows that *p*MPQ and *m*MPQ have two doubly charged ions: *m/e* 139 (140.5) and 146.5 (149.5). The latter probably represents $[M-15]^{++}$. The first should be $[M-30]^{++}$. Its relative abundance is 3.8%, and that of $[M-30]^{+}$ is 1.1%. Ion *m/e* 139 (140.5) is practically absent from the MS of *o*MPQ.

The tropylium ion, *m/e* 91, is relatively low in abundance in all three isomers. The ions *m/e* 90 (90) and 102 (102) are similarly found in O-TMS-Q's with an aliphatic side chain [9].

Gas chromatographic properties

In Table I methylene units (MU) [18] on two silicone phases are given for the O-TMS-Q derivatives of PPA, the three isomeric methylphenylglyoxylic acids and phenylglyoxylic acid (PGA), respectively. O-TMS-phenylquinoxalinol (from PGA) and the PPA derivative have nearly identical elution times on OV-1 as well as on OV-17. There is a striking chromatographic *ortho*-effect in the elution time of the MPQ's: *o*MPQ comes out even earlier than the non-

methylated compound. The trend in ΔMU values ($MU_{OV\text{-}17}$—$MU_{OV\text{-}1}$) as shown in the table is not consistently found with different OV-1 samples. On OV-1 all derivatives yield perfectly symmetrical peaks. Multiple peaks do not occur.

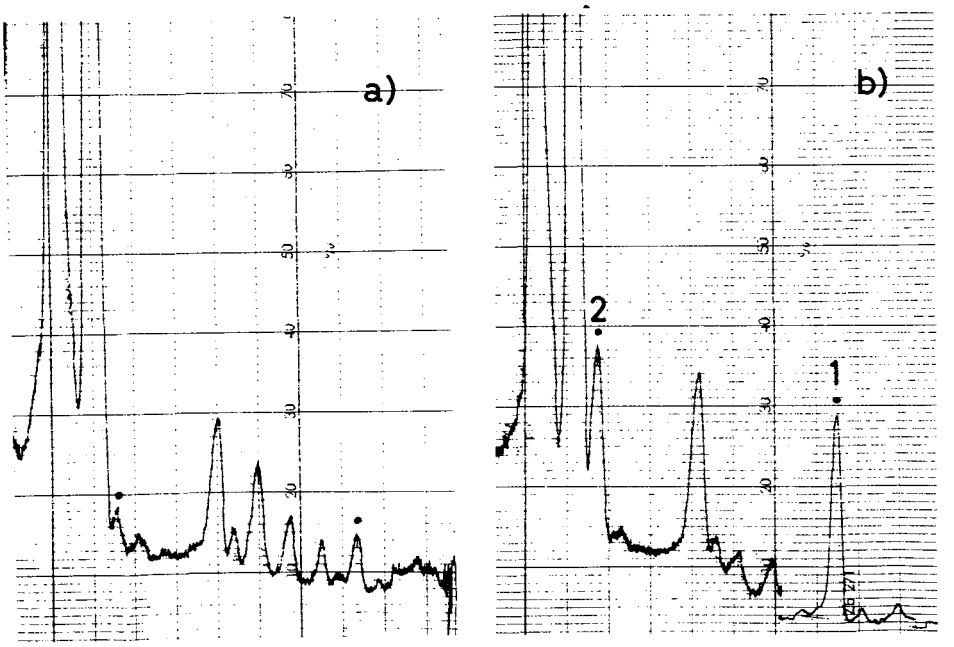

Fig. 2. Single ion monitoring of phenylpyruvic acid (2) in cerebrospinal fluid with α-ketocaprylic acid (1) as internal standard. O-TMS-Q procedure. The quadrupole mass spectrometer was focused on *m/e* 217. (a) 1 ml CSF was processed without added ketoacids. (b) To 1 ml CSF 5.3 nmoles of both ketoacids were added. Final sample volume 40 μl. 1 μl was used for GC—MS.

TABLE I

METHYLENE UNITS OF O-TMS-Q'S FROM AROMATIC α-KETOACIDS

Separation was performed with a Hewlett-Packard Model 7611 A gas chromatograph with 180 × 3 mm I.D. U-shaped glass columns. OV-1 and OV-17 both on 100—120 mesh Supelcoport. Temperature program from 60° to 180° at 2°/min. Carrier gas was nitrogen at 60 ml/min.

O-TMS-Q	OV-1	OV-17	ΔMU
o-Methylphenyl-	20.25	22.94	2.69
Benzyl-	20.63	23.31	2.68
Phenyl-	20.70	23.40	2.70
m-Methylphenyl-	21.66	24.38	2.72
p-Methylphenyl-	21.85	24.61	2.76

Single ion monitoring

Our earlier application of this technique with α-ketocaprylic acid as internal standard is shown in Fig. 2. Although partial overlap is observed between BQ and a peak of unknown identity, very high sensitivity is already obtained. With this method we showed in an untreated female patient with phenylketonuria that only traces of PPA (about 1 μmole/l) were present in her lumbar cerebrospinal fluid [19].

It is evident from Fig. 1 that greater sensitivity will be obtained by monitoring fragment *m/e* 308 or 293 with a methylphenylglyoxylic acid as internal standard. Monitoring these high masses also will improve accuracy of quantitative data.

DISCUSSION

To our knowledge, Narasimhachari [20] was the first to use isomeric internal standards for single ion monitoring. In the present paper we have evaluated this principle for the quantitative determination of PPA.

Because of its close proximity in the chromatogram *o*-methylphenylglyoxylic acid appears to be suited best as an internal standard for single ion monitoring of PPA. The stability of the derivatives is very high: down to 5 pmoles per injection we found no differential chromatographic loss of the PPA derivative when nitrogen-selective detection was used (unpublished data).

Our earlier data [8, 9] and the results of this report reveal that quantitative, α-ketoacid-specific profiling can be performed with GC—MS multiple ion detection of O-TMS-Q's on only four channels, namely *m/e* 217, 232, 245 and 308. Internal standards should be α-keto-*n*-valeric acid and *o*-methylphenylglyoxylic acid.

Such profiling studies eventually will prove helpful in interpretation of secondary metabolic derangements in phenylketonuria [21] as well as in the assessment of animal models for that disease [22, 23]. Our present studies are concerned with these topics.

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NOTE ADDED IN PROOF

Since this paper went to press we have established a GC—MS method for quantitative determination of PPA in the blood of patients with phenylketonuria. Dual ion monitoring at *m/e* 293 and 308 is performed with *ortho*-methylphenylglyoxylic acid as internal standard [24].

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QUANTITATION OF ADRENALINE AND NORADRENALINE FROM HUMAN PLASMA BY COMBINED GAS CHROMATOGRAPHY—HIGH-RESOLUTION MASS FRAGMENTOGRAPHY*

K. JACOB, W. VOGT, M. KNEDEL and G. SCHWERTFEGER

Institut für Klinische Chemie am Klinikum Grosshadern der Universität München, Postfach 701260, D-8000 München 70 (G.F.R.)

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SUMMARY

A gas chromatographic—high-resolution mass fragmentographic method for the simultaneous determination of adrenaline and noradrenaline from human plasma is presented. The catecholamines are separated by adsorption on alumina and converted by a selective, two-step procedure to the corresponding N-trifluoroacetyl-N-trimethylsilyl derivatives. The benzylic fragment $C_{16}H_{31}O_3Si_3$ (*m/e* 355.1568) of these derivatives is detected at a mass spectrometric resolving power of 5000. This high resolution detection was necessary to differentiate this fragment from others with the same nominal mass of 355 originating from the biological matrix and/or the bleeding from column and septum.

INTRODUCTION

There exists an increasing interest in the accurate and sensitive quantitative determination of the catecholamines adrenaline and noradrenaline in human plasma. Estimation of the concentration of circulating catecholamines is of importance in consideration of the pathogenesis of essential hypertension and the evaluation of stress factors in relation to cardiovascular diseases. Detection and localization of catecholamine-producing tumours such as neuroblastoma and pheochromocytoma can be achieved by means of plasma catecholamine measurements.

Analytical procedures for these estimations demand methods with a high

*Parts of the results described here were presented at "Gemeinsame Jahrestagung der Deutschen und Niederländischen Gesellschaften für Klinische Chemie", 9—11 March 1977, Aachen, G.F.R., and at the International Symposium "Mass Spectrometry and Combined Techniques in Medicine, Clinical Chemistry and Clinical Biochemistry", 14—15 November 1977, Tübingen, G.F.R.

degree of sensitivity and specificity since the plasma concentrations are extremely low. Among the detection methods which have been described for plasma catecholamines are fluorimetry, radiometry, gas chromatography (GC) and mass fragmentography. Fluorimetric procedures normally show a certain lack of specificity [1, 2], or need relatively large plasma samples [3]. The radioenzymatic double-isotope assay affords good reliability and specificity [4]. An increased sensitivity was obtained with tritiated S-adenosyl-methionine as methyl donor [5]. However, the experimental effort required for these methods is considerable. GC procedures with electron capture detection allow the required sensitivity [6], whereas the specificity is inadequate for the small amounts present in plasma. A GC method with a dual hydrogen flame detector has been reported, but the structure of the employed derivatives was not clarified [7].

Wang et al. [8] have published a low-resolution mass fragmentographic method using per-trifluoroacetyl derivatives. Drawbacks to this procedure, in addition to the low mass numbers of the detected fragments, are the instability and the poor GC separation of these derivatives.

Due to the high reliability and sensitivity of mass fragmentography we have developed another, improved method for plasma catecholamines using high-resolution mass fragmentography, which offers an outstanding degree of specificity.

EXPERIMENTAL

Materials

All reagents and solvents were of analytical grade and were obtained from E. Merck (Darmstadt, G.F.R.), unless otherwise specified. Noradrenaline was purchased from Fluka (Buchs, Switzerland), isoprenaline was a gift from Boehringer Ingelheim (Ingelheim, G.F.R.). Alumina (Woelm, neutral, activity grade I) was supplied by ICN Pharmaceuticals (Eschwege, G.F.R.) and treated as described by Von Studnitz [9]. N-Methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) and N-methyl-bis-(trifluoroacetamide) (MBTFA) were obtained from Macherey-Nagel (Düren, G.F.R.).

Procedure

Blood samples were collected (after a short rest) from the brachial vein, into 10-ml Li-heparinized tubes which each contained 5 mg sodium dithionite as antioxidant. The blood was immediately cooled with ice-water and centrifuged (4°, 15 min, 1000 *g*). A 2-ml volume of plasma was deproteinized with 0.16 ml of 70% perchloric acid and again centrifuged (4°, 20 min, 15000 *g*). The supernatant was transferred, 20 ng isoprenaline and 1 ml of 0.2 *M* Na_2-EDTA were added, and the pH was adjusted to 6 with 4 *M* NH_4OH. A 300-mg amount of alumina was neutralized with 0.1 *M* ammonium acetate, both the adsorption and elution of the catecholamines were performed as described by Wang et al. [8]. A 10-μl volume of a 0.1% solution of methyl orange in methanol was added to the eluate, which was concentrated at 30° by vacuum evaporation and dried in a vacuum desiccator over potassium hydroxide—phosphorus pentoxide for at least 8 h. The residue was derivatized in a 1-ml glass-

stoppered tube following the procedure of Donike [10, 11] by dissolving in 20 μl of trifluoroacetic acid and treating with 45—55 μl MSTFA (the exact amount depends on the colour change of the indicator from red to yellow) for 30 min at 80°. A 10-μl volume of MBTFA was added and after a 10-min treatment at ambient temperature the mixture was heated for 5 min at 80°. Of this solution 2 μl were used for mass fragmentography. The solutions can be stored in a desiccator for several days, provided that a possible colour change of the indicator is titrated with a slight excess of MSTFA.

Gas chromatography—mass spectrometry

GC—mass spectrometry (MS) analyses were performed on a double focusing mass spectrometer MAT 311 A, which was coupled to a gas chromatograph Varian 1440 (Varian, Bremen, G.F.R.). An all-glass coupling system with a one-stage glass frit separator was used. A device for removal of large quantities of the solvent was installed between the GC column and the separator. The peak matching decade served for the exact monitoring of the desired mass using perfluorokerosene as reference substance. GC—MS conditions are as follows: column, 2 m × 2 mm I.D., 4% OV-17 on Chromosorb W AW DMCS; carrier gas, He, 30 ml/min; injector 250°; column temperature 200°; connection capillary 240°; separator 250°; line-of-sight 160°; ion source 160°; ionization energy 70 eV; emission current 3 mA; electron multiplier 2.0—2.4 KV; resolving power 5000; detected mass 355.1568.

RESULTS AND DISCUSSION

Due to their high stability against oxidation and excellent GC properties we chose the N-trifluoroacetyl-O-trimethylsilyl (N-TFA-O-TMS) derivatives [10, 11]. They can be well separated on silicon phases and do not show adsorption effects either on the GC column or in the GC—MS coupling system. The derivatization has to be carefully performed in a two-step procedure under controlled silylation conditions as outlined by Donike [10, 11].

The mass spectra of the N-TFA-O-TMS derivatives exhibit weak molecular ions, whereas the base peaks are formed by very intense benzylic fragments at *m/e* 355, which we used for mass fragmentography. Detection of the fragment ion at *m/e* 355, and not the molecular ion, leads to the loss of important information concerning the molecular structure. However, this mutual ion allows the simultaneous detection of both catecholamines and the internal standard isoprenaline.

The N-TFA-O-TMS derivatives have been successfully applied to the low-resolution mass fragmentographic determination of catecholamines from tissues [10]. Hitherto plasma samples could not be assayed using this type of derivative because of the low content of catecholamines and the large amounts of interfering substances from the biological matrix.

The sensitive and selective low-resolution monitoring of the benzylic fragment is excluded by the presence of ions with the same nominal mass number (Fig. 1). These undesirable ions originate either from the silicon phases of the column and the septum or from the biological material. They are particularly intensified in the analyses of plasma samples, where sometimes even negative

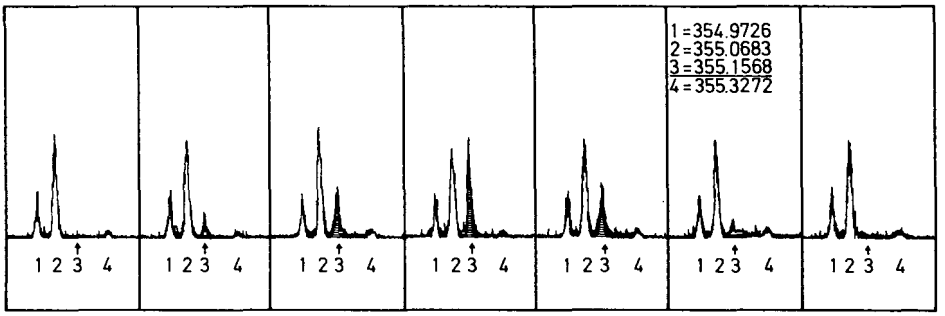

Fig. 1. Detection of N-TFA-O-TMS catecholamines in plasma. Sweep from mass 354.5 to 355.5 (R=5000). The expected signal of the benzylic fragment $C_{16}H_{31}O_3Si_3$ (*m/e* 355.1568) is indicated by an arrow (No. 3) and corresponds to the isoprenaline peak in the chromatogram.

peaks are observed. However, these difficulties could be overcome by increasing the resolving power of the mass spectrometer to 5000. Thus we were able to separate completely the monitored signal of the benzylic fragment $C_{16}H_{31}O_3Si_3$, with the precise mass of 355.1568, from the background. The impressive specificity of high-resolution mass fragmentography was previously shown in the detection of steroids by Millington et al. [12].

The comparison of the analyses of the same plasma sample with low-resolution detection (Fig. 2a, R = 1000, *m/e* 355) and high-resolution detection (Fig. 2b, R = 5000, *m/e* 355.1568) clearly demonstrates the superior specificity of high-resolution mass fragmentography. The low-resolution fragmentogram results in totally incorrect intensity ratios.

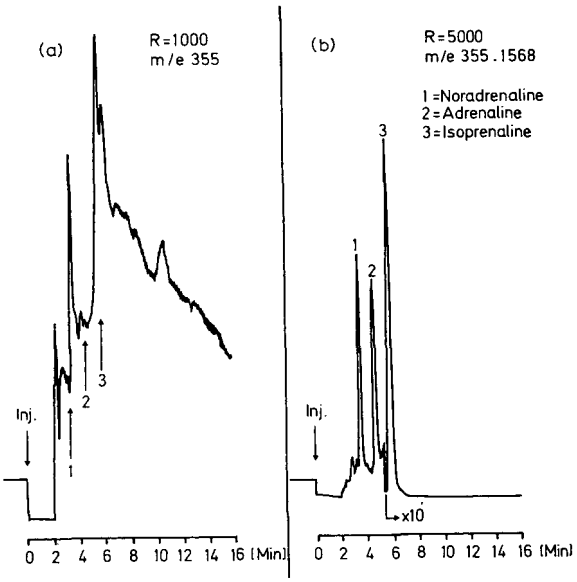

Fig. 2. Comparison of the detection of plasma catecholamines with low-resolution mass fragmentography, (a) R = 1000, *m/e* 355, and high-resolution mass fragmentography, (b) R = 5000, *m/e* 355.1568. The content of this sample was 0.2 ng/ml adrenaline and 0.7 ng/ml noradrenaline.

The detection limit was 2 pg of injected pure sample with a signal-to-noise ratio of 2 to 1. The coefficient of variation of the method is 16% (n = 6) at a catecholamine level of 0.2 to 0.7 ng/ml plasma. The standard curve for adrenaline shows a correlation coefficient of 0.9901 (Fig. 3). Plasma levels of healthy volunteers lay at 0.1 ng/ml for adrenaline and from 0.2 to 0.4 ng/ml for noradrenaline. These values correspond well with those obtained from the literature as determined by radioenzymatic assays.

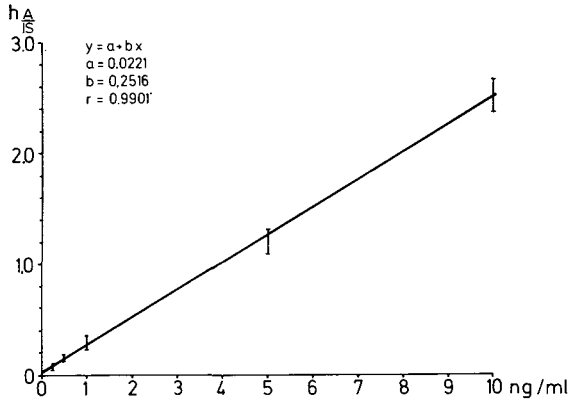

Fig. 3. Standard curve for adrenaline (N-TFA-O-TMS derivative).

By our method a pheochromocytoma could be diagnosed and localized before confirmation by surgical excision of the tumour. The determination of the catecholamine concentration in the vena cava inferior gave the following values.

TABLE I

VENOUS CATECHOLAMINE CONCENTRATIONS

Sample site	Noradrenaline (ng/ml)	Adrenaline (ng/ml)
V. cava inferior (superior part)	10.0	6.0
V. suprarenalis dextra	⩾ 15.0	6.5
Abouchement of vv. renales	7.5	1.9
V. cava inferior (bifurcation)	4.0	1.5
V. iliaca communis dextra	1.0	1.0

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Biomedical Applications

CHROMBIO. 178

QUANTITATIVE ANALYSIS OF PRAZEPAM AND ITS METABOLITES BY ELECTRON CAPTURE GAS CHROMATOGRAPHY AND SELECTED ION MONITORING

APPLICATION TO DIAPLACENTAL PASSAGE AND FETAL HEPATIC METABOLISM IN EARLY HUMAN PREGNANCY

H. NAU*, C. LIDDIARD, D. JESDINSKY and W. WITTFOHT

Institut für Toxikologie und Embryonalpharmakologie, Freie Universität, Garystrasse 9, D-1000 Berlin 33 (G.F.R.)

and

K. BRENDEL

Department of Pharmacology, College of Medicine, University of Arizona, Tucson, Ariz. (U.S.A.)

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SUMMARY

Methods have been developed for the determination of the benzodiazepine tranquilizer prazepam and its metabolites desmethyl diazepam, 3-hydroxy-prazepam and oxazepam by electron capture gas chromatography and selected ion monitoring with diazepam as the internal standard. The benzodiazepines were isolated from blood serum or homogenized tissue samples, either by extraction with ethyl acetate or on small Extrelut columns packed with porous silica. The concentrated extracts were directly injected into the gas chromatograph equipped with an electron capture detector. Following trimethylsilylation, analysis on a gas chromatography—mass spectrometry—computer system operated in the selected ion-monitoring mode was performed. Using 50—200 mg (μl) biological material, concentrations of prazepam and metabolites of 5 ng/g(ml) could be determined with signal-to-noise ratios of >10. Using 1 g(ml) samples, the same signal-to-noise ratios were obtained with 1 ng/g(ml) concentrations.

The methods developed were applied to the analysis of the diaplacental transfer of prazepam and desmethyl diazepam in early human pregnancy. Furthermore, prazepam metabolism in human fetal liver and cell cultures was studied.

*To whom correspondence should be addressed.

INTRODUCTION

It has been recognized in recent years that almost all foreign substances taken in by the mother including drugs, environmental contaminants, food additives, the constituents of cigarette smoke, alcohol, etc. cross the placenta, enter the fetal blood stream and may be stored in fetal tissues [1, 2]. It has also been demonstrated that from a relatively early stage (6—8 weeks of gestation) the human fetus can metabolize a variety of foreign compounds [1, 3—5].

While metabolism can lead to detoxification of harmful substances, it may also result in the production of toxic or pharmacologically active metabolites from otherwise innocuous compounds. Frequently such derivatives are more polar than the parent compounds and slowly cross the placenta back to the mother. Such transfer characteristics may result in an accumulation of metabolites in the fetus. It is therefore important to determine the concentrations of metabolites as well as those of the parent compounds in the fetus. In contrast to man, common laboratory animal species do not possess the ability to metabolize xenobiotics at such an early stage of gestation. Therefore, results obtained from studies with animals cannot be reliably extrapolated to the human situation and may underestimate the risk at hand, thus making studies on human subjects essential.

Of particular interest is the transfer of xenobiotics from mother to embryo during organogenesis (4—8 weeks of gestation) at which time the conceptus is most sensitive to teratological lesions. Although fetal tissue is available from therapeutic abortions during the first trimester, the amounts of clearly identifiable material obtained at this early stage of human gestation are often only in the milligram range. Therefore, analytical methods with high sensitivity are required to investigate such material.

This report describes analytical methods suitable for the study of the diaplacental transfer of prazepam (a benzodiazepine) as well as its metabolites during early human pregnancy and for in vitro investigations of the metabolism of prazepam in cultures of human fetal liver. A gas chromatograph equipped with an electron capture detector and a GC—MS—computer system used in the selected ion monitoring mode are shown to provide sufficient sensitivity and selectivity to achieve the experimental goals. Several results are reported as illustrative examples of the suitability of the analytical methods.

EXPERIMENTAL PROCEDURES

Solvents and reagents

Extrelut and pyridine ("getrocknet") were obtained from Merck (Darmstadt, G.F.R.), ethyl acetate, benzene and methanol (nanograde) from Byk-Mallinckrodt (Wesel, G.F.R.), Tri-Sil BSA formula P from Pierce (Rotterdam, The Netherlands), the GC column-packing material 3% SP-2250 on Supelcoport 100—120 mesh from Supelco (Bellefonte, Pa., U.S.A.).

Internal standard

A stock solution of 6 mg diazepam in 10 ml of methanol was prepared and

diluted 1:10 with methanol prior to storage in 1.5 ml glass vials at −25°; 10.0 µl of this dilution (= 60 ng diazepam) were added to each sample as early as possible (see below). We have used identical amounts of methyl-d_3-diazepam as internal standard for the analysis of the metabolites of various benzodiazepine drugs including medazepam (Nobrium) and diazepam (Valium), prazepam (Demetrin) by selected ion monitoring, hence the label with deuterium was necessary.

Standard calibration graphs were obtained by adding known amounts of prazepam and its metabolites (between 2 and 200 ng) to 200-µl portions of drug-free human serum containing the internal standard (60 ng); see Figs. 1 and 2. A linear least-square analysis of the peak height ratios vs. amounts of drug and metabolites added was made.

Biological material

Human fetuses (between 6 and 24 weeks of gestation) were obtained by hysterotomy, curettage, or prostaglandin-induced abortions for social or medical reasons. Maternal blood samples were taken at the time of the interruptions. For studies of the diaplacental passage of prazepam, the fetuses were kept frozen on dry ice until dissection began. The fetal material was allowed to thaw and the various tissues were carefully dissected and stored in 1.5 ml conical plastic semi-micro tubes (Eppendorf) at −25°. At the time of analysis, 10—200 mg of the frozen tissues, depending on availability, were weighed and transferred into Eppendorf tubes. A volume of 200 µl of deionized water and 60 ng internal standard were added and the samples were homogenized either with a conical PTFE pestle made to fit the conical tubes or by sonication at 0°. Both methods gave identical results.

For the preparation of fetal liver organ cultures and of isolated fetal liver cells, the livers were excised from the fetuses as soon as possible and then pro-

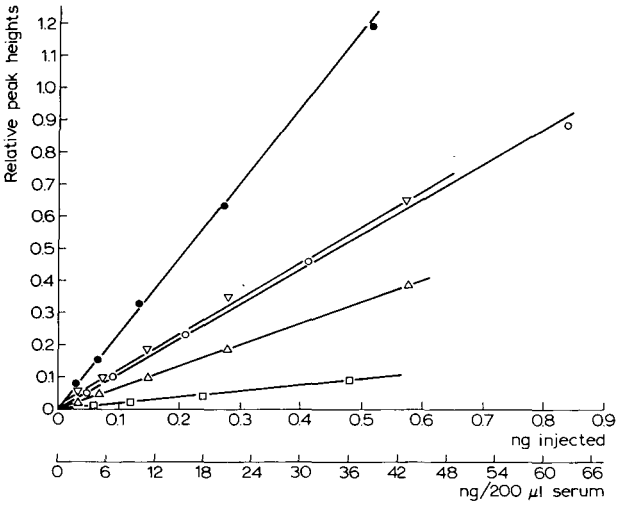


Fig. 1. Plots of the peak heights (relative to the peak height of diazepam, internal standard) of Pr and metabolites vs. amounts present in 200 µl serum as well as in injected samples (detection by electron capture GC). •, Pr-OH (TMS); ▽, Ox; ○, DD; △, Pr; □, Pr-OH.

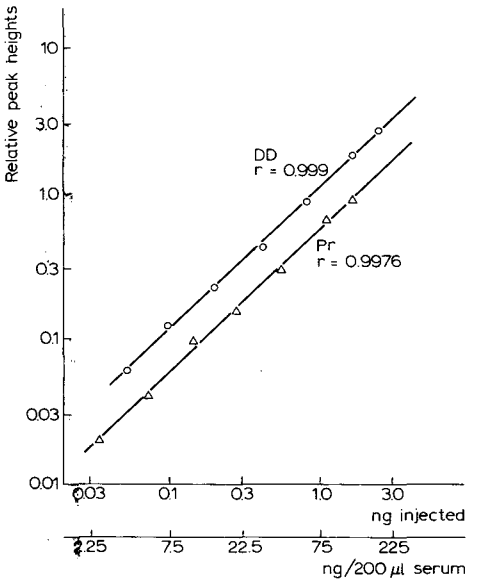


Fig. 2. Double logarithmic plots of the peak heights of Pr and DD (relative to the internal standard, diazepam) vs amounts present in 200 µl serum as well as injected samples (detection by electron capture GC).

cessed as described elsewhere in detail [6]. Prazepam 0.5—20 µg per ml incubation medium, was added to the final suspension of the fetal tissue and the cultures were incubated on a gyratory shaker (60 gyrations per min) in an atmosphere of 95% O_2/5% CO_2 at 37°. Samples were removed at selected time intervals, sonicated and frozen. At the time of analysis, 200 µl of the homogenized samples were transferred into 1.5 ml Eppendorf tubes and 60 ng of internal standard was added.

Extraction

Three methods for the isolation of prazepam and metabolites were explored:

(a) To 200 µl of the various samples to be analyzed (e.g. maternal serum, fetal tissue homogenates or homogenates of fetal liver cells or organ culture material, all of which contained 60 ng of internal standard), 1 ml of ethyl acetate was added. The Eppendorf tubes were tightly closed, shaken on a Vortex mixer for 2 min and centrifuged at 550 *g* for 2 min. A volume of 800 µl of the organic phases were transferred into 1.5-ml glass vials and the solvent was evaporated under a stream of nitrogen. Traces of remaining water were removed by the addition of 100 µl benzene and a repeat of the evaporation. Finally, 30 µl of pyridine were added, the vials were closed with a PTFE-lined rubber septum using a hand crimper, and 1 µl was injected into the gas chromatograph. If the samples were to be silylated, 30 µl of Tri-Sil BSA were added, and the vials were incubated at 55° for 30 min. Portions of 2 µl were injected into the gas chromatograph.

(b) The 200 µl samples to be analyzed were diluted with up to 200 µl of deionized water and poured into Pasteur pipettes (Fig. 3) filled with a weighed

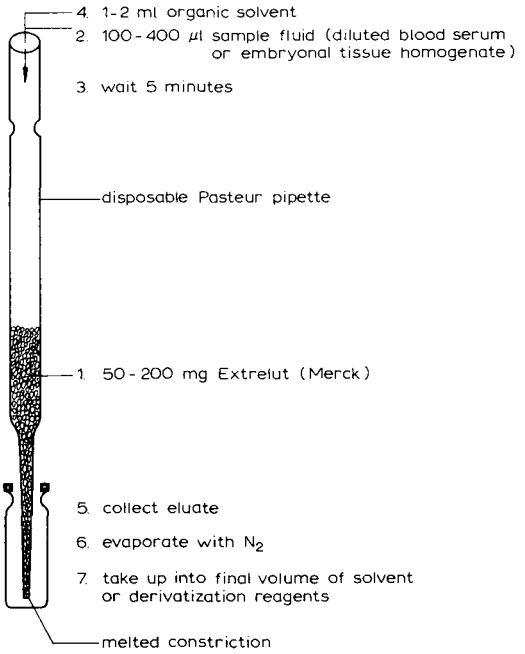


Fig. 3. Schematic representation of the isolation of benzodiazepines and their metabolites from small samples (5—200 mg) of human embryonal and fetal tissue (following homogenization) and 50—200 µl of serum or plasma (following dilution by factor of 2).

amount of Extrelut [7] in a proportion of 50 mg Extrelut for each 100-µl sample volume. After 5 min the columns were eluted with 1—2 ml of ethyl acetate, the eluate collected in 1.5-ml glass vials and further processed as described under (a).

(c) To the 200-µl samples, 100 µl of butyl acetate [8] were added and the contents of the tubes were Vortex mixed for 2 min. Following centrifugation, 2.5 µl of the supernatant butyl acetate phase were carefully withdrawn into a 10-µl syringe and injected into the gas chromatograph.

Gas chromatography

A Carlo-Erba 2300 gas chromatograph equipped with an electron capture detector was used. The detector (HT-20) was operated in constant current mode (Model 250). A 2-m glass column was used which was packed with 3% SP-2250 on Supelcoport 100—120 mesh. The oven temperature was kept at 260° for the simultaneous analysis of all prazepam (Pr) metabolites and at 280° for the determination of the trimethylsilylated 3-hydroxy-prazepam (Pr-OH TMS). The temperature of the injection port was 275° and of the detector 300°. Nitrogen (Linde, Munich, G.F.R.; purity 5.0) was used as carrier gas (20 ml/min) and scavenger gas between column and detector (40 ml/min).

Selected ion monitoring on a GC—MS—computer system

This system consisted of a Perkin-Elmer F-22 gas chromatograph which was

directly coupled via a 1/4 in. Swagelok union (drilled to 6 mm) to a single-stage Watson—Biemann separator and the ion source of a CH 7 A Varian-MAT mass spectrometer. A Varian SS-100 data system was used for switching the acceleration voltage and monitoring the intensities of up to 8 ions. The following ions were recorded for the analysis of the trimethylsilylated samples: *m/e* 287(M^+, internal standard); 324 (M^+, Pr); 342 (M^+, desmethyl diazepam (DD) TMS); 383 (M^+—29, Pr-OH TMS); and 429 (M^+, oxazepam (Ox) $(TMS)_2$). Calibration graphs are shown in Fig. 4.

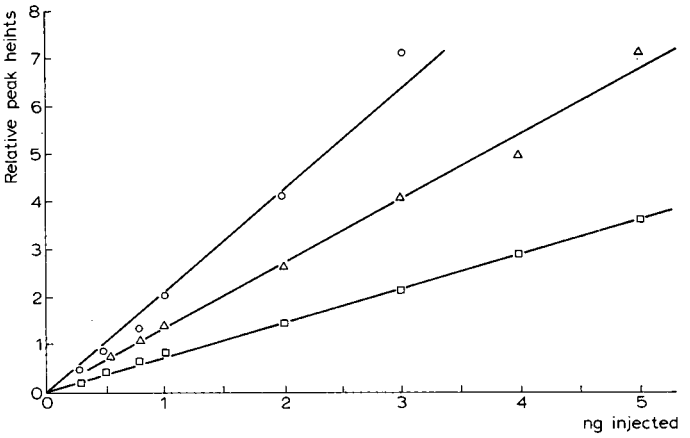


Fig. 4. Plots of peak heights of ions corresponding to Pr and metabolites (relative to ion *m/e* 287, methyl d_3-diazepam used as i.s.) vs. amounts present in injected samples (detection by selection ion monitoring). ○, DD (trimethylsilylated), r = 0.985; △, Pr, r = 0.9987; □, Pr-OH (trimethylsilylated), r = 0.9994.

RESULTS

Isolation Procedures

Three methods for the isolation of prazepam and metabolites from biological material have been used (see Experimental). The yields of the benzodiazepines studied obtained by method (a) (single-step ethyl acetate extraction) and method (b) (using small Extrelut columns) are compared in Table I. Higher yields were obtained with procedure (a) with the exception of Pr-OH whose recovery was higher with procedure (b). A comparison of the electron capture gas chromatograms of two samples which have been prepared with procedures (a) and (b) revealed that a lower baseline was obtained when the ethyl acetate extraction rather than the Extrelut column work up was used.

Method (c) was faster than either method (a) or (b) and resulted in high yields of all metabolites studied. However, the gas chromatographic base lines obtained are somewhat higher than those resulting from method (a). This method may be preferable if benzodiazepines are present in concentrations exceeding 50 ng/ml(g). Since high sensitivity was required in most of our experiments, we have routinely used method (a).

TABLE I

RECOVERY OF PRAZEPAM AND METABOLITES DURING ISOLATION PROCEDURES (a) AND (b).

Benzodiazepines	Recovery (%)	
	Ethyl acetate extraction*	Extrelut column**
Prazepam	93.5	80
Desmethyl diazepam	99.6	85
3-Hydroxy prazepam	83	93
Oxazepam	75	69
Diazepam (internal standard)	97.3	86

*Single-step ethyl acetate extraction.
**On small columns filled with porous silica (Extrelut).

Gas chromatography

Pr and its metabolites elute well-separated from each other and from the internal standard used (diazepam) on an SP-2250 column kept isothermally at 260° (Fig. 5). The detection limits of the compounds analyzed are presented in Table II. Pr-OH eluted relatively late (though as a well shaped peak) and therefore was not detected with sensitivity comparable to the other compounds. The volatility of this metabolite could be greatly enhanced by trimethysilylation, and the resulting TMS ether eluted conveniently just after Pr (Fig. 6). The lower limit of detection of this derivative was also comparable to Ox, DD, and Pr. Thus, if low levels of Pr-OH (<50 ng/g) were to be quantitated, the sample was first directly injected into the gas chromatograph to detect Ox, DD, and Pr (Fig. 6B). Then, the trimethylsilylating reagent was added and the resulting mixture injected for the quantitation of Pr-OH (Fig. 6C).

Selected ion monitoring

All metabolites of Pr could be quantitated in a single analysis using a GC—MS—computer system. The sample was trimethylsilylated and injected into the gas chromatograph. With the aid of the computer, the acceleration voltage of the mass spectrometer was switched rapidly to focus the molecular ions of the compounds to be analyzed sequentially at the electron multiplier detector. Since the molecular ions of the benzodiazepines studied are very abundant, high sensitivity was obtained which was comparable to the electron capture detector. The specificity of selected ion monitoring for the detection of benzodiazepines and their metabolites was even higher than electron capture GC, and all Pr metabolites could be quantitated in a single experiment either isothermally (Fig. 7B) or with linear temperature programming (Fig. 7A).

Calibration plots

Precisely measured amounts of Pr, DD and Pr-OH were added to 200-μl human serum samples in addition to 60 ng internal standard. The samples were then processed by the ethyl acetate extraction procedure (a) and analyzed

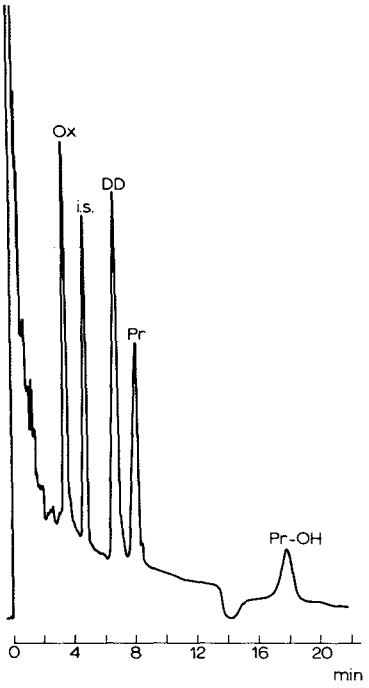


Fig. 5. Electron capture gas chromatogram (isotherm, 260° of a butyl acetate extract of human serum (extraction method, c; see Experimental); the injected sample contained 750 pg each of Ox, diazepam (internal standard), DD, Pr and Pr-OH.

TABLE II

LOWER DETECTION LIMITS AND RELATIVE STANDARD DEVIATIONS OF THE BENZODIAZEPINES DETERMINED BY ELECTRON CAPTURE GC

Signal-to-noise ratio = 2.

Benzodiazepines	Lower detection limit (pg)*	Relative standard deviation**	
		Day—day variation	Single-day variation
Diazepam	1.8	Internal standard	Internal standard
Prazepam	7.2	5.9	3.7
Desmethyl diazepam	4.1	4.2	2.8
Oxazepam	5.7	9.1	4.9
3-Hydroxy prazepam -TMS	29 1.5	7.5	5.9

*In terms of concentrations: 1—5 ng/ml serum or ng/g fetal tissue using 1000—50 μl (mg) biological material.

**Determined in 200-μl human serum samples during one month; concentrations of the benzodiazepines between 2 and 40 ng/200 μl serum.

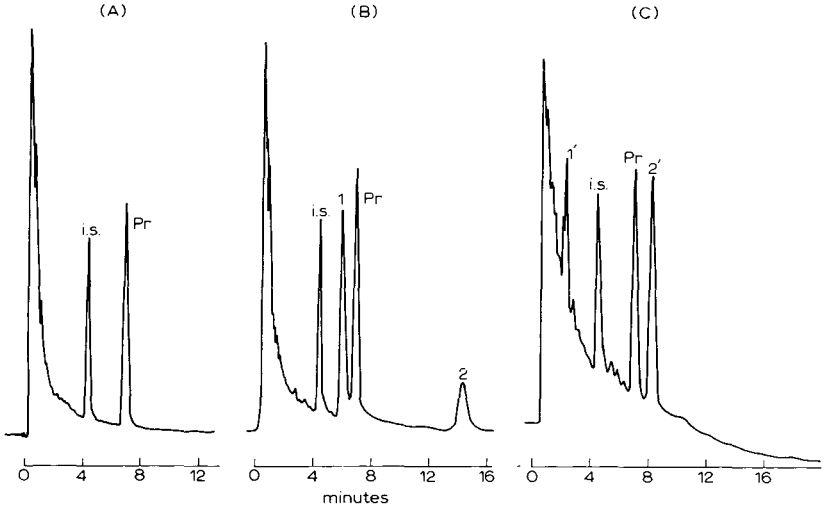


Fig. 6. Electron capture gas chromatograms of ethyl acetate extracts (extraction method a) of in vitro cultures containing Pr: (A) without liver; (B) with fetal liver (human fetus 10 weeks gestation), 1 = DD, 2 = Pr-OH; (C) sample B following trimethylsilylation, 1′ = DD, 2′ = Pr-OH, (both TMS).

by electron capture GC. Linear relationships between the relative peak heights referred to the internal standard vs. amounts added to the serum samples, were obtained for all compounds studied (Fig. 1). Double logarithmic plots of the relative peak heights of DD and Pr vs. amounts added, showed a linear range over more than three orders of magnitude. The plots covering two orders of magnitude, which we have used routinely for quantitation in our experiments, are shown in Fig. 2, where a slope of 1.0 indicates linearity in a double logarithmic presentation.

Comparable results were obtained on the GC—MS—computer system used in the selected ion monitoring mode (see Fig. 4).

DISCUSSION

The benzodiazepines are a thoroughly investigated class of drugs and a large selection of literature describing the analysis of these compounds exists [9, 10]. The analytical methods for Pr are, however, not as multifarious as those for many other benzodiazepines.

In pharmacokinetic studies involving Pr, DiCarlo and co-workers [11] made use of ^{14}C-ring-labelled Pr. The absolute level of activity in samples was measured by scintillation spectrometry. Pr and its metabolites were separated by thin-layer chromatography (TLC) and relative amounts were determined using a radioscanner.

Maier and Wehr [12] have reported a thin-layer and a GC method for the identification of Pr. The latter method relies upon the acid hydrolysis of Pr to the more volatile benzophenone. This method was, however, not suitable for our investigational goals since it did not allow a distinction between drug and metabolite(s).

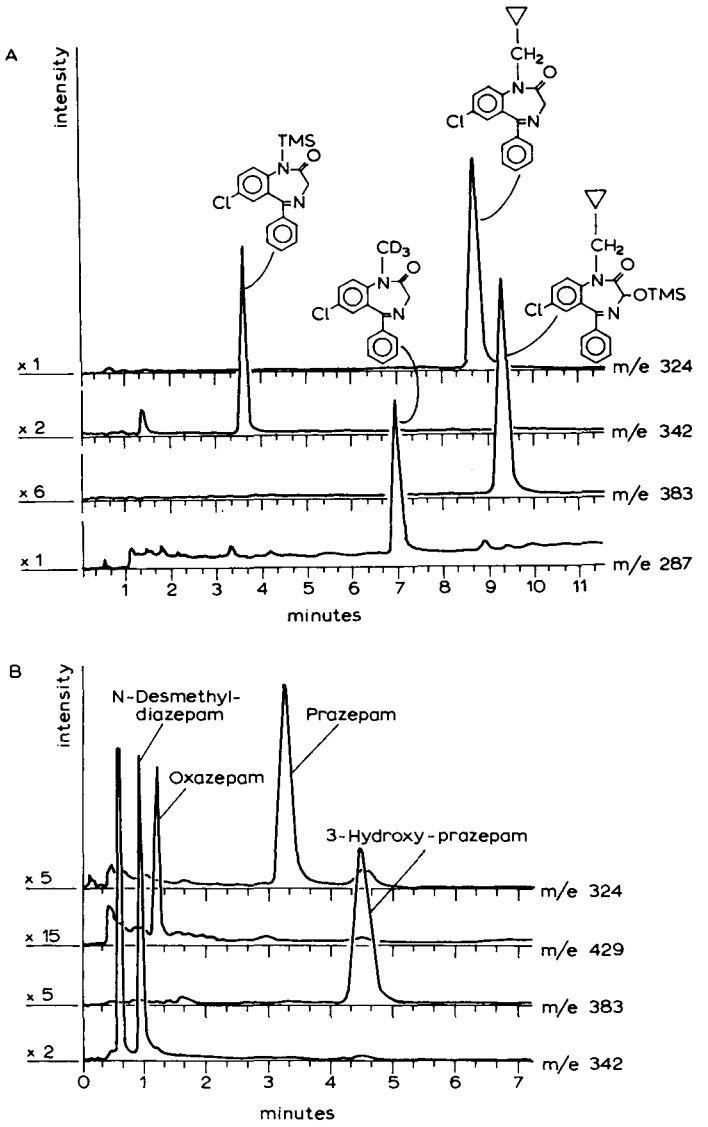


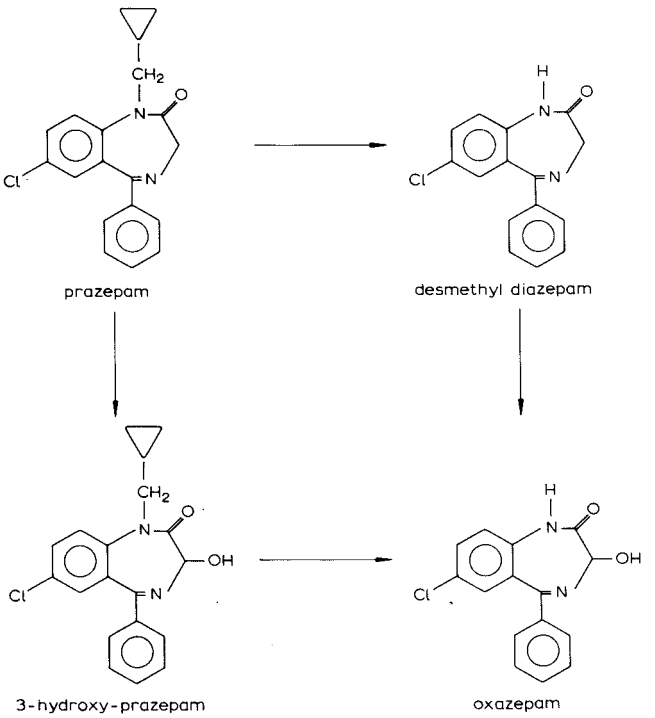
Fig. 7. (A) Selected ion monitoring (temperature program from 200 to 260° with 10°/min) of the trimethylsilylated ethyl acetate extract of Pr metabolites formed in cultures of human fetal liver fragments (fetus at 10 weeks). (B) Selected ion monitoring (isothermal at 260°) of the TMS ethyl acetate extract of Pr metabolites formed in cultures of isolated human fetal liver cells (fetus at 24 weeks).

The first GC method for the quantitation of other benzodiazepines also relied upon this acid hydrolysis step but has now been superseded by the GC separation of the intact benzodiazepines. The use of the electron capture detector for increased sensitivity and selectivity in the detection of both the intact benzodiazepines and their metabolites or the acid hydrolysis products is also well documented [9, 10]. However, to our knowledge, there is only

one example of the use of a GC—MS system operated in the selected ion monitoring mode for the analysis of the benzodiazepines pivoxazepam and 2'chloropivoxazepam [13]. The greater specifity which this method can afford is, however, vitiated since analysis follows acid hydrolysis to the benzophenone, where both drug and major metabolites result in the same product.

We found that Pr and its metabolites which have been identified in the human [11] (Scheme I) can be separated using a well conditioned SP-2250 or OV-17 column (Fig. 5) and quantitated with high sensitivity by an electron capture detector.

Scheme I



Although Pr-OH elutes much later than the other compounds from this column, it could also be quantitated, although with reduced sensitivity. Therefore, if low concentrations of Pr-OH (<50 ng/g) were to be determined, the samples were trimethylsilylated and then injected again (Fig. 6). The minimum-detectable quantities of all compounds studied in the injected samples were in the low pg-range (Table II). Blood serum containing 5 ng/ml and fetal tissue containing 5 ng/g of Pr and metabolites could be quantitated by our method with signal-to-noise ratios of >10 on the electron capture detector using 50—200 μl (mg) sample sizes. Lower concentrations (1 ng/mg and 1 ng/g, respectively) could be analyzed if 1 ml or 1 g biological material, respectively, were available.

Selected ion monitoring proved to be of comparable sensitivity for the quantitation of the benzodiazepines studied. We found that the selectivity of the technique for the determination of this class of compounds exceeded that of electron capture GC. Analysis of the trimethylsilylated sample was

sufficient to quantitate all Pr metabolites in a single experiment.

The day to day variation of our method is acceptable as expressed by the relative standard deviations in Table II. Although oxazepam (if it is not trimethylsilylated prior to injection) apparently rearranges to the corresponding quinazolin carboxaldehyde [14, 15], it could be quantitated with slightly higher standard deviations by electron capture GC (Table II). Following trimethylsilylation, the intact molecule with a molecular weight of 429 could be detected (Fig. 7B).

Much lower relative standard deviations result for samples analyzed on the same day. We routinely processed three spiked serum samples (containing between 4 and 40 ng of Ox, DD, Pr, PrOH per 200 μl) with 10—15 serum and fetal tissue samples per day.

Of the three extraction methods which were compared, we now routinely use method (a), the single step ethyl acetate extraction. Method (b) with the Extrelut columns is comparable with (a) regarding speed and ease, and led to higher recoveries of Pr-OH while all other compounds studied had poorer yields. Work is in progress to evaluate possible techniques to increase further the efficiency on the small Extrelut columns. Prepurification of the Extrelut may also result in more acceptable baselines necessary for high sensitivity work. Method (c) is faster and more convenient than (a) and (b) but not as sensitive; the limit of sensitivity was, for Pr and DD, 20—30 ng/ml which is in accordance with the findings of Rutherford [8] who used method (c) for the isolation of diazepam metabolites.

We have applied our method during the past year to the study of the diaplacental passage of Pr in early human pregnancy (Fig. 8) [16]. Fetal tissue

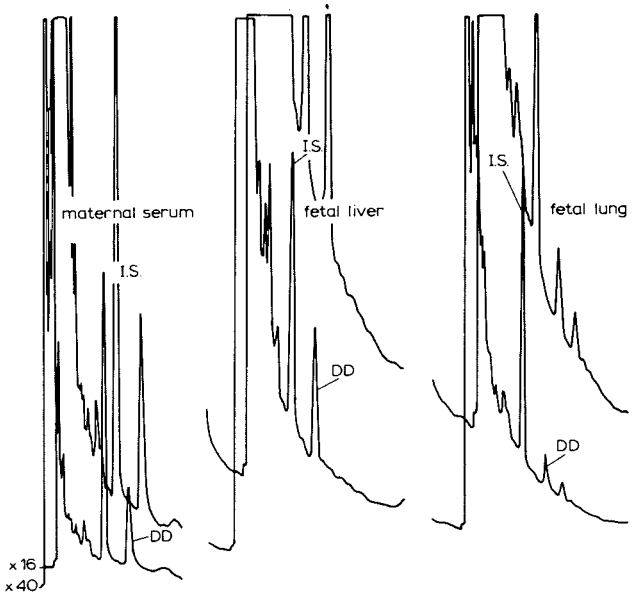


Fig. 8. Electron capture gas chromatograms of ethyl acetate extracts (extraction method, a) of maternal serum, human fetal liver and human fetal lung. The patient received 10 mg Pr 25 h 50 min prior to interruption of pregnancy (17 weeks gestation). DD concentrations were: maternal serum, 74 ng/ml; fetal liver, 151 ng/g; fetal lung, 39 ng/g.

samples were homogenized and extracted in the same manner as serum and plasma samples. Our results indicate that DD, the main metabolite of Pr, accumulates in the fetal liver (Fig. 8). In placenta and fetal heart, concentrations of DD were found which were comparable to the corresponding maternal blood levels, while the other fetal tissues contained much lower concentrations. Accumulation of DD in the fetal liver had previously been detected following maternal diazepam intake [17].

The methods presented in this paper have also been applied extensively to the study of Pr metabolism in human fetal liver in vitro. Both in liver organ cultures (Fig. 6) and cultures of isolated human fetal liver cells (Fig. 7) extensive metabolism of Pr was found. The main metabolites were DD and Pr-OH which were present in comparable amounts, and Ox was also detected (Fig. 7B), albeit in lower concentrations [6].

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AUTOMATED QUANTITATIVE GAS—LIQUID CHROMATOGRAPHY OF INTACT LIPIDS

I. PREPARATION AND CALIBRATION OF THE COLUMN

P. MAREŠ*, E. TVRZICKÁ and V. TAMCHYNA

Gastroenterological Research Unit I, Faculty of Medicine, Charles University, Prague (Czechoslovakia)

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SUMMARY

A method for quantitative gas chromatographic determination of plasma lipids (free cholesterol, cholesteryl esters and triglycerides) in the low concentration range is described. This method permits a determination of not only the lipid classes mentioned above, but also their fractions according to molecular weight, down to 10 ng, without previous derivatization. Special attention was devoted to the preparation of columns with high efficiency and minimal losses of the test substances. The best results were obtained with a glass column 0.5 m × 2.0 mm I.D., packed with 1% OV-1 on Gas-Chrom Q (100—120 mesh). The processing of results is fully automated, using an MDS-2400 computer and includes the calculation of a non-linear calibration plot for each substance analyzed, accuracy control of the measured values, tabulation of the f_{wr} values and the calculation for analyses of biological samples. For the calibration, the pure substances were used at 15 concentrations within a range of 10—1000 ng. The coefficient of variation calculated from 20 duplicate measurements of the calibration mixture did not exceed 5% for any component in the interval from 10 to 100 ng or 3% within range from 100 to 1000 ng.

INTRODUCTION

Direct gas chromatographic (GC) analysis of plasma lipids was first described by Kuksis et al. [1] more than ten years ago. Since then, great advances have been made not only in the equipment, but also in the field of computing technique. In 1975 it was again Kuksis et al. [2] who published their experiences with fully automated GC estimation of the plasma-lipid profile. In the development of GC equipment, supports and stationary phases, as well as in the quality of standards, great progress has been made during the last decade.

*To whom correspondence should be addressed at the following address: 4th Medical Clinic, Charles University U nemocnice 2, 12808 Prague 2, Czechoslovakia.

At present, the detection limit of high-molecular substances, such as lipids, is lower than 50 ng. However, quantitative analysis of such low concentrations is not carried out as a routine procedure. The main reason is the non-linear weight correction factor (f_w) vs. concentration plot for the substances analyzed [3], which was observed e.g. by Bezard and Bugaut [4, 5], even at higher concentrations. In a higher concentration range (of the order of thousands of ng), the f_w value is independent of the amount of lipid analyzed [6]; the concentration limit depends on the quality of the column and on the whole chromatographic system. With biological samples, the determination of much lower concentrations is necessary. This paper describes a computerized, fully automated procedure for the GC determination of neutral lipids down to 10 ng of each component. The sample preparation, accuracy and precision of the analysis of biological samples will be the object of the following communications.

MATERIALS

Triglycerides with carbon numbers 48, 50, 52, 54 and 60, purity 99% (tripalmitin, rac-glyceryl-1,3-palmitate-2-stearate, rac-glyceryl-1,3-stearate-2-palmitate, tristearin and triarachidin) were obtained from Supelco (Bellefonte, Pa., U.S.A.) and Sigma (St. Louis, Mo., U.S.A.), respectively. Cholesteryl esters with carbon numbers 31, 41, 43, 45 and 47 (cholesteryl butyrate, cholesteryl myristate, cholesteryl palmitate, cholesteryl stearate and cholesteryl arachidate) and free cholesterol were obtained from Applied Science Labs. (State College, Pa., U.S.A.). The OV-1 stationary phase and the Gas-Chrom Q support (100–120 mesh), were also supplied by Applied Science Labs. Isooctane (analytical grade) was provided by International Enzymes (Windsor, Great Britain). Chloroform, methanol, acetone and toluene, all analytical grade, were obtained from Lachema (Brno, Czechoslovakia). Helium of 99.99% purity was supplied by Messer (Griesheim, G.F.R.) and trimethylchlorosilan was provided by Merck (Darmstadt, G.F.R.).

Apparatus and operating conditions

All analyses were performed on a Perkin-Elmer F 30 gas chromatograph (Norwalk, Conn., U.S.A.) equipped with a dual column system with flame-ionization detection. Samples were injected by means of a Perkin-Elmer PS 4950 liquid autosampler into a 1/4 in. glass-lined injector thermostated to 300°. Oven temperature was programmed as follows: initial temperature 180°, programme rate 5°/min, final temperature 350°, detector temperature 350°. Helium flow-rate was 100 ml/min. The gas chromatograph was combined with a Perkin-Elmer Model 56 recorder, a Perkin-Elmer M-2 calculating integrator and a Teletype 33 ASR-FR (Teleprint, Frankfurt, G.F.R.) equipped with paper-tape puncher and reader. Data were processed by a computer MDS-2400 (32 K Byte core, 2 magnetic tape units, line printer, paper-tape reader) using a special programme.

METHODS

Preparation of the column

A glass column 0.5 m × 2.0 mm I.D. was rinsed with 150-ml volumes of chloroform, methanol and acetone and dried. Then the column was filled with a 10% solution of trimethylchlorosilane in anhydrous toluene, allowed to stand for 10 min and rinsed with toluene. The column was then treated for 5 min with methanol and rinsed with the same solvent to the neutral reaction. After drying in an oven, the column was packed immediately with 1% OV-1 on Gas-Chrom Q (100—120 mesh), using the combined effect of suction and vibration. The packing was prepared by the evaporation technique and pre-stabilized in a stream of helium, initially at a temperature programme 80—350°, 1°/min, then for 3 h at 350°. After testing the separation ability (by means of a standard mixture of triglycerides and cholesteryl esters), the column was further stabilized by 5 injections of 10,000-ng amounts of triolein. The reproducibility of the recovery was then checked by 5 analyses of the calibration mixture containing equal amounts of all substances analyzed (500 ng in 2 μl of injected sample). The respective f_w values calculated from these analyses did not exceed a 2% interval.

Calibration of the column

The column was calibrated by a mixture of equal amounts of cholesterol, cholesteryl esters with 41, 43, 45 and 47 carbon atoms and triglycerides with carbon numbers 48, 50, 52, 54 and 60. The calibration mixture was prepared from stock solutions containing 1 mg/ml of individual substances in a mixture isooctane—chloroform 80:20 (v/v). After drying, the mixture was dissolved in the internal standard solution (cholesteryl butyrate 200 ng/μl) and diluted standards were prepared according to Table I. All solutions were injected in duplicate by means of the PS-4950 autosampler; the volume of the injected samples was 2 μl.

Mathematical processing of the calibration curve

The f_w plot vs. the amounts of the individual components serves as a basis for mathematical processing of the calibration curves, with special consideration of the analytical use in the non-linear region of this plot. As the plot of f_w vs. the amount of the test substance is convex, a multilinear approximation of n—1 linear sections was used in order to simplify the problem of the calibration curve (n = 15, i.e. 14 sections). In Practice, the scatter of the measured values may cause oscillations of the calibration curve. To solve this problem the values measured were replaced mathematically by another set of values which meets the condition of a minimum scatter between these two sets, together with that of the convex character of the calculated plot. The f_w values were calculated from eqn. 1 [3].

$$f_w = \frac{\text{weight (\%)}}{\text{area (\%)}} \qquad (1)$$

The f_{wr} values can be calculated from the formula

$$f_{wr} = \frac{f_w}{f_{wis}} \tag{2}$$

where f_{wis} is the weight correction factor for the internal standard. From eqn. 1 and 2 we get the formula for calculation of f_{wr} values for individual components

$$f_{wri} = \frac{m_i A_{is}}{m_{is} A_i} \tag{3}$$

where f_{wri}, m_i and A_i are the weight correction factor, the weight and the peak area for component i, respectively. Analogously, m_{is} and A_{is} are the weight and the peak area for the internal standard.

Indicating the measured peak areas as x_i and the corresponding calculated f_{wr} values as y_i, the problem can be formulated as follows: for the set of points y_i, a corresponding set of points y'_i should be found under the following conditions: The value of the expression $z = (y_i - y'_i)^2$ is minimal and the system is described by n—2 inequalities

$$\frac{y_{i+1} - y_i}{x_{i+1} - x_i} \leqslant \frac{y_{i+2} - y_{i+1}}{x_{i+2} - x_{i+1}}$$

By meeting these conditions, the convex shape of the calculated plot is ensured. From the mathematical point of view, it is necessary to find the extreme of a function, under certain limitations. In this case the minimum quadratic function with n variables should be found with simultaneous validity of n—2 linear limitations. In practice, the problem can be solved by application of the saddle point theorem proved in 1951 by Kuhn and Tucker [7]. A modified algorithm

TABLE I

PREPARATION OF DILUTED STANDARDS

Sample No.	Injected amount (ng)
1	1000
2	760
3	578
4	439
5	334
6	254
7	193
8	146
9	111
10	85
11	64
12	49
13	28
14	16
15	10

of the simplex linear-programming method, described first by Wolfe [8], was used.

The programme output is in the form of a magnetic tape containing the coordinates of the optimized points and the slopes of their connecting lines, as well as a printed table which renders possible the control of the scatter of the measured points. The output further contains a table of the f_{wr} values for all the substances analyzed, which permits a check of the plot of f_{wr} vs. the peak area (or alternatively peak height) and manual calculation if necessary.

For triglycerides with carbon number of 56 and 58, the computerized calibration based on the linear interpolation of f_{wr} values between C-54 and C-60 is performed.

Evaluation of the analyses of biological samples

For the quantitative analyses of biological samples, an internal standard method was used. For calculation of the results, a special programme was prepared, based on the following formula

$$\text{mg/dl}_i = C \cdot f_{wri} \cdot A_i; \qquad C = \frac{10^{-4}\, V_t m_{is}}{V_p V_a A_{is}} \tag{4}$$

where C is the constant for each sample analyzed, V_t (μl) is the volume of internal standard solution, which was used for dissolving the sample before analysis, V_p (ml) is the corresponding volume of plasma, V_a (μl) is the volume of injected sample and m_{is} (ng) is the amount of internal standard in the injected sample.

RESULTS AND DISCUSSION

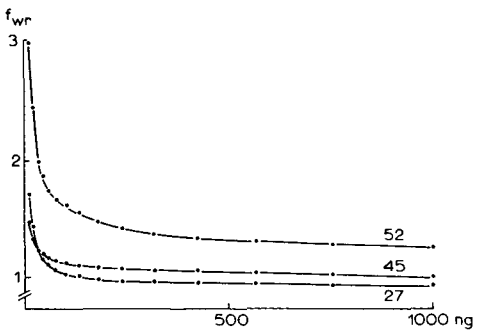
As observed by other authors, the f_w value depends on the amount and molecular weight of the substance analyzed [2, 5, 9]. However, different f_w values have been published for the same substances [4, 10] obtained under comparable conditions, but with different columns and apparatus. According to our present knowledge, a loss of the substances separated is caused by pyrolytic decomposition and irreversible sorption. It is also known that under strictly constant conditions, f_w values are highly reproducible [2]. From the chromatographic aspect, the f_w value increases with increased loading with stationary phase and with increased column length [3, 11]. The percentage of loading with stationary phase also influences the efficiency of the column. It is known from the literature, that columns with a higher percentage of loading need a longer time for stabilization than those with a lower one. A similar effect is also exerted by the column length. With such columns, relatively high f_w values are reached even after stabilization. For preparation of the column with the highest recovery of the test substances, lower loading with the stationary phase is necessary. The support should be highly inert. Chromosorb W HP, Chromosorb 750, Gas-Chrom Q and Supelcoport coated with 1—3% OV-1 or JXR were tested. Best results were obtained with glass column 0.5 m × 2.0 mm I.D., packed with 1% OV-1 on Gas-Chrom Q (100—120 mesh).

TABLE II

REPRODUCIBILITY OF THE GC DETERMINATION OF THE STANDARD MIXTURE

Each value represents average of three analyses; 2 μl of the standard mixture containing 100 ng/μl of each component were injected.

Compound (carbon number	Day to day variations		After 200 analyses	After 600 analyses
	1st day	2nd day		
27	202.1	198.7	201.9	213.7
41	204.0	196.8	203.0	208.7
43	203.9	196.5	203.3	206.9
45	203.4	197.6	202.6	209.0
47	202.9	195.9	198.5	215.9
48	201.8	199.7	200.9	210.2
50	200.9	198.9	197.9	213.7
52	200.2	199.5	201.9	216.5
54	200.7	201.2	204.8	216.8
60	202.5	201.5	205.9	223.2



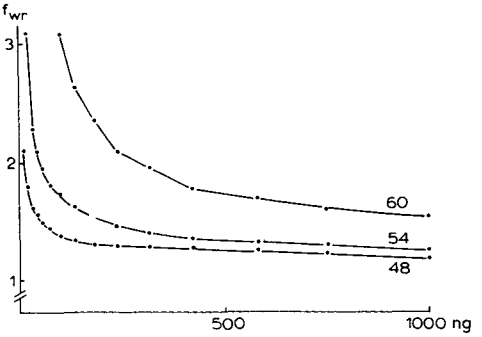


Fig. 1. The plot of f_{wr} vs. amount of the substance analyzed, for main components of the lipid profile. 27 = Free cholesterol; 45 = cholesteryl stearate; 52 = 1,3-stearate-2-palmitate.

Fig. 2. The plot of f_{wr} vs. amount of the substance analyzed, for triglycerides with different molecular weights. 48 = Tripalmitin; 54 = tristearin; 60 = triarachidin.

The efficiency of the column, expressed as $\Delta\ C_{48-54}$ [3], was sufficient. The column needed a relatively short time for stabilization.

The reproducibility of the calibration data plays an important role in the lipid analysis when measured in the region where f_w is independent of the amount of substance analyzed. This requirement is much more important in the non-linear region. The repeatability of the calibration data with time should also be checked as an important criterion of the reproducibility. Table II shows the results for repeated analyses of the calibration mixture (200 ng of each component) obtained with the column mentioned above. Similarly, the reproducibility of calibration was tested over the whole concentration range. It was confirmed that the quantitative lipid analysis in the non-linear

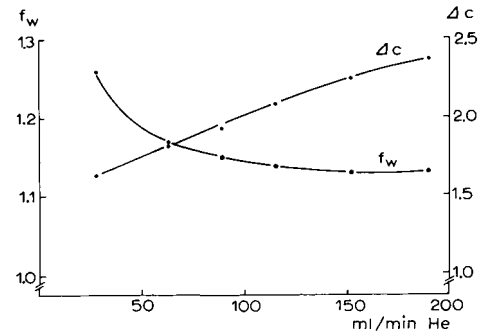


Fig. 3. The plot of f_w and ΔC vs. carrier-gas flow-rate.

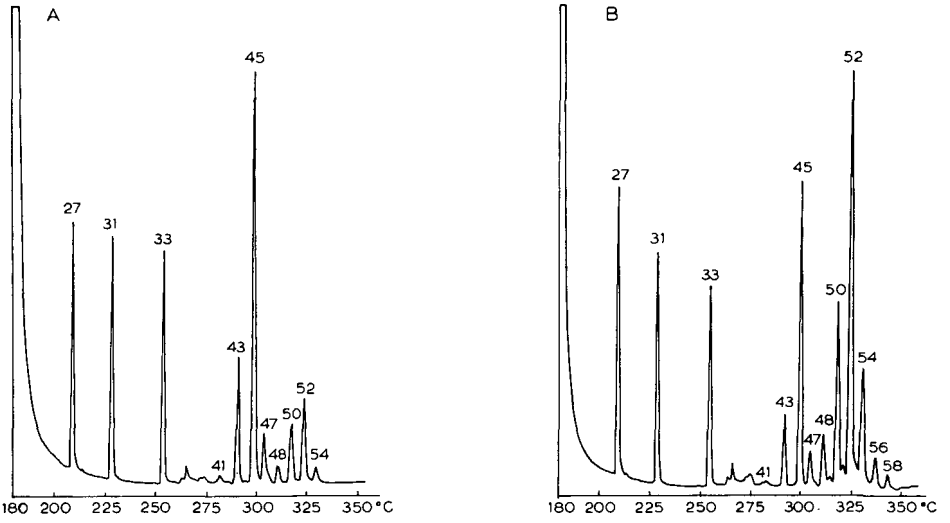


Fig. 4. Gas chromatograms of: (A) normal and (B) hyperlipidemic plasma neutral lipid. 27 = Free cholesterol; 31 = cholesteryl butyrate (internal standard); 33 = cholesteryl benzoate (standard for laboratory control); 41—47 = cholesteryl esters; 48—58 = triglycerides; sample volume = 2 μl; solvent = isooctane—chloroform (80:20, v/v); sensitivity = 1/64; chart speed = 5 mm/min; other analytical conditions are given in the text.

range of f_w values is sufficiently precise. The method described differs from that of the internal standard in that the f_w value is obtained from the f_w vs. peak-area calibration plot for the test substance. Figs. 1 and 2 shows these calibration plots for some individual substances.

As shown in Figs. 1 and 2, the higher the molecular weight of the substance, the more curved is the calibration plot. Furthermore, the parameters discussed previously, i.e. the quality of the column and the operating conditions also influence the shape of the calibration plot. It is known that the shape of the plot of f_w vs. the carrier gas flow-rate is the reverse of that for the separation ability vs. the flow-rate [3]. In our investigation, these conclusions were confirmed. Fig. 3 shows the optimization of the carrier gas flow-rate for the column described.

TABLE III

CALIBRATION OF THE COLUMN NO. 33

Date: 11-11-77; Carbon number: 54

Peak areas*		f_{wr}**		Optimised f_{wr}		Difference***		Difference (%)		Slope
A_1	A_2	f_1	f_2	of_1	of_2	df_1	df_2	f_1	f_2	
0.4	1.1	4.484	2.919	4.48400	2.91900	0.000	0.000	0.000	0.000	2.23571-
1.1	2.5	2.919	2.297	2.91900	2.29700	0.000	0.000	0.000	0.000	0.44429-
2.5	3.6	2.297	2.097	2.29700	2.09700	0.000	0.000	0.000	0.000	0.18182-
3.6	5.1	2.097	1.955	2.09700	1.95500	0.000	0.000	0.000	0.000	0.09467-
5.1	7.0	1.955	1.825	1.95500	1.82917	0.000	0.004	0.000	0.219	0.06623-
7.0	9.8	1.825	1.763	1.82917	1.75635	0.004	0.007-	0.219	0.399-	0.02601-
9.8	14.5	1.763	1.626	1.75635	1.63410	0.007-	0.008	0.399-	0.490	0.02601-
14.5	20.5	1.626	1.572	1.63410	1.56227	0.008	0.010-	0.490	0.640-	0.01197-
20.5	28.7	1.572	1.460	1.56227	1.46411	0.010-	0.004	0.640-	0.273	0.01197-
28.7	39.2	1.460	1.413	1.46411	1.41300	0.004	0.000	0.273	0.000	0.00487-
39.2	53.9	1.413	1.385	1.41300	1.38905	0.000	0.004	0.000	0.288	0.00163-
53.9	71.6	1.385	1.370	1.38905	1.36343	0.004	0.007-	0.288	0.513-	0.00145-
71.6	99.2	1.370	1.321	1.36343	1.32346	0.007-	0.002	0.513-	0.151-	0.00145-
99.2	134.7	1.321	1.272	1.32346	1.27206	0.002	0.000	0.151	0.000	0.00145-

*Integrator output divided by 5000.
**Calculated from measured values.
***Difference between f_{wr} optimized and f_{wr} measured.

TABLE IV

CALIBRATION OF THE COLUMN No. 33

Date: 11-11-77.

Area*	f_{wr27}	f_{wr41}	f_{wr43}	f_{wr45}	f_{wr47}	f_{wr48}	f_{wr50}	f_{wr52}	f_{wr54}	f_{wr56}	f_{wr58}	f_{wr60}
0.5	1.872	1.404	1.454	1.587	1.733	2.267	2.552	3.067	4.261	4.295	5.515	12.018
1.0	1.740	1.364	1.408	1.522	1.648	2.081	2.305	2.687	3.143	3.826	4.699	6.373
1.5	1.608	1.324	1.362	1.456	1.564	1.896	2.057	2.355	2.742	3.356	3.884	5.205
2.0	1.476	1.284	1.316	1.391	1.479	1.767	1.945	2.219	2.520	2.887	3.526	4.649
2.5	1.381	1.244	1.270	1.333	1.416	1.722	1.867	2.083	2.297	2.734	3.275	4.151
3.0	1.344	1.221	1.247	1.307	1.384	1.677	1.790	1.964	2.207	2.595	3.094	3.888
3.5	1.306	1.202	1.229	1.281	1.352	1.632	1.742	1.913	2.116	2.505	2.929	3.625
4.0	1.269	1.183	1.212	1.255	1.320	1.601	1.715	1.862	2.060	2.415	2.833	3.499
4.5	1.231	1.165	1.195	1.229	1.292	1.570	1.688	1.828	2.012	2.343	2.737	3.374
5.0	1.210	1.148	1.182	1.218	1.281	1.545	1.664	1.795	1.965	2.281	2.666	3.249
5.5	1.194	1.142	1.175	1.211	1.270	1.530	1.640	1.762	1.929	2.220	2.612	3.124
6.0	1.177	1.135	1.169	1.204	1.259	1.515	1.616	1.739	1.896	2.166	2.558	3.044
6.5	1.162	1.128	1.163	1.197	1.249	1.500	1.598	1.722	1.863	2.143	2.504	2.976
7.0	1.153	1.121	1.158	1.190	1.238	1.489	1.584	1.706	1.830	2.120	2.450	2.908
.	.	.	.	.	.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.	.	.	.	.	.
244.0	0.946	0.970	0.963	0.969	0.977	1.086	1.104	1.133	1.114	1.161	1.213	1.264
244.5	0.946	0.970	0.963	0.968	0.977	1.086	1.103	1.132	1.113	1.161	1.212	1.263
245.0	0.946	0.970	0.963	0.968	0.977	1.085	1.103	1.132	1.113	1.160	1.211	1.262
245.5	0.946	0.970	0.963	0.968	0.976	1.085	1.102	1.131	1.112	1.159	1.210	1.261
246.0	0.946	0.970	0.962	0.968	0.976	1.084	1.102	1.131	1.111	1.158	1.209	1.260
246.5	0.946	0.970	0.962	0.967	0.976	1.084	1.101	1.130	1.110	1.157	1.208	1.259
247.0	0.946	0.970	0.962	0.967	0.975	1.083	1.100	1.129	1.110	1.157	1.207	1.258
247.5	0.946	0.969	0.962	0.967	0.975	1.082	1.100	1.129	1.109	1.156	1.206	1.257
248.0	0.946	0.969	0.962	0.966	0.975	1.082	1.099	1.128	1.108	1.155	1.205	1.256
248.5	0.946	0.969	0.961	0.966	0.975	1.081	1.099	1.128	1.107	1.154	1.204	1.255
249.0	0.946	0.969	0.961	0.966	0.974	1.081	1.098	1.127	1.107	1.153	1.203	1.254
249.5	0.946	0.969	0.961	0.966	0.974	1.080	1.098	1.127	1.106	1.152	1.203	1.253
250.0	0.946	0.969	0.961	0.965	0.974	1.080	1.097	1.126	1.105	1.152	1.202	1.252

*Integrator output divided by 5000.

We chose computerized processing of the calibration data, because manual processing is very laborious and lacking in precision. The basic mathematical assumptions have been discussed above. In this way, 500 f_w values were obtained as a function of the peak area of the test substance. The programme permits two versions of data processing. As confirmed by Gold and Mathew [6], both the peak heights and their areas can be used for the quantitative analysis of lipids. The f_w vs. peak height plot is very similar to that of f_w vs. peak area. As mentioned above, mathematical processing permits the control of the accuracy of the calibration data, using a special optimization programme.

Table III shows an example of computerized calibration data for one compound; from this table the differences between the measured and optimized values and also between duplicate analyses of the same sample and the slopes of the individual linear regions are apparent. This method of data processing permits rapid control of the calibration accuracy. The difference between duplicate measurements of the same sample should not exceed 5% in relation to the mean f_{wr} value for each component. Analogously, this type of computer output serves as a rapid check of recalibration. As can be seen from Table II, the interval of recalibration is longer than 200 analyses. In practice, when the chromatograph is used daily, it represents a time interval of about 1—2 months. The calibration proper is automatic and takes 32 h. The calibration data are

recorded on a magnetic tape and used throughout the period of calibration validity for the evaluation of biological samples. The calibration is repeated if any of the substances display a deviation of f_{wr} values greater than 5% in any part of the plot. The problem of calibration stability will be discussed in the following part of this paper.

During the column life, the f_{wr} values decrease in the lower concentration range, especially in the case of triglycerides with higher molecular weight. These changes are smaller when the column is properly stabilized. Table IV illustrates part of the output of tabulated f_{wr} data in relation to the peak area; similar data were obtained for peak heights. Table V shows an example of the calculation for a biological sample. Gas chromatograms of normal and hyperlipidemic plasma neutral lipid are given in Fig. 4.

The proposed method permits on-line as well as off-line coupling with a computer. From economic aspects, the off-line coupling is more effective; it employs a conversion programme, using punched or magnetic tapes containing the input data in the ASC 11 code.

The method also permits the inclusion in the computerized programme of some additional parameters suitable for clinical purposes (e.g. various sums or ratios of measured values).

TABLE V

CALCULATION OF THE LIPID PROFILE

Analysis No. 999/0/0/8; Date: 8-11-77. V_t (μl) = 1000; V_p (ml) = 0.2000; V_a (μl) = 2.0; m_{is} (ng) = 400.00; A_{is}* = 48.6: all have the same meaning as in eqn. 4.

Lipid	Carbon No.	Area*	f_{wr}	mg/dl	Chol (mg)**
Cholesterol	27	26.2	1.009	54.39	
Cholesteryl esters	41	0.6	1.396	1.72	1.11
	43	12.8	1.115	29.37	18.19
	45	62.0	1.067	136.12	80.96
	47	6.5	1.249	16.70	9.59
Total cholesteryl esters				183.91	109.85
Total cholesterol					164.24
Triglycerides	48	0.8	2.156	3.55	
	50	4.1	1.709	14.42	
	52	17.1	1.538	54.11	
	54	5.1	1.956	20.53	
	56	0.3	4.483	2.77	
	58				
	60				
Total triglycerides				95.38	

*Integrator output divided by 5000.

**Content of cholesterol in individual ester fractions.

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CHROMBIO. 185

ISOLATION OF IMMUNOREACTIVE COMPONENTS FROM EXPERIMENTAL AND HUMAN TUMOUR TISSUES AND SERUMS BY HIGH-PERFORMANCE GEL CHROMATOGRAPHY

A. JANDOVÁ

2nd Gyn. Obst. Clin., Charles University, Prague (Czechoslovakia)

J. SANITRÁK

Laboratory Instruments Works, 162 03 Prague 6 (Czechoslovakia)

K. MOTYČKA

Institute of Haematology and Blood Transfusion, Prague (Czechoslovakia)

J. PEZLAROVÁ

Department of Clinical Biochemistry, KUNZ, 12808 Prague (Czechoslovakia)

and

J. ČOUPEK

Laboratory Instruments Works, 162 03 Prague 6 (Czechoslovakia)

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SUMMARY

A method for the rapid separation of proteinous fractions by high-performance gel chromatography was described. Homogenates from tumorous and healthy tissues and blood were eluted by saline on a column packed with rigid hydrophilic macroporous particles of O-glucose—Spheron 300. Fractions were collected and subjected to further analyses. Their antigenic activity was determined by the leucocyte adherence inhibition test method. For the specific immunoactive fractions a dependence of leucocyte adherence inhibition test values on the clinical state of sample donators has been found.

INTRODUCTION

In earlier studies dealing with the problem of separation of fractions possessing the lactate dehydrogenase (LHD) virus and enzymatic (1.1.1.27-L-lactate:NAD oxidoreductase) activity determined in the serum of mice of

various strains [1, 2], the question of application of the gel chromatography of biopolymers was solved by means of a new type of macroporous hydrophilic sorbent based on the copolymer of hydroxyethyl methacrylate and ethylenedimethacrylate, which proved to be satisfactory for the given purpose. In an investigation of other chromatographic applications of these sorbents [3], Borák and Smrž [4], Čech et al. [5], have shown that sorbents exhibit a certain hydrophobic interaction between the copolymer and the lipophilic part of molecules of the compounds under separation. This finding was used to advantage in the papers referred to above, in the separation based on hydrophobic chromatography [6]. The sorption effects, in addition to the molecular sieving properties of Spheron 1000 gel were exploited for the separation of complex, naturally-occurring mixtures of high-molecular-weight compounds like glycosaminoglycans and proteoglycans [7]. If, however, the only effect required from separation is that of the molecular sieve, the hydrophobic interaction of sorbents must be suppressed. This is why, quite recently, highly hydrophilic O-glycosyl derivatives of the basic hydroxyethyl methacrylate carrier have been synthesized and used in the affinity chromatography of lectins [8]. In addition to the function of a specific affinant, the high content of saccharide covalently attached to the internal pore surface (up to 20%, w/w) also causes high hydrophilicity of the carrier and suppresses undesirable hydrophobic sorptions. In this way, a rigid macroporous gel chromatographic material is formed, in which the excellent mechanical and hydrodynamic properties of macroporous Spheron gels are combined with the outstanding interaction characteristics of the Sepharose-type materials used in gel filtration for some time.

In this paper we report a practical application of O-glucose—Spheron in the rather demanding isolation of immunochemically active fractions obtained from homogenates of human and animal tissue and serum, both tumorous and non-tumorous. If a tissue is subjected to a preliminary treatment, consisting of mechanical disintegration of cells, followed by separation of cell fragments and subcellular particles, a clear or slightly opalescent supernatant is obtained, which contains a mixture of proteins and gives positive reaction in the leucocyte adherence inhibition (LAI)-test [9]. Halliday and Miller [9] discovered that in the presence of tumour antigens the adherence of leucocytes to the glass surface is inhibited and derived a semiquantitative evaluation method. Isolation of the active fraction with respect to the LAI-test, its characterization and subsequent treatment still remain an open problem. The method of high-performance gel chromatography provides an advantageous possibility of realization of the first step.

MATERIALS AND METHODS

O-Glycosyl—Spheron 300, a suspension copolymer of 2-hydroxyethyl methacrylate and ethylenedimethacrylate [10] with the exclusion limit of molecular weight 300,000 (tested using dextran standards from Pharmacia, Uppsala, Sweden) was carefully extracted successively with benzene, ethanol and water on a continuous apparatus for 8 h each time, washed with ethanol and ether, and dried. D-Glucose was bonded to the carrier, using an HCl—BF_3

mixture in dry dioxan as catalyst, under conditions described in ref. [6]. The gel, particle size 32—40 μm, was packed in suspension in 0.1 *N* NaCl solution containing 1% (w/w) of glucose into a column 0.8 × 120 cm and the column was calibrated with dextran standards and a mixture of proteins.

Chromatographic apparatus

The chromatographic equipment consisted of a high-pressure pump MP 2501 (Laboratory Instrument Works, Prague, Czechoslovakia), an injection valve, a column packed with O-glycosyl—Spheron, to which a differential refractometer R 403 (Waters Assoc., Milford, Mass., U.S.A.) and a UV spectrophotometric flow detector with variable wavelength (Optronica, Oberursel/TS, G.F.R.) were connected in series. The fractions were collected in an LKB Ultrorac 7000 fraction collector (Stockholm, Sweden).

Biological material

Tumorous and healthy tissues and blood were obtained with the kind consent of the Heads of thirteen medical institutes in Prague. The samples under investigation are listed in Table I. Homogenates from biological materials were subjected to centrifugation with cooling at 80,000 *g* for 30 min. Other methods of the centrifugation of homogenate are discussed elsewhere [11]. Serum samples were obtained by centrifuging blood for 30 min at 4000 *g* under cooling. The protein level was determined by employing the methods of Lowry and Folin [2].

The activity of E.C. 1.1.1.27-L-lactate:NAD oxidoreductase, was determined using a test kit from Boehringer [12], Mannheim, G.F.R.

Chromatography

A homogeneous supernatant was injected directly into the head of the chromatographic column in an amount of approximately 0.5 ml with a protein content from 3 to 10 mg/ml and eluted with a 0.1 *N* solution of NaCl containing 1% of glucose at a flow-rate of 100 ml/h at room temperature. The data from the differential refractometer and the spectrophotometric detector were recorded using a two-channel Philips PM 8010 recorder. The 340 nm wavelength used in the UV detection was chosen because it was also used to measure the activity of the above-mentioned isoenzyme. The fractions obtained, 2.5 ml in volume, were again analyzed for the protein content, subjected to the LAI-test [9]. The leucocytes were isolated from heparinized venous blood according to Holáň et al. [13]. After combining this suspension with the proteinous fraction investigated for its content of antigen in the SIAL® test-tubes, free cells were counted in triplicate before and after 2 h incubation at 37°(Bürker chamber). Values higher than 60% of non adhered leucocytes are regarded as positive. The activity of E.C.1.1.1.27-L-lactate:NAD oxidoreductase, was determined by the Boehringer test in all fractions [12].

RESULTS AND DISCUSSION

The effect of modification of the internal surface due to glycosylation is shown in Fig. 1, representing the calibration curve of polydextrans in the case

TABLE I

SAMPLES OF TISSUES AND SERA UNDER INVESTIGATION

Sample*	Diagnosis	
	Benign	Malignant
Human serum	10	11
Mouse serum	12	15
Endometrium	4	5
Cervix	10	11
Ovarium	10	14
Vagina	—	1
Placenta	8	—
Prostate	—	1
Mamma	4	5
Cerebrum	3	3
Cerebellum	1	1
Kidney	1	4
Stomach	—	1
Sigmoid colon	—	2
Lung	4	4
Dental periosteum	1	1
Cutis	1	4 (melanoblastoma) 3 (psoriasis)
Oculum	1	4 (melanoblastoma)
Os	2	3
Lymph node	2	4 (M. Hodgkin)
Embryonal tissue	8 (1 CEA)	—
Milk	10	—
Others	13	4
Σ	105	101

*Unless otherwise stated samples are of human origin.

of modified and unmodified Spheron 300. Both dependences show that the coating of the internal surface has only a small effect on the exclusion limit and specific pore volume. The covalent bond of glucose, which hydrophilizes the surface, does not influence greatly the molecular weight range of compounds under separation which can penetrate into the gel. An example of a gel chromatogram, where the results of refractometric and spectrophotometric analyses, the protein level after Lowry, the activity of E.C.1.1.1.27-L-lactate:NAD oxidoreductase and the results of the LAI-test have been indicated, is shown in Fig. 2. It provides evidence of the concentration of active components in fractions 12, 13, 14 with a maximum in fraction 13. Chromatographic experiments with detection at various wavelengths have revealed an interesting fact: in most samples of experimental and human tumorous tissues the LAI activity coincides with a maximum absorbancy at 340 nm. There are cases, however (Fig. 3), where the peak from the 13th fraction on the refractometer has no corresponding peak on the spectrophotometer, or in other words, is virtually nil. Such a situation has been observed with operated tumours after complex polychemotherapy, where residual tissue was obtained.

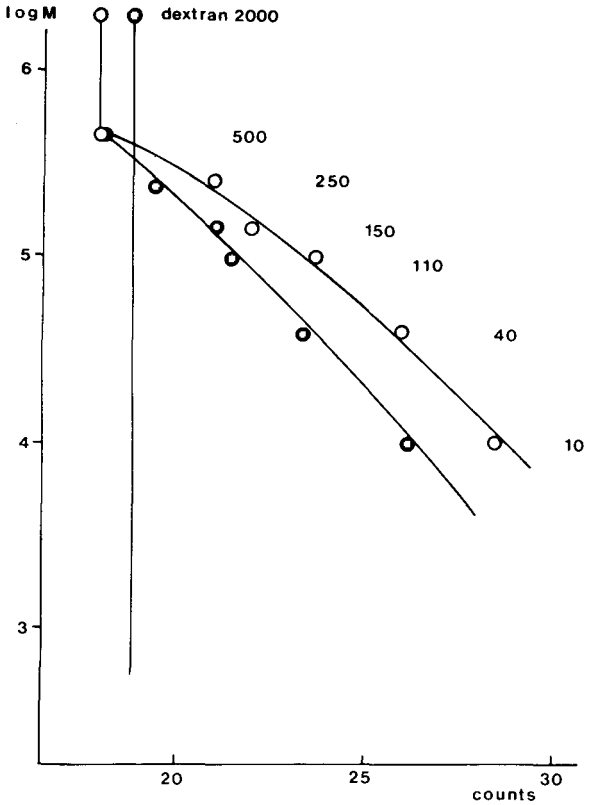

Fig. 1. Calibration curves of Spheron 300 and glucose—Spheron 300. Test mixture: polydextrans of defined molecular weight (M) from 20,000 to 2,000,000 (Pharmacia, Uppsala, Sweden); column: 1200 × 8 mm I.D.; flow-rate: 100 ml/h; eluent 0.1 *N* NaCl containing 1% glucose. ○ = Spheron 300; ● = glucose—Spheron 300.

In any case, the peak detected with the 13th fraction corresponds to the immunoactivity of the sample. These findings are the subject of further study.

A total of 206 separations of experimental and human tumorous tissues and healthy tissues and sera was performed. Proteinaceous fractions from tissues with a maximum UV absorbancy at 340 nm were collected and their antigenic activity was determined by the LAI-test. The correlation between the LAI values and histological findings is summarized, for the most representative groups of tumour diseases, in Table II.

The chromatogram shown in Fig. 4 shows the UV absorbancy and refractive index profiles of a protein fraction with a high content of tumour antigens (human carcinoma cervicis, sample Ce 11).

The method of fast gel chromatography described in this paper permits isolation of protein fractions characterized by a high immunological activity determined by the LAI-test. The activity of these fractions depends on the clinical state of the individual from whom the tissue was taken [9]. A fast and relatively convenient technique allows detection of enriched protein fractions, which are further examined as to the relationship between their

TABLE II

COMPARISON OF HISTOLOGY AND COMBINED GEL CHROMATOGRAPHY—L.A.I. TESTS

Diagnosis (carcinoma)	Number of patients	Histology	L.A.I.-test of the 13th fraction		%
			Positive	Negative	
Cervicis Endometrii Ovarii Vulvae	27	27 positive	25	2	92
Mammae Pulm. Recti Ventric. Others	42	42 positive	40	2	95
Healthy controls	55	55 negative	1	54	98.2

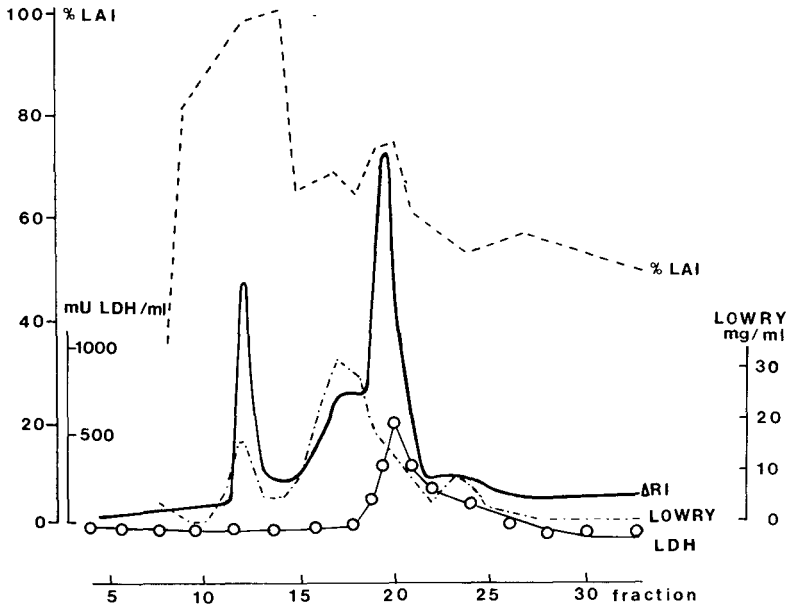

Fig. 2. Gel chromatogram of protein fraction from human carcinoma cervicis (C 13). Conditions: see Fig. 1.

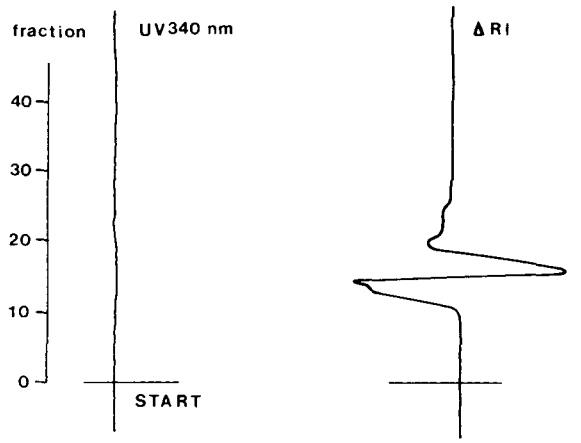

Fig. 3. Gel chromatogram of protein fraction from human carcinoma ovarii after operation and polychemotherapy. Conditions: see Fig. 1.

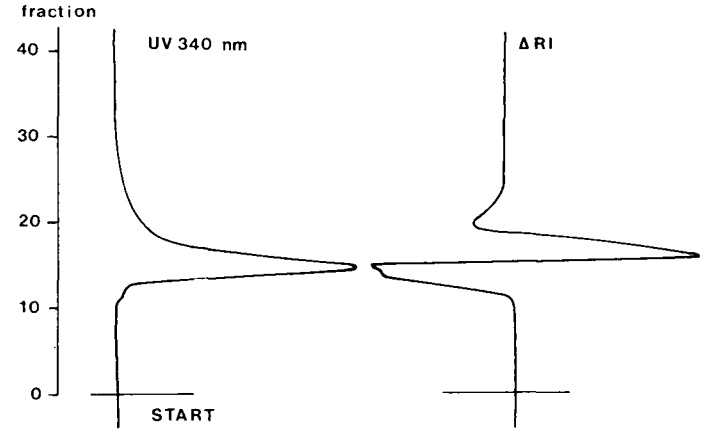

Fig. 4. Gel chromatogram of protein fraction of human carcinoma cervicis (Ce 11). Conditions: see Fig. 1.

composition, biological activity and antigenic character. When comparing the results of the LAI-test of crude homogenates or sera with those of chromatographically separated fractions (Fig. 2) the reliability of LAI confirmed histologically was in the latter case considerably higher.

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QUANTITATIVE HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY OF LIPIDS IN PLASMA AND LIVER HOMOGENATES AFTER DIRECT APPLICATION OF 0.5-μl SAMPLES TO THE SILICA-GEL LAYER

INGEBORG R. KUPKE* and SUZANNE ZEUGNER

Klinisch-chemisches Laboratorium der Universitäts-Kinderklinik, Moorenstr. 5, D-4000 Düsseldorf (G.F.R.)

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SUMMARY

Lipid profiles were determined by high-performance thin-layer chromatography (HPTLC) after direct application of 0.5 μl plasma from capillary blood to the silica-gel layer. Coefficients of variation for the fluorescence measurements were 2.1% for cholesterol, 1.5% for cholesterol esters, 2.8% for triacylglycerols, and 2.3% for phosphatidylcholine. The recovery of known amounts of lipid was 96—100%. A linear relationship between peak area and amount of lipid was found in the nmole range, corresponding to the amount of lipid in 0.125—0.75 μl Lipid-Trol, which served as the standard reference sample.

The plasma lipids of healthy subjects and of patients suffering from various illnesses were analyzed using reference methods and HPTLC. Identical values were obtained for cholesterol esters, triacylglycerols and phosphatidylcholine. Free cholesterol values determined by HPTLC were slightly lower (7%). The correlation between data obtained by reference methods and HPTLC was as follows: cholesterol, $r = 0.938$; cholesterol esters, $r = 0.964$; triacylglycerols, $r = 0.985$; phosphatidylcholine, $r = 0.938$. The separation and quantitation of liver lipids using HPTLC after direct application of the tissue homogenate to the silical-gel layer was carried out. Comparison with reference methods revealed that HPTLC gave higher cholesterol values (24%). The triacylglycerol concentrations, however, were identical under both methods and correlated satisfactorily ($r = 0.959$).

INTRODUCTION

Thin-layer chromatography (TLC) has been widely used for the determination of plasma lipids in various clinical situations [1—3]. However, lipid extraction prior to TLC is time-consuming and requires relatively large sample volumes. Therefore, efforts have been made to apply the plasma samples directly to the silica-gel layer. Whitner et al. [4], Buckley et al. [5], and Mantel et al. [6] succeeded in separating the neutral lipids of 10—20 μl serum using direct application of the sample to self-prepared thin-layer plates. With

*To whom correspondence should be addressed.

these systems, the phospholipids were not developed.

The direct application of 5—20 μl serum to commercial TLC plates has been described in a previous report [7]. Hydrochloric acid was used to denature the serum proteins on the plate. The quality of separation of the neutral lipids was satisfactory. The recovery of ^{3}H-label in free and esterified cholesterol of samples incubated with [^{3}H]-cholesterol prior to TLC was nearly 100%; the coefficient of variation was less than 2%. However, the phospholipids were not separated in this system.

It was the purpose of this study to develop a direct application procedure for quantitative high-performance TLC (HPTLC) in the nmole range. After the direct application of 0.5 μl capillary blood plasma to the silica-gel layer, the neutral and polar lipids were separated. Quantitation of the lipids was carried out by fluorescence measurement according to Segura and Gotto [8]. The results obtained for healthy subjects and various types of hyperlipidemic patients were found to correlate well with those determined by reference methods.

The same procedure was also applied to the separation of lipids in liver homogenates.

MATERIALS AND METHODS

HPTLC

HPTLC plates pre-coated with silica gel 60 without fluorescent indicator (10 × 20 cm; Merck, Darmstadt, G.F.R.) were used for nano-TLC.

Chemicals

All chemicals used were Merck analytical grade.

Reference methods

The results obtained by HPTLC were compared with the following reference methods: enzymatic determination of cholesterol [9]; enzymatic determination of triacylglycerols; alkaline hydrolysis of triacylglycerols and enzymatic determination of glycerol; phosphatide-phosphorus determination (Biochemical Test Combinations, from Boehringer, Mannheim, G.F.R.); cholesterol determination according to Liebermann—Burchard (Merck).

Standard reference samples

For standardization, commercial standard samples with known lipid concentrations were used: Lipid-Trol (Dade Div. Amer. Hosp. Supply Corp., Miami, Fla., U.S.A.); Precilip (Boehringer).

Instruments

For fluorescence measurement of the lipids separated by HPTLC, a Model KM 3 chromatogram spectrophotometer (Zeiss, Oberkochen, G.F.R.) equipped with a mercury lamp was used. Emission was recorded with a Servogor S recorder (Metrawatt, Nuremberg, G.F.R.). In a few cases, electronic integration was carried out with the Autolab System I (Spectraphysics, Santa Clara, Calif., U.S.A.). A Desaga Uvis lamp (Heidelberg, G.F.R.) served for visual examination

of the chromatoplates at 366 nm. The enzymatic triacylglycerol determinations were carried out on the LKB reaction rate analyzer (Copenhagen, Denmark) in conjunction with a Hewlett-Packard calculator (Loveland, Colo., U.S.A.).

Preparation of the biological samples

Capillary blood was collected in heparinized capillaries and centrifuged in an hematocrit centrifuge. The plasma samples not immediately used for lipid analysis were frozen in the capillaries.

A 1-g sample of fresh rat liver was homogenized with purified sand in a mortar in the cold. The volume was made up to a total of 5 ml with Tris buffer, pH 7.4; 10 mmole/l. The homogenates were centrifuged for 10 min at 78 *g* and the supernatants subjected to lipid analysis.

Direct application of the samples

The HPTLC plates were purified overnight in solvent system I (see below); a longer purification period is not advisable. After evaporation of the solvents with a hair dryer, the plates were activated for 1 h at 110°. A 15-μl volume of absolute methanol was applied by means of a micro-pipette to the silica gel layer 1.2 cm from the lower edge of the plate. As soon as the moisture was absorbed by the silica gel, 0.5 μl plasma or liver homogenate were applied on the methanol spot. A 10-μl quantitative microlitre sample dispenser (Elevitch; Hamilton, Reno, Nev., U.S.A.) fitted with a disposable sample tip 1 1/8 in.; Corning, Palo Alto, Calif., U.S.A.) was used to apply the samples. The dispenser with the tip containing the sample, was held in an upright position and the sample was pushed out so that a drop hung on the tip; this known amount of the sample was then applied to the methanol spot (the tip was held in position for 2 sec). The dispenser was then removed without releasing the piston. Immediately afterwards, the plasma spot was covered with a few μl methanol. It is important that this second application of methanol does not exceed the diameter of the sample spot. The spots were then dried under a stream of cold air. A total of 14 samples could be applied to one HPTLC plate by this procedure.

Separation of lipids

The chromatogram was developed in a saturated chamber in solvent system I (chloroform—methanol—water, 65:30:5). The solvent front was allowed to migrate a distance of 3.7 cm from the lower edge of the plate (3.5 min). After evaporation of the solvents under a stream of cold air, this first run was repeated and the solvents were again evaporated. With solvent system II (*n*-hexane—diethyl ether—acetic acid, 80:20:1.5) the neutral lipids were developed by migration of the solvent front up to 1 cm from the top of the plate (20 min). The plates were dried under a stream of warm air for approximately 1 h until the odor of acetic acid could no longer be detected.

Solvent system I was used for the development of a total of 4 plates, solvent system II for 2 plates.

Detection

The method used was a slight modification of that described by Segura and Gotto [8]: the HPTLC plates were transferred to a sandwich chamber (Desaga, Heidelberg, G.F.R.) containing $(NH_4)HCO_3$ on the bottom; 0.5 g were used for the development of 1 plate and 1.0 g for 2—4 plates. The plates were positioned 3 cm from the bottom, and the chamber (sealed with high vacuum grease) was placed in an oven and heated for 10 h at 150°.

Fluorescence measurements

The fluorescence of the lipid spots was scanned across the axis of development with the Model KM 3 chromatogram spectrophotometer (excitation at 366 nm, emission at 430 nm). The peak areas were taken as the product of peak height and the half-width of the peak. In a few cases, integration was carried out electronically with the Autolab System I.

Standardization of the HPTLC procedure

Free and esterified cholesterol in the standard reference samples, Lipid-Trol and Precilip, were determined enzymatically [9]. Triacylglycerols and phosphatidylcholine were obtained by preparative TLC [10] and, after dissolving the lipids in 1% Tween 80, the enzymatic determination of the triacylglycerols was carried out using the LKB reaction rate analyzer; the phosphatidylcholine was ashed prior to phosphorus determination.

Liver homogenates

For reference analysis, cholesterol was obtained by preparative TLC and determined according to Liebermann—Burchard; the enzymatic determination was carried out in the homogenate. For triacylglycerol analysis, free and total glycerol in the homogenate were determined enzymatically.

Comparison of HPTLC with reference methods

The lipids of healthy subjects and of hyperlipidemic patients (newborns, children and adults) were analyzed by HPTLC and by reference methods. The phosphatidylcholine was estimated as described for the standard reference samples. The results were examined by linear-regression analysis.

RESULTS AND DISCUSSION

The separation of lipids after the direct application of serum or plasma to the silica-gel layer usually gives poor results, due to the proteins in the sample [11]. In the present study, the separation of the lipids after direct application of plasma samples to the HPTLC plate was sharp and reproducible (Fig. 1), with coefficients of variation of the fluorescence measurements less than 2.8% for the lipids investigated (Table I). This precision of determination is consistent with data reported by Mlekusch et al. [12, 13] for the fluorometric determination of lipids preceded by lipid extraction. The relationship between peak area and the amount of lipid was found to be linear in the nmole range, corresponding to the amount of lipid in 0.125—0.75 μl Lipid-Trol (Fig. 2). In our experience, most of the lipid profiles to be determined in a clinical laboratory can be obtained using 0.5 μl plasma.

In order to standardize the HPTLC procedure, the lipid concentrations of Lipid-Trol and Precilip were evaluated by reference methods. The lipid concentrations in Lipid-Trol served as standards on the HPTLC plates and Precilip was used for accuracy control. The recovery of the Precilip lipids on the HPTLC plates was 96—100% of the reference values (Table II).

In order to gain insight into the responses of different types of plasma samples, up to 93 samples from newborns, healthy adults and various types

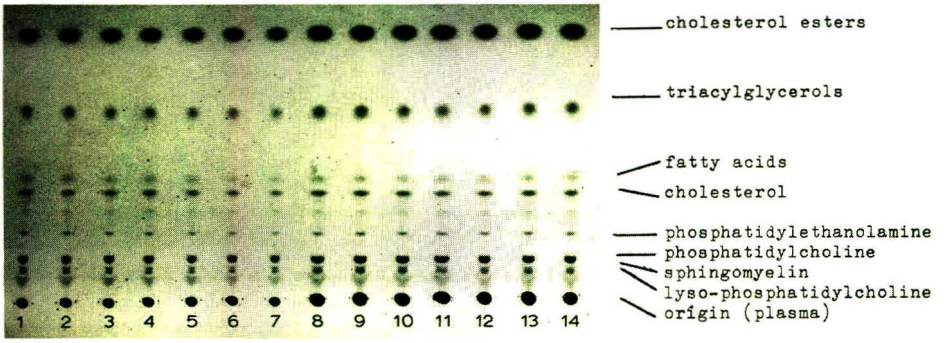

Fig. 1. Separation of plasma lipids by HPTLC (positions 1—7: 0.5 µl, positions 8—14: 1 µl Lipid-Trol). Visualization of the lipids with iodine vapor.

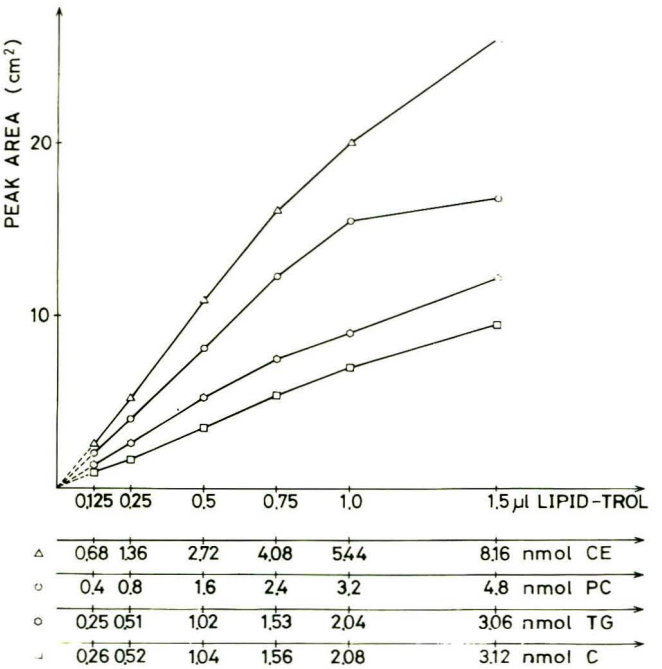

Fig. 2. Calibration curves for cholesterol esters (CE), phosphatidylcholine (PC), triacylglycerols (TG), and cholesterol (C).

TABLE I

RELATIVE RESPONSES OF LIPIDS AND PRECISION OF FLUORESCENCE MEASUREMENT

The lipids of Lipid-Trol were separated by HPTLC and the peak areas were taken as the product of peak height and half-width of the peak. The coefficients of variation (C.V., %) were calculated from 14 samples per HPTLC plate.

Lipid	Amount (nmole/spot)	Peak area (cm^2)	cm^2/nmole	C.V. (%)
Cholesterol	1.04	6.26±0.13	6.02	2.1
Cholesterol esters	2.71	15.74±0.23	5.82	1.5
Triacylglycerols	1.07	7.84±0.22	7.33	2.8
Phosphatidylcholine	1.6	8.7±0.2	5.4	2.3

TABLE II

STANDARDIZATION OF THE HPTLC PROCEDURE

The lipids of Lipid-Trol and Precilip were analyzed using the reference methods. The values obtained for Lipid-Trol served as standards and values of Precilip for accuracy control. Both standard reference samples were applied to each HPTLC plate. Mean values were obtained by analysis of the samples during 20 days. Numbers in parenthesis = coefficient of variation (%).

Lipids (mmole/l)	Lipid-Trol	Precilip		
	Reference method	Reference method	HPTLC	Recovery (%)
Cholesterol	2.08±0.10 (4.9)	0.87±0.08 (8.9)	0.87±0.07 (8.3)	100
Cholesterol esters	5.41±0.12 (2.3)	2.89±0.10 (3.4)	2.81±0.12 (4.3)	97
Triacylglycerols	2.14±0.08 (3.7)	0.84±0.03 (2.9)	0.81±0.03 (4.1)	96
Phosphatidylcholine	3.2 ±0.1 (3.2)	—	2.1 ±0.1 (4.8)	—

TABLE III

COMPARISON OF DATA OBTAINED FROM HUMAN SUBJECTS BY REFERENCE METHODS AND HPTLC

n = number of subjects investigated; P = error of probability according to t-test.

Lipids (mmole/l)	Reference method	HPTLC	P	n
Cholesterol	1.48±0.50	1.38±0.51	<0.0005	83
Cholesterol esters	3.35±1.16	3.30±1.05	<0.10	93
Triacylglycerols	1.03±0.66	1.05±0.68	<0.10	69
Phosphatidylcholine	2.7 ±0.6	2.7 ±0.5		22

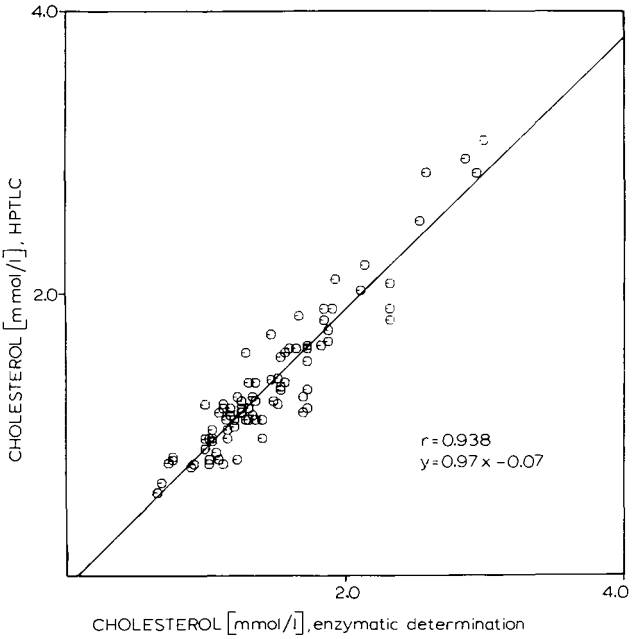

Fig. 3. Correlation between enzymatically-determined cholesterol and HPTLC cholesterol (n = 83); see Table III.

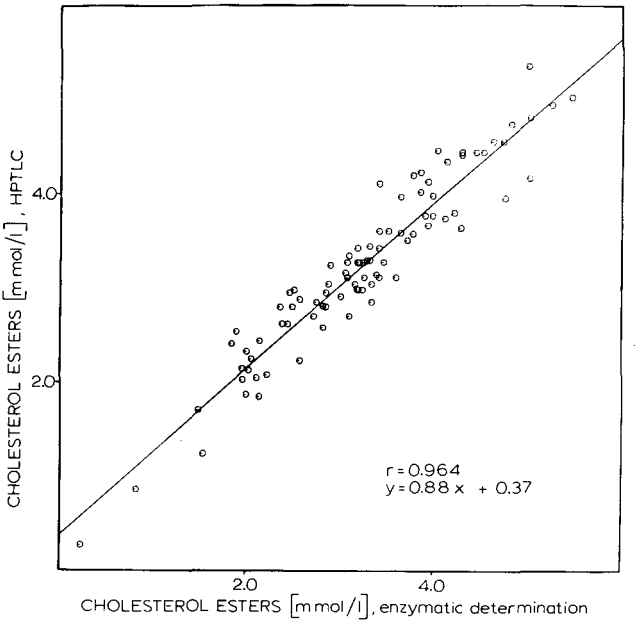

Fig. 4. Correlation between enzymatically-determined cholesterol esters and HPTLC cholesterol esters (n = 93); see Table III.

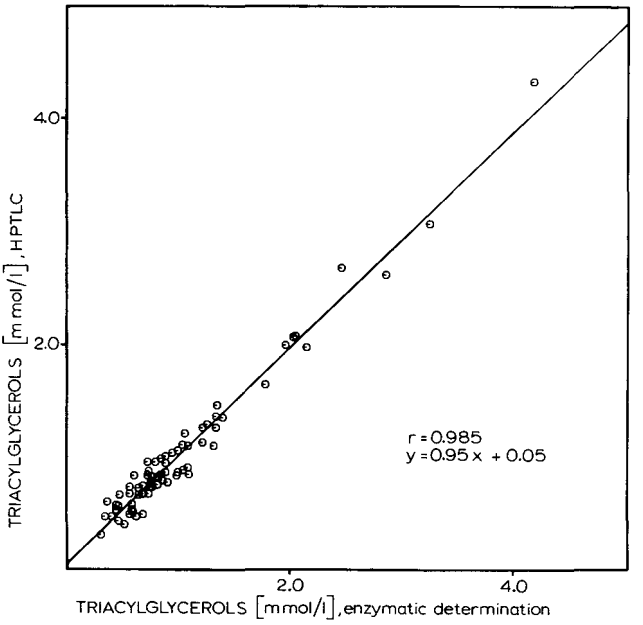

Fig. 5. Correlation between enzymatically-determined triacylglycerols and HPTLC triacylglycerols (n = 69); see Table III.

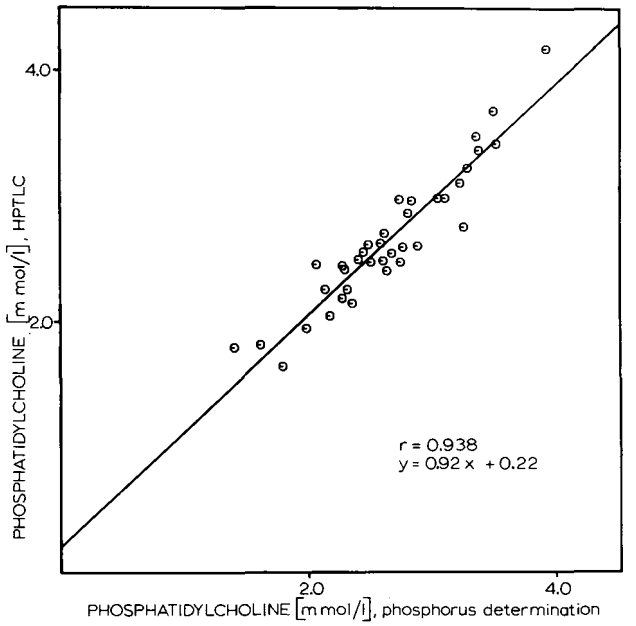

Fig. 6. Correlation between chemically-determined phosphatidylcholine and HPTLC phosphatidylcholine (n = 22); see Table III.

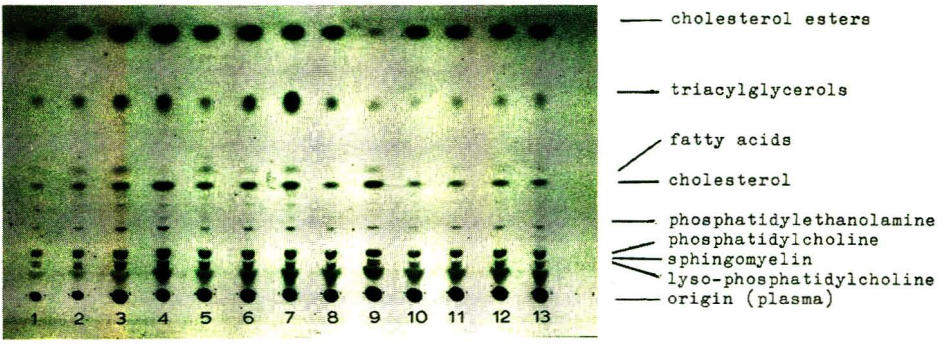

Fig. 7. Visual examination of lipid profiles of healthy subjects and different patients, compared with known lipid amounts in Lipid-Trol. The corresponding quantitative values are listed in Table IV.

of hyperlipidemic patients were analyzed by HPTLC and the results compared with those obtained using reference methods (Table III). The values obtained for cholesterol esters, triacylglycerols and phosphatidylcholine were identical. The HPTLC values for free cholesterol were 7% lower than those found by enzymatic determination. This result is possibly due to different fluorescent properties of sterols other than cholesterol present in the plasma, e.g. plant sterols. Linear-regression analysis of these data indicated good correlation between the reference methods and HPTLC for the lipids investigated (Figs. 3–6).

The lipid patterns of healthy subjects and various patients are presented in Fig. 7. By comparisons with the known lipid concentrations in Lipid-Trol

TABLE IV

DETERMINATION OF LIPIDS WITH REFERENCE METHODS

For visual examination, the lipids of 10 human subjects were separated by HPTLC and compared with known amounts of lipid in Lipid-Trol (see Fig. 7). The corresponding quantitative values (mmole/l) are listed here. (HLP = hyperlipoproteinemia.)

Position on HPTLC plate	Type of plasma sample	Cholesterol esters	Cholesterol	Triacyl-glycerols	Phosphatidyl-choline
1	Lipid-Trol	2.70	1.04	1.07	1.6
2	Lipid-Trol	5.41	2.08	2.14	3.2
3	Lipid-Trol	10.82	4.16	4.28	6.4
4	Type II HLP	17.3	5.4	2.46	—
5	Type II HLP	8.37	1.91	0.85	2.7
6	Type II HLP	8.68	2.53	2.52	3.5
7	Type IV HLP	8.94	2.97	7.36	3.5
8	Normal	4.48	1.81	1.19	2.8
9	Liver disease	0.64	3.44	0.85	—
10	Normal	3.27	1.29	0.43	2.2
11	Normal	4.01	1.55	0.58	2.7
12	Normal	3.80	1.66	0.92	2.8
13	Normal	4.28	1.32	0.92	2.5

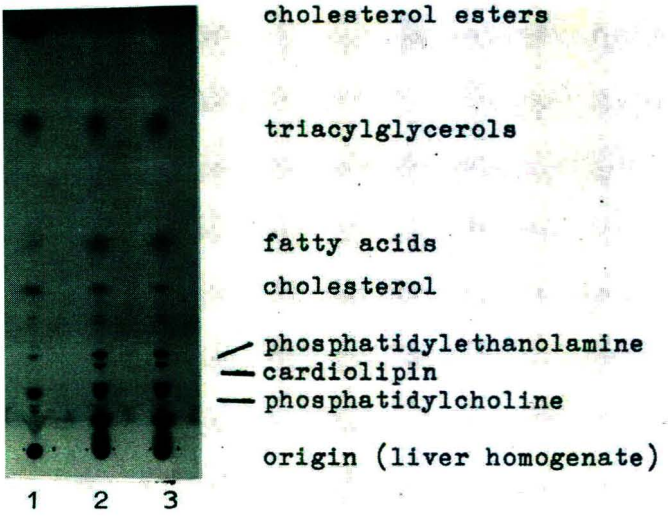

Fig. 8. Lipids of a liver homogenate (positions 2, 3), compared with Lipids-Trol (position 1).

present as standard on the HPTLC plate (positions 1—3), semi-quantitation of the lipids was possible. The values obtained by visual examination of the chromatoplate were in good agreement with data obtained by the reference methods (Table IV). One child (position 9) with liver disease associated with a severe icterus had practically no cholesterol esters, but had an increased free cholesterol content, reflecting the reduced lecithin—cholesterol acyltransferase activity found in liver parenchymatous damage [14, 15].

In order to ascertain whether this procedure can also be applied to lipid determinations of homogenized tissues, 0.5 μl of a liver homogenate were applied directly to the HPTLC plate and treated in the same manner as the plasma samples. As shown in Fig. 8, the separation of the lipids was as good as in the plasma samples. The coefficients of variation in the series (n = 10)

TABLE V

LIPID DETERMINATION IN LIVER HOMOGENATES

Liver samples from 6 rats were homogenized. For the Liebermann—Burchard reaction, cholesterol was obtained by preparative TLC. All enzymatic determinations were carried out on the homogenate. The data were examined by paired t-test statistics and linear-regression analysis.

Lipids (μmole/g liver)	Procedure					
	Liebermann—Burchard	Enzymatic	HPTLC	Enzymatic: HPTLC		
				P	$y = bx + a$	r
Cholesterol	4.10±0.65	3.90±0.45	4.85±0.75	<0.0005	$1.44x - 0.16$	0.866
Triacylglycerols	—	8.00±1.90	7.80±2.55	<0.30	$1.28x - 0.49$	0.959

were 3.3% for cholesterol and 5.7% for triacylglycerols. The concentrations of triacylglycerols and free cholesterol in liver homogenates from 6 rates were determined using the reference methods and HPTLC (Table V). The results obtained with the reference methods were consistent with data found by column chromatographic separation of lipids from rat liver [16]. Cholesterol values obtained with HPTLC were 24% higher than those found by enzymatic determination or the Liebermann—Burchard method. This difference was highly significant and the data showed relatively poor correlation (r = 0.866). These HPTLC values presumably reflect the existence in the liver of sterols with different fluorescence properties and/or the existence of sterols not detected by the reference methods. However, the relative measurement of liver sterols by HPTLC seems to be possible. The enzymatic and the HPTLC determination of triacylglycerols gave identical results and good correlation (r = 0.959). The results, although obtained from only 6 rat livers, suggest that at least the liver triacylglycerols can be estimated with satisfactory accuracy using the direct HPTLC procedure. Because of the very small quantity of tissue required, this method may be suitable for lipid analysis in tissue-biopsy material.

CONCLUSIONS

With the procedure described in this report, the plasma lipids of 0.5-μl samples of capillary blood can be quantitated in the nmole range without lipid extraction prior to HPTLC. This makes the method suitable for routine analysis and large-scale studies.

Liver homogenates can be analyzed by the same procedure. This makes it suitable for rapid lipid determination on small amounts of biopsy material.

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Biomedical Applications

CHROMBIO. 179

DIE BESTIMMUNG VON ALDOSTERON IN HARN UND PLASMA*,**

K. HERKNER, P. NOWOTNY und W. WALDHÄUSL

I. Med. Univ. Klinik, Abteilung für klinische Endokrinologie, Wien (Österreich)

(Eingegangen am 3. Februar 1978)

SUMMARY

Determination of aldosterone in urine and plasma

The method described permits an exact and rapid determination of aldosterone in urine and plasma. The reliability of the method is based on the separation of aldosterone from contaminating steroids by thin-layer chromatography. The mobile phase used was: cyclohexane—ethyl acetate (20:80). The steroids were extracted by dichloromethane. Plasma was extracted directly, and urine after hydrolysis with sulfuric acid (pH = 1). Recovery before radioimmunological analysis of aldosterone was 54.8 ± 7.2 (S.D.) % (n=40) for urine samples, and 39.1 ± 4.4 % (n=60) for plasma samples. The coefficient of variation for multiple determinations of aldosterone was for urine 8.2% for low (n = 10) and 14.5% for high (n=10) values; for plasma the respective values were 18.9% (n=10) and 7.8% (n=10). The sensitivity of the determination of aldosterone was for urine 0.04 μg per 24-h volume (n=10) and for plasma 4.4 ng per 100 ml (n=10). The method avoids pitfalls due to the cross-reaction of anti-aldosterone serum with other materials.

EINLEITUNG

Die bestimmung von Aldosteron aus biologischem Material erfolgt meist mit-

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**Folgende Trivialnamen wurden verwendet: 11β,21-Dihydroxy-18-oxopregn-4-en-3,20-dion = Aldosteron; Androst-4-en-3,17-dion = Androstendion; 11β, 17α,21-Trihydroxypregn-4-en-3,20-dion = Cortisol; 17α,21-Dihydroxypregn-4-en-3,11,20-trion = Cortison; 11β,21-Dihydroxypregn-4-en-3,20-dion = Corticosteron; 21-Hydroxypregn-4-en-3, 20-dion = Deoxycorticosteron; 17β-Hydroxyandrost-4-en-3-on = Testosteron; 3β, 17α-Dihydroxypregn-5-en-20-on = 17α-Hydroxypregnenolon; 17α-Hydroxypregn-4-en-3, 20-dion = 17α Hydroxyprogesteron; 3β-Hydroxypregn-5-en-20 = Pregnenolon; Pregn-4-en-3,20-dion = Progesteron; 17α,21-Dihydroxypregn-4-en-3,20-dion = Deoxycortisol.

tels radioimmunologischer Analyse [1], wobei in den meisten Fällen eine Vortrennung der mit organischen Lösungsmitteln extrahierten Proben notwendig ist.

Die ersten quantitativen Analysen von Aldosteron wurden von Neher und Wettstein [2] unter Anwendung physikochemischer Methoden berichtet. Kliman und Peterson [3] verwendenten für die Aldosteronbestimmung die Doppelisotopenderivatmethode. Für die Aussagekraft der radioimmunologischen Aldosteronbestimmung ist, je nach Art der Vortrennung, die Spezifität des verwendeten Antikörpers von Bedeutung. Es wurden jedoch auch hochspezifische Antikörper für radioimmunologische Verfahren entwickelt, die die Erfassung von Aldosteron aus dem Rohextract ohne vorherige Chromatographie erlauben [4—6] . Trotzdem überwiegen heute Methoden, die vor der radioimmunologischen Bestimmung eine Reinigung des Extraktes durchführen, wobei überwiegend die Papierchromatographie [7] und die Säulenchromatographie [8,9] verwendet wurden. Auch Vortrennungen mittels Verteilungschromatographie nach Periodat-Oxydation [10] , oder Dünnschichtchromatographie (DC) [11] sind bekannt.

In Voruntersuchungen konnte gezeigt werden, dass verschiedene Verfahren zur Bestimmung des Aldosterons zu unterschiedlichen Ergebnissen führen können [12] . Es wurde daher ein Analysengang gesucht, der ein exaktes und möglichst vollständiges Abtrennen des Aldosterons von anderen Steroiden ermöglicht und dadurch eine Störung des Radioimmunoassays durch möglicherweise kreuzreagierende Substanzen weitgehend ausschliesst.

MATERIAL

(1) Steroide

(a) Unmarkierte Steroide. Aldosteron (A 6628; Sigma, St. Louis, Mo., U.S.A.), 3β, 5α-Tetrahydroaldosteron (Ikapharm 2601), 3β, 5β-Tetrahydroaldosteron (Ikapharm 2606), 18-Hydroxy-Deoxycorticosteron (Q-1630; Steraloids, Pawling, N.J., U.S.A.) und 18-Hydroxy-Corticosteron (Ikapharm 1722).

(b) Markierte Steroide. Für die Untersuchung der Trenneigenschaften der einzelnen Laufmittel wurden die Steroide in tritiierter Form zugesetzt und mittels eines Dünnschichtscanners (Berthold LB) auf den Platten lokalisiert. Tritiiertes Aldosteron wurde als Zusatz zu den Proben für die Bestimmung der Aufarbeitungsverluste verwendet. Folgende Steroide wurden nach vorhergehender DC-Reinheitsprüfung eingesetzt: [1,2-^{3}H] Aldosteron (50 Ci/mmol), [7-^{3}H] Androstendion (3 Ci/mmol), [1,2-^{3}H] Cortisol (50 Ci/mmol), [1,2-^{3}H]-Deoxycorticosteron (40 Ci/mmol), [1,2-^{3}H]Testosteron (50 Ci/mmol), [7-^{3}H]-Dehydroepiandrosteron (17 Ci/mmol), [7-^{3}H] 17-α-Hydroxy-Pregnenolon (10 Ci/mmol), [7-^{3}H] 17-α-Hydroxy-Progesteron (10 Ci/mmol), [7-^{3}H] -Pregnenolon (17 Ci/mmol), [1,2-^{3}H] Progesteron (50 Ci/mmol) (alle Radiochemical Centre, Amersham, Gross Brittannien); 1,2-^{3}H-Deoxycortisol (40 Ci/mmol, NEN).

(2) Diverses Material und Reagentien

Alle Pipettierschritte erfolgten mit Brand-Pipetten. Polystyrolröhrchen (Nunc) wurden für die radioimmunologische Analyse verwendet.

CH_2Cl_2, C_2H_5OH, Cyclohexan und Essigsäureäthylester wurden in p.a. - Reinheitsgrad von der Fa. Merck (Darmstadt, B.R.D.) bezogen und ohne weitere Reinigung eingesetzt. Weitere Chemikalien: Bovine Serum Albumin (Calbiochem, Los Angeles, Calif., U.S.A.), Kieselgel-G Platten (20 × 20 cm; Merck), Dextran (Fluka, Buchs, Schweiz), Charcoal (Schwarz-Mann, Orangeburg, N.Y., U.S.A.), 2,5-Diphenyloxazolyl (PPO), 1,4-Bis-[2-(5-Phenyloxazolyl)] -Benzol (POPOP), Toluol (Packard, Downers Grove, Ill., U.S.A.), Triton X-100 (Serva, Heidelberg, B.R.D.).

Antisera gegen Aldosteron-Disuccinat (Lot 088, NIH) in einer Endverdünnung von 1:500,000 diente für die Bestimmung von Aldosteron im Harn. Antiserum gegen Aldosteron-Oxim (Institut für Pharmakologie der Univ. Heidelberg; PIH) in einer Endverdünnung von 1:500,000 wurde für die Bestimmung von Aldosteron im Plasma verwendet.

(3) Arbeitslösungen

Boratpuffer (BP): H_3BO_3 (8.25), NaOH (2.70 g), aqua (dest.) ad 1000 ml. (pH = 8.0 mit HCl). Serum-Albumin-Borat-Puffer (BPSAM): Bovine Serum Albumin (10.0 g), Methiolat (0.1 g), BP ad 1000 ml (pH = 8.0 mit HCl—NaOH). Aldosteron-Standardlösung: 10 µg Aldosteron pro ml Äthanol. Adsorptionslösung für die ungebundenen Steroide: (a) Radioimmunoassay von Aldosteron im Harn: Dextran (500 mg), Norit (500 mg), BP ad 100 ml (Suspension); (b) Radioimmunoassay von Aldosteron im Plasma: Dextran (100 mg/80 ml BPSAM)= "A"; Norit A (5.0 g/80 ml BPSAM) = "B". "A" und "B" werden im Verhältnis 3:1 gemischt. Szintillationslösung: POPOP (1.5 g) und PPO (55.0 g) werden in 3330 ml Triton X-100 und 6670 ml Toluol gelöst.

METHODIK UND ERGEBNISSE

(1) Aufarbeitung der Proben

(a) Aldosteron-18-Glucuronid im Harn. 0.5 ml des Tagesurines (24-Stunden-Menge; gesammelt mit 15 ml 6 *N* HCl) werden mit 1000 cpm [1,2-^{3}H] Aldosteron für die Ausbeutekontrolle versetzt und bei pH 1 (H_2SO_4 konz.) 20 Stunden im Dunkel bei Zimmertemperatur hydrolysiert. Die Aldosteronextraktion erfolgt anschliessend mit 7.5 ml CH_2Cl_2. Nach der Extraktion wird die organische Phase abgetrennt, und die CH_2Cl_2 Phase einmal mit 0.5 ml eisgekühlter NaOH (0.1 *N*), sowie zweimal mit eiskaltem Wasser (dest.) gewaschen und über Watte filtriert. Der gewaschene Extrakt wird unter Stickstoff zur Trockene eingeengt und in 50 µl CH_2Cl_2 resuspendiert, dünnschichtchromatographiert, die aldosteronhaltige Zone (1.5 × 1 cm) in der Folge von der Platte abgeschabt und mit 1 ml BPSAM eluiert. Aliquote dieses Eluates werden für die Bestimmung des Aufarbeitungsverlustes (200 µl) und für die radioimmunologische Bestimmung (3 × 100 µl) verwendet.

(b) Aldosteron im Plasma. 2 ml Heparinplasma werden mit 10 ml Wasser (dest.) und 1000 cpm [1,2-^{3}H] Aldosteron versetzt und mit 100 ml CH_2Cl_2 extrahiert. Danach wird die organische Phase zur Trockene eingeengt, in etwa 50 µl CH_2Cl_2 resuspendiert und der DC zugeführt. Die jeweilige Aldosteronzone (1.5 × 1 cm) wird von der Platte abgeschabt und mit 1.5 ml BPSAM eluiert.

Für die Erfassung der Aufarbeitungsverluste, sowie für die radioimmunologische Bestimmung werden je 400 µl eingesetzt.

(II Die Dünnschichtchromatographie

Die Abtrennbarkeit des Aldosterons von verschiedenen Steroiden wurde in vier Laufmittelsystemen untersucht (Tabelle I). Als Prüfsubstanz dienten 13 willkürliche gewählte, tritiierte Steroide aus der Mineralo-, der Glucocorticoid- und der Androgenreihe. Die Lokalisation von Aldosteron in unbekannten Harn- und Plasma proben erfolgte stets mittels eines parallel laufenden [1,2-^{3}H]Aldosterons (10,000 cpm). Die Detektion der markierten Substanz wurde mit einem DC-Scanner (Berthold LB-2723) durchgeführt. Die Charakterisierung der einzelnen Steroide erfolgte mittels ihres relativen, auf Progesteron bezogenen R_F-Wertes (R_{Po}).

Die beste Abtrennung von Aldosteron wurde mit dem System Cyclohexan-Äthylacetat (20:80) erzielt (WS-1; Fig. 1). In diesem Laufmittelsystem wurden auch Spironolacton (R_F = 0.45; R_{Po} = 0.54), 3β, 5α- und 3β, 5β-Tetrahydroaldosteron, 18-Hydroxy-Corticosteron und 18-Hydroxy-Deoxycorticosteron (R_F = 0.0—0.015; R_{Po} = 0.0—0.018) ausreichend von Aldosteron (R_F = 0.08; R_{Po} = 0.1) getrennt. Die Ausbeute der extrahierten und dünnschichtchromatographierten Proben ($\bar{x}$ ± S.D.) betrug für Harn 54.8 ± 7.3% (n = 40) und für Plasmaproben 39.1 ± 4.4% (n = 60).

(III) Die radioimmunologische Bestimmung von Aldosteron

(a) Aldosteron im Harn. Die radioimmunologische Bestimmung von Aldoste-

TABELLE I

DÜNNSCHICHTCHROMATOGRAPHIE VON STEROIDEN

Stationäre Phase; Kieselgel-G (0.25 mm), 20 × 20 cm. Mobile Phase: WS-1 = Cyclohexan—Äthylacetat (20:80); WS-2 = Cyclohexan—Äthylacetat (45:55); WS-3 = Benzol—Aceton (75:25); WS-4 = Benzol—Aceton (50:50).

Steroid	System WS-1		System WS-2		System WS-3		System WS-4	
	R_F	R_{Po}	R_F	R_{Po}	R_F	R_{Po}	R_F	R_{Po}
Aldosteron	0.08	0.10	0.02	0.03	0.07	0.10	0.41	0.46
Cortisol	0.23	0.27	0.06	0.10	0.15	0.23	0.57	0.64
Corticosteron	0.24	0.28	0.06	0.10	0.24	0.36	0.66	0.74
Cortison	0.28	0.33	0.075	0.12	0.22	0.33	0.58	0.65
11-Deoxycortisol	0.48	0.57	0.22	0.36	0.35	0.53	0.69	0.77
Deoxycorticosteron	0.55	0.65	0.28	0.46	0.51	0.87	0.79	0.88
Testosteron	0.61	0.73	0.37	0.61	0.48	0.73	0.76	0.85
17-α-Hydroxy-Pregnenolon	0.75	0.89	0.46	0.76	0.46	0.70	0.75	0.84
Androstendion	0.76	0.90	0.46	0.76	0.63	0.96	0.82	0.92
Dehydroepiandrosteron	0.77	0.92	0.49	0.81	0.54	0.83	0.77	0.86
17α-Hydroxy-Progesteron	0.80	0.95	0.46	0.76	0.54	0.83	0.78	0.87
Pregnenolon	0.83	0.98	0.59	0.98	0.55	0.84	0.80	0.89
Progesteron	0.84	1.00	0.60	1.00	0.65	1.00	0.89	1.00

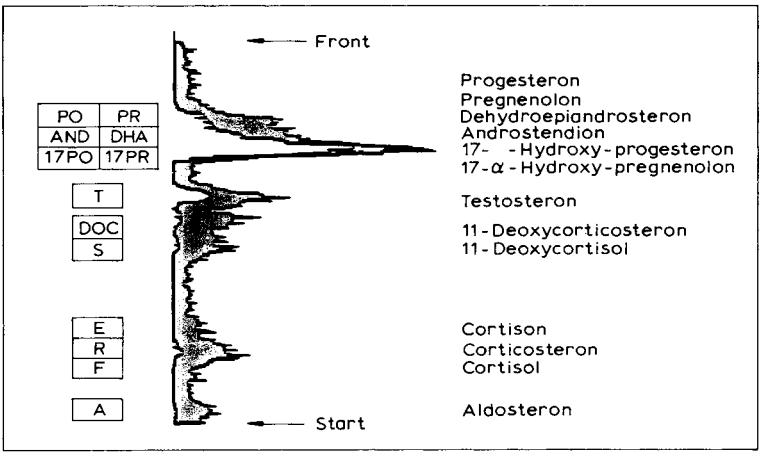

Fig. 1. Die Steroidtrennung im Laufmittelsystem: Cyclohexan—Äthylacetat (20:80).

ron in den Eluaten (je 100 µl; siehe Ia erfolgte dreifach in Polystyrolröhrchen. Die Eichkurve (Fig. 2) des radioimmunologischen Ansatzes umfasste die Absolutwerte 0, 20, 50, 100, 200, 400, 800 und 1000 pg Aldosteron in äthanolischer Lösung. Das Standardmaterial wurde bei 40° in einem Vakuumtrockenschrank zur Trockene gebracht und danach in 100 µl BPSAM aufgenommen. Anschliessend wurden 10,000 cpm [1,2-^{3}H] Aldosteron in 100 µl BPSAM und Antialdosterondisuccinatlösung (900 µl; 1:500,000) im Eisbad zugesetzt. Der Ansatz wurde 30 min bei 30° , sowie 60 min bei 4° inkubiert, und die Inkubation anschliessend durch Zusatz von 500 µl einer Tierkohle—Dextran-Suspension (siehe Material 3a) beendet. Die Proben wurden nach weiteren 10 min bei 3000 rpm und 4° zentrifugiert, der Überstand dekantiert und mit 10 ml Szintillationsflüssigkeit in einem Flüssigkeitsszintillationszähler (Packard, Modell 2450) gemessen. Die Berechnung der Aldosteronexkretion von je 24 h erfolgte entsprechend:

$$\frac{x.V}{A.5}\ \mu g \text{ Aldosteron pro 24 h}$$

wobei
x = unkorrigierter Wert (ng pro 0.5 ml), V = Harnvolumen (ml pro 24 h) und A = Ausbeute (%).

Aldosteron im Plasma. Hier wurden jeweils Doppelwerte des BPSAM-Extraktes (je 400 µl) aus der Aldosteronzone der DC eingesetzt. Die Messpunkte der Eichkurve (Fig. 2) wurden wie unter IIIa gehandhabt. Hier umfasste die Eichkurve die Mengen von 0, 5, 10, 20, 50, 100, 200, 400, 800 und 1000 pg Aldosteron absolut. Allen Proben wurde [1,2-^{3}H] Aldosteron (300 cpm; 10 pg) und Antialdosteron-Lösung (1 ml; 1:500,000) zugesetzt. Die Inkubationszeit betrug 15 h bei 4° und wurde durch Zusatz von 150 µl der Tierkohle—Dextran-Lösung (siehe Material 3b) beendet. Die weitere Aufarbeitung erfolgte wie unter IIIa beschrieben. Die Aldosteronkonzentration im Plasma wurde berechnet als:

$\frac{x.5}{A}$ = ng Aldosteron pro 100 ml

wobei

x = unkorrigierter Wert (pg pro 2 ml) und A = Ausbeute (%).

(c) Die Beurteilung des analytischen Systems. Die Wiederfinderate exogen zugesetzten Aldosterons ($\bar{x}$ ± S.D.) betrug für Harnproben 80.6 ± 9% (n = 32) und für Plasma proben 90.1 ± 4.2% (n = 13). Die Präzision der Methode wurde durch zehnfache Bestimmung der Aldosteronkonzentration in jeweils zwei Ansätzen für verschiedene Hormonkonzentrationen geschätzt (Tabelle II). Der mittlere Variationskoeffizient (V.K.) der Harnaldosteronbestimmung betrug für den unteren Messbereich 8.2% und für eine hohe Aldosteronkonzentration 14.5%. Bei der Bestimmung des Plasmaaldosterons betrug der mittlere V.K. bei niedriger Aldosteronkonzentration 18.9%, bei mittlerer Aldosteronkonzentration 10.9% und bei hoher Aldosteronkonzentration 7.8%.

Die Empfindlichkeit (2 S.D. × $100/\bar{x}$) der Methode betrug dementsprechend für niedere Aldosteronwerte 0.04 µg pro 100 ml (Harn), bzw. 4.4 ng pro 100 ml (Plasma) und für den oberen Messbereich 0.48 µg pro 100 ml (Harn), bzw. 21.5 ng pro 100 ml (Plasma).

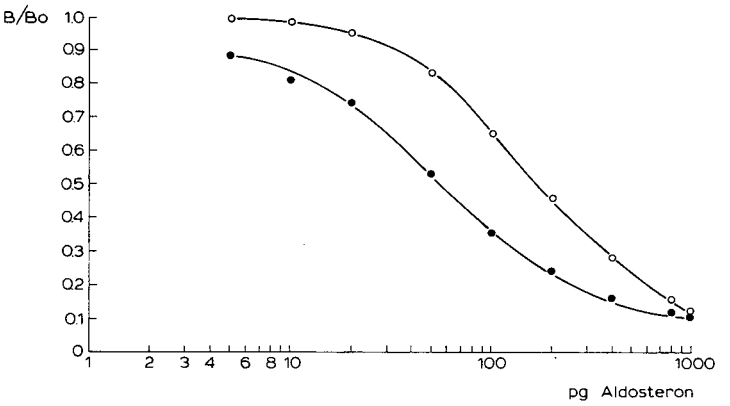

Fig. 2. Verlauf der Eichkurven für Aldosteron bei Verwendung des Antikörpers gegen (•) Aldosteron-Oxim (PIH; Plasma) und (○) Aldosteron-16,21-Disuccinat (NIH, LOT).

TABELLE II

ERGEBNISSE DER BESTIMMUNG VON ALDESTERON IN HARN UND PLASMA

Aldosteron im Harn (µg pro 100 ml)		Aldosteron im Plasma (ng/ml)	
Ansatz	x ± S.D. (n = 10)	Ansatz	x ± S.D. (n = 10)
A1	1.77 ± 0.21	A1	141.0 ± 13.0
2	1.53 ± 0.26	2	134.7 ± 8.5
B1	0.29 ± 0.02	B1	22.1 ± 3.4
2	0.21 ± 0.02	2	18.5 ± 1.2
		C1	11.0 ± 2.7
		2	12.8 ± 1.7

Die Kreuzreaktion der verwendeten Antikörper gegen Aldosteron-Disuccinat (NIH, Lot 088; Harn) und Aldosteron-Oxim (PIH; Plasma) mit verschiedenen im Plasma in höherer Konzentration vorkommenden Steroiden und deren Tetrahydroverbindungen ist in Tabelle III zusammengestellt. Eine starke Kreuzreaktion von Anti-Aldosteron-Oxim mit Tetrahydroaldosteron ist erkennbar.

TABELLE III

KREUZREAKTIONEN (%) EINIGER STEROIDE MIT DEN VERWENDETEN ALDOSTERON-ANTISERA

Antikörper gegen		
Steroid	Aldosteron-Disuccinat	Aldosteron-Oxim
Cortisol	<0.01	<0.1
Cortison	0.06	<0.1
Corticosteron	<0.01	<0.1
Deoxycortisol	0.06	<0.1
Deoxycorticosteron	<0.01	<0.1
3β, 5α-Tetrahydroaldosteron	1.00	7.0
3β, 5β-Tetrahydroaldosteron	0.20	7.0
Tetrahydrocortisol	<0.01	<0.1
Tetrahydrocortison	<0.01	<0.1
Tetrahydrodeoxycortisol	<0.01	<0.1
Tetrahydrodeoxycorticosteron	<0.01	<0.1

DISKUSSION

Die kovalente Bindung von Steroiden im Sinne eines Haptens an ein Trägerprotein ermöglichte es, Steroiden antigene Eigenschaften zuzuordnen [13]. Die radioimmunologische Bestimmung von Aldosteron verwendet die auf diese Weise gewonnenen Antikörper unterschiedlicher Spezifität als Trägerprotein des analytischen Systems, wobei der Mehrzahl der beschriebenen Methoden eine Vorreinigung der zu untersuchenden Proben durchgeführt wird [7—11]. Die Vorreinigung der Proben erscheint vor allem deswegen notwendig, weil die sogenannte Spezifität eines Antikörpers chemisch gesehen geringe Aussagekraft besitzt und nur bekannte Kreuzreaktionen ausschliessen kann. Da die Spezifität eines Antikörpers in der Mehrzahl der Fälle durch Beschreibung von Kreuzreaktionen mit handelsüblichen Steroiden festgestellt wird, bleibt jedoch unberücksichtigt, dass möglicherweise auch isomere Steroidformen, artverwandte Moleküle, unbekannte Metaboliten oder exogene Substanzen, wie Arnzei- und Lebensmittel mit dem Antikörper reagieren und damit zu falschen Messwerten führen können. In diesem Sinn fand sich bei dem verwendeten Antikörper gegen Aldosteron-Oxim eine starke Kreuzreaktion mit beiden isomeren Tetrahydroaldosteronen. Ebenso wurden bei Bestimmung von Aldosteron in biologischem Material mit verschiedenen Antikörpern unterschiedliche Messwerte für die untersuchten Proben beobachtet, die durchwegs höher lagen als die nach chromatographischer Reinigung gefundenen Werte [12]. Zudem ist auch zu be-

achten, dass ein und dasselbe kreuzreagierende Material in Abhängigkeit von der jeweiligen Antikörperbindung zu erheblichen Unterschieden der gewonnenen Messwerte führt [14].

Die vorliegende Methode der Aldosteronbestimmung versucht die Aussagekraft des radioimmunologischen Indikatorsystems unabhängig von einer allfälligen Kreuzreaktion des verwendeten Antikörpers mit anderen Substanzen, durch eine genaue Beschreibung des Steroidverhaltens in dem dem Radioimmunoassay vorgeschaltenen Reinigungsschritt zu verbessern. Die Anwendung einer derartigen Modifikation des analytischen Systems ist stets dann zu fordern, wenn eine absolute Aussage über das Verhalten eines einzeln Steroids, z.B. von Aldosteron, in biologischem Material gemacht werden soll. Auf diese Weise wird die Gefahr einer Verfälschung der Messergebnisse durch Fremdeinflüsse herabgesetzt, und eine Optimierung der analytischen Verhältnisse erreicht. Der analytische Aufwand ist vertretbar und erlaubt die Aufarbeitung von 80 Proben je Arbeitswoche und Arbeitskraft.

ZUSAMMENFASSUNG

Die beschriebene Methode ermöglicht eine genaue und rasche Bestimmung von Aldosteron im Harn und Plasma. Die Zuverlässigkeit der Bestimmung ruht auf der Abtrennung des Aldosterons von Verunreinigungen und anderen Steroiden mittels Dünnschichtchromatographie im Laufmitteln Cyclohexan—Äthylacetat (20:80). Aus dem Plasma werden die Steroide direkt, aus dem Harn nach Hydrolyse mit Schwefelsäure (pH = 1) mit Dichlormethan extrahiert.

Die Ausbeute des Analysenganges nach der Vorreinigung und vor der radioimmunologischen Bestimmung des Aldosterons beträgt 54.8 ± 7.3 (S.D.) % (n = 40) für Harnproben, und 39.1 ± 4.4% (n = 60) für Plasmaproben. Der Variationskoeffizient (V.K.) beträgt für wiederholte Bestimmung von Aldosteron im Harn im unteren Mess bereich 8.2% (n = 10) und für hohe Werte 14.5% (n = 10). Für Plasma bestimmungen sind die entsprechenden Werte des V.K. 18.9% (n = 10) und 7.8% (n = 10). Die Empfindlichkeit der Analysen im unteren Messbereich beträgt für Aldosteron im Harn 0.04 μg pro 24-h Volumen (n = 10) und im Plasma 4.4 ng pro 100 ml (n = 10). Der Analysengang vermeidet die Gefahr der fälschlichen Bestimmung von mit dem Antikörper im radioimmunologischen System kreuzreagierenden Substanzen (Steroide).

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Biomedical Applications

CHROMBIO. 173

DETERMINATION OF CYTOSINE ARABINOSIDE IN HUMAN PLASMA BY GAS CHROMATOGRAPHY WITH A NITROGEN-SENSITIVE DETECTOR AND BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY

JOHN BOUTAGY

MRC Unit and University Department of Clinical Pharmacology, Radcliffe Infirmary, Oxford, OX2 6HE (Great Britain)

and

DAVID J. HARVEY

University Department of Pharmacology, South Parks Road, Oxford, OX1 3QT (Great Britain)

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SUMMARY

A method for the determination of cytosine arabinoside in the plasma of leukemic patients being treated with this drug is described using either gas—liquid chromatography with a nitrogen-sensitive flame ionization detector or gas chromatography—mass spectrometry (GC—MS). To increase volatility, a double derivative of cytosine arabinoside was used, prepared by acetylation and subsequent methylation. Cytidine was used as internal standard for the GC procedure. GC—MS was performed with either cytidine as internal standard and detection by single-ion monitoring or by the use of [$^2H_3$] acetate-methyl derivative of cytosine arabinoside as internal standard and subsequent multiple-ion monitoring. Attempted extraction of cytosine arabinoside from plasma with various organic solvents was unsuccessful, but protein precipitation with ethanol or trichloroacetic acid followed by washing of the aqueous residue with organic solvents to remove as many of the interfering substances as possible gave satisfactory results. The minimum detectable quantity of pure cytosine arabinoside was similar for both techniques (approximately 500 pg). However, with GC using a nitrogen-sensitive detector, the lower limit of detection from plasma was found to be approximately 40—70 ng per ml plasma whilst GC—MS showed greater analytical selectivity with a detection limit in some cases as low as 1 ng per ml plasma.

INTRODUCTION

The study of the pharmacokinetics of the synthetic antitumour purine and pyrimidine nucleosides has been limited by lack of suitable assay techniques

capable of detecting and analysing small concentrations of these drugs in biological fluids free from interference from structurally related endogenous compounds. In particular, cytosine arabinoside (ara-C), which is one of the most useful agents for treatment of acute myeloid leukemia (AML), has been a difficult drug to assay in plasma and other tissues because of its high water solubility making selective extraction from plasma difficult and also because of its structural similarity to naturally occurring pyrimidine nucleosides.

The clinical pharmacology of ara-C has been principally studied using radiolabelled (tritiated) drug in selected patients [1—6]. However, because of the difficulty in using radiolabelled drugs in man, the alternative approach has been the development of a number of biological and related assay techniques for ara-C. These include microbiological [7—9], radioenzymatic [10], radioimmunoassay [11,12], and other bioassay techniques [13—16]. Whilst in some cases these techniques have a considerable degree of sensitivity (less than 50 ng/ml in plasma) they are subject to varying degrees of interference from endogenous substances.

In contrast there are few reports of physical and chemical techniques for measurement of ara-C in biological fluids. The first of these was an elegant technique by Furner et al. [17] using ultraviolet spectroscopy to determine simultaneously cyclocytidine, ara-C and uracil arabinoside in plasma or serum, but this technique lacked the detection sensitivity for plasma analysis at concentrations of ara-C resulting from therapeutic doses in man. (Detection sensitivity of this method was approximately 2 μg/ml.) In recent times, high-pressure liquid chromatographic techniques have had increasing use in separation and analysis of naturally occurring nucleosides but with only little application for determination of ara-C in biological fluids. Published reports using this technique [3,18] for ara-C determination give insufficient data as to the scope and sensitivity of the assay. Similarly, the application of gas chromatographic (GC) techniques for analysis of ara-C have received little attention, mainly due to the difficulty in applying these techniques to such highly polar compounds. One report, that of Pantarotto et al. [19] described a method using GC—chemical ionization mass fragmentography for determination of ara-C and other cytotoxic bases in mouse plasma using permethylated derivatives Although this approach was promising, only moderate detection sensitivity in plasma (0.1 μg/ml) was reported by these workers because of high background and the use of small plasma volumes.

In view of the lack of assay techniques based on physicochemical methods for direct and sensitive measurement of the free drug in plasma this report examines the application of gas—liquid chromatography (GLC) using a nitrogen-sensitive flame ionization detector (N-FID) for enhanced sensitivity, and a coupled mass spectrometer for enhanced selectivity, to the measurement of ara-C in the plasma of leukemic patients receiving this drug. Various extraction methods were investigated and derivatization was by formation of acetylmethyl derivative as previously described [20].

EXPERIMENTAL

Materials

Ara-C was kindly supplied by Upjohn (Crawley, Great Britain). Cytidine was purchased from Sigma (St. Louis, Mo., U.S.A.). Ethereal diazomethane was prepared from Diazald (Aldrich, Milwaukee, Wisc., U.S.A.). Catalyst solution of boron trifluoride was prepared by diluting 100 μl of boron trifluoride etherate (BDH, Poole, Great Britain) with 5 ml of diethyl ether. All solvents were re-distilled before use.

Gas chromatography

GC was performed on a Hewlett-Packard 5750G gas chromatograph with a nitrogen—phosphorus-sensitive FID. The chromatograph was linked to a Hewlett-Packard 3370 digital integrator for chart presentation and peak area determination. Glass columns (2 m × 4 mm I.D.) were packed with 3% SE-30 on 80—100 mesh Chromosorb W (Pierce, Rockford, Ill., U.S.A.) or 3% OV-17 on 100—120 Gas-Chrom Q (Applied Science Labs., State College, Pa., U.S.A.). The injection port temperature was 290° and the N-FID was maintained at 400°. The column oven temperature was 225° for the SE-30 column and 275° for the OV-17 column. Flow-rates of helium carrier gas were 45 ml/min, hydrogen 28 ml/min and air 180 ml/min. The height of the rubidium bromide crystal in the detector block was adjusted to give equal response to a 2-μl injection of azobenzene (10 ng) and octadecane (5000 ng) in hexane when the instrument was in the 'normal' mode. For all subsequent analyses the instrument was used in the 'high sensitive' mode.

Gas chromatography—mass spectrometry (GC—MS)

Single- and multiple-ion monitoring were performed with a V.G. Micromass 70/70F Mass Spectrometer interfaced to a Varian 2400 GLC instrument using 1 or 2 m × 2 mm columns packed with either 3% SE-30 or OV-17. The column oven temperature was set to give a retention time of 1.5 min (in the range of 230° for 1 m SE-30, to 265° for 2 m OV-17). The mass spectrometer was operated at 70 eV with an accelerating voltage of 4 kV and a trap current of 100 μA. GLC-interface and ion-source temperatures were 300 and 260°, respectively.

Procedure for plasma extraction

Blood (5 ml) was collected by an indwelling catheter before and at various time intervals after a single intravenous bolus of ara-C (2 mg/kg) into a lithium heparin tube containing tetrahydrouridine (final concentration 10^{-4}M). The samples were kept over ice until centrifugation. The blood was centrifuged at 2000 g for 10 min and the plasma was removed and stored at -20° until required. Before extraction the plasma was thawed and re-centrifuged to remove any suspended solids. Investigation into plasma extraction using various methods showed the following to be the most useful:

Method 1. Plasma (1 ml) was treated with absolute ethanol (6 ml) in a glass test tube and mixed thoroughly on a vortex mixer for 15—20 sec. The extract was centrifuged for 5 min, the supernatant was transferred to another tube and

was evaporated to near dryness at 60° under a stream of nitrogen. The residue was then redissolved in water (3 ml) and was extracted with isoamyl alcohol (2 ml) by mixing on the vortex mixer. After centrifuging, the top isoamyl alcohol layer (containing most of the yellow pigments) was removed and discarded. The aqueous residue was again extracted with isoamyl alcohol (2 ml) and the isoamyl alcohol was discarded. The aqueous residue was then extracted twice with 3-ml volumes of ether and the ether was discarded. The aqueous phase was then evaporated to dryness under a stream of nitrogen at 60° and the residue was derivatized as described below for GLC or GC—MS analysis.

Method 2. Plasma (1 ml) was treated with cold 15% (v/v) trichloroacetic acid (3 ml), thoroughly mixed on a vortex mixer for 15 sec, and was stood in ice for 30 min to allow complete protein precipitation. The mixture was then centrifuged for 10 min and the supernatant was transferred to a separate tube. The supernatant was then extracted four times with 3-ml volumes of ether (the ether extracts were discarded), then once with isoamyl alcohol (2 ml). The isoamyl alcohol was discarded. The remaining aqueous phase was then freeze-dried and the residue was derivatized as described below.

Method 3. Plasma (3 ml) was ultrafiltered by centrifugation at 0° (250 g for 1 h) through a conical ultrafilter (Amicon, Lexington, Mass., U.S.A.). 1 ml of water was added to 1 ml of the colourless ultrafiltered plasma and the mixture was then extracted twice with 2-ml volumes of ether. The ether extracts were discarded. The aqueous phase was then freeze-dried, and the residue was derivatized as described below.

Derivatization

To the dried plasma extract, acetic anhydride (60 μl) and dried pyridine (30 μl) were added and the mixture was stood at room temperature for 40 min. The acetylation mixture was then removed by evaporation at 50° under a stream of nitrogen. The residue was dissolved in water (1 ml) and chloroform (2 ml) was added. The mixture was thoroughly mixed on a vortex mixer for 20 sec and centrifuged. The top aqueous layer was removed and discarded. The chloroform extract was washed again with water (1 ml) and the aqueous phase was removed and discarded. The chloroform residue was then evaporated to dryness under a stream of nitrogen. The residue was dissolved in ethyl acetate (50 μl) and ethereal diazomethane (200—300 μl) was added together with boron trifluoride etherate in ether (1:50, 2 μl). The yellow mixture was stood at room temperature for approximately 30 sec and then evaporated to dryness under a stream of nitrogen. The residue was dissolved in ethyl acetate (20—40 μl) and aliquots (1—3 μl) were used for GLC or GC—MS analysis.

Calibration

Stock solutions (1 mg/ml) of ara-C and cytidine were prepared in methanol and serial dilutions were made. Varying amounts of ara-C (0.075—2 μg/ml) and a constant amount of cytidine (1 μg/ml) were added to plasma from normal subjects and a standard curve was constructed following extraction and derivatization as described above.

Recovery

Recovery of ara-C from plasma was determined by adding known amounts of ara-C and [^{3}H]-labelled ara-C (5-[^{3}H]cytosine-β-D-arabinoside, 15 Ci/mmol; The Radiochemical Centre, Amersham, Great Britain) to drug-free plasma to give final concentrations of 20, 200 and 2000 ng/ml and an activity of 0.01 μCi/ml. The plasma was then extracted by each of methods 1, 2 and 3, as described. The dried extracts were redissolved in methanol (0.5 ml) and Instagel (Packard) (8 ml) and the radioactive content was determined by liquid scintillation counting.

Reproducibility and stability

Eight aliquots of a mixture of pure ara-C (100 μg) and cytidine (100 μg) were derivatized to give the acetyl-methyl derivatives, as described above. The derivatives were dissolved in ethyl acetate (100 μl) in a stoppered tube and stood at room temperature for 24 hours. Aliquots (1 μl) were chromatographed immediately and again after 24 hours.

Storage

Plasma from patients receiving ara-C or drug free plasma containing known amounts of ara-C were assayed immediately and after storage at -20° for 7 days.

Deuterated derivatives of ara-C

[$^2H_3$]acetyl and [$^2H_3$]methyl analogues of the acetyl-methyl derivative of ara-C were prepared by substituting [$^2H_6$]acetic anhydride or [$^2H_2$]diazomethane, respectively, for the unlabelled reagents in the above preparations.

RESULTS AND DISCUSSION

Plasma extraction

Ara-C, unlike many of the clinically used drugs, presented a problem for selective extraction from plasma using conventional solvent extraction techniques. Clarke [21] has indicated that ara-C is extracted by organic solvents from aqueous alkaline solution, however repeated attempts at extraction of ara-C from aqueous solution (neutral or alkaline) with a variety of water immiscible organic solvents and mixtures of solvents gave poor recovery. This is not surprising since ara-C has a considerable degree of water solubility and is barely soluble in organic solvents. However, the recovery was greatly enhanced by saturation of the aqueous solution containing ara-C with ammonium sulphate and using polar solvents such as *n*-butanol or propan-2-ol, but considerable salt residues together with large quantities of undesired plasma components in the extract rendered this method unsuitable for GC work-up. Attempts using charcoal adsorption and elution with methanol or methanol—pyridine gave inconsistent recoveries. It was found that the best way to achieve sample clarification was by initial protein precipitation using ethanol or trichoroacetic acid solution, or by protein removal through centrifugation of the plasma through conical ultrafilters, and subsequent washing of the aqueous residue containing the ara-C with organic solvents to remove as many plasma compo-

nents as possible whilst leaving the ara-C in the aqueous phase. Lyophilization of the aqueous residue gave a suitable extract for derivatization.

Gas chromatography

Among the various chromatographic derivatives that were previously examined [20] the acetyl-methyl derivative was chosen because of the relative ease of preparation and because of the water stability of the nucleoside acetates enabling partial purification of the extract by extraction from the aqueous phase with chloroform. Furthermore, the acetyl-methyl derivative of ara-C could be well separated by GLC from the acetyl-methyl derivative of the major metabolite, uracil arabinoside, and from other structurally similar pyrimidine nucleosides on either OV-17 or SE-30. The N-FID gave enhanced detection sensitivity over the conventional flame ionization, with minimum detection of 500 pg for the derivative of the pure nucleoside with a 3:1 signal-to-noise ratio. Because of the close structural similarity to ara-C, cytidine (the epimeric ribonucleoside) was used as an internal standard since cytidine has similar solubility properties to ara-C and undergoes the same derivatization reaction.

The chromatograms from normal individuals or from leukemic patients prior to receiving ara-C (pre-dose plasma) contained many chromatographic peaks, mainly at earlier retention times than that of ara-C. Examples of chromatograms of drug-free plasma are shown in Figs. 1A and 1C. The chromatogram in Fig. 1B shows plasma from a normal individual to which 0.5 μg/ml ara-C was added. Analysis of plasma from normal individuals or from leukemic patients prior to receiving ara-C showed little or no response for the presence of endogenous cytidine, allowing cytidine to be used as internal standard. The chromatogram in Fig. 1D shows plasma from a leukemic patient who had received ara-C and to which cytidine (1 μg/ml) had been added as internal standard. The concentration of ara-C in that sample was 0.53 μg/ml. The tetrahydrouridine (a deaminase inhibitor) added to the blood on collection to prevent deamination of ara-C during storage did not give a chromatographic peak under the conditions used and hence did not interfere with the assay.

The percentage recovery of added ara-C from plasma by extraction methods 1, 2 and 3 is shown in Table I. For each recovery method the percentage did not vary significantly in the concentration range of 0.02—2 μg/ml. Furthermore the percentage recovery of ara-C and that of cytidine was the same since peak height ratios of extracted plasmas containing known amounts of both these compounds were the same as that of identical quantities of derivatized standards. After acetylation of the plasma extract, extraction with chloroform gave near quantitative recovery (98%) of the acetate into that solvent, hence there was little further loss of ara-C after the initial plasma extraction procedure.

To test the reproducibility of the derivatization reaction and relative stability of the derivatives, eight aliquots of 100 μg each of ara-C and cytidine were derivatized and 1-μg quantities were injected into the gas chromatograph. The mean peak height ratio of ara-C to cytidine was 1.19 ± 0.08 (standard deviation) from sixteen determinations. After standing at room temperature for 24 h the samples were re-analysed and showed little change in peak height ratio (mean 1.205 ± 0.053). An identical experiment using 0.5 μg each of ara-C and cytidine gave similar results.

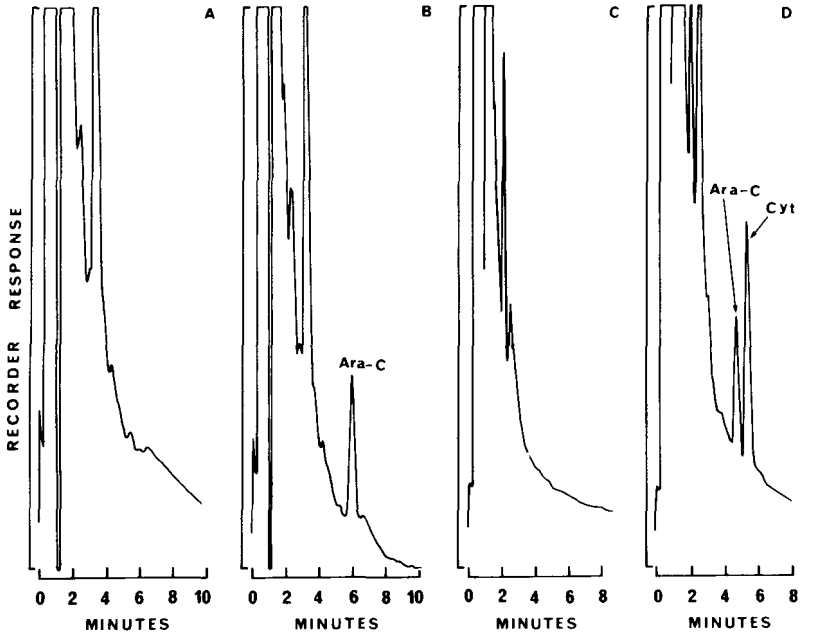

Fig. 1. Gas chromatography after derivatization of plasma extracts. Chromatograms A and B were obtained on SE-30 at 225° and were from drug-free plasma from a normal individual (A) and the same plasma after 0.5 μg/ml ara-C had been added (B). Chromatograms C and D were obtained on OV-17 at 275° and were from plasma of a leukaemic patient prior to receiving ara-C (C) and 10 min after a single dose of ara-C (2mg/kg) (D). In addition to ara-C, chromatogram D contains a peak produced by cytidine (1 μg/ml) added as an internal standard.

TABLE I

PERCENTAGE RECOVERY OF ARA-C FROM PLASMA

Extraction method	Mean percentage recovery ± S.D.	*n*
1	71 ± 5.4	9
2	84 ± 4	6
3	85* ± 4	6

* Recovery per ml of filtrate.

Ara-C in plasma showed no decomposition on storage in that samples of plasma from patients or drug-free plasma to which ara-C had been added showed no change in peak height response when assayed immediately or after storage at - 20° for seven days or longer.

For calibration, analysis of drug-free plasma containing known amounts of added ara-C showed a linear response (peak height ratio) up to 2 μg/ml with a minimum detection from one ml of plasma of 0.04—0.07 μg/ml (twice signal strength above background). The precision of the assay at 0.5 μg/ml of added

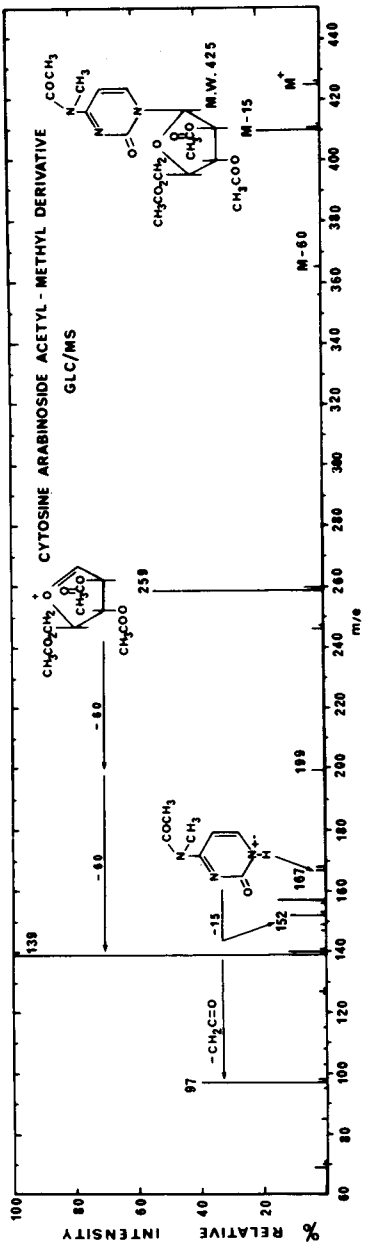

Fig. 2. 25-eV mass spectrum of the acetyl-methyl derivative of ara-C.

ara-C to drug-free plasma was 7.6% (coefficient of variation from ten determinations). This value increased at the lower concentrations. Each of methods 1, 2 and 3 for plasma extraction gave similar results, with little difference in background or sensitivity. Attempts at further clean-up of the plasma by prechromatography on ion-exchange columns or by thin-layer chromatography did not significantly improve the background response or increase the detection limit. Similarly use of either SE-30 or OV-17 showed no significant difference in sensitivity. Repeated injection over a period of 6—8 h into the chromatograph of plasma extracts gave diminished absolute response from the N-FID due to contamination of the rubidium crystal but the peak height ratios of ara-C to standard did not alter. Optimum detection response was readily regained by removing the rubidium crystal from the detector block and removing the accumulated deposits from the crystal.

Gas chromatography—mass spectrometry

Single-ion detection. The detection limit from plasma was improved by the use of the more selective mass spectrometer as the GC detector. Using single-ion monitoring for the $[M-15]^+$ ion it was possible to achieve a limit of 3—10 ng/ml from plasma and an absolute detection limit of 0.5 ng for the pure derivative. Although better sensitivity could be achieved with the drug alone by monitoring the more abundant ions in the lower mass range (*e.g.*, *m/e* 169; Fig. 2), when these ions were monitored in the presence of plasma extracts the increased background produced by endogenous plasma constituents at these masses resulted in poorer overall performance. At the mass of the $[M-15]^+$ ion (*m/e* 410) there was a dramatic improvement in the background at the retention time of ara-C with OV-17 compared with SE-30 (Fig. 3). However, a small residual peak was present and this limited the sensitivity of the assay to about 3—10 ng/ml. The nature of the compound producing this peak is unknown. More polar columns such as OV-25 and OV-210 were also investigated but these produced broad peaks with ara-C and no improvement in sensitivity [20]. OV-17 was thus used for measurement of ara-C in plasma. As with GLC, cytidine could still be used as an internal standard at levels of about 50 ng/ml as its spectrum also contained an ion at *m/e* 410. Fig. 4 shows the single-ion trace (*m/e* 410) from 0.5 μg/ml ara-C and 2 μg/ml of cytidine extracted from plasma. Fig. 5 shows a comparison between the plasma levels of ara-C found using an N-FID detector and by MS monitoring of *m/e* 410 in the plasma of an AML patient who had received a single dose of the drug (2 mg/kg). The ara-C was extracted using method 2. The limit of detection using N-FID was approximately 40 ng/ml whilst single-ion monitoring could achieve greater sensitivity (detection limit 20 ng/ml) and enabled measurement of ara-C for a longer period of time (60 min) after drug administration.

Multiple-ion monitoring. Below about 50 ng/ml, cytidine proved to be a poor internal standard for monitoring ara-C levels because of interference by endogenous compounds (probably cytidine itself) at the same retention time. At this level it was thus necessary to subtract the contribution of the endogenous substances. Also, as only one ion was monitored, the selectivity of the assay, in the presence of interfering compounds was poor at low levels. Thus

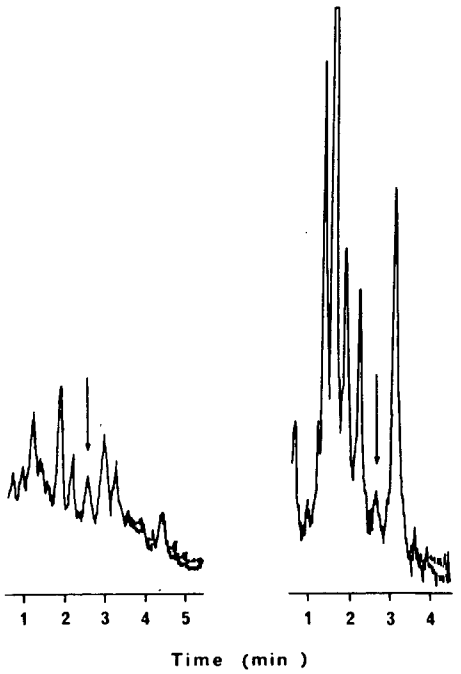

Fig. 3. Comparison of the single-ion traces of *m/e* 410 recorded from drug-free plasma on 3% SE-30 (left) and 3% OV-17 (right). The position at which ara-C elutes is indicated by the arrow. The amplifier gain was different during the recording of each trace; the compounds producing the major peak and the peak at the retention time of cytidine are of equal concentrations.

multiple-ion monitoring using a deuterated standard was used to overcome these difficulties. Deuterated ara-C itself was not available but the derivative incorporating four [$^2H_3$]acetate groups was readily prepared and was added as an internal standard and carrier to the plasma samples after conversion of the plasma ara-C to its unlabelled acetate derivative. An attempt to prepare the deuterated methyl analogue by reaction of ara-C with deuterodiazomethane give a mixture of [$^2H_1$]-, [$^2H_2$]-, and [$^2H_3$]- derivatives and was not investigated further. The recovery of ara-C from plasma before derivatization was measured radiochemically (Table I) and the plasma values found by multiple-ion monitoring were corrected accordingly. Monitoring was performed using four ions, the molecular and $[M-15]^+$ ions from both normal and deuterated derivatives *m/e* 425, 410, 437 and 419 ($M-CD_3$), respectively. Fig. 6 shows a typical trace recorded from a 1-m 3% OV-17 column; the deuterated standard can be seen eluting just ahead of the unlabelled derivative. The calibration curve of peak height ratio against concentration was linear over the range 1 ng to at least 100 ng.

Although ara-C could be detected in plasma at levels of around 1 ng/ml, the presence of endogenous compounds made quantitation difficult at levels below about 3—10 ng depending on the background level in the patient's plasma. When measuring the plasma levels following a single dose, a value for the background was obtained before administration of ara-C and this was then subtrac-

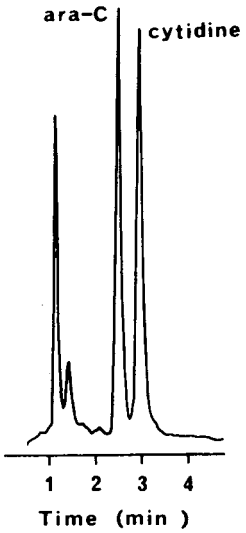

Fig. 4. Single-ion chromatogram (*m/e* 410) of ara-C (0.5 μg) and cytidine (2 μg) extracted from plasma and separated on 3% OV-17.

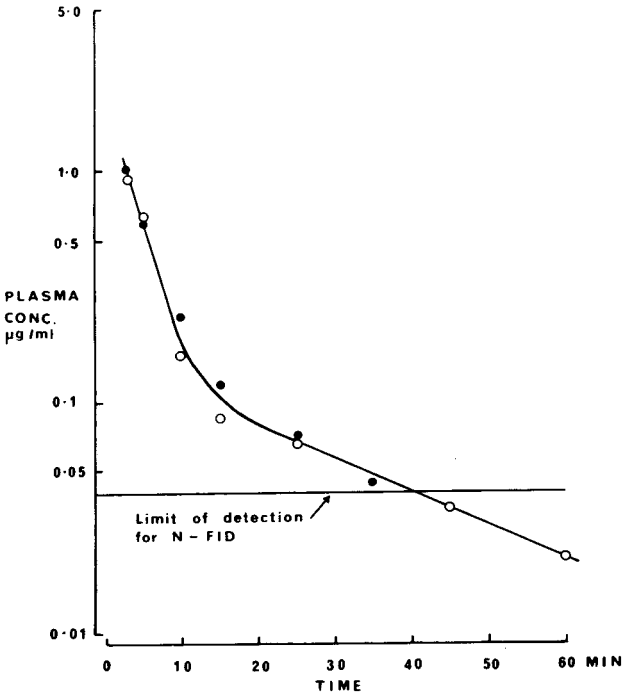

Fig. 5. Comparison of the plasma levels of ara-C from a patient who had received single dose (2 mg/kg) of the drug as measured by the N-FID (•) and by mass spectrometry (○, single-ion monitoring of *m/e* 410).

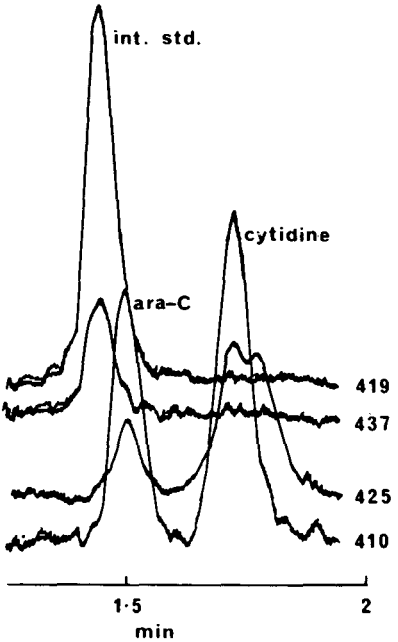

Fig. 6. Typical multiple-ion trace of the molecular and $[M-15]^+$ ions from the acetyl-methyl and $[^2H_3]$acetyl-methyl derivatives of ara-C extracted from plasma. The trace represents a plasma level of 47 ng/ml of ara-C and 50 ng/ml of internal standard and was recorded from the equivalent of 0.04 ml of plasma or about 2 ng injected into the chromatograph.

ted from the total value found after drug administration. In most cases, this method seemed to give a good value for ara-C levels as the height of the background ion was generally unchanged when the ara-C could no longer be detected. However, it was not possible at this stage to check if the concentration of the interfering compound remained unchanged in the presence of the drug. Fig. 7 shows a typical plasma level curve for ara-C found following a single dose and extraction using method 2. From this patient ara-C was detected in the plasma to 5 h after the dose was given, although at this level the contribution of ara-C present was only 20% of the total peak height and was therefore difficult to quantitate. At 6 h the height of the peak at the retention time of ara-C equalled that of the plasma sample taken before drug administration and thus it was assumed that the background did not change in the presence of the drug.

CONCLUSIONS

Of the three methods of detection discussed, all of which gave an equivalent detection limit of approximately 500 pg with pure drug, the N-FID was the least sensitive for measuring N-FID in plasma because of the presence of interfering substances rendering detection below 40—70 ng/ml difficult. MS considerably reduced the signal produced by these endogenous compounds at the retention time of ara-C giving an enhanced detection limit (1 ng/ml) in plasma.

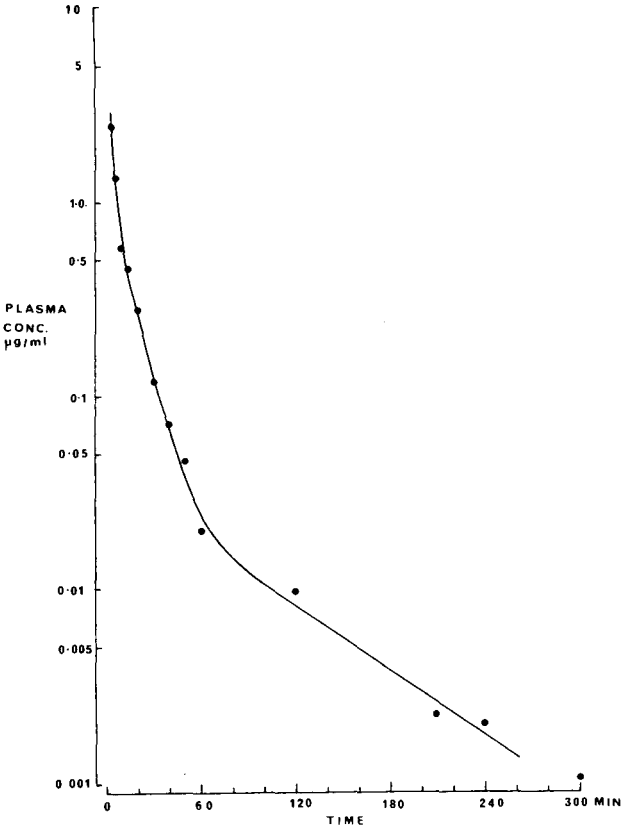

Fig. 7. Plasma levels of ara-C found using multiple-ion monitoring from the plasma of a patient who had received a single intravenous dose (2 mg/kg) of the drug.

With single-ion monitoring, problems arose with the choice of an internal standard at low concentrations of ara-C, multiple-ion detection on the other hand with the [$^2H_{12}$]acetyl-methyl derivative as internal standard and carrier enabled ara-C to be measured to a level of 3—10 ng/ml and detected at the 1-ng/ml level. This is a significant increase in the sensitivity of detection over the biological assay techniques and because of the specificity of GC—MS this method is suitable for pharmacokinetic studies of ara-C and for plasma monitoring of long-term infusion of this drug.

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CHROMBIO. 172

ROUTINE DIRECT INJECTION GAS—LIQUID CHROMATOGRAPHIC PROCEDURE FOR THE ANALYSIS OF VOLATILE HALOGENATED ANAESTHETICS IN WHOLE BLOOD USING A NEW EXTERNAL INJECTION PORT★

P.E. REID, D.E. BROOKS, Y.C. PANG and R. MUELCHEN

Department of Pathology, Faculty of Medicine, University of British Columbia, Vancouver V6T 1W5 (Canada)

and

K.M. LEIGHTON

Departments of Anaesthesia and Pharmacology, Faculty of Medicine, University of British Columbia, Vancouver V6T 1W5 (Canada)

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SUMMARY

This communication describes the design and construction of a new external injection port for the direct gas—liquid chromatographic analysis of volatile compounds in whole blood. Aliquots (4—40 μl) of EDTA anti-coagulated blood containing the volatile compound and a weighed quantity of the internal standard, isobutanol, were injected into the disposable glass wool filter of the heated injection port. The volatiles released were led by means of the carrier gas stream directly onto the chromatographic column. Typical data are presented from chromatography performed with dual 6 ft. × 2 mm I.D. glass columns containing Chromosorb 101 programmed from 110—180° at 6°/min and the external injection port maintained at 180°. The method eliminated the problems usually associated with direct injection methods and permitted the accurate analysis of halothane, methoxyflurane, diethyl ether and ethanol over the approximate range 1—100 mg%. Using this analytical procedure the distribution of halothane between the cells and plasma of human blood at 4° was found to be 2.0 ± 0.2.

INTRODUCTION

Gas—liquid chromatographic procedures for the quantitative analysis of

★A preliminary account of this work was presented in a paper delivered before the 20th Annual Meeting of the Canadian Federation of Biological Societies, Calgary, Alberta, Canada, June 21—24, 1977. Proc. Can. Fed. Biol. Soc., 20 (1977) 173, Abstract No. 710.

volatile anaesthetics in blood or other fluids [1—31], are of two types: either indirect, in which the anaesthetic is separated from the blood prior to injection, or direct, in which the gas chromatography is performed on a sample of whole blood. The indirect techniques (head space methods [1—7], solvent extraction [8—22] and distillation [23,24]) are time consuming [1,2,6,28,30,31], require complicated pre-treatments [29] involving a potential loss of the agent [30] and may be difficult to apply to small samples [30]. Furthermore, solvent extraction procedures may be complicated by the deposition of lipid materials on the columns [2] and by the production of solvent peaks of large area which may interfere with the estimation of the anaesthetic peak and/or result in prolonged elution times [11,14,16]. Direct injection methods [25—31], in contrast, are rapid and apparently simple [16] and can be used with small samples of blood [1]. They may, however, suffer from problems associated with contamination of the columns with non-volatile blood components [2,6,30] and subsequent baseline drift due to the slow elution of such components [2,30], clogging of the syringes used for injection [6,30], distortion and broadening of the peaks [2,6,16,30], ghost peaks [6,30] and poor reproducibility [1,2].

In this paper we describe the design and construction of an external injection port which allows the injection of a small sample of whole blood into the pre-heated carrier gas stream of the gas chromatograph. Flash evaporation removes the non-volatile components and only the volatile components enter the column. This external injection port in combination with the use of an internal standard avoids the problems discussed above and permits the rapid analysis of halothane, diethyl ether, methoxyflurane and ethanol in whole blood over the approximate range of 1—100 mg%.

MATERIALS AND METHODS

Halothane (Hoechst Pharmaceuticals, Montreal, Canada), methoxyflurane (Abbot Laboratories, Vancouver, Canada) and diethyl ether (spectroanalysed; Fisher Scientific, Montreal, Canada) were used without further purification. Isobutanol (reagent grade; Mallinckrodt, St. Louis, Mo., U.S.A.) was dried over anhydrous potassium carbonate and purified by fractional distillation [32]. Ethanol was dried by refluxing with magnesium turnings and purified by distillation [32]. All other chemicals employed were of reagent grade or better. Silicone rubber O-rings 3/16 in I.D. and 5/16 in O.D. HT8 low bleed septa were obtained from Applied Science, State College, Pa., U.S.A. Chromosorb 101, 103, 105, and 107 were purchased from Johns Manville, Denver, Colo., U.S.A. and Reacti-vials and Mininert valves from Pierce, Rockford, Ill., U.S.A.

The external injection port

Design

The design of the external injection port is shown diagrammatically in Fig. 1; Fig. 2 shows detailed engineering drawings. The external injection port consists essentially of a heat reservoir and a rotary valve both of which

contain channels for directing the flow of carrier gas. Both the reservoir and rotary valve are constructed of brass, their respective plane face plates being separated by a 1/32-in. Teflon gasket against which the valve rotates. Mounted on the front of the rotary valve are: (a) a spring which can be used to tighten the valve against the Teflon gasket to achieve a gas tight seal, (b) a gas chromatographic injection port of conventional design sealed with a septum and (c) a pair of handles which allow rotation of the valve. The reservoir is maintained at 180° with a heater set into a well drilled into the body of the reservoir; a similar well contains a thermometer. The base of the port is attached, by a gas tight seal, to the injection port system of the gas chromatograph and can be readily modified for use with a variety of instruments. The carrier gas enters the port through a Swagelok fitting sealed into the side of the reservoir; this requires a diversion of the carrier gas stream prior to the point where it enters the original injection port of the gas chromatograph.

Fig. 3 illustrates the flow paths of the carrier gas within the external injection port when the rotary valve is in the "on" operational and the "off" non-operational positions. In the "on" position the carrier gas passes successively through the fixed channels a and b located, respectively, in the body of the heat reservoir and the rotary valve. It then proceeds via the fixed channel c located in the reservoir, into a glass U-tube (3/16 in. O.D.) connecting c with the fixed channel d located in the body of the reservoir. The carrier gas then passes through fixed channels e and f located, respectively, in the rotary valve and the reservoir and enters the gas chromatography column through the base of the injection port. The U-tube is located on the left side (see Fig. 2) and is sealed into position with silicone rubber O-rings lightly lubricated with a high-temperature vacuum grease. Before insertion the ends of the U-tube were also lubricated with the same vacuum grease. A lever attached to the reservoir (see Fig. 2) prevents the ejection of the U-tube when the valve

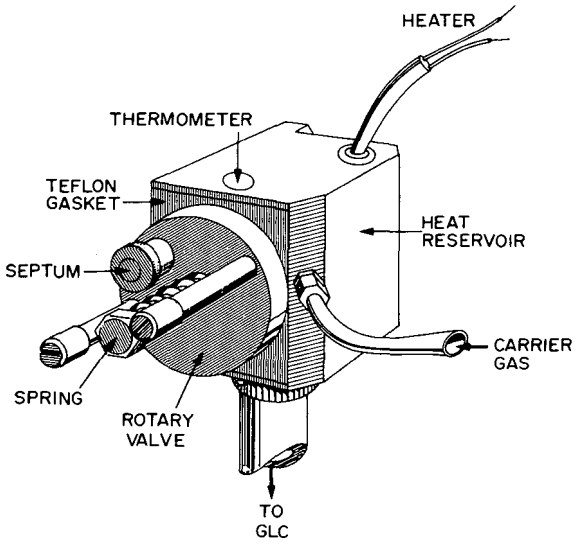

Fig. 1. Diagrammatic representation of the front view of the external injection port.

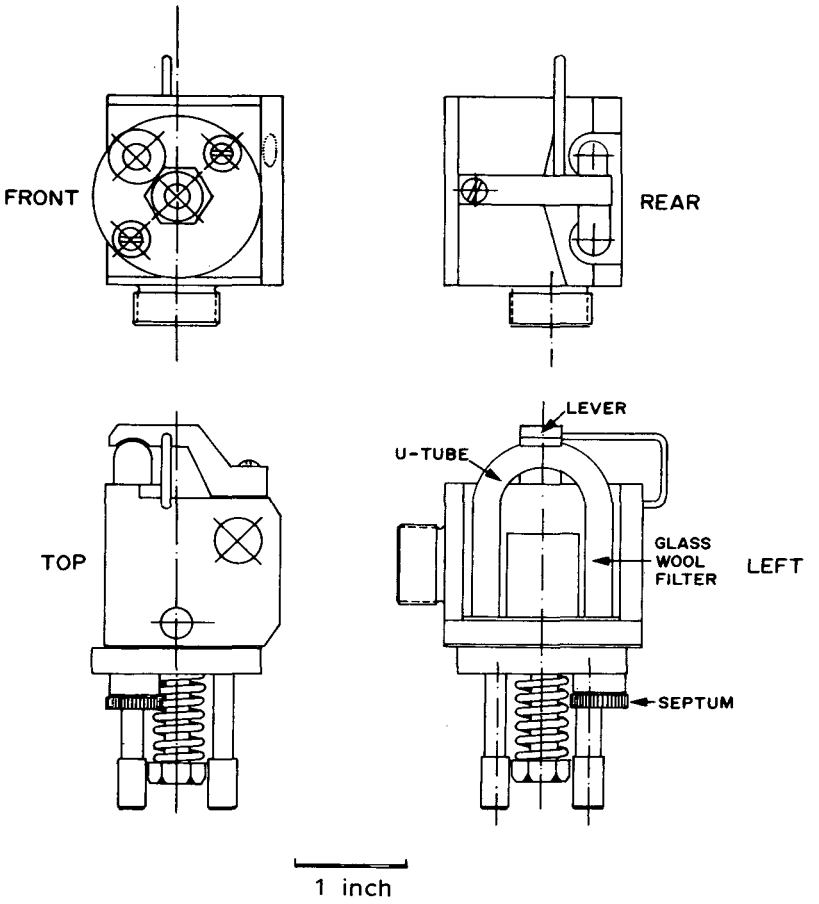

Fig. 2. Engineering drawings of the external injection port.

is in the "on" position (approximate pressure 20—25 p.s.i.) and also serves to maintain a gas tight seal. Holes situated in the appropriate positions in the Teflon gasket allow free passage of gases between the fixed channels in the reservoir and the rotary valve. When the valve is in the "on" position the needle of a Hamilton gas chromatographic syringe will pass through both the septum of the injection port mounted on the front of the valve and the Teflon gasket and enter the U-tube permitting injection directly into the pre-heated carrier gas stream. When the valve is in the "off" position the U-tube is by-passed and the carrier gas passes directly into the gas chromatograph through channels a, b and f. This enables the U-tube to be changed simply and rapidly without interfering with the gas flows in the chromatograph.

Operation

Prior to the first operation of the external injection port the apparatus is maintained at 180° with a pressure on the Teflon gasket of 15—20 pounds for

24—36 h. Under these conditions the Teflon gasket softens and is moulded into the shape of the inside surfaces of the rotary valve and heat reservoir. This ensures a good seal and provided the reservoir is maintained at 180° remoulding is only necessary when a new gasket is fitted.

With the rotary valve in the "on" position a sample of blood (4—40 μl) is injected directly into a loose glass wool filter plug inserted into the U-tube. To avoid clogging of the needle of the syringe by coagulated blood components injection was accomplished with a chaser technique in which the blood sample in the syringe barrel is separated from a plug of water by a bubble of air. When the analysis is completed the rotary valve is switched to the "off" position and the U-tube is replaced. On switching to the "on" position the equipment is ready for another injection. Changing the U-tube occupies between 15 and 60 sec.

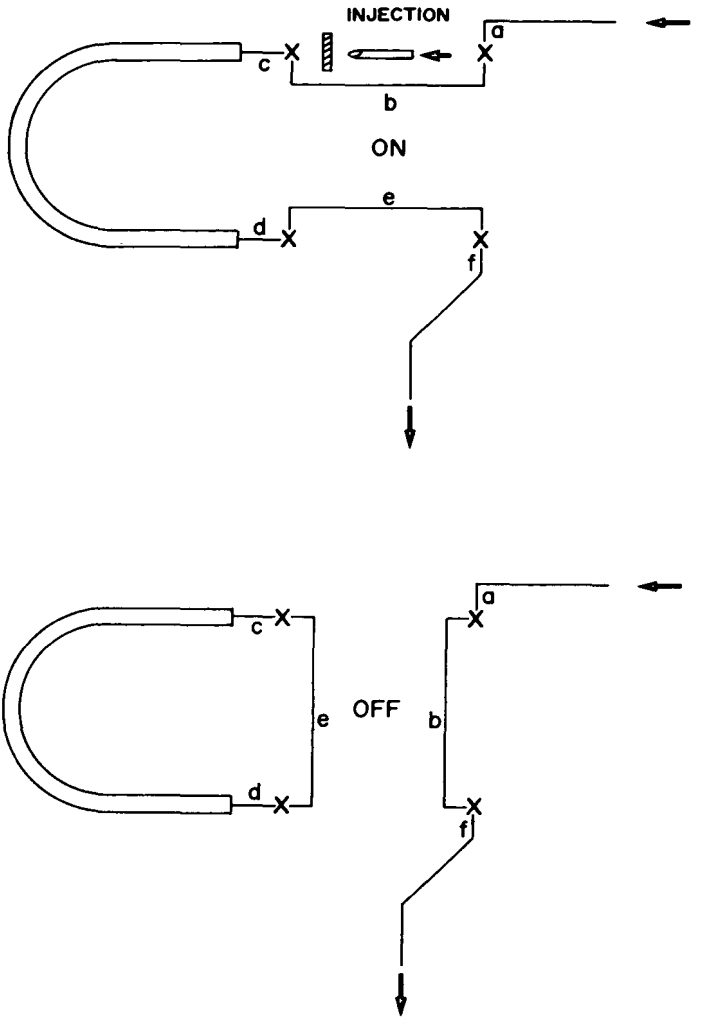

Fig. 3. Schematic carrier gas flow diagram of the external injection port.

Gas chromatography

Gas chromatography was performed on a Hewlett-Packard 7610A gas chromatograph fitted with dual flame ionisation detectors using dual 6 ft. × 2 mm I.D. glass columns containing Chromosorb 101 (80—100 mesh) programmed from 110—180° at 6°/min. The injection port of the chromatograph was maintained at 200°, the external injection port at 180° and the flame ionisation detector at 250°. Gas flow-rates employed were as follows: carrier 25 ml/min; auxillary 35 ml/min, hydrogen 50 ml/min and air 470 ml/min. Both helium and nitrogen could be used as carrier gas. Range resistance of the electrometer was set at $10^8\ \Omega$.

Peak areas were measured with a Hewlett-Packard 3370B electronic integrator operated at Up and Down slope sensitivities of 0.1 mV/min. Injection volumes were chosen to yield a minimum anaesthetic peak area of $5 \times 10^4\ \mu V$ sec.

Analytical methods

Response factors were determined by the gas chromatographic analysis of solutions of known composition containing the agent and the internal standard isobutanol with the equation:

$$\text{Response factor} = \frac{\text{peak area agent}}{\text{peak area isobutanol}} \times \frac{\text{wt. isobutanol}}{\text{wt. agent}}$$

Analyses of blood samples were performed on EDTA anti-coagulated blood using Reacti-vials each equipped with a magnetic stirring bar and a Mininert valve. Water was added to the vial such that the remaining volume, when the vial was filled to the rim, was 0.5 ml. A standard solution (50 μl) containing an accurately known quantity of isobutanol in water (approximately 260 mg per 100 g) was added and the vial was completely filled with the blood sample to be analysed (450 μl). The quantities of blood and internal standard employed were established by weighing the vial at the appropriate times. The samples were mixed and gas chromatography was performed on aliquots of 4—40 μl. The concentration of the anaesthetic was calculated from the formula:

$$\text{Anaesthetic concentration} = \frac{\text{peak area anaesthetic} \times \text{wt. I.S.} \times \text{concentration I.S.}}{\text{peak area I.S.} \times \text{wt. blood sample} \times \text{response factor}}$$

where I.S. = internal standard.

The effect of storage on the halothane concentrations of blood samples was tested as follows. Reacti-vials (1.0 ml), containing a glass bead, were filled with blood samples, containing an accurately known quantity of halothane, in such a manner that no air bubble was trapped in the vial. The samples were stored at 4° for 7 and 14 days, thoroughly mixed and analysed by gas chromatography.

RESULTS

An external injection port temperature of 180° was found to be necessary to maintain a gas tight seal between the Teflon gasket and the face plates of

the rotary valve and the heat reservoir. Under these conditions the glass wool plug served the dual function of providing a large, heated surface area for evaporation of the volatiles and of a trap for the non-volatile components. The rate of evaporation was presumably controlled by both the pre-heating of the carrier gas stream in the body of the heat reservoir and the dispersion of the injected blood sample. When liquid was deposited on a portion of the U-tube free of glass wool the fluid beaded and evaporated slowly, a phenomenon accompanied by peak broadening on gas chromatography. Successive injections of volumes of blood up to a total of 10 μl could be made before it was necessary to insert a fresh U-tube but use of samples of between 10 and 40 μl necessitated the insertion of a fresh U-tube after each injection. The O-rings used to seal the U-tube into the port gave some bleeding problems when new. These could be eliminated, however, by heating the rings for 16 h at 160° before insertion into the port. The effective life of the rings was improved by light lubrication with a high-temperature vacuum grease. Routinely the septum of the injection assembly, mounted to the front of the rotary valve, was changed once a week; it could, in any case, be used for at least 35—40 injections.

Preliminary experiments were conducted to evaluate the use of methanol, ethanol, *n*- and isopropanol, *n*-, *sec*-, *tert*- and isobutanol and isoamyl alcohol as internal standards for the estimation of halothane and methoxyflurane on one or more of the column packings Chromosorb 101, 103, 105, or 107 under either isothermal or temperature-programmed conditions. Isobutanol was found to be a cheap and convenient internal standard since it could be readily purified, was soluble in blood at the concentrations employed and had a retention time intermediate between that of halothane and methoxyflurane; it also proved to be a good internal standard for both ethanol and diethyl ether. The use of a temperature programme resulted in the splitting out of the water peak from the halothane peak permitting accurate measurements of peak area. Chromosorb 101 was the preferred packing material since it gave little or no bleed and was very stable; the column has been in routine use for more than 12 months. Furthermore Chromosorb 101 can be employed, under the same operating conditions, for the rapid analysis of halothane, methoxyflurane, ethanol and diethyl ether. Using the conditions specified above the system exhibited little or no baseline drift. Such drift when it occurred could be corrected by a single blank program run or by a short period of conditioning at 180°. Table I lists the sensitivity of the flame ionisation detector and the characteristics of the electrometer and integrator when halothane, methoxyflurane, ethanol, diethyl ether and isobutanol were analyzed as described above. Fig. 4 shows sample chromatograms obtained from these analyses.

Table II shows the results obtained by analysis of blood samples containing accurately known weights of halothane, methoxyflurane, diethyl ether and ethanol over the approximate concentration range 1—100 mg%. As can be seen excellent recoveries were obtained over the entire concentration range for all the compounds investigated. Reacti-vials proved to be efficient for storing blood samples containing halothane for periods as long as two weeks at 4°. Such storage permits efficient staging of analyses when the experimental design requires multiple samplings.

TABLE I

PERFORMANCE CHARACTERISTICS OF THE FLAME IONISATION DETECTOR, ELECTROMETER AND ELECTRONIC INTEGRATOR

These performance characteristic data correspond to peaks A—E of the sample chromatograms in Fig. 4.

Performance characteristic	Isobutanol	Halothane	Methoxy-flurane	Diethyl ether	Ethanol
Concentration (mg%)	11	98	52	60	56
Volume of injection (μl)	15	15	40	10	10
Detector output at maximum (A)	2.56×10^{-10}	3.44×10^{-10}	4.18×10^{-10}	6.5×10^{-10}	9.61×10^{-10}
Integrator input at maximum (mV) (=electrometer output)	2.56	3.44	4.18	6.50	9.61
Peak area (μV·sec)	7.422×10^{4}	1.363×10^{5}	9.543×10^{4}	1.222×10^{5}	2.354×10^{5}

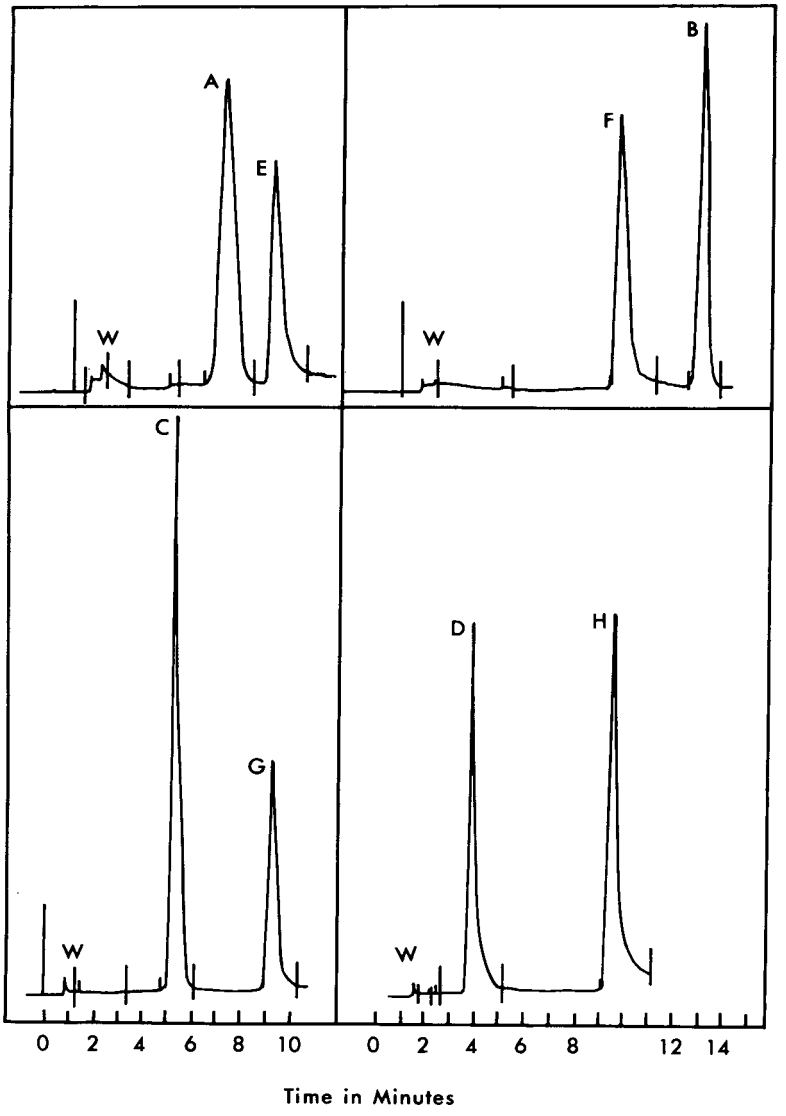

Fig. 4. Sample chromatograms from the analysis of halothane (A), methoxyflurane (B), diethyl ether (C) and ethanol (D) using, in each case, isobutanol (E, F, G, H) as internal standard. The water peak in each chromatogram is labelled W. The vertical lines crossing the baselines are event markers arising from the integrator.

As an illustrative example of the general usefulness of this gas—liquid chromatographic procedure, in Table III are included the results of a preliminary study of the equilibrium distribution of halothane between the cells and plasma of human blood at 4°. This experiment was performed as follows. To Reacti-vials completely filled with EDTA anti-coagulated human blood was added, with a microlitre pipette whose tip was located near the base of the vial, a known volume of a solution of halothane in 0.85% saline equal to

TABLE II

GAS–LIQUID CHROMATOGRAPHIC ANALYSIS OF WHOLE BLOOD SAMPLES CONTAINING KNOWN CONCENTRATIONS OF HALOTHANE, METHOXYFLURANE, DIETHYL ETHER AND ETHANOL

	Compound added							
	Halothane		Methoxy-flurane		Diethyl ether		Ethanol	
Response factor (isobutanol = 1)	0.207 ± 0.008		0.242 ± 0.009		0.924 ± 0.001		0.797 ± 0.024	
Retention time (min)*	6.21 ± 0.20		12.31 ± 0.13		5.36 ± 0.15		3.21 ± 0.08	
Relative retention time (Isobutanol = 1)	0.697		1.397		0.608		0.382	
	Added (mg%)	Found (mg%)	Added (mg%)	Found (mg%)	Added (mg%)	Found (mg%)	Added (mg%)	Found (mg%)
	102.7	105.2	110.4	111.3	111.6	115.3	119.7	121.7
	102.3	103.6						
	94.1	90.9						
	93.5	97.3						
	92.8	90.4						
	54.7	53.1	54.7	54.2	67.4	66.6	62.2	63.6
	15.3	15.0					24.4	23.5
	14.9	13.8						
	14.2	14.3						
	13.2	12.2						
	11.4	12.1	10.3	10.7	12.7	11.9	11.5	11.7
	4.7	4.3	5.0	4.9	5.2	5.5		
	1.2	1.0	0.7	0.8	1.0	0.7		
t**	0.26		0.69		0.5		0.9	
d.f.***	12		4		4		3	
	N.S.§		N.S.		N.S.		N.S.	
After 1 week storage	18.8	17.8						
After 2 weeks storage	18.4	18.6						

*In these studies the retention times for isobutanol were 8.91 ± 0.56, 8.81 ± 0.56, 8.81 ± 0.56 and 8.41 ± 0.48 for analysis of halothane, methoxyflurane, diethyl ether and ethanol respectively.
**Paired t test [39]. Differences between quantities added and found were not significantly different from zero.
***d.f. = degrees of freedom.
§N.S. = not significant.

exactly one tenth of the volume of the vial. The vial caps were immediately replaced thus displacing any excess blood and insuring a bubble free sample (i.e., no head space). The vials were rotated slowly at 4° for various time periods and were then centrifuged at 2800 g for 15 min. The plasma was analysed for halothane and the concentration of the anaesthetic in the cellular fraction calculated from the equation:

$$[\text{blood}_{\text{halo}}] = 0.9\ Hc\ [\text{cell}_{\text{halo}}] + (1—0.9\ Hc)\ [\text{plasma}_{\text{halo}}]$$

where

$[blood_{halo}]$ = added concentration of halothane in blood (29.7 mg%)

$[plasma_{halo}]$ = concentration of halothane in plasma as determined by gas chromatography

Hc = volume fraction of undiluted blood occupied by cells

0.9 = dilution factor due to addition of the saline solution of halothane to blood

$[cell_{halo}]$ = concentration of halothane in the cellular component of blood

From this equation the distribution of halothane between cells and plasma can be calculated.

The halothane solution was prepared by saturating 0.85% saline at 37° with the anaesthetic. Under these conditions the saturation concentration of halothane as determined by gas chromatography was 297±8 mg% (n=5); at 4° the value obtained was 481±12 mg% (n=4). Possible losses of halothane were estimated in separate control experiments by omitting the centrifugation step and determining the concentration of halothane in the whole blood after the period of equilibration. Statistical analysis of these data (n=9) indicated that there was no difference between the quantity of halothane added and that found by gas chromatographic analysis. Significant haemolysis did not occur during the period of time used in these equilibration experiments.

TABLE III

DISTRIBUTION OF HALOTHANE BETWEEN THE CELLS AND PLASMA OF EDTA ANTI-COAGULATED HUMAN BLOOD AT 4°

Samples A and B were obtained from the same individual on two separate occasions; they had haematocrits of 0.489 and 0.486 respectively. Sample C, obtained from a different individual, had a haematocrit of 0.507. Assuming that equilibrium is achieved after 16 h then the mean ratio:

$$\frac{[cell_{halo}]}{[plasma_{halo}]} = 2.0 \pm 0.2 \ (n = 11).$$

Sample A		Sample B		Sample C	
Incubation time (h)	$\frac{[cell_{halo}]}{[plasma_{halo}]}$	Incubation time (h)	$\frac{[cell_{halo}]}{[plasma_{halo}]}$	Incubation time (h)	$\frac{[cell_{halo}]}{[plasma_{halo}]}$
3.5	0.6	19.5	1.8	18.0	1.8
13.0	1.1	20.5	2.1	18.5	2.4
16.5	1.9	22.0	2.3	20.5	2.0
17.5	2.0	22.0	1.8	21.5	2.4
		23.0	1.9		

DISCUSSION

Direct injection methods for the gas chromatographic analysis of volatile anaesthetics in blood can be divided into three general classes: (a) those in which the sample is injected into the injection port of the chromatograph [25,26,29], (b) those in which injection is made into a removable glass liner inserted into the injection port [27,30], and (c) those in which injection is made into a heated pre-column device isolated from the columns and then, after a period of time, the volatiles are swept onto the column by a stream of carrier gas via a switching valve [28,31]. The analytical system described here appears to combine all the advantages of these systems without any of their disadvantages. Thus it permitted the rapid, direct, quantitative analysis of the blood concentrations of four volatile compounds on a stable, readily available column packing. The difficulties associated with baseline drift [2,30], ghost peaks [6,30], interference from water [6,30], poor reproducibility [1,2], contamination of the columns with non-volatile components [2,6,30], and non-uniform evaporation which necessitated a preheating period of the sample within the pre-column device [28,31] were not encountered. The use of an internal standard coupled with the measurement of peak areas, rather than peak heights, obviated the need for calibration curves and eliminated problems associated with measurements of broadened or distorted peaks. Reference to Table II demonstrates that, with our procedure, accurate analyses can be performed over a wide range of concentrations.

The external injection port can be easily constructed from inexpensive materials and has proved to be reliable and simple to operate for routine purposes. Installation requires a minimum of modification of existing chromatographs and the port can be readily adapted for use with other chromatographs. Furthermore the U-tube assembly appears to offer distinct advantages over previous systems [28,31] since it is easily accessible and can be rapidly changed without interrupting the carrier gas flow.

The equilibrium distribution of halothane between the cells and plasma of human blood at 4° determined in this study, 2.0±0.2 (see Table III), differs from that reported by Han and Helrich [33] who found that 70% of the halothane was in the plasma. The latter value was, however, estimated indirectly from calculations based upon the Ostwald solubility coefficients of halothane in plasma and cells at 38°. Moreover the Ostwald solubility coefficients were determined after shaking the samples with liquid halothane and mercury for 1 h.

Our data would indicate that the solubility of halothane in blood should increase with haematocrit. Experimental observations on this relation are, however, in conflict. Mapleson et al. [34] and Steward et al. [35] found that in rabbits and dogs, respectively, halothane solubility decreased as the haematocrit decreased. Furthermore, Steward et al. attribute solubility variations to the greater solubility in the cells. Cowles et al. [36], however, found that haematocrit did not affect the solubility of halothane in dog blood. Similar discrepancies have been noted with studies of human blood. Several investigators [29,33,36] have reported a decrease in solubility with an increase in haematocrit while other studies have shown that the solubility of halothane

is independent of the haematocrit [37,38], that halothane is less soluble in blood of low haematocrit [19,37] or more soluble in blood of high haematocrit [38]. On the other hand, other factors affect solubility, including lipid concentration [4,38] and the albumin-to-globulin ratio [37]. In view of the results discussed above it is impossible to assess fully the significance of published data in this area. Our preliminary experiments do however demonstrate that the gas chromatographic procedure described here provides a facile method of examining this interesting controversy. Experiments designed to study this problem in more detail are currently in progress.

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ANALYSIS OF LORAZEPAM AND ITS GLUCURONIDE METABOLITE BY ELECTRON-CAPTURE GAS—LIQUID CHROMATOGRAPHY

USE IN PHARMACOKINETIC STUDIES OF LORAZEPAM

DAVID J. GREENBLATT*, KATE FRANKE and RICHARD I. SHADER

Clinical Pharmacology Unit, Massachusetts General Hospital Boston, Mass. 02114, and the Psychopharmacology Research Laboratory, Massachusetts Mental Health Center, Boston, Mass. 02115 (U.S.A.)

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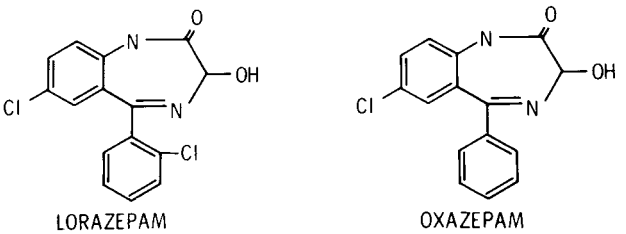
SUMMARY

This paper describes a rapid and sensitive method for analysis of lorazepam and its glucuronide metabolite in plasma and urine following therapeutic doses of lorazepam in humans. After addition of the structurally related benzodiazepine derivative, oxazepam, as the internal standard, 1-ml samples of plasma or urine are extracted twice at neutral pH with benzene (containing 1.5% isoamyl alcohol). The combined extracts are evaporated to dryness, reconstituted, and subjected to gas chromatographic analysis using a 3% OV-17 column and an electron-capture detector. Lorazepam glucuronide in urine is similarly analyzed following enzymatic cleavage with Glusulase. The sensitivity limits are 1—3 ng of lorazepam per ml of original sample, and the variability of identical samples is 5% or less. The applicability of the method to pharmacokinetic studies of lorazepam is demonstrated.

INTRODUCTION

Lorazepam (Fig. 1) is a 3-hydroxy-1,4-benzodiazepine derivative extensively used as a sedative and antianxiety agent in clinical practice [1, 2]. The major metabolic pathway of lorazepam in humans involves conjugation of the



LORAZEPAM OXAZEPAM

Fig. 1. Structural formulae of lorezepam and oxazepam.

* To whom correspondence should be addressed.

3-hydroxy substituent to glucuronic acid, yielding a water-soluble glucuronide metabolite that is excreted in urine [3—6]. Other minor metabolites have been identified, but none is of quantitative importance.

Several gas chromatographic methods are available for quantitation of lorazepam and lorazepam glucuronide in body fluids following therapeutic doses of lorazepam in humans. One approach involves acid hydrolysis of the benzodiazepine nucleus to the corresponding benzophenone derivative [4, 7]. This method is used successfully, but the extraction, derivatization, and clean-up steps are time-consuming and lead to considerable sample loss, due to aliquot taking. Quantitation of lorazepam is also possible using electron-capture gas—liquid chromatography without prior derivatization [8—10]. This report describes a rapid and sensitive method for assay of lorazepam and its glucuronide metabolite requiring minimal clean-up and no derivatization. The applicability of the method is illustrated in a study of the pharmacokinetics and bioavailability of lorazepam in a healthy human volunteer.

EXPERIMENTAL

Apparatus and chromatographic conditions

The analytic instrument is a Hewlett-Packard Model 5750 gas chromatograph equipped with a ^{63}Ni electron-capture detector (ECD). The detector is operated in the pulsed mode with a pulse interval of 150 μsec. The column is coiled glass, 6 ft. × 4 mm I.D. packed with 3% OV-17 on 80—100 mesh Chromosorb W HP (Supelco, Bellefonte, Pa., U.S.A.). The carrier gas is ultra-pure helium (Matheson Gas Products, Gloucester, Mass., U.S.A.) at a flow-rate of 50 ml/min. The purge gas is argon—methane (95:5), at a flow-rate of 80 ml/min. Operating temperatures are: injection port, 300°; column, 280°; detector, 320°. Before being connected to the detector, a new column is conditioned at 325° for 4 h with no carrier flow, followed by 48 h of conditioning with a carrier flow as described above.

At the beginning of each working day, the column is primed by injection of 2 to 3 drug-free "blank" plasma extracts.

Reagents

The following reagents are used: pesticide grade certified benzene (Fisher Scientific, Fair Lawn, N.J., U.S.A.), certified isoamyl alcohol (Fisher), analytical reagent grade toluene (Mallinkrodt, St. Louis, Mo., U.S.A.), absolute ethanol (IMC Chemical Group, Terre Haute, Ind., U.S.A.), and reagent grade KH_2PO_4 (Mallinkrodt). Solvents are used without further distillation.

Reference standards

Pure samples of lorazepam and the structurally similar benzodiazepine derivative, oxazepam (Fig. 1), were kindley supplied by Wyeth Labs. (Radnor, Pa., U.S.A.). A 10-mg amount of each compound is dissolved in 2—3 ml of absolute ethanol, then diluted to 100 ml with double-distilled water. The stock solutions are stored in amber bottles at 4°, and are stable under these conditions for up to 1 year. Working standards, containing 0.25—1.0 μg/ml, are prepared as needed by appropriate dilution with double-distilled water.

Preparation of samples: intact lorazepam in plasma

Oxazepam serves as the internal standard. A 50-μl volume of stock solution (1.0 μg/ml) containing 50 ng of oxazepam is added to a series of conical 40-ml centrifuge tubes equipped with PTFE-lined screw-top caps. A 1-ml sample of "unknown" plasma is added to each of the tubes. Calibration standards are prepared by adding 12.5, 25, 37.5, and 50 ng of lorazepam to consecutive tubes. Drug-free control plasma, preferably taken from the experimental subject prior to lorazepam administration, is added to each of the calibration tubes and to one additional drug-free blank. Calibration standards are analyzed together with each set of unknowns.

Extraction procedure

A 6-ml volume of benzene (containing 1.5% isoamyl alcohol) is added to all tubes. The tubes are agitated gently in the upright position on a vortex mixer for 30 sec, then centrifuged at room temperature for 10 min at 400 *g* (Portable Refrigerated Centrifuge Model PR-2; head No. 269; International Equipment, Boston, Mass., U.S.A.). The organic layer is transferred to a conical 13-ml centrifuge tube. The procedure is repeated, and the combined organic extracts are evaporated to dryness at 40° under conditions of mild vacuum. Care is taken to ensure that the residue is rinsed from the sides of the tubes. The final dry residue is redissolved in 50 μl of toluene (containing 15% isoamyl alcohol), of which 1—3 μl is injected into the chromatograph.

Lorazepam and lorazepam glucuronide in urine

Analysis of intact lorazepam in 1 ml of urine proceeds exactly as described above for plasma.

Lorazepam glucuronide in urine is quantitated using the aqueous remainder following extraction of intact lorazepam. The aqueous phase is washed with 10 ml of ether, centrifuged, and the organic phase aspirated and discarded. The sample is heated to 40° under conditions of mild vacuum for 15 min to ensure removal of any remaining organic solvent. A quantity of 0.1 ml of each urine sample is transferred to another 40-ml conical centrifuge tube; 1.0 ml of 1 *M* KH_2PO_4 buffer (pH approximately 4.6) is added. Enzymatic cleavage of lorazepam glucuronide is achieved by addition of 30 μl of Glusulase (Endo Labs., Garden City, N.Y., U.S.A.), a commercial preparation of snail intestinal juice containing approximately 175,000 units of β-glucuronidase and 35,000 units of sulfatase per ml. The resulting mixture is agitated gently, then incubated for 12—18 h at 37° in a temperature-controlled incubation room. Following incubation, the pH of each tube is adjusted back to 7.0 by addition of approximately 0.65 ml of 1 *N* NaOH. A quantity of 100 ng of oxazepam, the internal standard, is added to each tube, and lorazepam (25, 50, 75, 100, 150 and 200 ng) is added to the calibration tubes. (Larger amounts of standards are used for analysis of lorazepam glucuronide in urine since high concentrations of this metabolite are present in most samples.) Extraction then proceeds as described for intact lorazepam in plasma, except that 8—10 ml of solvent are used for each extraction to avoid emulsion formation.

Clinical pharmacokinetic study

A healthy 23-year-old female volunteer participated after giving written informed consent. Single 4-mg doses of lorazepam were administered on three occasions in a cross-over study, with at least one week elapsing between trials. Modes of administration were: 5-min intravenous infusion, deltoid intramuscular injection, and oral ingestion in the fasting state with 100 ml of water. Multiple venous blood samples were drawn during 48 h, and all urine collected in divided samples for 72 h, following each dose. Concentrations of intact lorazepam in plasma, and of lorazepam and lorazepam glucuronide in urine, were quantitated as described above.

Plasma lorazepam concentrations following each mode of administration were analyzed by iterative weighted non-linear least-squares regression analysis as described in detail elsewhere [3, 11, 12]. The following pharmacokinetic variables were determined following intravenous injection of lorazepam: distribution half-life, elimination half-life, total volume of distribution, and total clearance. Following oral and intramuscular dosage, the following variables were determined: lag time prior to the start of absorption, absorption half-life, and elimination half-life. The systemic availability (completeness of absorption) of oral and intramuscular lorazepam was determined from the

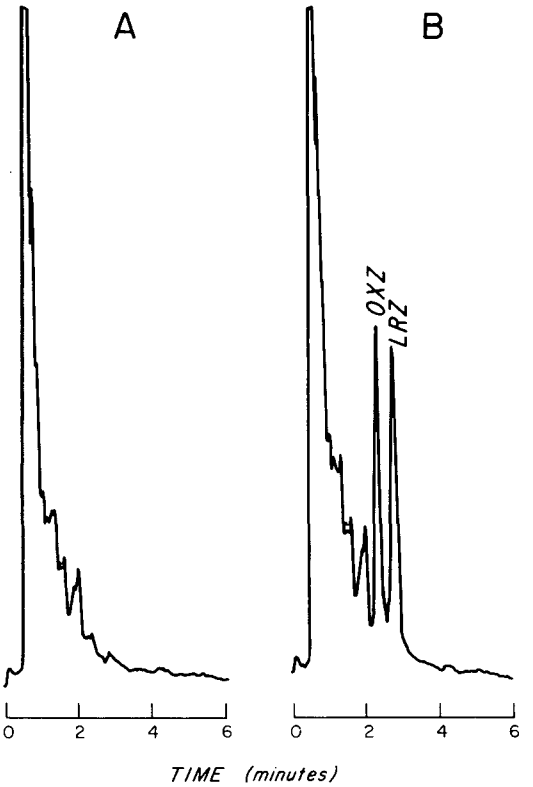


Fig. 2. A,Chromatogram of a drug-free control plasma extract. B, The same sample to which was added 50 ng/ml of oxazepam (OXZ) and 25 ng/ml of lorazepam (LRZ).

total area under the plasma concentration curve (AUC) calculated from the pharmacokinetic function, and upon 72-h urinary excretion of lorazepam glucuronide. AUC and 72-h excretion after the two extravascular modes of administration were compared with those observed following intravenous injection of the same dose [11, 13, 14].

RESULTS

Evaluation of the method

Under the described conditions, the retention times of oxazepam and lorazepam are 2.4 and 2.7 min, respectively (Fig. 2). The chromatographic peaks probably do not correspond to the intact compounds, but rather to quinazoline carboxaldehyde derivatives formed by on-column rearrangement [10, 15–18].

The relation between plasma lorazepam concentration and the lorazepam-to-oxazepam peak height ratio is linear up to at least 50 ng/ml. Analysis of 34 standard curves constructed on different days over a period of 6 months indicated that the correlation of peak height ratio and lorazepam concentration is always 0.99 or greater. The day-to-day coefficient of variation in the slope of the calibration curves was 8.6%.

The sensitivity limit of the method is 1—3 ng of lorazepam per ml of original sample. Coefficients of variation for identical samples were: at 25 ng/ml,

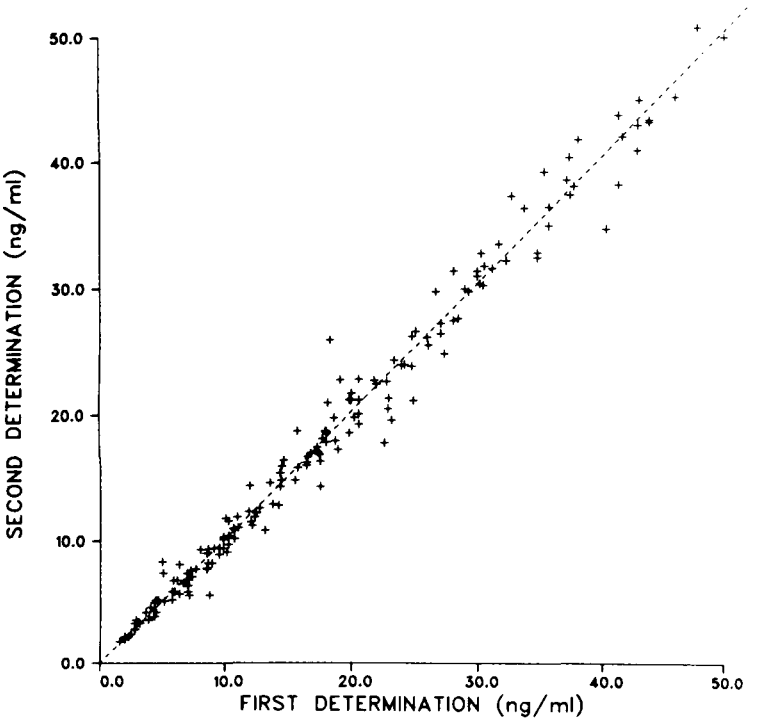


Fig. 3. Relation between lorazepam concentrations measured in a series of 171 duplicate plasma samples. The overall correlation coefficient is 0.99.

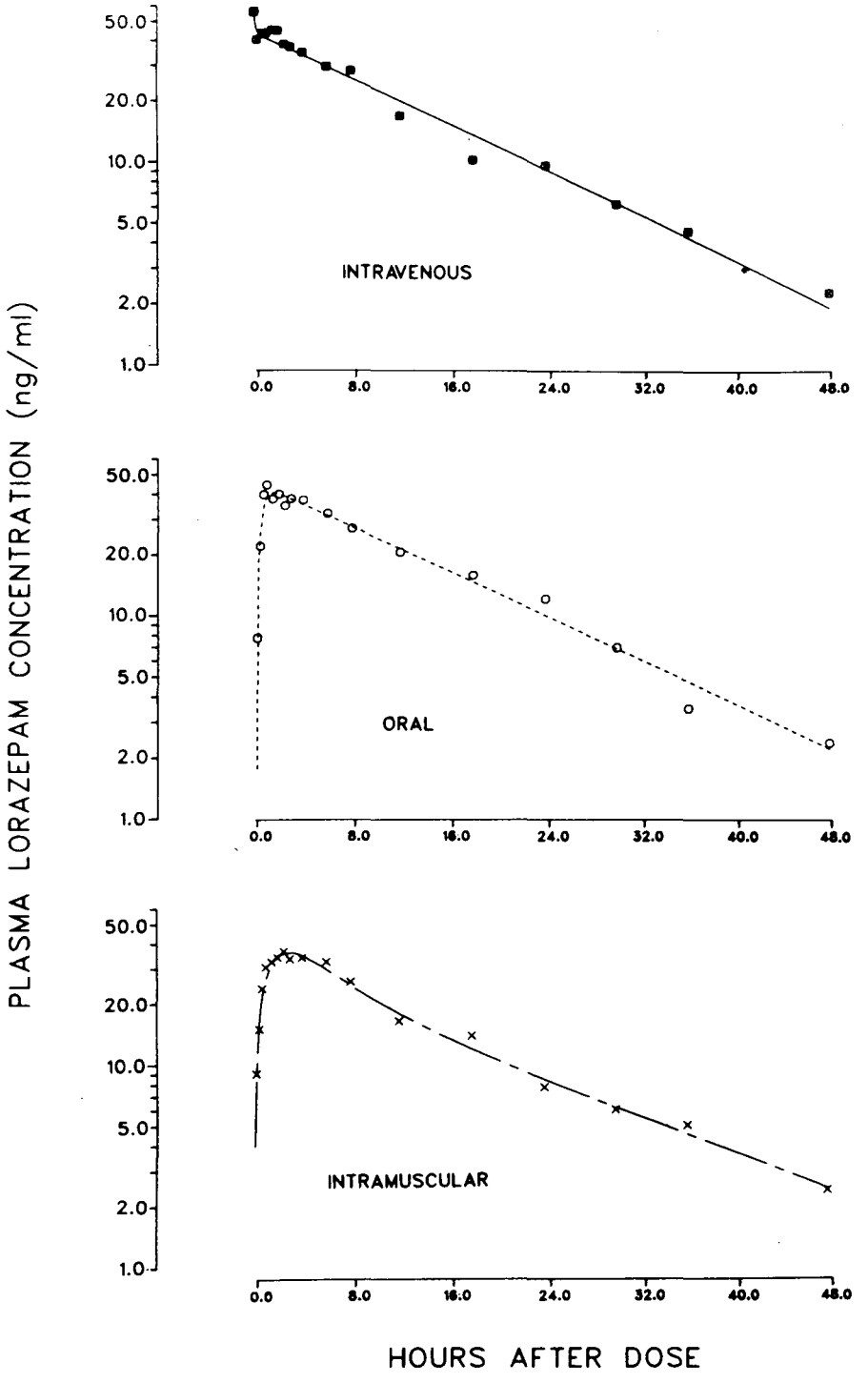


Fig. 4. Plasma lorazepam concentrations, and pharmacokinetic functions determined by least-squares regression analysis, following single 4-mg doses of lorazepam administered by three different routes to a healthy volunteer.

2.8% (n = 10); at 12.5 ng/ml, 4.5% (n = 8); at 6.25 ng/ml, 4.3% (n = 11); at 2.5 ng/ml, 5.1% (n = 10). Residue analysis indicated that extraction of both oxazepam and lorazepam is more than 95% complete.

A series of 171 plasma samples from pharmacokinetic studies, each analyzed in duplicate, was assessed to determine the replicability of identical samples (Fig. 3). The correlation coefficient between duplicate samples was 0.99, with an overall mean deviation of 5.2%.

Pharmacokinetic study

Fig. 4 shows plasma lorazepam concentrations, together with pharmacokinetic functions, following intravenous, oral, and intramuscular administration of lorazepam to the volunteer subject. Disappearance of lorazepam from plasma following intravenous infusion proceeded with two exponential phases, with an apparent elimination half-life during the terminal or "beta" phase of 10.6 h (Table I). The total volume of distribution was 1.62 l/kg, indicating reasonably extensive drug distribution. Following oral administration, a short lag time elapsed prior to the start of absorption, after which absorption proceeded as an apparent first-order process with a half-life of 14.3 min. A peak concentration of 44.4 ng/ml was measured in the sample drawn 1.0 h after the dose; elimination thereafter proceeded with a half-life of 10.8 h. After intramuscular injection, no lag time was observed, but the absorption phase had a somewhat longer half-life of 73.2 min. A peak level of 36.2 ng/ml was measured in the 2.5-h sample. The apparent elimination half-life was 13.9 h, slightly longer than following intravenous and oral administration. Based

TABLE I

PHARMACOKINETIC VARIABLES FOR LORAZEPAM

Variable	Route of administration		
	Intravenous	Oral	Intramuscular
Distribution half-life (min)	5.1	—	—
Elimination half-life (h)	10.6	10.8	13.9
Total volume of distribution (l/kg)	1.62	—	—
Total clearance (ml/min/kg)	1.77	—	—
Lag time prior to start of absorption (min)	—	11.3	0
Peak plasma concentration (ng/ml)	—	44.4	36.2
Time of peak concentration (h after dose)	—	1.0	2.5
Apparent absorption half-life (min)	—	14.3	73.2
72-h excretion of lorazepam glucuronide (% of dose)	94.6	82.4	72.2
Systemic availability (%)			
Based on AUC	—	100	93.4
Based on urinary excretion of lorazepam glucuronide	—	82.4	72.2

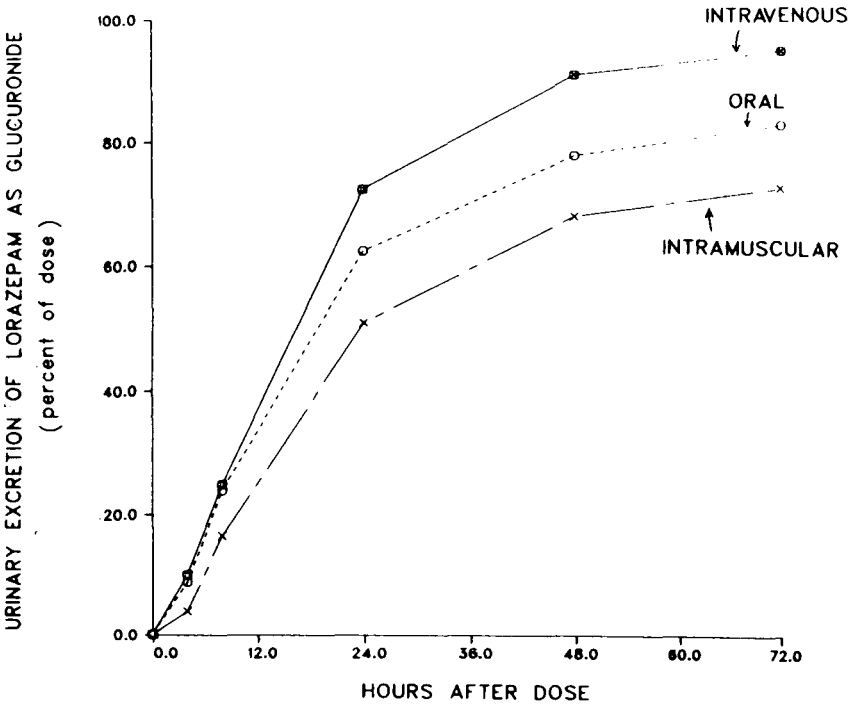


Fig. 5. Urinary excretion of lorazepam glucuronide during 72 h after single 4-mg doses administered by three different routes to the healthy volunteer subject.

on AUC, the systemic availability of oral lorazepam was 100%, and 93.4% following intramuscular injection (Table I). Based upon 72-h urinary excretion of lorazepam glucuronide following each mode of administration, the systemic availability of oral lorazepam was 82%, and that of intramuscular lorazepam 72% (Fig. 5). Urinary excretion of intact lorazepam accounted for less than 0.4% of the dose following all three routes of administration.

DISCUSSION

This report describes a rapid and sensitive method for quantitation of lorazepam in plasma, and of lorazepam and its glucuronide metabolite in urine. The method utilizes direct extraction of lorazepam together with the internal standard, evaporation of the organic solvent, and injection of the redissolved residue directly into the chromatograph. Blank samples of plasma and urine are consistently free of contaminants in the areas corresponding to retention times of oxazepam amd lorazepam. Therefore, extensive clean-up procedures are unnecessary. Since chromatographic peaks corresponding to both compounds are Gaussian, peak heights rather than peak areas were used to quantitate detector response [19], thereby making an electronic integrator unnecessary. Studies utilizing gas chromatography combined with mass spectroscopy indicate that the chromatographic peaks correspond to quinazoline carboxaldehyde derivatives of oxazepam and lorazepam, formed by on-column

rearrangement and loss of a molecule of water [10, 15—18]. This thermal rearrangement does not influence the reliability of the method. Standard curves are always linear and have similar slopes from day to day. Furthermore, peak height ratios following repeated injection of the same sample do not vary by more than 2%. It is evident that the same technique can be used for quantitation of oxazepam, simply by reversing the roles of oxazepam and lorazepam.

The use of this method obviates the need for acid hydrolysis of the benzodiazepine derivatives to corresponding benzophenones. Although acid hydrolysis allows good sensitivity and specificity, it involves multiple steps, is time-consuming, and results in sample losses due to aliquot taking. In our experience, the hydrolysis method also requires redistillation of solvents. A detailed comparison of intact-drug *versus* acid hydrolysis techniques for quantitation of nitrazepam, another benzodiazepine derivative, is reported by Kangas [20].

Several aspects of the procedure require further mention. An appropriate choice of internal standard is critical for reliable and reproducible quantitation of lorazepam. Howard et al. [8] for example, utilize flunitrazepam as an internal standard for analysis of lorazepam, but coefficients of variation for identical samples exceed 10%. Our use of desmethyldiazepam as an internal standard for lorazepam analysis also yielded unacceptably large variability. Only oxazepam has served as an acceptable internal standard in our experience, presumably because its characteristics of extraction and of on-column rearrangement are similar to those of lorazepam. Other 3-hydroxy benzodiazepines having N-1 alkyl substitutions (such as temazepam and 3-hydroxyprazepam) are less suitable than oxazepam since they do not rearrange on-column [16]. Addition of isoamyl alcohol to the extracting solvent also appears critical, since it greatly reduces problems of inconsistent and poor recovery, and/or adsorption of the compounds onto glassware. Finally, not all brands of disposable plastic syringes are suitable for pharmacokinetic studies of lorazepam. The rubber plungers contained in Monoject syringes (Sherwood Medical Ind., St. Louis, Mo., U.S.A.) are contaminated with a large and as yet uncharacterized electron-capturing substance having a retention time similar to that of oxazepam. The contaminant is transmitted to all biological fluids collected in these syringes, and complicates quantitation of detector response to oxazepam and lorazepam. Syringes produced by Becton Dickinson & Co. (Rutherford, N.J., U.S.A.), contain much smaller quantities of this contaminant and are suitable for use in pharmacokinetic studies.

Our method is readily applicable to studies of the pharmacokinetics and bioavailability of lorazepam in humans. Consistent with previous reports [3—7, 12, 21, 22] we observed an elimination half-life of lorazepam in the range of 10—15 h. Lorazepam clearance was accomplished mainly by conjugation to glucuronic acid, followed by urinary excretion of lorazepam glucuronide [3—7, 12 21, 22]. More than 90% of an intravenous dose of lorazepam was recovered in the urine as the glucuronide metabolite. Absorption of oral lorazepam was rapid and nearly complete; intramuscular lorazepam absorption was less rapid and slightly less complete. Further studies are needed to es-

tablish within- and between-subject variability in the pharmacokinetics and bioavailability of lorazepam.

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Note

Quantitative analysis of human and rabbit tear cholesterol by gas—liquid chromatography

RENNIE RUIZ and PHOEBE DEA*

Department of Chemistry, California State University at Los Angeles, Los Angeles, Calif. 90032 (U.S.A.)

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Recently, the importance of serum cholesterol levels has resulted in numerous investigations of clinical methodologies. Gas—liquid chromatography has proved very successful in analyzing sterols in serum and blood samples [1—6]. However, very little has been discussed in terms of cholesterol in tears. It was only recently that the presence of cholesterol could be demonstrated in tear samples [7—9]. In the work reported, standard enzymatic and/or colorimetric determinations were employed. However, with the well-documented success seen with the analysis of serum cholesterol, a gas—liquid chromatographic method with suitable sampling and processing techniques would be more specific and sensitive for the determination of total tear cholesterol levels. We report here the development of an analytical method which employs small tear volumes and allows for the detection of low cholesterol levels applicable to human as well as rabbit tear analyses.

EXPERIMENTAL

Chemicals

Carbon tetrachloride (Spectrograde) was fractionally distilled and dried over molecular sieves. The petroleum ether was the 30—60° boiling point fraction. Alcoholic potassium hydroxide was prepared by diluting to vol. (50 ml) a 3-ml aliquot of 33% aqueous potassium hydroxide with isopropyl alcohol. Cholesterol (Matheson, Coleman & Bell, East Rutherford, N.J., U.S.A.) and 5α-cholestane (Aldrich, Milwaukee, Wisc., U.S.A.) were used without further purification. N,O-Bis-(trimethylsilyl)-acetamide (BSA; Pierce, Rockford, Ill., U.S.A.) and hexamethyldisilazane (HMDS; Pierce) were used as silylating agents.

*To whom correspondence should be addressed.

Instrumentation

The chromatograms were obtained with a Perkin-Elmer 3920 temperature-programmable gas chromatograph with a hydrogen flame-ionization detector. A 6 ft. × 2 mm I.D. glass column was packed with 3% OV-17 (Pierce) on Chromosorb W HP (80—100 mesh). The column was initially conditioned at 325° overnight with the carrier gas on. The column temperature was programmed linearly with the initial temperature at 265° and a rate of 1°/min. The temperature of both the injector and detector ports was 300°. Helium was used as the carrier gas at a flow-rate of 42 ml/min. The column was silylated with 1 μl HMDS and baked from 265—300° at a rate of 32°/min between injections. When not in use, the column was maintained at 200°.

Tear sample preparation

With the use of micro-capillaries, tear samples were collected from the inferior lacrimal punctum and lower cul-de-sac of female rabbits (New Zealand White) as well as human subjects of either sex. A sample of 1 μl of human tear or 2 μl of rabbit tear is added to 1.0 ml of the alcoholic potassium hydroxide. After vortex mixing, the saponification is allowed to proceed for 1 h at 60°. The mixture is then allowed to cool and 1.0 ml of water is added. After mixing, 1.0 ml of petroleum ether is added, vortex mixed and centrifuged for 10 min. A 0.9-ml volume of the petroleum ether layer is withdrawn and a second 1.0-ml portion of petroleum ether is added, mixed and centrifuged for 10 min. A second 0.9-ml aliquot is withdrawn and the two petroleum aliquots combined. The petroleum ether is evaporated off and the silylated derivative of cholesterol is prepared by adding 25 μl of BSA, followed by mixing and incubating at 50° for 10 min. At the end of the incubation period, the excess silylating agent is evaporated off (hot water bath under a stream of nitrogen), the residue cooled and reconstituted with a 10-μl aliquot of a 28-ppm 5α-cholestane solution in carbon tetrachloride.

RESULTS AND DISCUSSION

Because of the low concentration of cholesterol in tear samples and the smaller tear volume used as compared to plasma samples, the method necessitates the use of a glass column as well as preparing a silylated derivative of cholesterol. A stainless-steel column resulted in excessive binding (approximately 50 ppm) as compared to the glass column where binding is observed experimentally at less than 2 ppm for the silylated derivative of cholesterol. Silylation resulted in easily-detectable, symmetrical peaks with no tailing. The retention time of the silylated derivative of cholesterol was observed to be 5.3 min. The silylated cholesterol peak in tears was confirmed by the retention time of silylated cholesterol, varying the column temperature and column length, as well as quantitating the cholesterol levels in plasma samples. The internal standard, 5α-cholestane, whose similar structure to cholesterol resulted in its use, gave a single peak with a retention time of 3.0 min. Typical chromatograms from 1-μl injections of prepared human and rabbit tear samples are shown in Figs. 1 and 2, respectively.

The cholesterol concentration of the prepared tear sample was determined

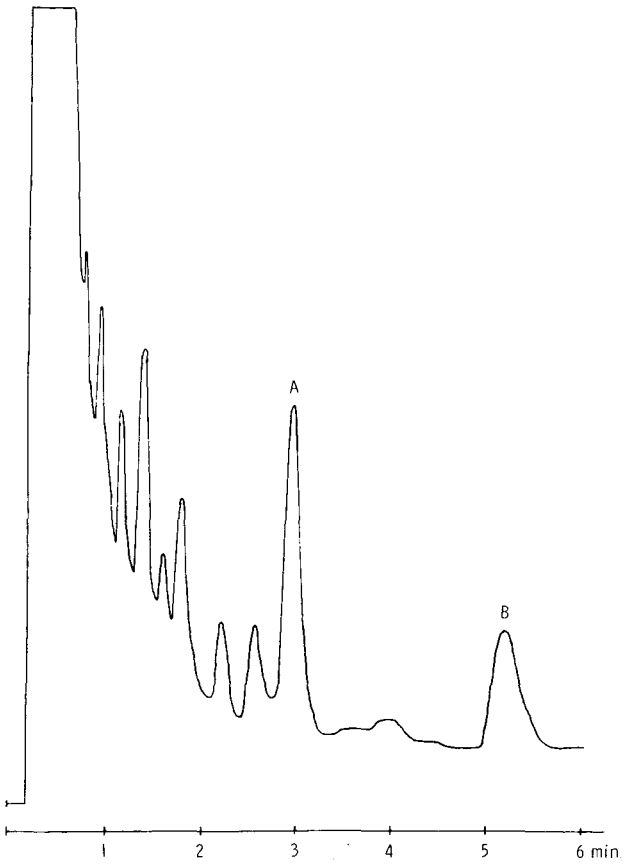

Fig. 1. Gas chromatogram of 1-μl injection of prepared human tear sample. A = 28 ppm of 5α-cholestane; B = silylated cholesterol, calculated to be 219 ppm.

by comparing the silylated cholesterol—5α-cholestane peak-area ratio with a prepared standard calibration curve. A linear relationship was observed when the ratio of the silylated cholesterol to the 5α-cholestane peak area was plotted against the cholesterol concentration for the standards prepared. Results of a linear-regression analysis yielded a slope of 0.0339 with a y intercept of 0.0479 and a coefficient of linearity (r) of 0.983. The tear cholesterol concentration was determined by applying the appropriate dilution factor as based on the extraction and dilution procedure. The results for cholesterol levels in human and rabbit tears are summarized in Table I.

The range of tear cholesterol level in eight human subjects was found to be 65—225 ppm. This is within the range of 80—370 ppm reported by Van Haeringen and Glasius [8] using a colorimetric determination of cholesterol. Our results also showed a wide range and variability from subject to subject as was evidenced by Van Haeringen. The range found for the rabbit population was lower, being 19.3—124 ppm, with a mean of 60.3 ppm. This is the first report on rabbit tear cholesterol levels presumably due to the lack of a sensitive method for cholesterol determination in microsamples. Within one standard deviation of the mean, all but two rabbit samples fell within a range of 35.6—76.7 ppm.

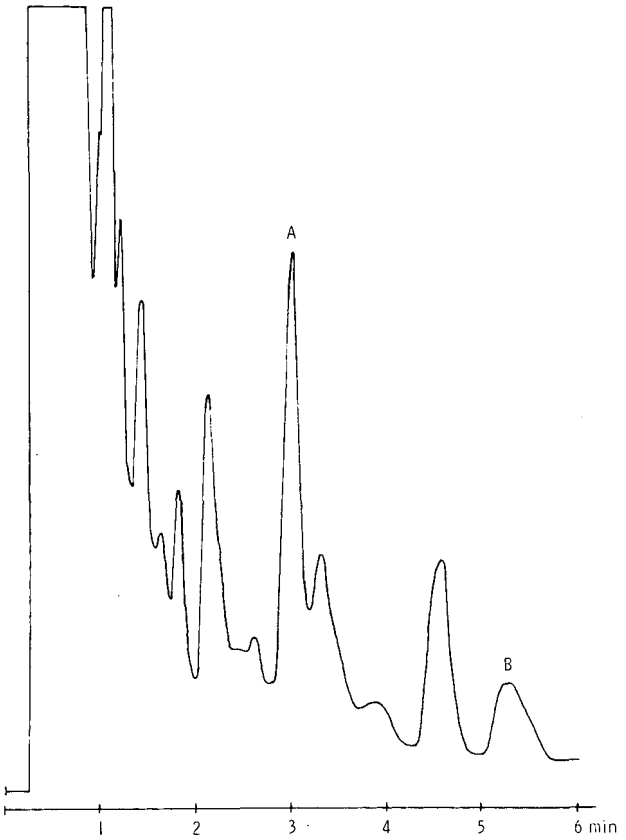

Fig. 2. Gas chromatogram of 1-μl injection of prepared rabbit tear sample. A = 28 ppm of 5α-cholestane; B = silylated cholesterol, calculated to be 60.3 ppm.

Tear cholesterol could be related to plasma levels, and thus this could be due to the more uniform cholesterol intake from the given batch of rabbit food, as compared with the expected wider range of human food consumption.

From the data in Table I, an estimate of the precision for the method can be determined by the daily variation observed. For the rabbit tear values, an average variation of ±7.0% was observed, while human tear samples gave an average variation of ±1.2%. The smaller variation observed for human samples is due to the much higher cholesterol levels for human samples compared with rabbit samples. Minor peaks in some rabbit samples also impaired complete baseline resolution of the internal standard resulting in the larger variation observed.

Cholesterol recovery was performed by adding a known amount of cholesterol to assayed tear samples. The total cholesterol was then extracted and assayed. The multiple extractions and the long incubation period promised better efficiency for the handling of microsamples as compared with the gas chromatographic determination of serum cholesterol studies by Blomhoff [2] where the incubation period was 15 min at 55°. The longer incubation period employed in this study allows for a complete hydrolysis of the tear lipids into

TABLE I

EXPERIMENTAL LEVELS OF TEAR CHOLESTEROL.

Unless specifically indicated, values are the mean of 2—6 determinations ± standard deviation.

Tear samples	Subject	Cholesterol (ppm)
Human	1	194 ± 2
	2	222 ± 4
	3	137*
	4	103 ± 12
	5	91*
	6	147*
	7	207 ± 7
	8	65*
Rabbit	1	58.6 ± 2.3
	2	65.2 ± 4.7
	3	19.3 ± 2.3
	4	48.3*
	5	59.9 ± 2.3
	6	46.4 ± 5.8
	7	124.0 ± 7.0
	8	35.6 ± 4.6
	9	76.7 ± 6.9

*Only one sample available.

water-soluble fatty acids thereby increasing the efficiency of the petroleum ether extraction. The total recovery of cholesterol in this study was found to be 99.5%.

A measure of the sensitivity was determined by a serial dilution of a silylated cholesterol standard. Setting the chromatograph at the most sensitive detection level, a 1.0-ppm silylated cholesterol standard, diluted 10,000 times, was prepared and injected into the column. The results observed were an easily-detectable cholesterol peak with the smallest solvent response achievable experimentally. The sensitivity is calculated to be 10^{-4} ppm or 0.1 ng/ml cholesterol.

CONCLUSION

Thus presented is a technique for total-tear cholesterol determination whose order of specificity far outweighs that achievable for a colorimetric determination. More importantly, the gas—liquid chromatographic technique proved to be fast as well as sensitive and applicable to human and rabbit tear sample sizes as small as 1 μl. The use of minimum tear volumes decreases the time and physical stimulation of the eye during tear collection.

In addition to being a more specific method for clinical studies, this method makes possible the study of tear cholesterol in popular laboratory models such as the rabbit.

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Note

Gas chromatographic head-space analysis of clinically interesting ketone bodies

PETR HRADECKÝ and PŘEMYSL JAGOŠ

Department of Diagnostics, Therapy and Prophylaxis, University of Veterinary Medicine, Brno 61242 (Czechoslovakia)

JAROSLAV JANÁK

Institute of Analytical Chemistry, Czechoslovak Academy of Sciences, Brno 66228 (Czechoslovakia)

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The estimation of ketone bodies in biological fluids is an important part of the clinical and preclinical diagnosis of energy-metabolism disturbances in animals and man. Clinically, the term ketone bodies encompasses acetone, acetoacetic acid, isopropanol and 3-hydroxybutyric acid. Acetone and acetoacetic acid may be clinically termed oxidized ketone bodies and their reduced forms isopropanol and 3-hydroxybutyric acid (hydroxy-compounds) similarly termed reduced ketone bodies. The sum of oxidized and reduced ketone bodies is then termed total ketone bodies.

To prove levels of oxidized ketone bodies the classical colour reaction with nitroprusside [1] known as Legal-probe is still widely used. More recently, acetoacetic and 3-hydroxybutyric acids have been determined photometrically [2, 3] or fluorimetrically [4] after their mutual enzymic conversion. The most frequently used determination of free acetone is the photometric one [5—8]. The volatility of isopropanol and acetone, to which all other ketone bodies mentioned may be converted in a relatively simple manner, has facilitated their succesful determination by several workers [9—14] using gas chromatography. The head-space technique of analysis [15—17] has provided a further improvement on the methods available for the solution of this problem [10, 12, 18].

In this research note we have given the conditions under which we were successful in the application of gas chromatographic head-space analysis to the expeditious control of ketone-body levels in a broad range of cattle body fluids.

MATERIALS AND METHODS

As acid standards, we used a 1 *M* solution of ethylacetoacetate in 5 *M* KOH, stored at 4°, and the sodium salt of DL-3-hydroxybutyric acid (BDH, Poole, Great Britain). For analysis, there were three proven reagents: (i) alkaline reagent, 5 *M* KOH with 2 m*M* butanone as internal standard; (ii) acidic reagent, 5 *M* H_3PO_4 with 2 m*M* butanone; (iii) oxidative reagent, 0.1 *M* $K_2Cr_2O_7$ in 5 *M* H_3PO_4. We recommend storage of the reagents with butanone at room temperature as reproducibility of the results deteriorates with the use of cooled reagents. The acetone standard solutions stored at 4° are not stable after one week.

In separate stages of the analysis the following were pipetted:

Free acetone: 0.1 ml of sample without deproteination, plus 0.1 ml of alkaline reagent, equilibration for 15 min before GC analysis.

Oxidized ketone bodies: the same procedure, with acidic reagent replacing the alkaline one.

Total ketone bodies: 0.1 ml of sample without deproteination plus 0.1 ml of acidic reagent, heated in a boiling water bath for 3—5 min; after cooling to room temperature 0.5 ml of oxidative reagent is added as rapidly as possible through the septum, using a polypropylene syringe; the bottle set is immediately returned to the boiling water bath for 40 min and equilibrated for 15 min before GC analysis.

Samples and reagents were dosed with a micropipette into 15-ml dry test-bottles (biochemical test-bottles e.g. by Boehringer, Mannheim, G.F.R.) to which approximately 0.5 g of crystalline potassium sulfate had previously been added. The sets of bottles capped with rubber septa and perforated covering-stoppers were equilibrated in a thermostated water bath (70°). Samples of 1.5 ml of vapour were taken for analysis using a water-jacketed and temperature-controlled (70°) 2-ml injection syringe (Fig. 1) and injected into

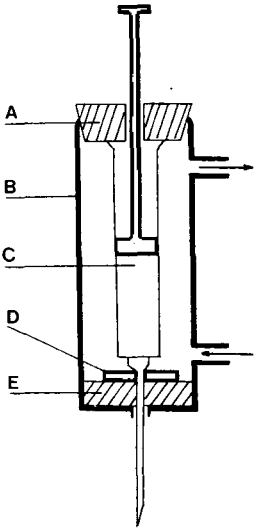

Fig. 1. Sampling device: A = rubber stopper, B = glass thermostating jacket, C = injection syringe, D = metal ring, E = rubber septum.

the gas chromatograph (CHROM 4, Laboratory Equipment, Prague, Czechoslovakia). Flame-ionization detection was used and experimental conditions were as follows: glass column 120 × 0.3 I.D.; Porapak R resin (100—120 mesh); column temperature, 180°; injector temperature 185°; carrier gas, nitrogen; flow-rate, 57 ml/min. The retention time of butanone was 3.2 min and the retention ratio of acetone 0.47. For quantitation, the peak-height ratios of acetone—butanone were used following prior confirmation of the full acetone recovery by the standard-additions method [19]. The calibration curve was without a blank value (passed the zero) and was linear at least to a 100 m*M* concentration of acetone. This limit includes all practical clinical cases.

RESULTS AND DISCUSSION

The method for the determination of physiological ketone-body levels has to be extremely sensitive. Higher sensitivity of the gas chromatographic headspace analysis may be achieved through increasing the acetone vapour tension by using a higher temperature (70°) and by the addition of an inorganic salt (K_2SO_4) to the reaction mixture. The salt simultaneously suppresses the influence of different matrices (sample composition). During the determination of total ketone bodies the sulfate must not be completely dissolved at 100° as the conversion of 3-hydroxybutyric acid to acetone would be drastically reduced. The sulfate addition is advisable particularly for high-protein samples which may be reproducibly, analyzed without deproteination. Variations in the amount of sulfate from 0.5 to 1.6 g do not influence the results, therefore weighing is unnecessary. Addition of K_2SO_4 is also desirable if the standard addition method [17, 19] is used, the results of which are, by nature, independent of matrix differences.

The optimal reagent compositions were found, by trial and error, from wide ranges of component concentrations. The alkaline reagent ensures sufficient stability of acetoacetic acid for at least an hour during which time series of 12—14 samples may be analyzed. The acidic reagent with phosphoric acid breaks the sample buffer system and decreases the pH to a point where acetoacetic acid decarboxylation is rapid and quantitative at equilibration temperature and there is no risk of conversion of the 3-hydroxybutyric acid to crotonic acid [5]. Sulfuric acid works more slowly and the samples must be heated in a boiling water bath. Consequently, for the determination of total ketone bodies, the decarboxylation step must be performed before the addition of oxidative reagent in order to prevent decomposition mechanisms other than that to acetone (loss of about 90% of acetoacetic acid) [20].

The composition of the acidic and oxidative reagents ensures quantitative conversion of reduced ketone bodies to acetone. Older methods [22] have used exclusively the dichromate—sulfuric acid mixtures, compositions of which were determined using the 3-hydroxybutyric acid standard solutions. But the degree of conversion was only acceptable for a relatively low and narrow concentration range of sulfuric acid and dichromate, and could be incorrectly, applied to the biological samples. A higher amount of eaily-oxidizable compounds decreases the oxidative capacity of the reagent substantially and the

composition of the reaction mixture leaves the optimal range. As a consequence, the actual conversion of the ketone bodies to be estimated decrease and the use of previously estimated standard factors leads to lower results. The most expressive decrease was found with milk samples. The oxidative reagent with phosphoric acid fully converts the ketone bodies present both in the standard and the milk sample. The conversion using an analogous reagent with sulfuric acid produces the value of 75% for the standard and about 50% for the milk sample.

Further, the parameter of time must be taken into consideration. After addition of oxidative reagent to the sample it is necessary to heat the reaction mixture as quickly as possible in a boiling water bath. At room temperature, 3-hydroxybutyric acid decomposes to products other than acetone [20, 21]. A long contact (days) with acidic oxidant causes loss of ketones probably due to complex-formation [20]. Both effects are much more evident in the case of oxidative reagent with sulfuric rather than phosphoric acid.

Vapour sampling and injection with the described device is simple and with the use of internal standard also surprisingly precise. Duplicate vapour sampling from the same bottle gives only negligible differences between the results of the first and second analyses. The syringe may be inserted into the bottle empty or a volume of air, equal to the volume of vapour withdrawn [10, 17] may be added, but the chosen procedure must be consistent.

The use of butanone as internal standard is fully justified in the case of human samples [14], but its presence in the body fluids of other species always has to be verified. Table I contains the natural butanone concentrations found in some stages of the body-fluid analyses of healthy and ketotic late-pregnancy cows together with the values for the relative increase of the total butanone concentration over the 2 m*M* butanone used as internal standard. At coincident stages of analysis, the calibration factors obtained with standard solutions have to be divided by the appropriate value for the relative increase in order to correct for the natural butanone background. It is true that this background changes slightly in cases of ketosis but the changes are negligible for our purpose [20].

TABLE I

NATURAL BUTANONE CONCENTRATIONS AND THE VALUES OF RELATIVE INCREASE OF THE TOTAL BUTANONE CONCENTRATIONS OVER THE 2 m*M* OF INTERNAL STANDARD

Sample	Number of samples	Determined ketone bodies	Natural butanone m*M* ($\bar{x}$ ± S.D.)	Relative increase
Urine	24	Oxidized	0.078±0.017	1.04
Urine	24	Total	0.38 ±0.05	1.19
Blood plasma	36	Total	0.133±0.008	1.065
Amniotic fluid	18	Total	0.053±0.007	1.025
Allantoic fluid	20	Total	0.087±0.007	1.04
Foetal serum	18	Total	0.117±0.007	1.06
Foetal urine	12	Total	0.052±0.008	1.025

A serious problem is caused by the presence of additional compounds which can decompose to acetone or other interfering substances. Among these belong especially glycides (glucose, fructose, lactose), lactate and disinfectants. The strongest interference occurs with alkaline reagent, as these compounds then give acetone values almost comparable to physiological levels [20]. This problem cannot be solved by subtraction of the correction factor as samples anticipated to contain high amounts of glycide (e.g. foetal fluids) often give acetone values much lower than the corresponding glycide standard and vice versa. Samples without provable amounts of glycide (urine) sometimes give higher values for free acetone than for oxidized ketone bodies. We found that the use of acidic and oxidative reagents is almost free from this interference. As the determination of free acetone is of questionable diagnostic value [23] we recommend, for current diagnostic analyses, the determination of oxidized and total ketone bodies only. The interference of disinfectants is rare and often may be overcome by changing the analytical conditions [18].

The presented method permits precise determination of total and/or reduced ketone-body amounts. Older methods converted isopropanol and 3-hydroxybutyric acid to the acetone in different degrees and the precise calculation was not possible unless the separate determination of isopropanol was made [24]. With our procedure, the reproducibility expressed as coefficient of variation of a six-times-repeated analysis of the same sample is better than 2.5% for both standard and biological samples. This precision, together with the total conversion of the reduced ketone bodies to acetone, is especially valuable in the case of analyses of non-traditional samples (milk, foetal fluids) which were poorly reproducible using older methods.

In Table II are given some physiological levels of oxidized and total ketone bodies in body fluids of late-pregnancy cows.

Besides direct measurements of the concentrations of ketone bodies, the ratio of oxidized to total ketone bodies concentrations (O:T) expressed as a percentage may be of greater diagnostic value. This ratio is more sensitive and rapidly exceeds the physiological range during bovine ketosis [20, 23].

TABLE II

PHYSIOLOGICAL LEVELS OF OXIDIZED AND TOTAL KETONE BODIES IN BODY FLUIDS OF LATE-PREGNANCY COWS

Sample	Number of samples	Ketone bodies mM ($\bar{x}$ ± S.D.)	
		Oxidized	Total
Urine	7	0.039±0.007	1.01±0.16
Blood plasma	9	0.063±0.016	1.23±0.16
Amniotic fluid	8	0.034±0.006	0.33±0.03
Allantoic fluid	9	0.030±0.005	1.06±0.10
Foetal serum	8	0.023±0.002	0.64±0.06
Foetal urine	4	0.028±0.009	0.55±0.17

CONCLUSIONS

For ketone-body determination, gas chromatographic head-space analysis was used successfully. The matrix effect of different biological samples was suppressed by addition of crystalline potassium sulfate together with the use of concentrated reagents and internal standard. The optimized reagents comprised 5 *M* KOH, 5 *M* H_3PO_4, and 0.1 *M* $K_2Cr_2O_7$ in 5 *M* H_3PO_4. As the internal standard 2 m*M* butanone was used.

For determination of oxidized and total ketone bodies 0.2 ml of the biological sample without deproteination is sufficient. Reproducibility of the method is better than 2.5% and stoichiometric conversion of isopropanol, 3-hydroxybutyric and acetoacetic acids to acetone and total recovery of acetone is reached with all samples mentioned. The method was succesfully verified in serial analyses and is suitable for diagnosis of ketotic states in animals and man. The mean physiological values for different body fluids of late pregnancy cows are given.

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Note

Convenient system for the simultaneous separation of 11-deoxycortisol and aldosterone by Sephadex LH-20 multiple column chromatography

W.G. SIPPELL*, GISELA PUTZ and MARIANNE SCHEUERECKER

University of Munich Medical School, Children's Hospital (Dr. von Haunersches Kinderspital), Department of Paediatrics, Division of Paediatric Endocrinology, Lindwurmstrasse 4, D-8000 München 2 (G.F.R.)

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In routine separations of multiple adrenal steroids on Sephadex LH-20 columns [1], an overlapping zone of 10 to 30% was constantly found between the elution peaks of 11-deoxycortisol (S) and aldosterone (A) when using solvent systems and column dimensions which allow the simultaneous and automated isolation of a variety of both very unpolar (e.g., progesterone) and very polar (e.g., cortisol) steroids from the same plasma extract [2] prior to radioimmunoassay. Such an overlapping zone between the peaks of two tritiated steroids leads either to very low recoveries of both steroids if the overlap is eliminated or to erroneously high recoveries in one and erroneously low recoveries in the other steroid when no fraction between the two peaks is omitted. The use of differently labelled S and A was disadvantageous, too, because of the low specific activity of the ^{14}C-labelled compound whereby large amounts of steroid would have been introduced into the sensitive radioimmunoassay system.

A variety of organic solvent systems was therefore tested in combination with different LH-20 column dimensions in order to obtain a complete and convenient separation of S and A. The present communication reports a simple, rapid and highly practicable chromatographic system for the separation of these adrenal steroids on Sephadex LH-20 which to our knowledge has not yet been described to date.

*To whom correspondence should be addressed.

EXPERIMENTAL

Tritiated steroids, all with a specific radioactivity of 40—60 Ci/mmole, were purchased from New England Nuclear (Dreieich, G.F.R.) and were repurified every three months on Sephadex LH-20 using methylene chloride—methanol (98:2) as solvent. Sephadex LH-20 was obtained from Deutsche Pharmacia (Freiburg, G.F.R.). Analytical-reagent grade solvents (E. Merck, Darmstadt, G.F.R.) were used without further purification.

Sephadex LH-20 was allowed to swell overnight in the solvent system methylene chloride—acetone (80:20) and then poured into borosilicate-glass columns (44 × 1.1 cm I.D.) with a ground-in connector at the top and a frit (Schott, Mainz, G.F.R.) having 40—90-μm wide pores as gel support at the bottom. Below the frit, a PTFE stopcock was attached. With the stopcock permanently open, the gel was allowed to settle by gravity. A total of ten columns was packed at the same time; a gel height of 40 cm was reached in all columns after ca. 40 min. After packing, all columns were rinsed with one gel volume (38 ml) of the solvent and were left stoppered until use.

Radioactive steroids or the unseparated, dried S and A fractions from the initial chromatographic step [2] were applied to the almost dry top of the gel in two 250-μl portions of the solvent delivered by means of 1-ml tuberculin glass syringes. The columns were eluted by gravity flow with the solvent from a graduated, cylindrical (100 ml) reservoir which had been attached on top of the columns immediately after sample application [3]. Starting from the 60-ml mark, the eluate could be conveniently fractionated by volume as indicated by the actual solvent level in the reservoir. For routine separations, when ten columns were eluted together, the collection limits for S and A were appropriately marked on the calibration scale of each reservoir.

For localization studies, 1-ml fractions of the eluate were collected into miniature scintillation vials (16 × 54 mm; Zinsser, Frankfurt, G.F.R.) and were then counted with 5 ml of a toluene-based scintillation fluid in a Nuclear Chicago Isocap 300 scintillation counter (Searle, Heusenstamm, G.F.R.).

RESULTS AND DISCUSSION

With the solvent system methylene chloride—acetone (80:20), a complete separation of S and A from each other was obtained on 40-cm Sephadex LH-20 columns (Table I).

Resolution of the less-polar steroids tested was incomplete, whereas more-polar steroids like cortisone could not be eluted in a distinct peak. The 3-ml overlap zone between corticosterone and S did not create any problems since these two steroids were already completely separated from each other by a prior chromatographic step [2]. As the elution flow-rate averaged 40 ml/h, the separation of S and A could be completed within ca. 75 min.

In Fig. 1, the elution profiles of tritiated S and A from the ten parallel columns are shown. Both location and width of the steroid peaks show only limited variation. Nevertheless, the collection limits of each column were individually established by fraction-to-fraction analysis during the first elution. Due to a slight shrinkage of the gel column of ca. 2.5 cm after 14 elu-

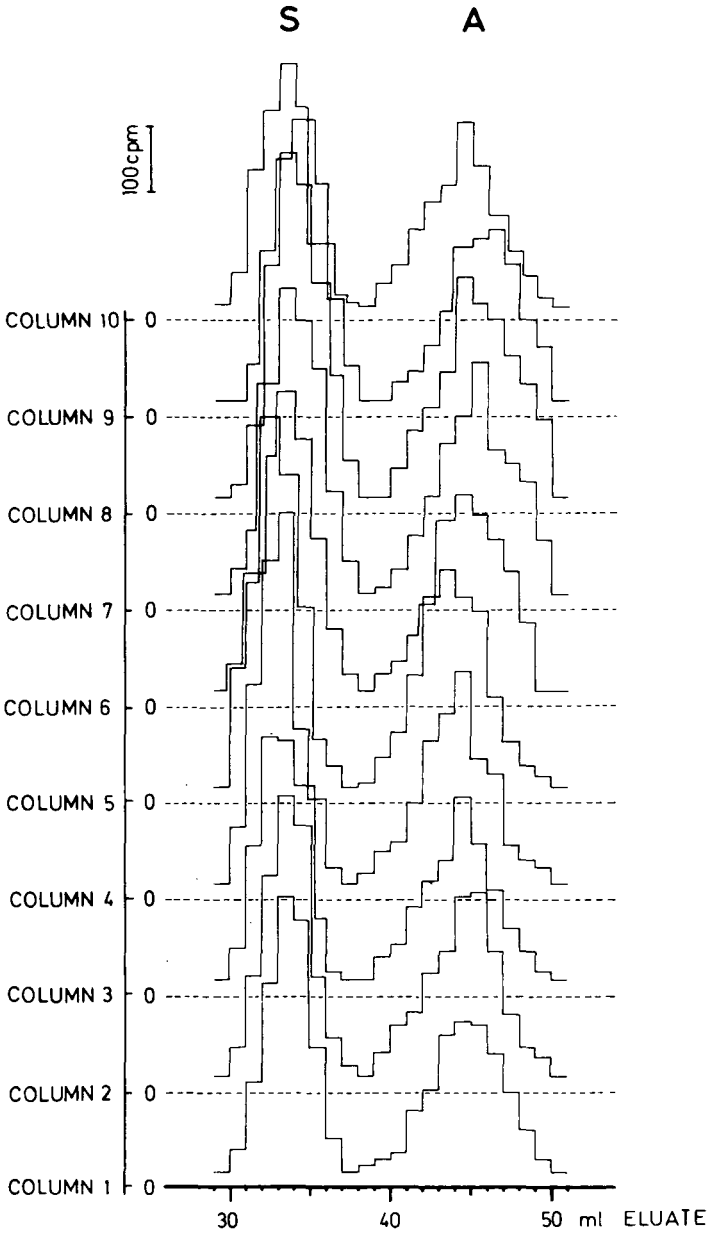

Fig. 1. Inter-column variation of the chromatograms of tritiated 11-deoxycortisol (S) and aldosterone (A) obtained at the 15th elution of ten LH-20 columns which had been packed at the same time.

tions, the elution volumes of S and A decreased by 2—4 ml, as can be seen by comparing the data in Table I with those in Fig. 1 which were obtained at the 15th elution of the columns. Because of this shifting, S and A were collected 2—4 ml earlier, as soon as the recoveries of tritiated S and A were

TABLE I

ELUTION VOLUMES AND COLLECTION LIMITS OF PLASMA STEROIDS ISOLATED ON FRESHLY PACKED 40-cm SEPHADEX LH-20 COLUMNS

Steroid	Elution volume (ml)	Collection limits* (ml)	Limits marked on reservoir*,** (ml)	Fraction pool volume collected* (ml)
Progesterone	19	(17—21)	(43—39)	(4)
11-Deoxycorticosterone	22	(19—24)	(41—36)	(5)
17-Hydroxyprogesterone	26	(23—28)	(37—32)	(5)
Testosterone	29	(27—31)	(33—29)	(4)
Corticosterone	32	(29—35)	(31—25)	(6)
11-Deoxycortisol	36	32—39	28—21	7
Aldosterone	48	44—52	16—8	8
Cortisone	>60	—	—	—

*Data for incompletely separated steroids are shown in parentheses
**Originating from a starting volume of 60 ml.

found to be slightly decreasing which usually occurred after 10—15 elutions. All columns were packed afresh after about 30 elutions using either fresh or re-purified [4] Sephadex LH-20.

Recoveries of tritiated S and A after extraction and two subsequent chromatographies were 61.4 ± 7.8% and 55.7 ± 7.7% (mean of 120 two-fold elutions ± S.D.), respectively, with mean coefficients of variation of 11.2 and 10.5% between ten parallel pairs of columns.

Among the ten different solvent systems tested, methylene chloride—acetone (80:20) provided by far the fastest, most reliable and economical separation of S and A. The consistently high steroid amounts recovered allowed sensitive radioimmunological quantification of these steroids in the same small plasma sample [5].

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Note

Two-dimensional separation of glycopeptides and charged oligosaccharides on silica thin layers

E. MOCZAR

Laboratoire de Biochimie du Tissu Conjonctif, Faculté de Médicine, Université Paris XII, 6, rue du Général Sarrail, 94000 Créteil (France)

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The composition of the carbohydrate moieties of the glycoproteins is subject to variation under environmental influences, while the amino acid sequence of their polypeptide chain is genetically determined. Variations of the carbohydrate chains are characteristic for some pathological alterations of serum glycoproteins [1]. The glycans of cell-surface glycoproteins are modified during malignant transformation [2]. These glycans seem to be involved in cell association phenomena and in tissue differentiation [3, 4]. Thus, the study of the carbohydrate chains of glycoproteins has gained increasing importance in biomedical research.

With few exceptions the carbohydrate portion of each pure glycoprotein is a mixture of heteroglycans. The proteolytic hydrolysis of a glycoprotein produces a mixture of glycopeptides, from which fractions can usually be isolated differing in their carbohydrate composition. Several methods have been described for the separation of glycopeptides, involving ion-exchange [5] or exclusion chromatography [6, 7], paper [7] or thin-layer electrophoresis [8]. The latter excepted, these methods are insufficiently sensitive for the detection of glycopeptides with less than 1 μg of sugar constituents, and their resolving power is often insufficient. No thin-layer chromatographic (TLC) methods were described for the separation of glycopeptides containing more than 3—4 sugar residues. Such glycopeptides and oligosaccharides could be separated by paper chromatography [9], but the method is time-consuming (migration times of several weeks or months).

In the present study, a rapid micro-method is described for the separation of glycopeptides obtained by the pronase digestion of glycoproteins, containing ten or more sugar units. The same method can also be used for the two-dimensional separation of charged oligosaccharides.

MATERIAL AND METHODS

TLC sheets of "Selecta 1500" (Schleicher and Schüll, Dassell, G.F.R.) were used for the separations. Fibrinogen and pronase were obtained from Sigma (St-Louis, Mo., U.S.A.), ovalbumin from Koch-Light (Colnbrook, Great Britain) and [1-^{14}C]acetic anhydride (25.5 mCi/mole, in 20% benzene solution) from CEA (Gif-sur-Yvette, France). Insoluble gastric mucin [10] from donors of blood group A was a gift of Prof. D. Waldron-Edward, McGill University, Montreal, Canada.

Preparation of the glycopeptides

Crude pronase digests were prepared as follows: 1 mg of glycoprotein was suspended in 400 μl of water containing 20 μg of pronase and 2 μl of 25% ammonium hydroxide solution, the mixture was incubated for 48 h at 50°. The solution was dried in a vacuum desiccator and dissolved in 100 μl of water, 500 μl ethanol was added, the mixture was allowed to stand for 20 h at 4° and the glycopeptides precipitated were centrifuged. The precipitate was redigested with pronase as described above. A 100-μl volume of 50% trichloroacetic acid was then added, the mixture centrifuged, the supernatant extracted three times with 3 ml diethyl ether and lyophilized.

Glycopeptides were prepared by pronase digestion and purified by Sephadex G-25 and G-50 column chromatography [11, 12]. The glycopeptide mixture obtained by the pronase digestion of the fibrinogen (fibrinoglycopeptides) was treated with 0.1 *N* sulfuric acid at 80° [12] to remove the sialic acid groups.

Chromatographic separation of glycopeptides and oligosaccharides

TLC was performed on silica thin-layer sheets (Selecta 1500: 20 × 20 cm) in the following solvent systems:

(A) *n*-Propanol—nitromethane—acetic acid—water (7:2:2:2);
(B) *n*-Propanol—nitromethane—water (4:3:3);
(C) Ethanol—nitromethane—acetic acid—water (5:3:3:3);
(D) Ethanol—*n*-butanol—0. 1 *N* HCl (10:1:5) [13];
(E) Ethanol—*n*-butanol—0. 5 *N* triethylamine borate buffer pH 8.5 (6:1:3).

Fingerprinting

Crude pronase digests, purified glycopeptides or oligosaccharide mixtures containing 0.2—2 μg of carbohydrate constituents were applied 2—3 cm from the low edge of the 20 × 20 cm sheets, in the proximity of the median line. It is possible however to realize two fingerprints on the same plate, applying the samples 3 and 12 cm respectively for the anodic side of the sheet. Dinitrophenyl (DNP)-alanine was used to indicate the electrophoretic migration rate. The electrophoretic step was carried out in acetic acid—pyridine—water (10:1:89) at pH 3.8 [8], and 10—20 V/cm for 2—3 h. DNP-alanine migrates 6—7 cm under these conditions.

The plates were dried overnight at room temperature and developed perpendicularly to the direction of the electrophoretic migration by solvent A or C. Plates are chromatographed with solvent A by the ascending method,

until the solvent reaches the upper edge of the sheet. Solvent C is used for the fingerprinting of the glycopeptides in a continuous flow chamber [14]. Migration time 18—30 h.

Detection of the carbohydrate constituents was carried out by the sulphuric acid char, or using the orcinol—sulphuric acid reagent [8].

The plates were exposed for 2—3 days to Kodak Kodirex films and developed with Kodak LX14 developer.

Partial alkaline degradation of the gastric mucin

A 10-mg amount of insoluble gastric mucin from blood group A individuals [10] was suspended in 1 ml of 0.33 *M*, 1% KBH_4 containing sodium hydroxide solution. The mixture was stirred for 6 days at room temperature, neutralized with Amberlite CG-120 (H^+) and centrifuged. The supernatant was lyophylized.

Radioacetylation of glycopeptides

A 0.5-mg amount of the lyophylized pronase digest was dissolved in 200 μl water, and 100—200 μCi [^{14}C]acetic anhydride in 100 μl acetone were added (about 1 μl of the benzene solution of the anhydride). The mixture was shaken vigorously, allowed to stand 1 h at room temperature, and evaporated to dryness in a vacuum desiccator over potassium hydroxide and phosphorus pentoxide. N-Acetylation realized under these conditions is sufficient for radioactive labelling but is not quantitative. The reaction can be completed by non-labelled acetic anhydride as described [12].

RESULTS AND DISCUSSION

The R_F values of some oligosaccharides and glycopeptides in the solvents described are shown in Table I. The separation of oligosaccharides for 2—8 sugar units can be realized with solvents A, B and E. Solvents C and D may be used for the separation of the glycopeptides. The best results were obtained with solvent C using a continuous-flow chamber. Most of the glycopeptides studied have a tendency for tailing in solvent D.

The fingerprints of the fibrinoglycopeptides and of the desialofibrinoglycopeptide are shown in Fig. 1. The carbohydrate in the fibrinogen is linked to two different segments of the polypeptide chains. The pronase digestion of this protein yields glycopeptides with Asp-Lys and Gly-Gly-Asp-Arg sequences [15, 16]. The oligosaccharide chains linked to the aspartic acid residues of these peptides are heterogenous. The average heterosaccharide is branched and contains 4 to 5 residues of N-acetylglucosamine, 4 residues of mannose, 3 residues of galactose and 1 to 2 residues of sialic acid linked through 2—3 and 2—6 linkages [16, 17]. Fibrinoglycopeptides contain the same structural elements as the heterosaccharide chains of the other serum glycoproteins [18].

The fingerprint of the desialofibrinoglycopeptides shows two major sugar-containing spots (Fig. 1a) indicating the presence of two major glycopeptides with different electrophoretic and chromatographic mobilities. The difference in the electrophoretic behaviour of these glycopeptides can be explained by

TABLE I

R_F VALUES OF OLIGOSACCHARIDES AND GLYCOPEPTIDES ON SILICA THIN-LAYERS (SELECTA 1500)

Chromatographed by the ascending method, migration: 18 cm. nm = No migration, n.t. = not tested.

Sample	Solvent				
	A	B	C*	D*	E
Maltose	0.42	0.52	0.85	n.t.	n.t.
Lactose	0.39	0.46	0.79	n.t.	n.t.
Raffinose	0.26	0.41	0.71	0.82	0.53
Stachiose	0.11	0.26	0.61	0.70	0.35
Bovine fibrino-glycopeptides	n.m.	n.m.	(0.02—0.07) 0.12	(0.2—0.28) 0.32	n.m.
Bovine desialo-fibrinoglycopeptides	n.m.	n.m.	(0.03—0.1)	(0.29—0.38)	n.m.
Bovine fibrinoglyco-peptides N-acetylated	n.m.	n.m.	(0.10—0.14) 0.22	(0.25—0.32) 0.43	n.m.
Ovalbumine glyco-peptides	n.m.	n.m.	(0.10—0.19)	0.21; 0.29; 0.32	n.m.

*R_F values in parentheses are the lower and the upper limits of incompletely separated zones, otherwise they correspond to well-defined spots.

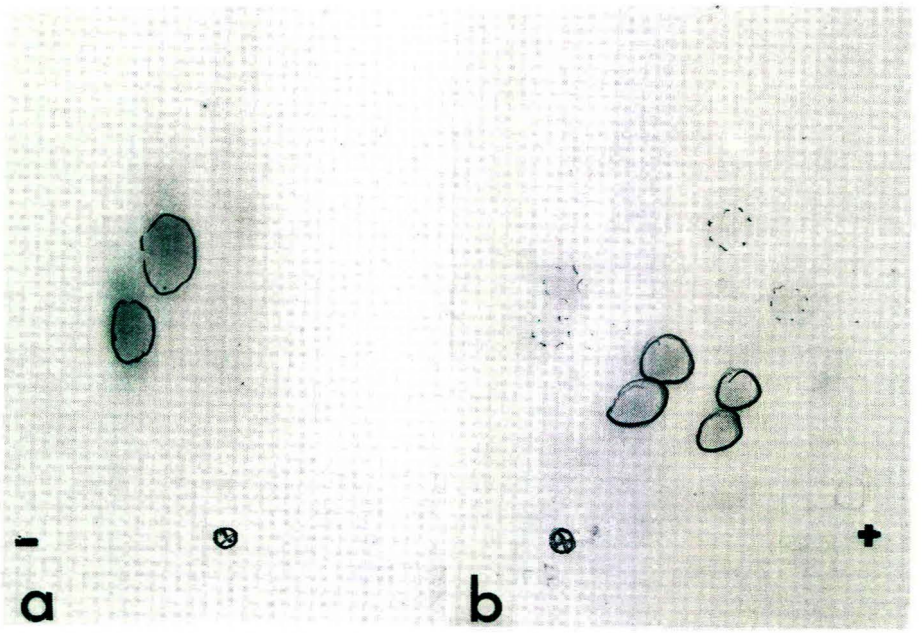


Fig. 1. Fingerprint of (a) desialo and (b) "native" fibrinoglycopeptides on Selecta 1500 sheets 20 × 20 cm. Electrophoresis: pyridine-acetate pH 3.8, 10V/cm. 2.5 h. Chromatography: solvent C, 24 h in a horizontal continuous flow chamber. Detection: 0.1% orcinol in 1 N H_2SO_4 in 80% ethanol, heating at 110° for 5 min.

the difference of the two peptide chains, hence the sialic acid was removed.

The fingerprint of the "native" fibrinoglycopeptide (Fig. 1b) is more complex. It exhibits the presence of 4 major carbohydrate-containing components indicating the increased heterogeneity of the heterosaccharide chains, explained partly by the variation of their degree of sialysation. This finding is consistent with the actual concept of the microheterogeneity of the carbohydrate moieties of glycoproteins [19].

Several minor carbohydrate-containing peptides can be detected on the fingerprint of both native and desialysed, fibrinoglycopeptides. This phenomenon can be explained by the presence of incomplete sugar chains [19] or of incompletely digested glycopeptides [16].

The fingerprint of the radioacetylated fibrinoglycopeptide mixture and its radioautogram are presented in Fig. 2. Each sugar-containing spot is radioactive. The electrophoretic as well as the chromatographic migration properties of the glycopeptides are changed by the N-acetylation. The sensitivity of the method can be 10—100-fold improved by the radioacetylation technique.

Detection of the non-glycosylated peptides by ninhydrin reagent, as well as the localisation of the non-glycosylated N-acetyl peptides on the autoradiograms, indicate that these peptides migrate in the chromatograms much faster

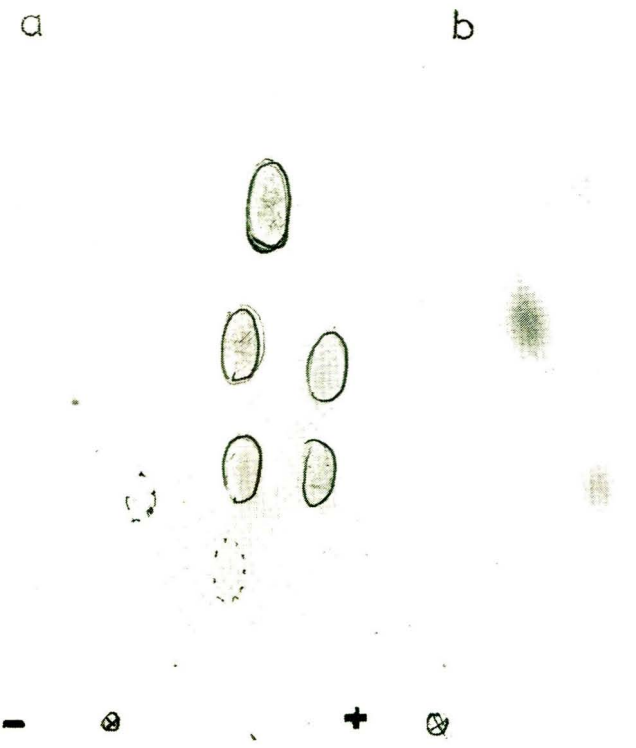
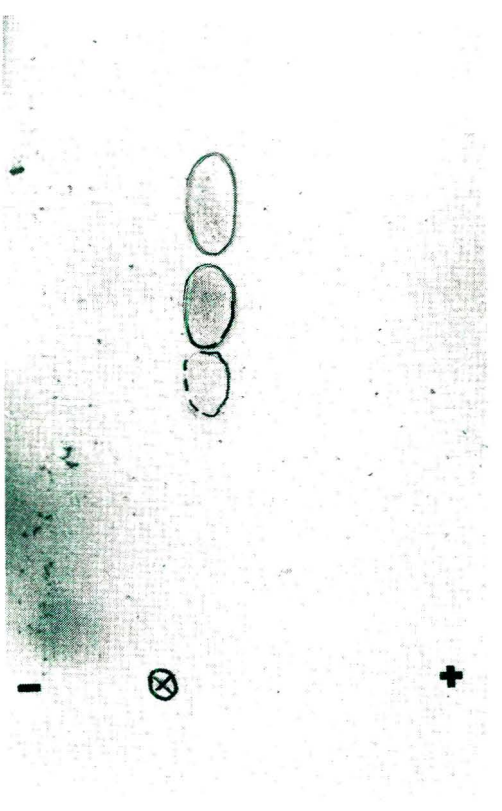


Fig. 2. Fingerprint of the N-acetylated ([1-^{14}C]acetic anhydride) "native" fibrinoglycopeptide (a) and its autoradiogram (b). Electrophoresis, chromatography and detection: see Fig. 1. Autoradiography on Kodirex films, exposure 3 days.

than glycopeptides (R_F between 0.8—1.0, in solvent C).

Pronase digestion of ovalbumin yields mannose and N-acetyl-glucosamine-containing oligosaccharides linked to aspartic acid [20]. The presence of two minor and three major carbohydrate chains was demonstrated in the glycopeptide mixture. The major fractions contained glucosamine and mannose in ratios of 4:6, 2:6 and 2:5, respectively [20]. The presence of three major carbohydrate-containing spots on the fingerprint of the ovalbumin glycopeptides (Fig. 3) is consistent with these results. The identity of the electrophoretic mobility of these substances can be explained by the identity of the amino acid moiety (aspartic acid only) and by the absence of sialic acid in the carbohydrate fraction.

The differences in the chromatographic migration of the sialic acid-containing and the sialic acid-free fibrinoglycopeptides (Table I, Fig. 2) as well as the galactose-free ovalbumin glycopeptides (Fig. 3) suggest that the chromatographic behaviour of the glycopeptides studied is strongly influenced by the structure and composition of the carbohydrate chains.



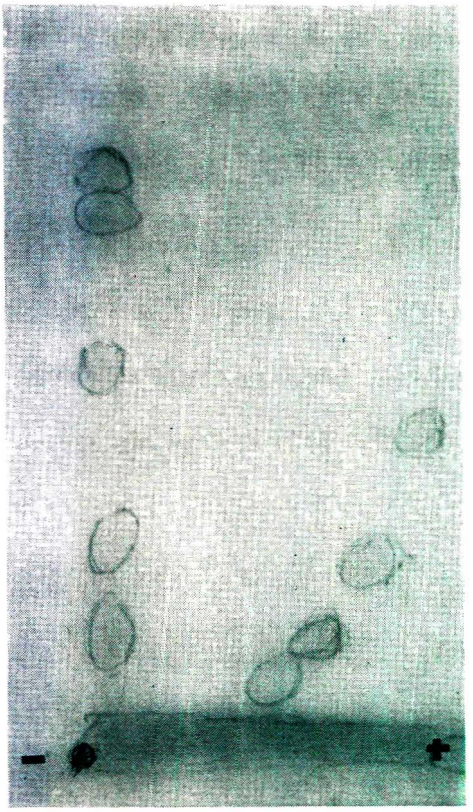


Fig. 3. Fingerprint of the ovalbumin glycopeptides. Experimental conditions: see Fig. 1.

Fig. 4. Fingerprint of the partial alkaline degradation products of the insoluble gastric mucin from blood group A individuals. Electrophoresis and detection as described in Fig. 1. Chromatography: solvent A ascending method, migration 18 cm.

However, much more work is needed with glycopeptides of exactly known structure before reliable correlations can be established between the structure of the sugar chain and the chromatographic mobility of glycopeptides.

Fig. 4 shows the fingerprint of the oligosaccharides obtained by the partial degradation of an insoluble gastric mucin in the presence of alkaline borohydride. Neutral and acidic oligosaccharides with a reduced aldehyde group were liberated from these mucins under the conditions used (peeling effect) [21]. A satisfactory two-dimensional separation of these oligosaccharides was obtained. The non-degraded macromolecular carbohydrate components exhibit electrophoretic mobility but they are not displaced by the solvents used in the chromatographic step.

The method is especially useful for studies of the variations of the carbohydrate chains of glycoproteins during development, aging, malignant transformations or other pathological conditions.

In such cases, the amino acid sequence of the polypeptide does not change, and in consequence, the peptide chains of the glycopeptides obtained by proteolytic digestion are identical.

Thus, only the composition of the carbohydrate moieties of these glycopeptides determines their electrophoretic and chromatographic mobilities.

As fingerprints prepared from the purified glycopeptide mixtures or from the crude pronase digest are identical, aliquots of these digests can be used directly for the characterization of the glycans of the glycoproteins.

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CHROMBIO. 191

Note

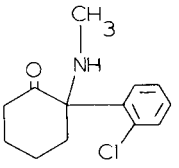
Rapid gas chromatographic analysis of plasma levels of ketamine and major metabolites employing either nitrogen selective or mass spectroscopic detection

JOHN N. DAVISSON

College of Pharmacy and Health Sciences, Northeast Louisiana University, Monroe, La. 71209 (U.S.A.)

(Received January 2nd, 1978)

Ketamine [2-(*o*-chlorophenyl)-2-(methylamino)cyclohexanone] (I), a parenteral anesthetic with unusual pharmacological properties [1], has been studied by numerous investigators. Many groups [2—4] have sought to clarify therapeutic relationships between this compound and its major metabolites. Two biotransformation products that have received much attention include 2-(*o*-chlorophenyl)-2-aminocyclohexanone (II) and 6-(*o*-chlorophenyl)-6-amino-



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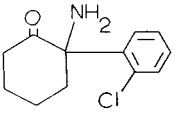

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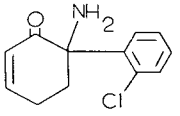

III

cyclohex-2,3-en-1-one (III). Most investigations of this type have focused upon biodisposition and metabolism. Thus suitable chemical analyses of these compounds remain essential to these experiments.

Most researchers employ modifications of the gas chromatographic (GC) assays developed by Chang and Glazko [4, 5] and Lo and Cumming [6]. Additionally, a high-pressure liquid chromatographic (HPLC) method has been described [7]. All of these analytical processes require derivatization and/or extensive extraction and concentration steps.

This report offers new analytical methods for I—III based upon either the convenience and sensitivity of computer assisted integrated gas chromatography—mass spectroscopy (GC—MS) or the advantages of GC separation

coupled with selective nitrogen detection. Additionally, these procedures simplify methodology and reduce assay time. Finally, qualitative identification of compounds is greatly facilitated by examination of MS data.

EXPERIMENTAL

Supplies

Compounds I, II, III and diphenhydramine were supplied courtesy of Parke Davis & Co. (Detroit, Mich., U.S.A.). All solvents and additional chemicals were standard reagent-grade materials.

Gas chromatography

GC separations were made with a Perkin-Elmer 3920 B gas chromatograph equipped with a phosphorous—nitrogen (P—N) detector. The column consisted of a 6 ft. × 2 mm I.D. glass tube packed with 3% SP-2300 on Supelcoport (100—120 mesh; Supelco, Bellefonte, Pa., U.S.A.). Nitrogen carrier gas flow-rate was maintained at 40 ml/min, hydrogen flow-rate at 5 ml/min and air flow-rate at 100 ml/min. The rubidium bead current control was set at 500. Injection port and interface temperatures were 270°, and the column oven operated isothermally at 220°.

A Hewlett-Packard 5720 gas chromatograph equipped with a Ni^{63} detector was employed to compare the results of this study with the electron capture method [5].

Mass spectroscopy

Mass spectra were recorded with a Dupont Instruments Dimaspec 321 gas chromatograph—mass spectrometer interfaced with a DuPont Instruments 320 data system. Source temperature was 225° with ionization at 75 eV, and the accelerating voltage maintained over the range of 12,300—600 eV. The jet separator was operated at 225°. Other GC parameters were identical to those noted above except that helium was employed as the carrier gas.

Procedure

Male Swiss—Webster mice (20—30 g) were injected intraperitoneally with 125 mg/kg ketamine hydrochloride dissolved in normal saline. After 15 min, animals were sacrificed by decapitation and blood was collected in a small beaker. Plasma was obtained by standard centrifugation methods and saved for analysis. Routinely 0.4—0.5 ml of plasma per animal was obtained by this process.

For a normal assay procedure 0.1—1.0 ml of plasma was transferred to a glass stoppered centrifuge tube. To this was added 0.10 ml of 4 *N* NaOH solution, 0.1 ml of diphenhydramine hydrochloride solution (equivalent to 25 μg/ml free base) and sufficient distilled water to produce a final volume of 1.2 ml. Methylene chloride (2.0 ml) was added and the tube was shaken by hand for approximately 30 sec. Separation of the layers was facilitated by centrifugation, and the organic (lower) phase was transferred to a conical centrifuge tube. After evaporation of the methylene chloride under a stream of nitrogen at 20°, 0.05 ml of acetone was added and the tube vortexed for a few seconds.

A 1—2 μl volume of this solution was injected into the gas chromatograph.

Standard curves were obtained as described above except that known quantities of compounds I—III (0.4—10 μg) were added to plasma samples obtained from animals that had been injected with normal saline.

RESULTS AND DISCUSSION

Under the conditions employed for GC analysis of various samples, excellent separation of all four compounds (I, II, III, and diphenhydramine, IV) was achieved (Fig. 1). Relative retention times for compounds I—III were 2.5, 3.5 and 5.5 min respectively. The average GC analysis time of approximately 10

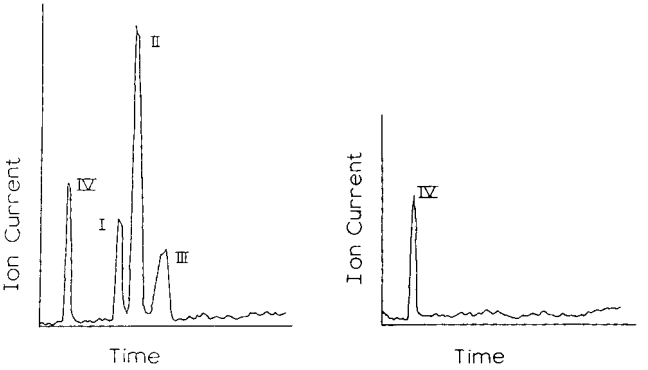

Fig. 1. Reconstructed gas chromatograms of compounds I, II, III and IV (diphenhydramine). Column: 6 ft. × 2 mm I.D., 3% SP 2300 on Supelcoport (100—120 mesh) at 240°. The injection mixture (left chromatogram) was obtained initially from 0.2 ml of plasma containing 4.0 μg I and III, 10 μg II and 2.5 μg IV, while the other chromatogram resulted from a plasma sample containing only the internal standard (IV).

min, permitted rapid processing of several samples. As can be seen no significant interfering peaks were observed. Similar separations were obtained using an instrument equipped with a P—N detector.

Qualitative identification was facilitated by examination of mass spectral data. All compounds showed extensive fragmentation which was consistent with known chemical structures. Compounds I and II displayed very weak molecular ions while the M^+ ion of III was highly significant in the mass spectrum. Base peaks for all compounds contained chlorine as evidenced from the relative intensities of ions due to isotope distribution (Table I).

Quantitation of compounds I—III was accomplished by two methods, the most expeditious of which utilized the available software contained in the GC—MS data-acquisition system. This essentially involved integrating total ion currents produced by compounds I—III and comparing each of these to the analogous response shown by the internal marker, diphenhydramine.

Standard peak-height ratio methods were used to quantitate data obtained in experiments employing the P—N detector equipped instrument. Results were essentially the same as those obtained through the computer analyses described above. Since the P—N detector is available to more laboratories, the

TABLE I

RELATIVE INTENSITIES OF MAJOR IONS IN THE MASS SPECTRA OF COMPOUNDS I—III

See Experimental for conditions

Compound	*m/e*	Relative intensity
I	180	100
	182	35.3
	209	26.9
	211	9.4
	237 M	0.9
	239 M + 2	0.3
II	166	100
	168	34.8
	195	26.9
	197	8.9
	223 M	0.2
	225 M+ 2	0.07
III	153	100
	155	32.2
	221 M	40.4
	223 M + 2	13.2

data illustrated (Fig. 2) was compiled from experiments using this type of instrumentation. As can be noted, standard curves for compounds I—III were linear in the range 0.8—10.0 μg/ml. Other experiments showed that this range of linearity could be extended to at least 40 μg/ml.

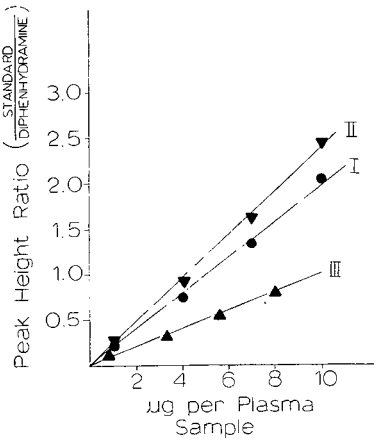

Figure 2: Standard curves for ketamine and major metabolites. • = Ketamine (I); ▼ = N-demethylated product (II); ▲ = N-demethylated cyclohexenone analog (III). Known quantities of I—III were added to 0.2 ml plasma. After addition of 2.5 μg diphenhydramine (IV) to each sample, GC analysis was carried out using a P—N detector equipped instrument. Ratios of standards to internal marker (IV) were calculated. Points represent mean of three determinations. Regression lines were calculated by least-squares method.

Percentage recovery of I—III was determined by extracting 1.0 μg of each from plasma samples. Peak-height ratios of compounds I—III versus internal marker were compared with those obtained from acetone solutions of similar concentrations. Four determinations resulted in mean per cent recoveries for I, II, and III of 97±2.4, 98±1.9 and 93±3.2, respectively.

To establish the utility of this method for application to in vivo studies, mice were injected with ketamine (see Experimental) and plasma samples analyzed for the presence of compounds I—III. The results (Table II) indicated that all compounds could be readily identified and quantitated, using these procedures. In addition to plasma samples, liver and brain specimens were also subjected to analysis. Again this method proved quite satisfactory for monitoring compounds I—III in these tissues. Similar findings resulted when laboratory rats were used as test animals.

TABLE II

PLASMA CONCENTRATIONS OF COMPOUNDS I—III IN ANIMALS

Measured for male, Swiss-Webster mice (n = 8), 25 min after receiving ketamine (125 mg/kg) intraperitoneally.
Values determined by peak-height ratio method from data obtained using P—N detection. No significant differences were noted when computer analysis of peak areas of reconstructed gas chromatograms were quantitated.

Compound	Concentration (μg/ml)
I	8.5 ± 1.16
II	13.9 ± 1.28
III	2.58 ± 0.21

Analytical methods for ketamine and its major metabolites described in this paper offer rapid and accurate procedures for the separation, identification and quantitation of these compounds. Several advantages over published methods can be cited, a major difference is that no expensive and lengthy derivatizations, such as those required for electron capture detection methods [5], are necessary. Additionally, extensive extraction, back extraction, and concentration steps are eliminated. Similar advantages can be noted for this method when compared with HPLC analysis [7]. In general one plasma sample can be processed and assayed in approximately 15 min with sensitivity comparable to other methods.

Computer assisted GC—MS processing greatly facilitates identification and quantitation. The extensive fragmentation of compounds I—III suggests that chemical ionization would probably be valuable.

Finally, it should be noted that use of a nitrogen-selective GC detector provides similar results to GC—MS computer methods. The latter allows for more rapid analyses but the former may be more attractive for economic reasons. However, both offer advantages over previously published methods.

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Note

Determination of cytosine arabinoside in human plasma by high-pressure liquid chromatography

ROSS W. BURY* and PETER J. KEARY

Department of Medicine (St. Vincent's Hospital), University of Melbourne, Fitzroy, Victoria 3065 (Australia)

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Cytosine arabinoside (1-β-D-arabinofuranosyl-cytosine; ara-C) is a pyrimidine analogue with significant anti-tumour activity and has been reported to be therapeutically effective in the treatment of acute myeloblastic leukaemia and other haematological malignancies [1—4].

The anti-metabolite properties of the drug apparently result from selective inhibition of DNA polymerase and incorporation into DNA (or RNA) following the formation of arabinosyl-CTP [5]. However, ara-C is not effective in all treated patients and Baguley and Falkenhaug [6] found that there was a significant correlation between failure of treatment and rapid clearance of the drug from the plasma. If an effective blood concentration is the sole determinant of efficacy, kinetic data could be used to establish the most appropriate dosage schedules. Nevertheless, the availability of active arabinosyl-CTP at tumour sites in appropriate concentration will ultimately determine efficacy and the relationship of this to ara-C blood concentrations needs to be explored.

Previous analytical methods for ara-C in blood have involved radioactive drug administration and subsequent measurement of radioactivity [6, 7], biological assay involving tissue culture [6, 8] or paper chromatography [7, 9]. The methods are complex and most are relatively insensitive. Wan et al. [7] reported that high-pressure liquid chromatography (HPLC) may prove useful in measurement of ara-C but provided little detail of methodology and other characteristics of their assay. The use of a rapid, simple and sensitive HPLC procedure for ara-C is now described in which plasma protein is removed by precipitation and a buffered aliquot of supernatant is injected directly into the chromatographic system.

*To whom correspondence should be addressed.

METHODS AND MATERIALS

Blood specimens (10 ml) were collected in heparinized tubes containing 100 μg tetrahydrouridine (THU), which prevents deamination of ara-C [5]. These were immediately stored at 4°. Plasma samples (2 ml) were pipetted into tubes containing 100 μl 4 *M* trichloroacetic acid. After mixing for 20 sec on a vortex mixer, these were centrifuged for 20 min at 0° to separate the precipitated protein. A 1-ml volume of supernatant was immediately transferred to another tube and 20 μl 5 *M* ammonium formate buffer (pH 7.0) was added to achieve a final pH of approx. 3.5. Aliquots of 100 μl were then injected into the chromatograph.

Analyses were performed on a cation-exchange column (Whatman Partisil PXS 10/25 SCX; 250 × 4.6 mm) using 0.01 *M* ammonium formate (pH 4.8) at a constant flow-rate of 1.0 ml/min as the mobile phase. A single-piston high-pressure pump (Altex model 110), a sample injection valve containing a 100-μl loop (Chromatronix) and a fixed-wavelength ultraviolet detector with a 20-μl flow cell (Altex model 150) formed the basis of the chromatograph.

Absorbance of the effluent from the column at 254 nm was monitored at a sensitivity of 0.005 a.u.f.s. Peak heights were used for quantitation of the assay.

To prevent contamination of the ion-exchange column by plasma constituents remaining after the protein removal step, a pre-column (50 × 4.6 mm) containing reversed-phase packing (Partisil ODS, 10 μm; Reeve Angel) was incorporated into the system. Ara-C was not appreciably retained by the pre-column. Significant increases in perfusion pressure necessitating a change of pre-column (at 3000 p.s.i.) did not occur until about 100 samples had been passed through the system.

All chemicals used were analytical grade and water was doubly distilled. Ara-C and THU were kindly donated by Upjohn (Kalamazoo, Mich., U.S.A.).

Calibration curves were derived from pooled blood bank plasma.

RESULTS AND DISCUSSION

Typical chromatograms of plasma samples (Fig. 1) show that control samples are free from contaminating peaks. Ara-C was eluted in 11 min. The major metabolite of ara-C, 1-β-D-arabinofuranosyl uracil (ara-U) was eluted with the large initial mass of endogenous material. All calibration curves for ara-C passed through the origin and were linear from the detection limit of 20 ng/ml plasma to the maximum concentration used (1 μg/ml). Concentration of ara-C in the plasma as a result of the precipitation of plasma protein led to a high recovery of the drug throughout the concentration range listed above (mean recovery 109.5%; S.E.M. 0.7%; n=10) in comparison to standard aqueous solutions of ara-C. The coefficient of variation for the analysis was determined to be 1.7% (n=10) at a concentration of 200 ng/ml.

Blood samples from six hospitalised leukaemic patients receiving constant intravenous infusions of ara-C over 7 days were taken twice between days 2 and 7 to determine the plasma levels likely to be obtained in a steady-state situation (Table I). There was no apparent correlation between the dose of ara-C admin-

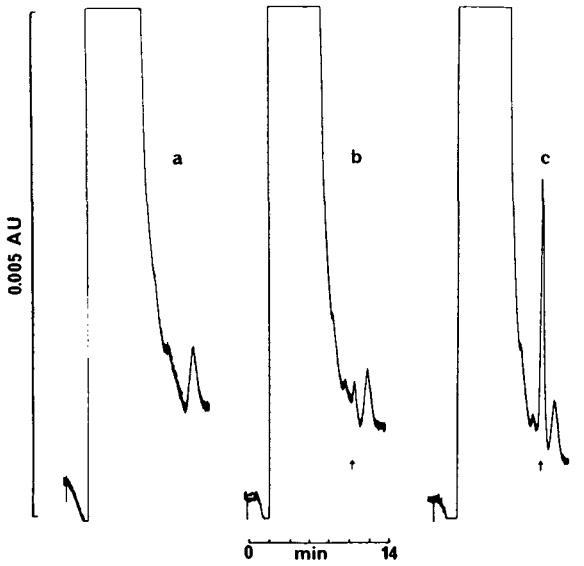

Fig. 1. Typical chromatograms for ara-C in deproteinized plasma. The ordinate represents absorbance units (AU). The arrows indicate the peaks eluting as ara-C. a, blank; b, 50 ng/ml ara-C; c, 500 ng/ml ara-C.

TABLE I

STEADY-STATE PLASMA CONCENTRATIONS OF ARA-C IN SIX PATIENTS WITH ACUTE MYELOID LEUKEMIA

Patients received constant intravenous infusion of ara-C for 7 days. Blood samples from each patient were taken on separate days. Each level represents the mean of duplicate estimations.

Patient	Surface area (m^2)	Dose (mg/m^2/day)	Ara-C plasma concentrations in steady state (ng/ml)
E.C.	1.9	70	241, 223
J.T.	1.6	70	168, 170
R.B.	1.6	70	88, 79
L.P.	1.4	70	76, 80
R.T.	1.6	130	105, 114
B.J.	1.9	150	153, 135

istered daily and the plasma concentration attained in these patients. This is probably a reflection of pharmacokinetic variability and emphasizes the difficulties in establishing appropriate dosage schedules. Routine measurement of ara-C in plasma may prove useful in this regard.

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Note

High-pressure liquid chromatographic determination of cimetidine sulphoxide in human blood and urine

R.M. LEE and P.M. OSBORNE

Department of Biochemistry, Smith Kline & French Laboratories Ltd., Research Institute, Welwyn Garden City, Hertfordshire (Great Britain)

(First received January 10th, 1978; revised manuscript received April 21st, 1978)

Cimetidine [1] an H_2-antagonist of histamine, has been introduced recently as Tagamet® for the treatment of gastric and duodenal ulcers. The major identified metabolite of cimetidine is the sulphoxide [2], which is eliminated from the body mainly by renal excretion. A satisfactory method for the determination of cimetidine in blood and urine has been published [3] and this can now be complemented by a method for the determination of the sulphoxide metabolite in blood and urine using high-pressure liquid chromatography (HPLC) to separate the components of the extract and UV extinction to monitor the column effluent. This method differs in several important respects from that published [3] for the assay of cimetidine itself, but can be used for cimetidine analysis by a simple modification of the solvent system.

METHODS

Extraction procedure

All blood samples were heparinized at collection, frozen (—20°) as soon as possible thereafter and stored at this temperature until thawed immediately prior to extraction. Urine samples were frozen within 30 min of collection and similarly stored.

The procedure for extraction of cimetidine sulphoxide was the same for blood and urine samples and is only described for blood samples.

A 3-ml sample of the blood to be assayed was made alkaline (pH 9.0) by the addition of 1 ml of *N* carbonate buffer, which contained a suitable amount (about 2 μg) of internal standard (N-cyano-N′methyl-N″-(3-(4-imidazolyl)-propyl)guanidine; compound SK&F 92374)*. A 4-ml volume of 1-octanol was then added to the samples in 15-ml polythene tubes, which were stoppered and rotated for 15 min on a blood-mixer. After 15 min the octanol layer was

*Available from SK&F Labs., Welwyn Garden City, on request for the analysis of cimetidine sulphoxide.

separated cleanly by centrifugation, removed and stored. A second 4-ml volume of octanol was added and the process repeated, so that the combined octanol extracts (total volume 7 ml) could be re-extracted with 3 ml of 0.02 *N* HCl by the same rotary mixing and centrifugation technique. After removal of the octanol by aspiration, the acid layer (2.8 ml) was transferred to clean tubes, and 200 μl of ethanol added and mixed before saturating the whole with solid potassium carbonate (ca. 5 g). This had the effect of "salting-out" the ethanol into a discrete layer which could be removed after centrifugation and stored at −20° prior to HPLC separation and analysis.

Chromatography

The following HPLC conditions were employed:Column: LiChrosorb Si 60; 5-μm particle size; 25 cm × 3.2 mm I.D. Solvent: acetonitrile—methanol—water (distilled)—ammonium hydroxide (0.88 sp.gr.); (250:20:6:1.5, v/v). Flow-rate: 1 ml/min maintained by a constant flow system (Waters Assoc. 6000). Injection: 10—20 μl of the ethanol extract were introduced onto the column via an Altex variable-volume loop injector. Detection: the UV absorption of the column effluent was monitored by a variable wave-length detector (Perkin-Elmer LC55) set at 228 nm. Recording: peaks were recorded on a conventional chart recorder and the areas under them integrated by means of an Infotronics CRS 309 integrator which compensated for baseline drift. Retention: the retention times of compounds of interest were determined by the chart speed of the recorder, and by the programme of the integrator which was reset at the time of each sample injection.

RESULTS

The relationship between peak height, peak area and the amount of cimetidine sulphoxide applied to the HPLC column is given in Table I; it was found

TABLE I

CORRELATION BETWEEN PEAK AREA OR PEAK HEIGHT AND CIMETIDINE SULPHOXIDE*

Determined by HPLC—UV analysis. Background peak heights were less than 1 mm, and integrator counts associated with background were not greater than 500. The peak height and area values are the means of four observations, together with their standard deviations.

Concentration of cimetidine sulphoxide in ethanol (mg/l)	Cimetidine sulphoxide in 20-μl injection (ng)	Peak height (mm)		Peak area (relative counts)
		Range	Mean	Mean
0.65	13	5—5	5 ± 0	2010 ± 94
1.30	26	9—11	10 ± 1	3970 ± 198
2.60	52	19—21	21 ± 1	8020 ± 301
5.20	104	38—41	40 ± 2	14940 ± 727
10.40	208	76—83	78 ± 4	29520 ± 356
20.80	416	158—162	161 ± 2	62400 ± 1496

*Concentrations up to mg/l.

TABLE II

TEST OF REPRODUCIBILITY OF CIMETIDINE SULPHOXIDE EXTRACTION AND ASSAY FROM BLOOD AND URINE SAMPLES

Injection volume onto column 15 μl from a total of 200 μl.

Sample No. (2 ml)	1 mg/l in Blood				5 mg/l in Urine			
	Sulphoxide		Internal standard		Sulphoxide		Internal standard	
	Peak ht. (mm)	Area (rel. count)	Area (rel. count)	Area ratio	Peak ht. (mm)	Area (rel. count)	Area (rel. count)	Area ratio
1	29	9251	47719	5.1	109	35657	44641	1.25
2	26	8706	45417	5.2	104	33473	38168	1.14
3	24	8822	47148	5.3	112	35341	43867	1.24
4	25	9321	48334	5.2	115	36479	45164	1.24
5	26	8662	45126	5.2	102	31922	37561	1.18
6	29	9294	46732	5.0	92	29554	37244	1.26
7	27	8206	48092	5.9	105	34247	42887	1.25
8	30	9270	48787	5.3	106	32672	40664	1.24
Mean	27	8967	—	5.3	106	33668	—	1.22
Range	24—30	—	—	5.0—5.9	92—112	—	—	1.14—1.26
S.D.	2	—	—	0.3	7	—	—	0.04

to be linear over the range 13—416 ng/20-μl injection, with a minimum reliable estimation of 10 ng. Higher concentrations were not investigated, but the assay was accurate for 200-μl ethanol extracts containing 0.1—4.2 μg of cimetidine sulphoxide.

The results of extracting known quantities of cimetidine sulphoxide from blood and urine samples are shown in Table II. The standard curve was linear for cimetidine sulphoxide blood concentrations between 0.5 and 5.0 mg/l, and the reproducibility (Table II) at 1 mg/l was excellent (S.D. = 5.6% of

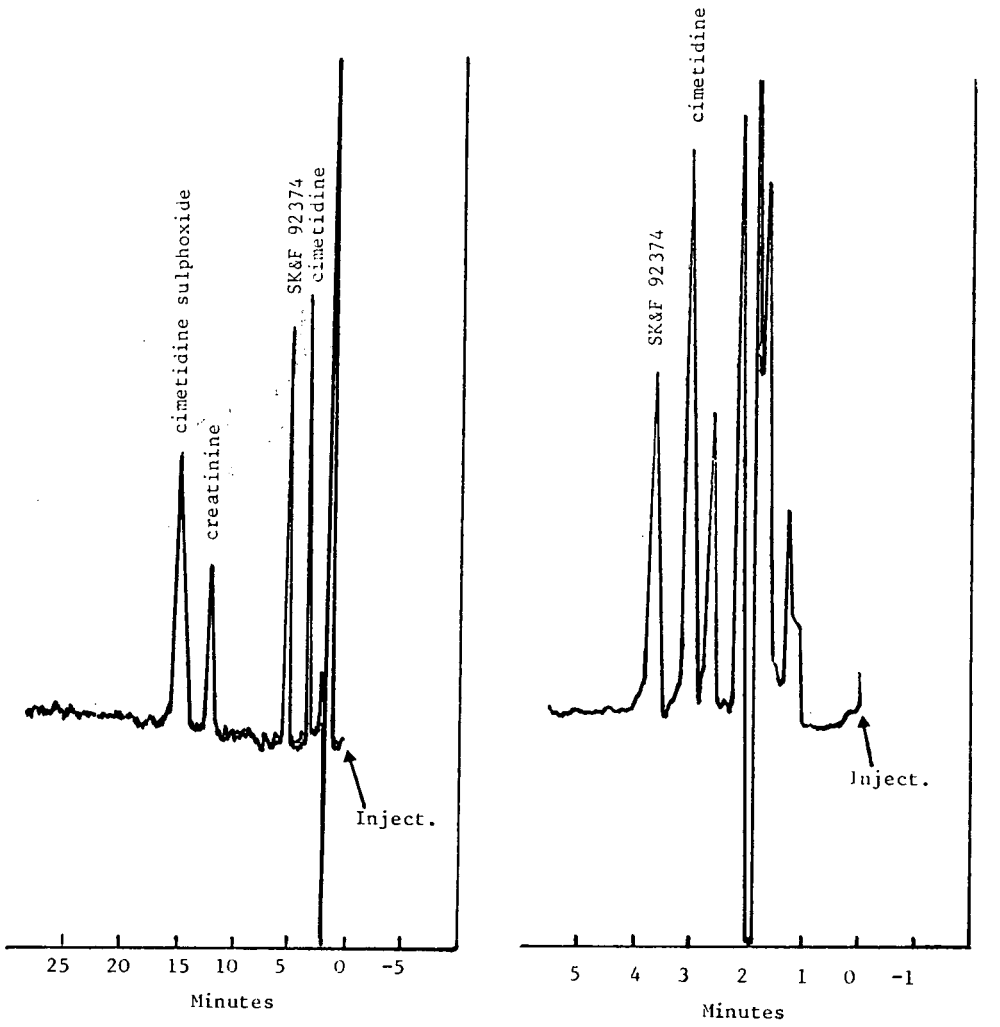

Fig. 1. Injection of a mixture of known quantities of cimetidine, SK&F 92374, creatinine and cimetidine sulphoxide dissolved in ethanol. Solvent system: acetonitrile—methanol—water—0.88g ammonia 250:20:6:1.5, v/v).

Fig. 2. Injection of an ethanol extract of a blood sample from a patient receiving cimetidine, using SK&F 92374 as internal standard. Solvent system: acetonitrile—methanol—water—0.88g ammonia 200:30:6:1.5, v/v).

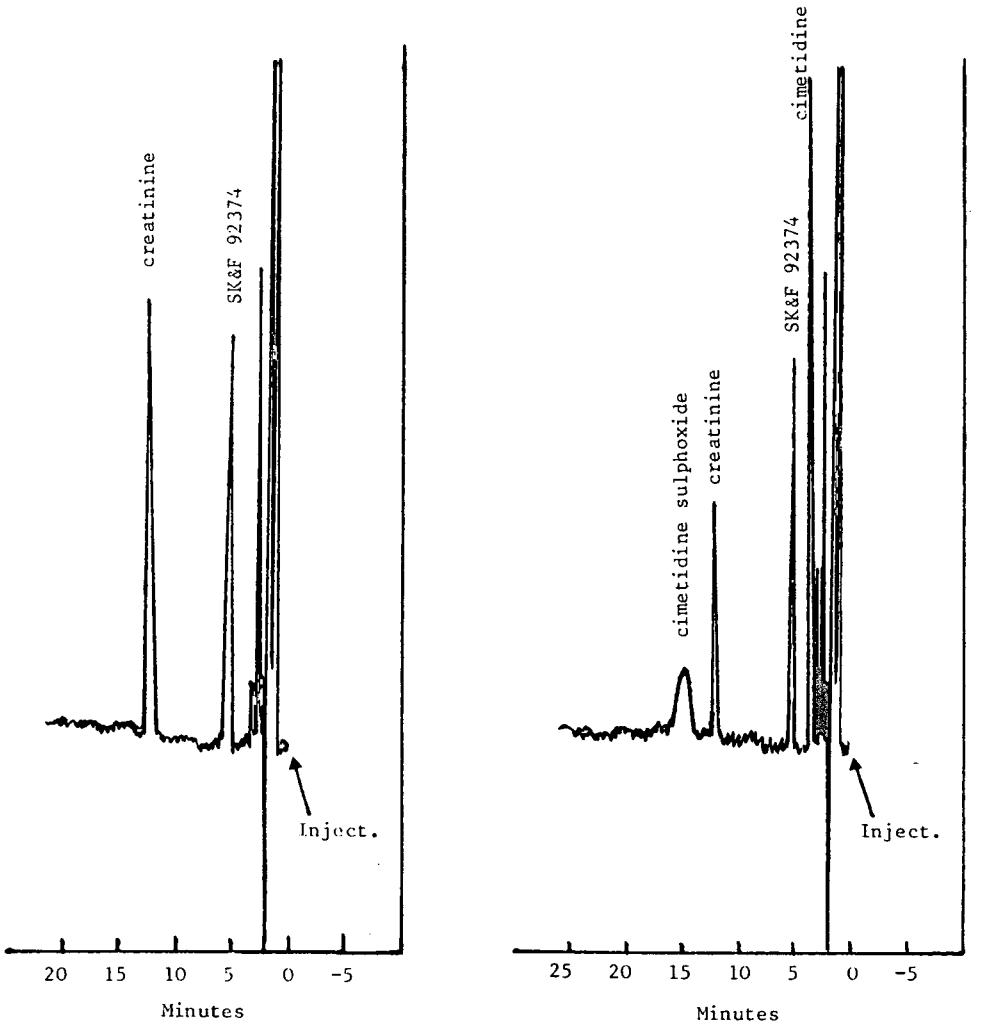

Fig. 3. Injection of an ethanol extract of a blood sample from a patient with renal failure but not receiving cimetidine. Solvent system as Fig. 1.

Fig. 4. As Fig. 2, but from a patient receiving cimetidine.

mean for peak area determinations, about 8% of mean for peak height measurement).

For urine, the standard curve was linear between 1 and 8 mg/l. Reproducibility at 5 mg/l was again excellent, with a standard deviation of less than 4% of the mean for peak area estimations. Theoretically, the complete extraction of 2 μg of cimetidine sulphoxide from a blood sample would result in a concentration of 10 mg/l in the ethanol available for HPLC. A 15-μl injection onto the column would contain 150 ng, giving a peak area of 21,400 counts (Table I). In practice, the count for 8 such samples was an average 8967, giving a recovery of 42%.

The recovery from urine was 31% for the extraction of a 2-ml sample containing 10 μg of cimetidine sulphoxide. With a minimum reliable determination of 10 ng injected onto the column, the assay is limited to concentrations of cimetidine sulphoxide greater than 0.2 μg/ml if 2-ml samples are available for extraction.

The analysis of cimetidine sulphoxide in clinical samples is complicated by the presence of cimetidine itself, and frequently creatinine. The retention times for the HPLC system described herein were 3, 5, 12 and 14 min for

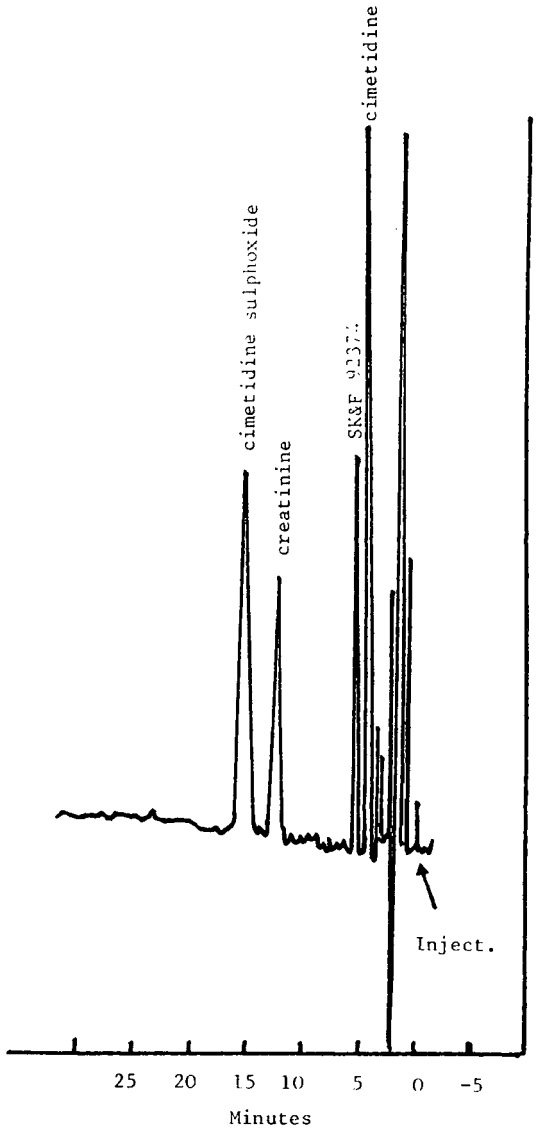

Fig. 5. Injection of an ethanol extract of a urine sample from a healthy volunteer, who had received cimetidine intravenously 2 h previously. Solvent system as Fig. 1.

cimetidine, internal standard, creatinine and cimetidine sulphoxide respectively (Fig. 1).

The separation of cimetidine from indigenous blood components was not always complete under these conditions, and is better performed using the same solvents in the proportions 200:30:6:1.5 with a flow-rate of 1.5 ml/min (pressure approx. 2500 p.s.i. at the pump). This system gave retention times of approximately 3.0 min for cimetidine and 3.5 min for the internal standard (Fig. 2).

The use of metiamide as internal standard [3] is quite inappropriate under the conditions used for sulphoxide analysis, as solvent systems for clean separation of metiamide [3] gave a long retention time for cimetidine sulphoxide which emerged as a wide-based peak of insufficient height for sensitive assay.

Blood samples taken from patients with renal failure usually contained high creatinine concentrations (Fig. 3). When these same patients were on a regimen of cimetidine (200 mg on three occasions during the day, plus 400 mg at bedtime), the blood samples taken 2—3 h after a 200-mg dose gave chromatograms as in Fig. 4, with peak height and area corresponding to about 0.5 mg/l cimetidine sulphoxide.

For the sequential analysis of cimetidine itself in these samples, it was necessary to allow the creatinine peak to appear before applying the next sample to the column, otherwise the absorption by creatinine at 228 nm seriously distorted the peaks due to cimetidine or internal standard in the following sample.

In blood samples from healthy volunteers or from patients taking cimetidine but free from renal failure, no creatinine or cimetidine sulphoxide has been found, although the lower limit of detection for the latter compound was 0.2 mg/l compared to 0.05 mg/l for cimetidine.

The analysis of cimetidine sulphoxide in urine is illustrated in Fig. 5, where the chromatogram is similar to that obtained from blood except that the cimetidine sulphoxide peak is much more in evidence (corresponding to about 3 mg/l) and was observed in the urine of healthy volunteers after oral administration of cimetidine (200 mg).

Using this technique, it would be possible to detect and quantify cimetidine sulphoxide in blood samples from cases of renal failure, and to follow the output of cimetidine sulphoxide in the urine of healthy volunteers and patients with kidney disease.

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Note

Rapid quantitative method for the simultaneous determination of carbamazepine, carbamazepine-10,11-epoxide, diphenylhydantoin, mephenytoin, phenobarbital and primidone in serum by thin-layer chromatography

Improvement of the buffer system

N.T. WAD and H. ROSENMUND

Department of Clinical Chemistry, University Hospital Zürich, CH-8091 Zürich (Switzerland)

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This note describes the modification of our published thin-layer chromatographic (TLC) method [1, 2] using a different buffer system. With the previously used buffer (0.3 *M* NaH_2PO_4) two unwanted substances from serum were extracted together with the drugs (see peaks 7 and 9 in Fig. 1). Under these conditions peak 7 can combine with the phenobarbital-peak and disappear due to small changes in the composition of the chromatographic solvent or due to unknown influences. Using a phosphate buffer almost saturated at room temperature with ammonium sulfate [500 g of $(NH_4)_2SO_4$ dissolved in 1 l of 0.3 *M* NaH_2PO_4; 500 μl of this buffer are added to 300 μl serum], peaks 7 and 9 disappear (Fig. 2). Since these two unnecessary substances are no longer extracted, the above-mentioned interference with phenobarbital is eliminated. The new buffer (pH = 3.9) elevates the recovery of primidone significantly (peak 1 in Figs. 1 and 2). The other drugs remain as previously reported [1].

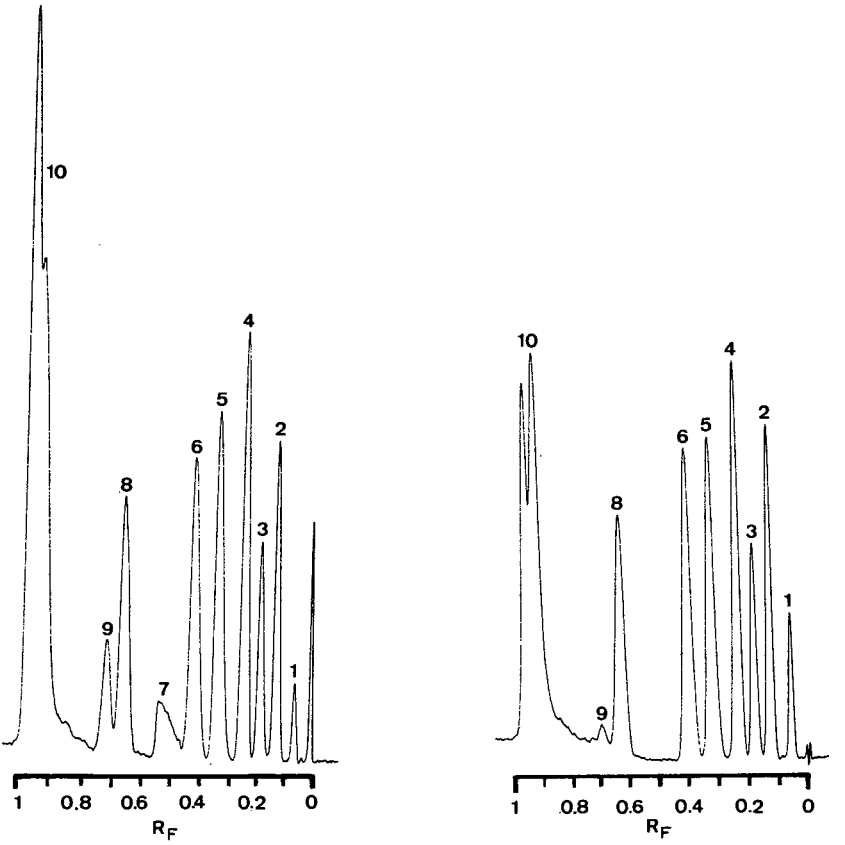

Fig. 1. Results obtained from a scan at 215 nm of a serum extract after using 0.3 *M* NaH_2PO_4 and TLC separation in chloroform—acetone (87:13). The serum contained 8.2 mg/l each of carbamazepine-10,11-epoxide (2), caffeine (3), carbamazepine (4) and 16.5 mg/l each of primidone (1), diphenylhydantoin (5), phenobarbital (6), mephenytoin (8). Peaks 7 and 9 are unidentified serum peaks 10 is the solvent front.

Fig. 2. Results obtained from a scan at 215 nm of an extract from the same serum and on the same TLC plate as in Fig. 1 after using 0.3 *M* NaH_2PO_4 which was almost saturated with $(NH_4)_2SO_4$.

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Book Review

Methods of Protein Separation, Vol. 2, edited by Nicholas Catsimpoolas, Plenum Press, New York, London, 1976, XVIII + 326 pp., price £18.60, US$ 35.40, ISBN 0-306-34602-8.

With increasing needs to isolate and characterize proteins of defined purity, the judicious selection of the most appropriate systems optimized for high resolution with good yields is all important. The past few years have witnessed dramatic improvements in available options for protein separations. This book, the second in the series, is a collection of essays reviewing the present state of the art of some chromatographic and electrophoretic systems. Of the four articles on chromatography, one deals with scanning gel chromatography, two with hydrophobic absorption chromatography and one on sievorptive chromatography. Three articles deal with theoretical and practical aspects of charge and size separations on polyacrylamide gel and one on electrophoresis in carrier free solution.

Ackers contributes a brief, but satisfying review of the potential applications of scanning gel chromatography in defining molecular parameters of macromolecules and in investigating interacting systems such as proteins with one another and with ligands or substrates. However, the article might have been improved by a short review of the theoretical background of gel chromatography.

Chrambach, Jovin, Svendsen and Rodbard review at length the theory and practical aspects of charge and size separations of proteins by electrophoresis in polyacrylamide gel. Understandably, though perhaps overoptimistically, they advance the technique as the universally applicable method of choice for obtaining information about the distribution of proteins in complex mixtures and of their size and charge characteristics. They offer many valuable practical considerations for optimizing this system, but unfortunately, give few examples to support their contention about the primary place of this method for analytical and preparative separations of proteins. Surprisingly, they make only passing reference to two dimensional electrophoretic systems which have recently proved so valuable in analysing complex protein mixtures. This article is followed by a useful comparison by Rodbard of the relative merits of gel filtration and gel electrophoresis for estimating molecular weights of proteins. The statistical analyses of R_F and molecular weight are particularly instructive and should be required reading for all those tempted to assign

absolute molecular-weight values to protein bands separated by SDS-polyacrylamide gel electrophoresis.

The articles of Hjerten, Hofstee and Kirkegaard on gel chromatography through hydrophobic and ion exchange interactions deal with potentially useful, but not well established, methods for fractionating macromolecules through various interactions with substituted agarose and dextran gels. Kirkegaard's discussion of macromolecules that interact separately or together with the gel matrix and can be differentially eluted by effecting their dissociation with buffer fronts of different mobilities seems promising but of limited applicability.

Individually, many of the articles are self sufficient, though the contents of some of those on gel chromatography overlap. These might have been better presented in a comprehensive manner in only one or two articles. As with many devotees of specialty techniques that have only limited applicability, some authors have succumbed to the temptation of overstating the potential of their pet technique for general application. The general quality of the hard backed book is good though some articles suffer from poor reproduction of close-typed Tables. It lacks a simple list of contents. The book will be a useful guide for graduate students and researchers in general biochemical areas.

Boston, Mass. (U.S.A.) J.W. DRYSDALE

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4 E. C. Horning, J.-P. Thenot and M. G. Horning, in A. P. De Leenheer and R. R. Roncucci (Editors), *Proc. 1st Int. Symp. Quantitative Mass Spectrometry in Life Sciences, Ghent, June 16–18, 1976*, Elsevier, Amsterdam, Oxford, New York, 1977, p. 1.

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