ISSN 0021-9673

VOL. 143 NO. 5 SEPTEMBER 1, 1977
(Biomedical Applications, Vol. 1, No. 5)

JOURNAL OF CHROMATOGRAPHY
BIOMEDICAL APPLICATIONS

ELSEVIER SCIENTIFIC PUBLISHING COMPANY
AMSTERDAM

PUBLICATION SCHEDULE FOR 1977

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

MONTH	J	F	M	A	M	J	J	A	S	O	N	D
Journal of Chromatography	130 131	132/1 132/2 132/3	133/1 133/2	134/1 134/2	135/1 135/2	136/1 136/2 136/3	137/1 137/2	138/1 138/2	139/1 139/2	140/1 140/2 140/3	142 144/1	144/2 144/3
Biomedical Applications	143/1		143/2		143/3		143/4		143/5		143/6	
Chromatographic Reviews				141/1				141/2				141/3

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Printed in The Netherlands

VOL. 143, No. 5 JOURNAL OF CHROMATOGRAPHY SEPTEMBER 1, 1977
(Biomedical Applications, Vol. 1, No. 5)

CONTENTS

Membrane Separation Processes

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

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

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

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

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P.O. Box 211, Amsterdam, The Netherlands

Distributor in the U.S.A. and Canada:
ELSEVIER/NORTH-HOLLAND, INC.,
52 Vanderbilt Ave., New York, N.Y. 10017

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Journal of Chromatography, 143 (1977) 425
Biomedical Applications

CHROMBIO. 073

Editorial

One of the most significant functions that chromatographic methods hold in clinical biochemistry is the profiling of body fluids. The review by E. Jellum published in this issue presents this topic most skilfully. The profiling approach often results in highly complex chromatograms, consisting of tens to hundreds of peaks. Many of these are still unknown, but are likely to be identified in the future. We are of the opinion that information on the biochemical composition of body fluids should be coordinated and regularly updated, and that this could become one of the goals of the new section of *Journal of Chromatography, Biomedical Applications*. We are greatly indebted to Dr. Egil Jellum who has offered to provide once a year, a list of normal compounds actually seen in the various chromatographic profiles of human body fluids.

We would like to invite the readers to submit any information on new compounds of biological fluids they succeed in identifying when working with gas chromatography and sooner or later also with high-performance liquid chromatography to:

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Rikshospitalet,
Oslo 1, Norway

KAREL MACEK

Journal of Chromatography, 143 (1977) 427—462
Biomedical Applications

CHROMBIO. 058

REVIEW

PROFILING OF HUMAN BODY FLUIDS IN HEALTHY AND DISEASED STATES USING GAS CHROMATOGRAPHY AND MASS SPECTROMETRY, WITH SPECIAL REFERENCE TO ORGANIC ACIDS

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(Received February 14th, 1977)

CONTENTS

1. INTRODUCTION

One of the trends in modern biomedicine is the increasing understanding that many, if not all, diseases may be linked in some way with deviations from, or alterations in, one or more of the several thousand chemical reactions that normally take place inside the cells and body. It does not seem unreasonable to assume that if one were able to identify and determine the concentrations of all compounds inside the human body, including both high- and low-molecular-weight substances, one would probably find that almost every known disease would result in characteristic changes in the biochemical composition of the cells and of the body fluids. Such a "total" analysis is, of course, impossible to carry out at present. However, the promising results achieved during the last few years have substantiated the importance of multicomponent analyses. Chromatographic profiling of the body fluids in particular has been useful both for diagnostic purposes and for obtaining new information about the biochemical reactions that take place inside the body in healthy and diseased states.

Most workers have favoured the use of gas chromatography (GC) for separation purposes, combined with mass spectrometry (MS) to identify the individual components in the body fluids. It is, however, important to bear in mind that GC and GC—MS can handle only volatile constituents and compounds that can be converted into volatile derivatives. Estimates have indicated that, because of this limitation, gas-phase analytical methods can only detect about 20% of the total number of substances present in complex biological materials [1,2]. The needs for alternative analytical methods are therefore obvious. High-performance liquid chromatography (HPLC) is considered to be one such alternative [3—6], although its methodology has not yet been

advanced to the stage where, e.g., mixtures of hundreds of different proteins, lipids, polysaccharides, nucleic acids, etc., can be separated. Recent advances, however, such as the computer-operated multi-wavelength array spectrometer detector [7,8], the coupling of HPLC to mass spectrometers [9—11] and new areas of application [12—14], indicate that HPLC will find its place beside GC—MS, where it is badly needed for multi-component analyses of high-molecular-weight and/or temperature-labile compounds. Isotachophoresis [15] and high-performance thin-layer chromatography (HPTLC) [16] may also become of considerable value in the analysis of body fluids in the future, as these techniques also have the potential of determining many of the widely different substances present in biological materials. Rapid-scanning fluorescence spectroscopy [17] and ion chromatography [18] are two recent techniques that might prove to be useful for the analysis of biological materials.

At present, however, it is clear that GC, and in particular GC—MS, are the major techniques for multi-compound analyses of the body fluids. Therefore, only these gas-phase methods will be considered here. The profiling approach is applicable to many different classes of biological compounds, from volatiles to complex carbohydrates, and several reviews have appeared recently. Volatiles were reviewed by Politzer et al. [19] and it is striking that the methodology has now been advanced to the stage where only 50—100 μl of body fluid is required for a complete recording of all of the volatiles present [20]. The scope of MS in clinical chemistry was reviewed by Roboz [2] and by Lawson [21], and the profiling approach in the study of microorganisms is considered in a recent book edited by Mitruka [22]. Comprehensive reviews on GC [23] and MS [24] discuss instrumentation, applications and experimental results.

The analyses of amino acids by paper (PC) and ion-exchange chromatography have proved over the last 25 years to be of great clinical value. In a similar manner, chromatographic profiling of organic acids in particular has become of diagnostic importance and has led to an improved understanding of many human disorders. It is therefore the purpose of this paper to review recent advances in organic acid profiling of human body fluids in healthy and diseased states.

2. RECENT ADVANCES IN INSTRUMENTATION PERTINENT TO CHROMATOGRAPHIC PROFILING

A. GC and combined GC—computer

Many modern GC instruments, available from over 500 manufacturers [23], are suitable for analyses of the widely different, often labile constituents of body fluids. All-glass systems seem to be preferred for this type of separation and packed GC columns with some type of silicone oil as the stationary phase (e.g., SE-30, OV-17, OV-22) have so far predominated. However, when the purchase of a new instrument is considered, many workers in the field now agree that the instrument of choice should be designed to handle glass capillary columns also, as these columns are becoming increasingly useful for profiling work. Excellent thermostable, wall-coated open-tubular glass capillary columns

suitable for the analysis of body fluids are now commercially available from several manufacturing companies. The glass capillary column not only offers greatly improved separation, but as the peaks are very sharp and as adsorption on the column wall is negligible, increased sensitivity is actually obtained. However, as was proposed in a round-table discussion on biomedical applications of GC—MS held during the 7th International Conference on Mass Spectrometry in Florence in 1976 [25], one should not advocate the view that packed columns are obsolete for the analysis of body fluids. Indeed, many problems, e.g., routine analyses of a given drug or of certain metabolites, can be carried out equally well on packed columns. Recent results (e.g., refs. 26—31), on the other hand, show that when one is dealing with *multi-component* analyses, where the aim is to separate and identify as many constituents in the body fluids as possible, then capillary columns are superior and give considerably more information.

Computerized GC has undergone major developments during the last 2—3 years. On-line mini- and micro-computer systems, ranging from programmable calculators to advanced time-shared systems, now offer the analyst a number of possibilities, such as adaptive program interaction, integrator/calculator functionality, instrument automation, automatic data acquisition and data reduction, real-time control of the sampling system, digital flow and temperature controllers, automatic peak detection, automatic baseline correction, calculation of retention indices, prediction of component resolution, detection and measurement of overlapping peaks and pattern recognition techniques (for references, see ref. 23). For example, a fully automated high-resolution GC system suitable for chromatographic profiling of the volatile constituents in body fluids was recently described [30]. It incorporates repetitive sampling on to a glass capillary column and simultaneous flame-ionization and nitrogen-sensitive detection prior to data acquisition and computer handling. Other sophisticated, automated and computerized GC systems have also been designed for studying volatiles (e.g., ref. 31) and for studying plasma lipid profiles [32]. Computerized GC has also proved useful for the chromatographic profiling of microorganisms [22].

Although automated high-resolution GC methods combined with powerful computer evaluation techniques are likely to find an increasing number of applications in the biomedical field, these methods cannot provide positive identification of individual GC peaks. Retention times alone are unfortunately not sufficient for identification purposes and should be used with caution when one is dealing with biological fluids. These materials, particularly urine, are so complex that even when highly efficient capillary columns with nearly 100,000 theoretical plates are used, many, if not most, of the GC peaks are still not resolved, but consist of a mixture of two or even more metabolites [25]. Much of the diagnostic value of chromatographic profiling ultimately depends on the identification of "disease-specific" compounds and it is therefore clear that methods in addition to GC must be sought. It is fully recognized to-day that MS at present is the best choice for provinding such information.

B. Combined GC—MS—computer

Excellent reviews on MS and on GC—MS—computer methods have been written, e.g., by Burlingame et al. [24, 33], Brooks and Middleditch [34], Horning et al. [35], Roboz [2], Lawson [21] and Politzer et al. [19] and, in addition, several books (e.g., refs. 22, 36—39) are available.

Both electron-impact ionization and chemical ionization techniques are now widely used. The availability of mini- and more recently micro-computers with suitable disc-oriented operating systems, graphic display units and electrostatic printer/plotter units as well as the continually increasing performance/cost ratios have greatly improved and facilitated the technique of MS [24]. Even the physical sizes of the equipment have changed recently, and a complete GC—MS system that can be placed on a laboratory bench-top is now commercially available.

It is now possible, both with quadrupole instruments and with the newer magnetic sector mass spectrometers, to carry out the fast scanning (about 1 sec) required for tandem operation with capillary columns, making the use of these high-resolution columns even more attractive. Selected ion monitoring (SIM), also called mass fragmentography and multiple-ion detection, has become completely computerized in recent years, i.e., software-operated in contrast with the traditional hardware-based systems (e.g., ref. 40). The high sensitivity and specificity of SIM is well recognized and this technique is now widely used for qualitative and particularly for quantitative analyses of a variety of endogenous metabolites and of exogenous compounds, e.g., drugs and drug metabolites, that occur in physiological fluids (for example, see the review by Falkner et al. [41] and the books cited in refs. 36—39, 42 and 43).

Some novel techniques of interest for multi-compound analysis include elemental composition chromatography (accurate mass chromatography) [24, 44], field ionization MS combined with computer techniques [45] and ultramicro-scale automatic MS analyses [46]. In the first of these methods, glass support-coated open tubular columns have been coupled to a high-resolution (dynamic resolution about 10,000) mass spectrometer—computer system. The scan cycle time is about 10 sec for the mass range 60—800 and several hundred high-resolution mass spectra can be obtained during a chromatographic run [44]. In the second method [45], extracts of the biological material (e.g., urine) are introduced into the mass spectrometer without prior chromatographic separation. The instrument is fitted with a heatable field ionization source. This type of ionization results in minimal fragmentation of the components in the mixture and therefore produces molecular weight profiles of the analysed samples. Using suitable computational methods, the system could readily differentiate between normal urine and urine from patients with infectious hepatitis [45].

The third system utilizes a mass spectrometer, coupled to automatic sample preparation devices and an electro-optical ion detector/computer system capable of detecting many ion species simultaneously [46].

Some recent advances in pyrolysis MS [47] are of interest. Thus, it is now possible to obtain reasonably reproducible "fingerprints" of complex biological samples such as whole cells, using either the Curie-point principle or the carbon dioxide laser beam for flash heating of the samples. This method is already

of value for characterizing multi-compound mixtures such as bacteria [22, 47].

During the last few years, much attention has been focused on the problem of interfacing liquid chromatography (LC) with MS. Several different systems are now in use, e.g., the method developed in Horning's laboratory [9], where a portion of the vaporized LC solvent is forced through an atmospheric-pressure ionization source [48]. In the system used by McLafferty et al. [10], about 1% of the LC effluent is introduced into the ion source of a chemical-ionization mass spectrometer. The vaporized solvent serves as the reagent gas for the sample. A third system utilizes a moving-wire or, more recently, a moving-belt interface. The set-up consists of an auxiliary vacuum chamber through which the wire or belt continually carries aliquots of the LC column effluent, and where the solvent is removed. The sample is further transported into the ion source of a quadrupole instrument where the final vaporization and ionization take place (e.g., ref. 11).

C. Library search of mass spectra and computer evaluation of chromatographic profiles

Apart from pattern recognition techniques that have been of great value in, for example, analyses of volatiles (see p. 430) and for bacterial classification [22], most of the work on profiling ultimately ends up with the problem of identifying certain peaks on the gas chromatogram. Manual interpretation of the corresponding mass spectra is often difficult and time consuming. The visual comparison of unknown spectra with a catalogue of known mass spectra often gives valuable clues with regard to the structure. The collection entitled "Mass Spectra of Compounds of Biological Interest", compiled by Markey et al. [49] and available through the National Technical Information Service, U.S. Department of Commerce, Springfield, Va. 22161, U.S.A., is very useful. The "Registry of Mass Spectral Data" [50], containing about 25,000 spectra, and the "Eight Peak Index" [51], which contains over 31,000 entries, are also valuable although most of the spectra in these collections relate to non-biological compounds. Many sophisticated computer programs, including interactive library searching, heuristic techniques and learning machine techniques, have been published [24, 52, 53]. At least two such search programs are now available over international computer networks. One system, McLafferty et al.'s self-training, interpretative and retrieval system for mass spectra (STIRS) [54], including probability based matching [55], is available through TYMNET [56]. The second system, developed by Heller at al. [57], is available through the General Electrics computer network, and in Scandinavia is available through SCANNET [58]. Several systems [59, 60] are now in operation for the rapid computerized identification of compounds in complex biological mixtures by GC—MS. The system used in Biemann's laboratory has been widely used for drug analyses [61] and the system developed by Sweedy et al. [60] has been used, for example, for the rapid characterization of urinary organic acids. Sjövall's group (e.g., ref. 62) has been used advanced computer systems for several years for the interpretation of steroid profiles. The system of Jellum et al. [63] has been designed to recognize anomalies in multi-compound mixtures, and is in principle able to detect the presence of abnormal com-

pounds as well as the lack of normal constituents. Although the first version of the program needs refinements, the approach has already proved useful [64].

In many instances the need for computer evaluation of the chromatographic profiles may seem unnecessary. This may be true when one is dealing with the diagnosis and detection of metabolic disorders that lead to gross metabolic changes. Other clinical conditions, however, may result in more subtle, yet perhaps important, changes in the metabolic patterns. It seems reasonable to assume that the best way to detect these minor alterations will be by means of advanced computer methods. For further discussion on computers and MS, see refs. 24, 52 and 53.

3. TREATMENT OF THE HUMAN BODY FLUIDS PRIOR TO CHROMATOGRAPHIC SEPARATION

A. Collection, storage and transport of specimens

Although increasing in number, there are still few clinical laboratories and hospitals that have at their disposal combined GC—MS—computer equipment. Many more laboratories, however, are equipped with simple gas chromatographs, and much useful screening of body fluids for abnormal metabolites can be carried out by GC alone. However, when a patient presents with an abnormal GC peak, the structure and identity of the substance concerned normally needs to be verified by MS and the sample can be submitted to a specialized laboratory. Transport is also necessary when clinicians have to rely on specialized laboratories for carrying out the complete chromatographic profiling. It is, of course, always important that the samples should undergo as little change as possible from the time of collection until they reach the place where the multi-component analyses are to be carried out. Recommendations for collection, storage and transport of physiological fluids for chromatographic profiling have been made [65]. Blood samples from fasting patients and urine collected in the morning appear to be preferred, and no preservatives should be added. The urine and serum samples should be frozen and stored at -20°C or preferably at an even lower temperature so as to prevent decomposition of certain metabolites, e.g., peptides [see 25]. When morning urine samples are analysed, quantitative results are usually expressed relative to the creatinine content. For more exact quantitative data, 24-h urine samples should be collected. Care must be taken to obtain information about drug intake and particular dietary habits [66—69]. Whenever unusual peaks are noted on gas chromatograms, especially in laboratories that lack GC—MS—computer facilities, it is a good rule to suspect the peaks of being drugs or drug metabolites [65]. This suspicion can sometimes be confirmed either by repeating the fluid collection from the patient at a later date when drug therapy has been discontinued, or by obtaining samples of physiological fluids from suitable control subjects who are receiving the same drugs as the patient. One should also realize, however, that many metabolic disorders are of intermittent types [70].

B. Isolation of organic acids from urine

Three principal methods for the isolation of the acidic constituents of urine prior to derivatization and GC are currently in use. The first is based on solvent extraction, usually with diethyl ether and/or ethyl acetate. Batchwise extractions using manual or mechanical shaking are normally carried out, although these procedures are not quantitative and polar compounds such as di- and tricarboxylic acids and polyhydroxy acids are poorly extracted [71]. More quantitative extraction is obtained by continuous extraction overnight, e.g., in a Soxhlet apparatus. Improved extraction is also obtained by saturating the sample with salts, e.g., sodium chloride before extraction. This treatment, however, precludes the use of the remaining aqueous phase for further analyses of, e.g., carbohydrates, amino acids and conjugates [1].

A good solvent should have a high volatility, a high solvent power with low water solubility and a high stability, and it should be commercially available in a high-purity analytical or chromatographic grade. In a recent investigation [72], isopropyl chloride was proposed as the solvent of choice for the extraction of volatile compounds in biological fluids. From the physicochemical characteristics (b.p. 35.7°, dielectric constant 9.82 (diethyl ether 4.3) and water solubility 1.3% (diethyl ether 6%), it appears that isopropyl cloride should also be suitable for the extraction of organic acids.

The second method for the isolation of the acidic constituents is based on anion exchange, as first suggested by Horning and Horning [73] and since used in many laboratories. Ion-exchange methods give quantitative recoveries of the organic acids in urine. Comparative studies on solvent-extraction and ion-exchange procedures have recently been carried out [71]. DEAE-Sephadex appears to be the most widely used ion-exchange material, although other resins, e.g., Dowex 3, are also suitable [74]. After capture on the resin, the acids are usually eluted with aqueous pyridinium acetate (e.g., refs. 66, 73) before lyophilization. In order to avoid any loss of the more volatile acids during the lyophilization step, it has been suggested that the column should be eluted with hydrochloric acid followed by neutralization of the eluate with sodium hydrogen carbonate, before lyophilization [75]. The direct silylation of the resulting sodium salts then follows [75, 76]. Thompson and Markey [71] introduced a clean-up step prior to ion exchange on DEAE-Sephadex. Their method involves removal of the interfering inorganic sulphate and phosphate as insoluble barium salts, followed by oxime formation, ion exchange, silylation and GC. Lawson et al. [66], however, warned that many organic acids are coprecipitated in this method, and that several organic acids would decompose when the alkaline barium hydroxide is added.

A third, more special, method, limited to the isolation of the more volatile constituents, makes use of either steam distillation [77—79] or vacuum distillation [80] to isolate the organic acids from the biological materials.

The problem of choosing between the solvent-extraction and anion-exchange methods often arises. It has been the experience of several groups that if the problem concerns screening for gross metabolic disorders, the detection of which does not require quantitative separation procedures, then solvent extraction is applicable. Even metabolic disorders that result in the excretion of

polar compounds such as pyroglutamic acid [81] or glutaric acid [82] are easily detected by solvent-extraction methods. On the other hand, very polar and highly water-soluble compounds such as tetronic and deoxytetronic acids are poorly extracted into organic solvents and may remain undetected unless ion-exchange methods are used. For quantitative work (e.g., refs. 66—68) and work involving subtle changes in the chromatographic profiles, the use of anion-exchange procedures is not only recommended, but is essential. Some laboratories therefore prefer always to use the quantitative methods, whereas others, particularly those engaged in diagnosis and studies of metabolic disorders, have found it convenient to be able to use both isolation procedures. Solvent extraction is used in emergency cases, e.g., on samples from severely ill acidotic children, in order to obtain a qualitative answer as soon as possible. Anion-exchange methods, which are more laborious and time consuming, are subsequently used for quantitative extraction and assay of the pathological metabolites.

C. Isolation of organic acids from protein-containing fluids

Urine is the only human body fluid that normally is devoid of proteins. All other body fluids (and tissue homogenates) contain small (in amniotic fluid, spinal fluid) or large amounts of protein (in serum, seminal fluid, synovial fluid, etc.). Urine, spinal fluid [83] and amniotic fluid [84] can therefore be extracted directly as discussed above, whereas most other physiological samples usually are handled so as to avoid interference by the proteins. If non-destructive methods (gel filtration, dialysis, membrane filtration) are used to remove the proteins prior to the GC analyses, the tightly protein-bound and/or water-insoluble low-molecular-weight substances (e.g., long-chain fatty acids) will be lost. Several organic acids have a tendency to become more or less protein bound, indicating that removal of protein by non-denaturing methods should be used with caution if the aim is to obtain a total organic acid profile. Other classes of metabolites, e.g., carbohydrates and amino acids, are not protein bound, and membrane filtration through a Millipore PSAC filter, for example, yields a protein-free eluate that is ideally suited for, e.g., carbohydrate profiling by GC methods [29]. Protein precipitation with, e.g., sulphosalicylic acid followed by extraction of the organic acids by organic solvents is sometimes used, although certain organic acids may be coprecipitated with the denatured protein. Direct extraction of serum, e.g., with the ion—solvent pair ammonium carbonate—ethyl acetate, is widely used for the extraction of drugs, but this method obviously does not extract the organic acids.

A convenient method for obtaining profiles of total organic acids in protein-containing body fluids and tissue homogenates makes use of ethanol as a precipitating agent (e.g., refs. 1, 2, 85 and 86). After removal of the proteins by centrifugation and removal of the ethanol in vacuo, the resulting aqueous solution can be treated as if it were urine.

Fatty acids and lipids are usually extracted by special methods, e.g., Folch extraction. The literature on the analyses of these compounds in biological fluids is vast. Procedures for their extraction and analysis by GC and GC—MS

methods can be found, for example, in three issues of Journal of Chromatographic Science (Sept. 1975, Oct. 1975 and Jan. 1976) devoted to this topic. Packed GC columns containing liquid phases such as DEGS, DEGA, EGSS-X, Silar 5C and 10C or Apolar 10C, are used extensively for the separation of fatty acids, usually as methyl esters. Methods for the separation of underivatized fatty acids have also been published [87, 88]. Highly efficient glass capillary columns are used to separate *cis* and *trans* isomers [89]. A review on the analysis of lipids in general has recently appeared [90].

4. DERIVATIZATION OF THE ORGANIC ACIDS

The "organic acid fraction" of biological fluids contains mono- and polycarboxylic acids, mono- and polyhydroxy acids, keto acids, phenols, phenolic acids and conjugates of the organic acids, particularly with glycine. Numerous publications have dealt with methods for preparing volatile derivatives of the different organic acids and the most widely used approches are discussed below.

A. Silylation, oximation

Trimethylsilyl (TMS) ethers and esters are probably the most popular derivatives used for studying the organic acids (e.g., ref. 85). TMS derivatives are comparatively easy to prepare, safe to handle and most of them have excellent chromatographic properties. Further, deuterium-labelled TMS derivatives can readily be prepared with commercially available silylating reagents. The latter approach is frequently of great value in elucidating the structures of unknown metabolites by GC—MS. The collections of mass spectral data (see p. 432) contain more information on TMS derivatives than on any other type of derivative. The most widely used silylating reagents are bis(trimethylsilyl)trifluoroacetamide (BSTFA) and bis(trimethylsilyl)acetamide (BSA) (with or without pyridine, trimethylchlorosilane or other catalysts), which form derivatives with the carboxyl groups, hydroxyl groups and phenol groups of organic acids. Sodium salts are generally more difficult to silylate than the free acids, although recent investigations [75, 76] have shown that BSA in the presence of trimethylchlorosilane and/or hydroxylamine converts the sodium salts directly into volatile TMS derivatives of the organic acids. Certain metabolites, particularly keto acids, have a tendency to yield multiple derivatives, and there are problems also with the silylation of short-chain dicarboxylic acids [74] and with *o*-hydroxyhippuric acid (salicyluric acid [91]). In both instances the corresponding methyl esters appear to be better. Previously it was observed that N-acylglycines also gave multiple peaks after trimethylsilylation, but this effect can now be avoided [92]. The GC behaviour of 20 N-acylglycines of clinical interest is described in refs. 92 and 93. Keto acids are more readily determined if double derivatives are prepared, particularly oxime—TMS [94], methoxime—TMS [95] and ethoxime—TMS derivatives [66]. Other O-substituted oxime—TMS derivatives, e.g., benzyloximes, have also been considered by Lawson et al. [96] although they prefer ethoxime—TMS derivatives for the determination of urinary organic acids in general [66—68]. Alipathic 2-keto acids can also be determined with *o*-phenylenediamine and BSTFA, yielding

O-TMS—quinoxalinol derivatives that are suitable for GC analysis [97]. The separation of the many biologically occurring keto acids is best acieved by using highly efficient glass capillary columns [98]. Combined silylation and methylation also yields derivatives with excellent chromatographic properties, and this procedure has been successfully for many years by Horning and co-workers (e.g. refs. 35 and 95).

Combined silylation and ethylation has been the preferred derivatization method in studies on the simultaneous determination by selected ion monitoring of the levels of four acid metabolites of catecholamines in urine, serum and cerebrospinal fluid [99].

B. Methylation

The methyl esters of organic acids also have excellent chromatographic properties and can readily be prepared. The mass spectra of methyl esters are usually simple, and are often easier to interpret than those of the corresponding TMS derivatives. Usually it is therefore easier to predict the fragmentation pattern of a methyl ester than that of a silyl ester/ether. This is frequently very useful in profiling work, because on the basis of a predicted mass spectrum one is often able, using mass chromatography, to search for the presence in a body fluid of a given metabolite, even if the authentic compound and/or information on its GC—MS behaviour are lacking.

In several laboratories, therefore, (e.g., refs. 1, 85, 100—102) methylation techniques are frequently used. Despite the hazards associated with diazomethane, it still appears to be the preferred methylating agent, the method being smooth, simple and rapid. This method, like silylating procedures, may also lead to certain by-products, artifacts and unexpected results. For example, more than one derivative is usually formed by the action of diazomethane on keto acids; pyrazolines are formed as by-products during the methylation of fumaric and aconitic acids; and an artifact resembling 3-hydroxypropionic acid is formed if water is present during the methylation [104]. Diazomethane reacts not only with carboxyl groups, but also methylates the hydroxyl groups of phenols and the thiol group of thiols. Thus, a number of important phenolic acids, e.g., *p*-hydroxyphenyllactic acid, may yield one or two derivatives depending on the temperature and length of treatment with diazomethane. For further discussion on this topic and on other artifacts and pitfalls associated with the chromatographic profiling of body fluids, see p. 439 and refs. 103, 104.

Alternative methylation procedures include esterification with methanol/hydrochloric acid, methanol/thionyl chloride or methanol/boron trifluoride and on-column methylation [105]. In the last method, the organic acids are converted into their trimethylanilinium salts by the addition of trimethylanilinium hydroxide, and subsequent pyrolysis of the salts in the injection port of the gas chromatograph generates the methyl esters [105, 106]. This method, combined with neopentylidene protection of the amino groups, is also suitable for the rapid derivatization and analysis of amino acids by GC [107].

Selective esterification of the carboxyl group can be achieved by the classical

method of treating the organic acids with alcohol in the presence of an acidic catalyst. Subsequent removal of excess of alcohol and acid before injection into the gas chromatograph often results in loss of the more volatile esters.

An alternative method for the selective derivatization of the carboxyl group involves addition of silver nitrate to an aqueous extract of the organic acids, followed by lyophilization and treatment of the dry silver salts with ethyl iodide (or methyl iodide) dissolved in pentane [108, 109]. The ethyl (or methyl) esters will be formed in the pentane solution, which can be injected directly on to, e.g., a capillary GC column.

The relative merits of the various approaches considered in this chapter are frequently discussed. There is no simple answer to the problem, as all methods, including solvent extraction, ion exchange, trimethylsilylation and methylation. have their advantages and disadvantages. Most workers, however, will agree with what Stokke [110] pointed out in a recent editorial, namely that the main point is to become familiar with one method, and to learn its pitfalls and to be able to use alternative procedures when required.

5. SPECIAL METHODS FOR SHORT-CHAIN VOLATILE ACIDS

Short-chain (C_2—C_8) monocarboxylic acids are volatile and therefore require no derivatization. Special methods for handling these acids, several of which are associated with metabolic disorders, have been published. In one of these [77—79] the volatile acids are separated after steam distillation (on a column containing neopentyl glycol adipate—orthophosphoric acid), or after vacuum distillation [80]. Other methods (e.g., refs. 111—113) utilize solvent extraction followed by separation on columns containing, e.g., Porapak [111] or FFAP (Carbowax 20M—nitroterephthalic acid [113]). In another method [114], the urine is mixed with Dowex 50-H^+ and the free acids released are separated on the porous polymer Chromosorb 105. In the method of Remesy and Demigne [115], as used by Wysocki et al. [116], the acids are separated on a column packed with 10% SP-1200 containing 1% of orthophosphoric acid on Chromosorb W. Special GC methods have been devised for the determination of acetate [117] and for propionic and methylmalonic acids [118].

The GC determination of the latter two acids in the presence of each other requires special care, as a portion of the free methylmalonic acid may become decarboxylated to yield propionic acid in the injection port of the gas chromatograph (e.g., ref. 119). The finding of large amounts of propionic acid in, e.g., the urine of patients with methylmalonic aciduria, may therfore be misleading. Possibly the simplest means of overcoming this problem is to use two different temperatures on the inlet block of the gas chromatograph, as suggested by Frenkel and Kitchens [118]. Their method is based on the spontaneous and quantitative decarboxylation of methylmalonic acid at 225° to give propionic acid. By utilizing another substituted malonic acid (ethylmalonic acid) as an internal standard, accurate quantitation is possible. Endogenous propionic acid is then measured at 130°, a temperature at which methylmalonic acid does not decarboxylate [118]. The use of ethylmalonic acid as an internal standard may be questioned, as it has been described as a normal con-

stituent of human urine (although present in small amounts) [120] and may occur in considerable amounts in, for example, patients with Jamaican vomiting sickness (p. 450) or with glutaric aciduria, type II (see Table 1). An alternative procedure was used by Stokke et al. [121] in their investigation of a patient with combined methylmalonic acidemia and β-hydroxy-*n*-valeric acidemia. Propionic and methylmalonic acids were first separated on buffered TLC plates so as to avoid loss of the volatile acids. The bands containing the two acids (as sodium salts) were subsequently analysed separately by GC (propionic acid as the free acid, methylmalonic acid as the methyl ester).

6. ARTIFACTS AND PITFALLS

The application of GC and GC—MS—computer methods to the analysis of body fluids is associated with many difficulties and pitfalls. There are problems of a technological nature (instrument breakdown), of a chemical nature (e.g., contaminated solvents, contamination from column bleeding and environmental contamination such as plasticizers), and problems related to the handling of the biological samples. Spiteller and Spiteller discussed several of these aspects in their book [122], which also contains the mass spectra of numerous commonly occurring contaminants. Plasticizers in biological materials have also been studied by other workers (e.g., refs. 123 and 124). Perry and Hansen [103] and more recently Jellum et al. [104] have reviewed the most usual pitfalls associated with the chromatographic profiling of biological materials using GC and GC—MS methods. Firstly, there are problems with collection and storage of the samples. Depending on the container used, contamination may occur from chemicals not only from plasticizers but also from rubber stoppers [104, 125], added preservatives and added anticoagulants [103, 104] (e.g., heparin contains benzyl alcohol as a stabiliser; see also ref. 126). Secondly, numerous problems exist with sample work-up and with the derivatization methods, e.g., formation of artifacts and multiple derivatives. The production of artifacts (e.g., crotonic acid formed from β-hydroxybutyric acid [127]) and the formation of unexpected compounds in the GC column (e.g., 5-hydroxycoumaran from homogentisic acid [104] and 3-methylcrotonic acid from 3-hydroxyvaleric acid [128]) may lead to serious errors. Decarboxylation reactions (e.g., of methylmalonic acid to yield propionic acid (p. 438) and of phenylpyruvic acid to yield phenylacetic acid [129]) during sample work-up and GC may lead to erroneous results. New compounds formed by trans-esterification processes [130] and artifacts produced by bacteria (e.g., benzoic acid [131, 132]) are potential sources of error. In methods where the biological material is made alkaline to prevent loss of volatile acids, one should realize that part of the glucose may be converted into acetate [133]. Perhaps the most serious problems arise from artifacts due to dietary factors and intake of drugs (e.g., refs. 103 and 104). Particular attention should be paid to drugs that are metabolized to give organic acids that one normally associates with metabolic disorders. An example is the anticonvulsant sodium dipropylacetate, which leads to increased urinary excretion of propionic acid [134], 2-oxodipropylacetic acid and 2-(*n*-propyl)glutaric

acid [135]. In general, many problems can be avoided if correct and complete information about drug intake always accompanies the samples submitted for chromatographic profiling. (See also a recent review on difficulties and pitfalls in the interpretation of screening tests for the detection of inborn errors of metabolism [136].)

7. NORMAL ORGANIC ACID PROFILES OF VARIOUS BODY FLUIDS

A. Normal patterns in urine from adults and newborns

A table of the urinary organic acids identified prior to 1973 was compiled by Markey et al. [137]. Since then, many new metabolites have been identified by GS—MS methods. More recent lists of the prevailing urinary acidic metabolites in normal adults [66, 138] and in newborns [139] have been reported. The list of Björkman et al. [139], showing the qualitative and quantitative pattern of organic acids in urine from newborns, also contains the characteristic mass spectral fragments of each compound. Comparison of the organic acids in adults and newborns shows that the latter urine contains large amounts of succinic, fumaric, 2-ketoglutaric and 3-hydroxy-3-methylglutaric acids. These acids are present in only small amounts in adults. Hippuric acid, on the other hand, is a minor component in urine from newborns and a major constituent of adult urine. Knights et al. [138], using glass capillary columns and MS, identified over 30 of the peaks in the chromatogram. They examined pooled urine specimens from controls and post-partum subjects; no major differences were observed.

B. Normal patterns in other body fluids and tissues

Most papers have dealt with urine, and less information exists on the organic acid composition of other body fluids. *Amniotic fluid*, at a mean gestational age of 17.6 weeks (range 15—20 weeks), contains lactate as the dominating water-soluble acid and smaller amounts of pyruvate, 3-hydroxy-butyrate, succinate, 2-oxoglutarate, citrate, 2-ketoisovalerate, 2-ketoisocaproate, malate and *p*-hydroxybenzoate [84]. Cholesterol [140] and fatty acids, the determination of which is important in connection with respiratory distress syndrome, are present (see p. 451). 2-Hydroxybutyrate is also found [141] in amniotic fluid, probably as a consequence of the high lactate content (see p. 445). The concentration of 2-hydroxybutyric acid in this fluid is about 100 times lower than that of the predominant acid, lactic acid.

Few normal organic acid profiles of *serum* have been published. In general, the fatty acids dominate whereas the water-soluble organic acids are present in small amounts owing to high renal clearance. Many organic acid profiles of sera from patients with metabolic disease have been reported (see Table 1).

Cerebrospinal fluid (CSF) contains, apart from the fatty acids (e.g., ref. 142), various other metabolites such as 3-methoxy-4-hydroxyphenylethanol, 5-hydroxyindoleacetate, citrate, homovanillate, 3,4-dihydroxyphenylacetate, 3,4-dihydroxyphenylethanol and 3-methoxy-4-hydroxyphenyl glycol [83]. Catecholamine metabolites are now frequently assayed in CSF by means of

selected ion monitoring with stable isotope-labelled compounds as internal standards. Several tryptophan metabolites in CSF have been determined by single-ion monitoring [143]. Lactate, succinate, citrate and maleate are normal constituents in CSF from newborns. Variations in the chromatographic profile of neutral and acidic metabolites in CSF from newborns and infants with different neurological disorders have been reported [144]. Further work, however, is required in order to determine whether the alterations seen are sufficiently specific to be used as major diagnostic criteria [144].

Capillary GC—MS methods have been utilized to evaluate the organic acid profile in *human saliva* [145]. Lactic, 2-hydroxyisocaproic, succinic and phenylacetic acids, 2,6-dibutylcresol (an antioxidant present in food), phenyllactic, *p*-hydroxyphenylacetic and *p*-hydroxyphenylpropionic acids, several fatty acids and cholesterol were identified in unstimulated saliva.

Seminal fluid also contains several organic acids of the type found in urine, with citric acid and fatty acids as dominating compounds (Jellum, unpublished results). *Synovial fluid* has been examined with both packed [146] and capillary columns [147].

Plasma from haemodialysis patients has been profiled with respect to volatiles [148]. *Dialysis fluid* from nephrectomized patients [149] contains organic acids of the same type as normally found in urine, plus additional compounds associated with uremia.

Little information on organic acid profiles of tissues exists, whereas certain other classes of compounds have been determined by GC methods. Thus, the volatile constituents of lung, brain and liver from rats have been assessed. There were marked differences, and it appears that certain volatile constituents may be characteristic of a particular tissue [150]. The volatiles that produce the odours of the human vagina [151], the amino acid composition of human nails [152] and the content of polyols and aldoses in cataractous human lens tissue [153] have recently been measured by GC and MS methods. Snedden and Parker [154] investigated the presence of altered purine metabolites in skeletal muscle of normal and gouty individuals before and after allopurinol therapy.

With the advent of glass capillary columns, increased sensitivity of the instruments and improved computer handling of the data, it is to be expected that the analysis of small (1—3 mg) tissue biopsies will become feasible and important in the future.

C. Identification of new normal metabolites

Lawson et al. [66] have identified some new polyhydroxy (aldonic and deoxyaldonic) acids in addition to those previously reported by Horning and Horning [73]. Thompson et al. [155] also reported on the occurrence of polyhydroxy compounds in urine and identified 4-deoxyerythronic, 4-deoxythreonic and 2-methylglyceric acids [155] as new normal metabolites. Fell et al. [156] determined 2-deoxytetronic acid in blood.

Many dicarboxylic acids have been identified as constituents of normal urine. Petterson and Stokke [157] found that a series of 3-methyl branched short-chain (C_4—C_8) dicarboxylic acids are excreted in small amounts, except

3-methyladipic acid, which often is present in considerable amounts (0.1—0.2 mmole per 24 h) in urine. Lindstedt et al. [158] have shown that 3,4-methylenehexanedioic acid (cyclopropaneadipic acid) is a normal urinary constituent (10 mg excreted per 24 h). They also identified an acetylenic compound (5-decynedioic acid) [159] and a series of *cis*- and *trans*-mono-unsaturated aliphatic dicarboxylic acids in normal urine [160]. Considerable amounts of cyclopropaneadipic acid have been found in urine after the intake of a certain cheese [104].

The presence of various furan-containing carboxylic acids in urine is well established. These metabolites appear to be of dietary origin [161]. Certain fructose-containing solutions designed for intravenous feeding may contain considerable amounts of furan derivatives, formed during heat-sterilization of the fructose solution. Patients receiving such mixtures will excrete large amounts of furan mono- and dicarboxylic acids in their urine [162].

Mamer and Tjoa identified 2-ethylhydracrylic acid in urine [163]. The compound stems from the degradation of isoleucine.

Duncan et al. [164] noted that in most of the GC profiles obtained on methylation of human urinary extracts, a peak, sometimes large and sometimes small, appeared immediately after hippuric acid (methyl ester). GC—MS and nuclear magnetic resonance (NMR) spectroscopy provided conclusive evidence that this peak was due to β-(*m*-hydroxyphenyl)hydracrylic acid. This compound was first identified in human urine in 1957 by two-dimensional paper chromatography [165] and is of dietary origin. Wadman et al. [166] have reported that β-(*p*-hydroxyphenyl)hydracrylic acid was present in the urine from a patient with gastrointestinal disease. The *para*-isomer is apparently not a normal urinary constituent.

Human urine also contains highly variable amounts (from non-detectable to a peak comparable in size to that of hippuric acid) of 4-hydroxycyclohexane-1-carboxylic acid of unknown origin [167]. The TMS derivative of this compound has a molecular weight of 288. An unknown compound with the same molecular weight was recently found in a patient with α-ketoadipic aciduria [168] and was tentatively suggested to be 1,2-butenedicarboxylic acid. As the retention times of this compound and of the newly recognized cyclohexanecarboxylic acid are similar, Bindel et al. [167] suggested that Przyrembel et al. [168] may in fact have isolated the cyclohexane compound from their patient.

Nearly all of the compounds mentioned above were separated on packed GC columns. A normal organic acid profile on such columns yields about 40—80 peaks. If the separation is carried out instead on modern glass capillary columns, at least three times as many peaks are seen [64]. Most of these peaks are still unidentified. In the future, the identification of a wide range of further metabolites should become possible.

D. Quantitative ranges and effects of individual variation and diet on the chromatographic profiles

Factors that influence the chemical composition of human body fluids have been the subject of numerous investigations in the last few decades. A

few years ago, Young and co-workers [169, 170] reported on the effects of a chemically defined diet on a wide range of urinary and serum metabolites. Witten et al. [171] also used a palatable standard diet and measured the means and standard deviations of the excretion rates of individual urinary organic acids, using GC—MS methods. In the latest and most comprehensive studies carried out by Chalmers and co-workers [66—68], both the effects of individual variations and of diet were studied. All three research groups observed that the coefficient of variaton for the excretion of the major and consistently excreted metabolites were large for all of the subjects studied. Extreme dietary alterations produced relatively small changes in the patterns or amounts of metabolites excreted, but large individual within-subject variations were observed [67]. The data of Chalmers et al. [67] therefore indicate that variations in the ranges of excretion depend mainly on individual metabolic alterations rather than on dietary factors. In a survey of 420 normal subjects on an unrestricted diet, quantitative ranges and frequency distribution patterns of the urinary organic acids were assessed [68]. Histograms of excretion values were prepared for all metabolites and could be allocated to four groups: (a) unimodal distributions with detectable values in almost all subjects (examples: tetronic, 2- and 4-deoxytetronic, citric and *cis*-aconitic acids); (b) apparently unimodal distributions with a number of values below the level of detectability (examples: *p*-hydroxyphenylacetic, glucaric and 3-deoxytetronic acids); (c) clearly bimodal distributions, a unimodal sub-group + a block of undetectable values (examples: 3-hydroxyisovaleric, hippuric and glucuronic acids); and (d) irregular distributions with a majority of undetectable values (examples: benzoic, succinic and 2-ketoglutaric acids).

It is important to bear in mind results such as those referred to above when discussing "pathological amounts" of normal metabolites present in healthy and diseased persons.

8. APPLICATION OF GC—MS PROFILING TO DISCOVER NEW METABOLIC DISORDERS

Widespread amino acid screening programmes in the 1950s and early 1960s using paper and ion-exchange chromatography resulted in the discovery of numerous amino acidemias and amino acidurias. In 1963, Klenk and Kahlke [172] used GC and MS methods to separate and identify an unknown metabolite (phytanic acid) present in a patient who had died of Refsum's disease [173]. Since then, gas-phase analytical methods have become increasingly important for diagnosis and studies of metabolic disorders. Chromatographic profiling in general is a complex, time-consuming, highly specialized and expensive technique that is not suitable for mass screening. Apart from research tasks, these methods are therefore usually applied to patients who are particularly suspected of having a metabolic disease, rather than to large population groups. In general, the signs and symptoms to look for include peculiar smells from the body and body fluids of the patients, a hereditary history of similar diseases in the family, lasting metabolic acidoses, mental retardation, failure to thrive, disturbances in pigment development, severe vomiting in early life and involuntary movements [174—176]. Major deviations in the ex-

cretion of end-products (e.g., urea) of the various metabolic pathways, and disagreement between the sums of the amounts of cations (Na^+, K^+, Mg^{2+}, Ca^{2+}, etc.) and anions (Cl^-, SO_4^{2-}, PO_4^{3-}, etc.) in a body fluid (e.g., an "anion gap"), are signals to be aware of.

Chromatographic profiling using GC and GC—MS have resulted, up to early 1977, in the discovery of 23 different, new diseases. In addition, about 50—60 previously described inborn errors can also conveniently be diagnosed by means of GC—MS techniques. In Table 1 the new metabolic disorders are listed, together with the characteristic (disease-specific) compounds that occur in each instance.

In general the diseases shown in Table 1 were recognized because of the occurrence of pathological metabolites, identifiable by means of GC—MS. Subsequent biochemical investigations, such as enzyme studies on biopsies, on cells grown in tissue culture, metabolic studies using stable and/or radioactive isotopes, dietary studies and loading experiments, are required in order to pin-point and, if possible, to treat the metabolic defect. Studies of this character, and the finding of additional cases with the same disorders as listed in Table 1, have been described in numerous publications. In many of these studies, GC and MS were utilized, e.g., to follow changes in the profiles after a loading test. In 1974, Gompertz [177] reviewed inborn errors of organic acid metabolism, and a year later Tanaka [178] prepared an even more comprehensive chapter (with 264 references) on the same topic (disorders of organic acid metabolism). These two excellent publications review in detail what is known about the biochemistry of many of the disorders listed in Table 1, and consider both the first patients and the additional cases that have been reported. Therefore, no attempt is made in this review to go into details about the biochemical defects behind the diseases shown in Table 1.

9. APPLICATION OF GC—MS PROFILING TO INVESTIGATE KNOWN METABOLIC DISORDERS AND OTHER HUMAN DISEASES

Profiling techniques using GC and MS have been used extensively not only to study the new disorders shown in Table 1 (see also refs. 177, 178), but also to diagnose and to obtain more biochemical information on several other previously known metabolic errors and other defined clinical conditions. In books edited by Stanbury et al. [70], Nyhan [205], Hommes and Van den Berg [206] and particularly in refs. 1, 176 and 207 are tabulated numerous inborn errors that can be diagnosed by means of gas-phase analytical methods. Approximately half of the 200 metabolic disorders that are recognized today can be studied by such methods. These techniques are of considerable value for confirming the diagnosis of patients suffering from any one of these diseases. The GC—MS methods are therefore used routinely in several specialized hospital laboratories and other institutions for the examination of specimens from patients suspected of suffering from metabolic disease.

Some of the diseases and clinical conditions that have recently been studied by means of GC—MS techniques are discussed below.

A. Ketoacidosis

This clinical condition has many causes and has been subjected to numerous investigations by means of chromatographic profiling techniques. The volatiles in the urine and breath from patients with diabetic ketosis have been separated and identified, e.g., by Zlatkis et al. [208] and Liebich and Al-Babbili [209] (for a review of volatiles, see ref. 19). Pettersen and co-workers [157, 210—212] and Landaas [213—215] focused their attention on the organic acids. It was found that ketoacidosis leads to increased excretion of adipic and suberic acids [210], whereas the levels of several 3-methyl branched-chain dicarboxylic acids, previously not recognized as normal constituents of human urine [157], were not raised significantly during ketosis. In uncorrected juvenile diabetics there were considerable amounts of adipic and suberic acids, which disappeared on insulin therapy [211]. The formation of the C_6 and C_8 dicarboxylic acids involved an initial omega-oxidation of long-chain fatty acids followed by beta-oxidation [212]. Landaas has shown that ketoacidosis also results in enhanced excretion of 3-hydroxyisovaleric acid [213] and of 3-hydroxyisobutyric and 2-methyl-3-hydroxybutyric acids [214]. These new metabolites stem from the degradation of leucine, valine and isoleucine and accumulate in ketoacidosis because acetoacetate and 3-hydroxybutyrate impair their further metabolic breakdown [215].

B. Lactic acidosis

This condition is known to accompany anaerobic metabolism and is also known to be associated with many diseases (e.g., glucose-6-phosphatase deficiency, fructose-diphosphatase deficiency, methylmalonic acidemia and pyruvate carboxylase and dehydrogenase deficiencies (see review by Tanaka [178] and a recent article [216] describing lactic acidosis in three siblings due to a double enzyme defect). GC—MS has been used to investigate many cases of lactic acidosis and Landaas and Pettersen [217, 218] showed that there is a close relationship between the urinary excretion of 2-hydroxybutyric acid and the occurence of lactic acidosis. The main factor responsible for the increased formation of 2-hydroxybutyric acid appears to be an increased $NADH_2/NAD$ ratio [218] (2-hydroxybutyrate, incidently, is a metabolite also seen in oast-house disease or methionine malabsorption syndrome, and is a normal constituent of amniotic fluid [141]).

C. Maple syrup urine disease (MSUD)

MSUD of various types and degrees of severity is one of the best known and most widely studied metabolic disorders, and has been extensively examined by means of paper chromatography and ion-exchange techniques. Chromatographic profiling using GC—MS methods [79, 97, 98, 219] has yielded additional information on the disease. For instance, clinical attacks result not only in increased excretion of the branched-chain 2-keto and 2-hydroxy acids, but also give rise to an accumulation of all of the metabolites (see above) associated with ketoacidosis and lactic acidosis. Altogether 15

TABLE 1

NEW METABOLIC DISORDERS DISCOVERED BY CHROMATOGRAPHIC PROFILING OF BODY FLUIDS USING GC AND/OR MS METHODS

Disorder	Disease-specific metabolites detected by GC—MS	References*
Refsum's disease** (phytanic acid storage disease)	Phytanic acid	Klenk and Kahlke, 1963 [172]; Eldjarn, 1965 [179]; Steinberg et al., 1965 [180]
Isovaleric acidemia	Isovaleric acid, isovalerylglycine, 3-hydroxyisovaleric acid	Tanaka et al., 1966 [77]
Methylmalonic aciduria Type I Type II Type III Type IV	Methylmalonic and propionic acids, homocystine and occasionally methylcitric acid	Oberholtzer et al., 1967 [181]; Stokke et al., 1967 [182]; Levy et al., 1970 [183]; Ando et al. 1972 [184]
Type IV? Methylmalonic and 3-hydroxy-*n*-valeric acidemia	Methylmalonic, propionic, 3-hydroxy-*n*-valeric and odd-chain fatty acids	Stokke et al., 1973 [121]
Propionic acidemia Type I: biotin-unresponsive Type II: biotin-responsive***	Propionic, 3-hydroxypropionic, methylcitric, 3-hydroxy-3-methylglutaric and 3-hydroxy-butyric acids and propionylglycine	Hommes et al., 1968 [78]; Gompertz et al., 1970 [185] Barnes et al., 1970 [186]
3-Methylcrotonyl-CoA carboxylase deficiency Type I: Biotin-unresponsive	3-Methylcrotonylglycine, 3-hydroxyisovaleric acid	Eldjarn et al., 1970 [187]
Type II: Biotin-responsive***	3-Methylcrotonylglycine, tiglylglycine, 3-hydroxyisovaleric, 3-methylcrotonic, methylcitric and 3-hydroxypropionic acids	Gompertz et al., 1971 [188]; Chalmers et al., 1974 [189]
Type III: ?	2-Oxoglutaric and 3-hydroxyisovaleric acids, not 3-methylcrotonylglycine	Finnie et al., 1976 [190]
Pyroglutamic aciduria (5-oxoprolinuria, glutathione synthetase deficiency)	Pyroglutamic acid (2-oxoproline)	Jellum et al., 1970 [81]; Hagenfeldt et al., 1974 [191]

Acyl CoA-dehydrogenase deficiency? (congenital dicarboxylic aciduria)	Saturated C_8—C_{14} dicarboxylic acids, C_{10}—C_{14} *cis*-5-monounsaturated dicarboxylic acids and C_{10} and C_{12} *trans*-3-monounsaturated dicarboxylic acids	Borg et al., 1972 [192]; Lindstedt et al., 1976 [160]
2-Methylacetoacetic and 2-methyl-3-hydroxybutyric aciduria	2-Methylacetoacetic, 2-methyl-3-hydroxybutyric acids and tiglylglycine	Daum et al., 1973 [193]; Hillman et al., 1972 [194]
D-Glyceric acidemia		
Type I	D-Glyceric acid and glycine	Brandt et al., 1974, 1976 [195—197]
Type II	D-Glyceric acid	Wadman et al., 1975 [198]
2-Ketoadipic aciduria	2-Ketoadipic, 2-hydroxyadipic and 2-aminoadipic acids	Wilson et al., 1975 [199]; Przyrembel et al., 1975 [168]
Glutaric aciduria		
Type I	Glutaric, 3-hydroxyglutaric and glutaconic acids	Goodman et al., 1975 [82]; Gregersen et al., 1977 [202]
Type II	Glutaric, (no glutaconic), lactic, isobutyric, isovaleric, propionic, 2-methylbutyric, ethylmalonic and 3-hydroxybutyric acids and several metabolites associated with lactic- and keto-acidoses	Przyrembel et al., 1976 [200]
Carnitine deficiency	Adipic, pimelic and suberic acids	Kapati et al., 1975 [201]
Non-ketotic dicarboxylic aciduria (carnitine deficiency?)	Adipic, suberic and sebacic acids and suberylglycine	Gregersen et al., 1976 [100]
3-Hydroxy-3-methylglutaric aciduria	3-Hydroxy-3-methylglutaric, 3-methylglutaconic, 3-hydroxyisovaleric and 3-methylglutaric acids (not 3-methylcrotonic acid [116, 128])	Faull et al., 1976 [203]; Wysocki et al., 1976 [116]; Faull et al., 1976 [128]
Glyceroluria	Glycerol	McCabe et al., 1977 [204]

*Only the first published references on the first patients are cited.
**Classified and described clinically by Refsum [173] many years before GC and MS methods were used to discover the accumulated metabolite.

*** A recent investigation [292] indicates that some of the patients with biotin-responsive propionic acidemia and 3-methylcrotonyl-CoA carboxylase deficiency may suffer from a combined carboxylase deficiency due to a defect in the transport and/or metabolism of biotin rather than the synthesis of the apoenzymes.

metabolites [98] were excreted by MSUD patients during attacks. A glass capillary column was required in order to separate these closely similar compounds. The roles of the newly recognized additional metabolites in MSUD and their possible contribution to the clinical picture are not yet known.

D. Chromatographic profiling of urine from mentally retarded patients

Many of the known inherited metabolic diseases lead to mental retardation and a likely place to start a systematic search for new inborn errors might therefore be in institutions for mentally retarded children. Jellum et al. [111] reported the initiation of such studies in 1971 using a comprehensive GC—MS system capable of detecting organic acids, aldehydes, ketones, amino acids carbohydrates and conjugates. The system was designed primarily for qualitative and semi-quantitative analyses. In 1972, they briefly reported [1] that after analysing urine from more than 450 mentally retarded children no indication of unknown metabolic diseases had been found.

In 1975, Watts et al. [207] reported on a comprehensive study of urinary organic acids in specimens from 1778 mentally retarded patients in comparison with 420 age- and sex-matched controls. Quantitative extraction and determination of the various organic acids were carried out. Approximately 5% of the patients had an abnormal organic aciduria, of which 1% was due to phenylalanine metabolites in cases of phenylketonuria, about 1% was due to increased excretion of benzoic acid and about 1% of the patients showed raised urinary excretion of 2-ketoglutaric and citric acids. Gompertz [220] pointed out that excretion of benzoic acid may be due to bacterial infection of the urine, as Perry and Hansen [103] had shown earlier. If the benzoic acid excreters and the phenylketonuria cases were excluded from the results described by Watts et al. [207], the incidence of organic aciduria in the mentally retarded patients fell to 1.95% [220]. In a reply to Gompertz's letter [220], Chalmers et al. [221]. who were aware of excretion of benzoic acid in urinary tract infections, discussed the occurrence of benzoic aciduria further and the potential effects of drug therapy on the organic acid profiles.

The main conclusion to be drawn from the studies discussed above is that the known organic acidurias do not account for a significant proportion of the children who have survived long enough to be admitted to institutions for mentally retarded patients. On the other hand, it is not unreasonable to assume that many cases of mental retardation patients must be due to an underlying biochemical defect. The problem is the failure of GC—MS to detect these biochemical abnormalities. Gas-phase analytical methods are probable not the most suitable, and the unsatisfactory results obtained by systematic chromatographic profiling emphasize the need for alternative methods, e.g., procedures for detecting changes in the patterns of medium- and/or high-molecular weight body constituents.

E. Stroke patients

Lin and Horning [222, 223] have developed a method for the concurrent analyses of long-chain fatty acids, cholesterol and tocopherols [222] in plasma,

using glass capillary columns. Their method was applied to a study of plasma samples from stroke patients and the results [223] indicated the existence of altered lipid metabolism and altered phosphatidylcholine structures. The changes may be due to depletion of α-tocopherol and linolenic acid.

The clinical value of these profiling techniques is not yet known but, as Lin and Horning suggest [223], it may be possible to define conditions of minimal biochemical risk and high risk of thrombotic disorders by use of these GC analyses.

F. Glycogen storage diseases

Samples from patients with this disease have been examined by GC—MS methods, and elevated levels of C_8—C_{10} dicarboxylic acids were found in the urine [224].

G. Hyperprolinemia

Further studies using GC—MS on patients with hyperprolinemia, type II, showed the presence of considerable amounts of a new metabolite, the glycine conjugate of pyrrole-4-carboxylic acid [225]. The organic acid profile also contained an additional unknown derivative of the pyrrolecarboxylic acid and very large amounts of succinic acid.

H. Formiminoglutamic aciduria

This previously recognized metabolic disorder has been subjected to further studies, and GC—MS proved useful for the identification of the abnormal urinary metabolite (formiminoglutamic acid) [226] and for its determination using multiple ion detection [227]. Analyses showed that this disorder also leads to increased urinary excretion of hydantoin-5-propionic acid [228].

I. Congenital dicarboxylic aciduria

Further studies [160] on samples from a child who had died from a new disorder, possibly Acyl-CoA dehydrogenase deficiency, showed that impaired β-oxidation resulted in the production not only of lactic acid and saturated dicarboxylic acids, but also of a series of unsaturated dicarboxylic acids. Their metabolic origin, methods for their chemical synthesis and GC—MS data have been described [160]. This investigation also showed that normal urine contains small amounts of the same dicarboxylic acids.

J. Prenatal diagnosis of methylmalonic acidemia

The usual procedure for obtaining a prenatal diagnosis involves withdrawal of amniotic fluid, growing of the foetal cells in tissue culture followed by the assay of the suspected enzyme. A direct GC method has been used in the prenatal diagnosis of methylmalonic acidemia by measuring methylmalonic acid in urine and in amniotic fluid from pregnant heterozygous women at risk [74].

K. Jamaican vomiting sickness

This condition has been extensively investigated by GC—MS, particularly by Tanaka [178]. The disease is caused by a plant toxin, hypoglycin A. Urine from intoxicated patients contains methylenecyclopropylacetic, 2-ethylmalonic 2-methylsuccinic and other dicarboxylic acids [229—231].

L. Phenylketonuria

The classical metabolic disorder phenylketonuria (PKU) continues to be studied, now by advanced GC—MS—computer methods. Chalmers and Watts [232] carried out quantitative studies on the urinary excretion of phenylglycollic, 2-hydroxyphenylacetic, phenyllactic, phenylpyruvic, 4-hydroxyphenyllactic and 4-hydroxyphenylpyruvic acids in over 40 cases of PKU. Pollitt [233] found unusual amounts of phenylpropionic acid in the urine of a PKU patient. This acid apparently arose from the action of gut bacteria on poorly absorbed phenylalanine. It was pointed out that differences in gut flora may be a contributing factor to the apparent heterogeneity of PKU. Wadman et al. [234] described a new, probably inherited variant of phenylalanine metabolism in two sisters, 12 and 14 years old, both in normal mental health. They had a permanently increased excretion of the typical PKU metabolites, in spite of normal phenylalanine concentrations in the blood and urine. GC has been used to determine phenylalanine and its metabolites in serum and urine of various hyperphenylalaninemic subjects, their relatives and controls [235]. New chemical-ionization MS methods have also been applied to analyses of samples from PKU patients [236]. GC analyses seem suitable for the detection of heterozygotes for PKU. Profiles are determined after a dose of phenylalanine [237].

M. Gastrointestinal disorders

Van der Heiden and co-workers [238—240] described the excessive urinary excretion of *p*-hydroxyhippuric acid and other *p*-hydroxyphenyl compounds in patients with gastrointestinal disorders. These metabolites originated from intestinal bacterial metabolism of non-absorbed tyrosine, which also appeared to be the precursor of *p*-hydroxyphenylhydracrylic acid [166]. Normal urine contains about 50—100 mg of volatile phenols per day (mainly *p*-cresol, phenol and smaller amounts of 4-etylphenol), all of which are the products of the metabolism of tyrosine in the gut by bacteria. GC methods have recently been used to study the urinary excretion of such phenols in patients with ileostomy, colostomy and diverticular disease [241]. Several patients with gastroenteritis were found to have increased plasma and urine levels of pyroglutamic acid [242]. This acid is the characteristic metabolite in the disease pyroglutamic aciduria (glutathione synthetase deficiency) (see Table 1). The source of this acid in the patients with gastroenteritis, however, proved to be a low-lactose food, Nutramigen, which had been used in treatment of the patients. Certain patients with gastrointestinal dysfunction excrete salicyluric acid even though they do not receive salicylates [91].

N. Disorders related to catecholamine metabolism

Determinations of catecholamines and their metabolites (e.g., homovanillic acid, vanillylmandelic acid and 3-methoxy-4-hydroxyphenylglycol) are of considerable importance in certain psychiatric and neurological disease and in diseases that involve increased activity in the peripheral adrenergic neurons, e.g., hypertension and tumours such as phaechromocytoma and neuroblastoma. GC—MS methods have been extensively used to diagnose and investigate such disorders. The use of stable isotopes in particular has opened up new possibilities for the study of the turnover and metabolism of catecholamines. Sjöquist and coworkers [243, 244] devised mass fragmentographic methods for the determination of 4-hydroxy-3-methoxymandelic acid and 4-hydroxy-3-methoxyphenylglycol in urine, cerebrospinal fluid, serum and brain. Narasimhachari et al. [99] measured homovanillic and isohomovanillic acids, and some additional metabolites from L-DOPA by a similar selected ion monitoring method. Rapid and simple GC procedures for the determination of homovanillic and vanillylmandelic acids [245, 246], 4-hydroxy-3-methoxyphenylglycol [246, 247], 3,4-dihydroxyphenylglycol [248] and 3,4-dihydroxyphenylacetic acid [249] in urine have been described. Wadman et al. [250] reported the application of GC and TLC methods to the study of urinary catecholamine metabolites in normal and reconvalescent children without neurological tumours and in 150 cases with neuroblastoma, ganglioneuroma or phaeochromocytoma. Zambotti et al. [251] used GC—MS to study the catecholamine metabolites in human amniotic fluid, and concluded that the assay of 4-hydroxy-3-methoxyphenylglycol in amniotic fluid is likely to be of importance in the prenatal diagnosis of congenital neuroblastoma. Free and conjugated 3-methoxy-4-hydroxyphenylglycol and vanillylmandelic acid in human ventricular fluid have provided some quantitative information on the role of the latter metabolite in human central nervous system catecholamine metabolism. Selected ion monitoring was used in this study [252].

O. Respiratory distress syndrome

Assessment of human foetal lung maturity in utero is based on the determination of the lung surfactant in the amniotic fluid. This may be expressed as lecithin or as the ratio of lecithin to sphingomyelin (L/S ratio). The most widely used methods involve extraction of the phospholipid fraction from amniotic fluid, followed by TLC [253]. L/S ratios above 2.0 indicate adequate maturity and little chance that the newborn child will develop respiratory distress syndrome. Several reports, however, indicate that L/S ratios may be of doubtful prognostic value in diabetic pregnancies, and in the critical region with L/S ratios around 1.5—2.0.

GC methods have therefore been taken into use to measure more accurately the fatty acids derived from lecithin hydrolysis. Rapid and convenient transesterification of aminotic lipid extracts, using sodium methoxide and/or methanol—boron trifluoride prior to GC have been used by several authors, e.g. by Lindback [254-256] and by Cooper and Brush [257] who also used MS.

Since dipalmitoyl lecithin is the major alveolar surfactant, GC methods for the specific measurement of lecithinpalmitic acid in amniotic fluid have been worked out. In the latest one of these [258] a hexane—2-propanol—sulphuric acid system was used to obviate major interferences from triglycerides and free fatty acids. Lecithin palmitic acid values, as determined by GC, exceeding 8 mg per liter of amniotic fluid, indicate that the infants are unlikely to develop respiratory distress syndrome. Results obtained by the above groups and other investigators [259, 260] indicate that amniotic fluid lipid profiles may be of some clinical value in asessing maturation of the foetal lung.

P. Bacterial and viral infections

The first attempts to classify microorganisms by GC profiling techniques were made by Abel et al. in 1963 [261]. Since then, many investigators have established that GC and GC—MS, particularly if combined with computer statistics and numerical taxonomy, are valuable supplementary methods in bacterial and viral classification. Two principally different approaches were made. The first involves the analysis of the chromatographic profiles of the microorganisms and their growth environment after in vitro cultivation and isolation. This approach has proved successful and is in common use. Comprehensive work-up procedures for the determination of, e.g., fatty acid profiles and carbohydrate profiles have been described (e.g., refs. 22, 262 and 263). Pyrolysis methods are also much used (e.g., ref. 47).

The second approach involves the direct analysis of the infected material, i.e., body fluids or tissues, without cultivation of the infectious agents. This approach was introduced by Mitruka et al. [264] and is based on the detection of bacterial and viral metabolites among a multitude of host specific compounds. The technique appears to be very attractive owing to its rapidity, and is under consideration by several laboratories [146, 265—267]. Problems regarding the unstandardizable host background have not yet been solved. Mitruka [22] recently edited a book on the application of GC in microbiology in which all work prior to 1975 is discussed. The latest overview of the topic was prepared by Jantzen [263].

Q. Miscellaneous

Although it is outside the scope of this paper to review the GC—MS analysis of steroids, carbohydrates, amino acids and peptides, it seems appropriate at this stage to emphasize that many metabolites belonging to these chemical classes are disease-related and therefore of diagnostic value. Increased urinary excretion of oligosaccharides and/or glycopeptides is found in the diseases aspartyl-glucosaminuria, mannosidosis, glycogen storage disease types II and III and GM_2-gangliosidosis. GC—MS profiling techniques have been developed to diagnose and study these disorders (e.g., refs. 268, 269) and some other lysosomal diseases have also recently been studied by GC—MS (e.g., ref. 270). Carbohydrate profiles have been recorded in seminal fluid from normal and sterile men [29].

Profiling of amino acids and peptides for diagnostic purpose is carried out

mainly by methods other than GC—MS, e.g., thin-layer, paper and ion-exchange chromatography. Some recent advances in GC—MS methodology, particularly on-column derivatization [107], the use of direct chemical-ionization MS [236] and the use of deuterated amino acids as internal standards in conjunction with computerized GC—MS systems [271—273], have led to rapid, highly sensitive and specific alternatives to the traditional methodology. The new methods have been used to study patients with phenylketonuria, cystinuria and maple syrup urine disease [236], and to study a child [272] and a man [274] with defects in collagen metabolism. Both patients excreted considerable amounts of dipeptides, particularly glycylproline. Such dipeptides may be associated with several disorders [275], e.g., prolidase deficiency [272].

The profile approach to the study of inborn errors of metabolism is also relevant to steroid analysis. Several defects in steroid metabolism, e.g., 3β-hydroxy-steroid dehydrogenase deficiency [276], steroid 21-hydroxylase deficiency [277] and congenital adrenal hyperplasia [278], have been studied by GC—MS methods. The major advance in recent years has been the introduction of open-tubular glass capillary columns, which permit the separation of all major physiologically occurring steroids. Steroid profiles, using high-resolution separation methods, have been obtained from normal males and pre- and post-menopausal females and from patients with congenital adrenal insufficiency, adrenal tumours and Cushing's disease [279, 280]. Advanced computerized GC—MS equipment [279, 281] and semi-automated GC methods [280] greatly facilitate such studies.

10. USE OF STABLE ISOTOPES

Many countries are now reluctant to permit the administration of radioactive isotopes to human patients. The use of stable isotopes and GC—MS for in vivo studies on metabolic pathways in healthy and diseased states is an alternative approach that is likely to increase rapidly in importance. Several international conferences on stable isotopes have already been arranged. A considerable number of deuterium-labelled and ^{13}C-labelled metabolites are already commercially available, and new compounds are regularly added to the various catalogues. A review of the biomedical applications of stable isotopes has recently been published by McCloskey [282]. Curtius et al. [283] administered deuterium-labelled amino acids to patients with phenylketonuria, hyperphenylalaninemia and oligophrenia of unknown genesis. More recently they have made an elegant study of the metabolism of tyrosine in the human intestine by means of stable isotopes and GC—MS [284]. Mamer and Tjoa [285] used a deuterium-labelled precurser to explain the production of a new acidic metabolite in normal human urine, viz., 2-ethylhydracrylic acid [163]. Apparently it is produced in a new, minor catabolic pathway from isoleucine. Stable isotopes and GC—MS techniques have been valuable, e.g., for studies on steroid metabolism (e.g., ref. 286), for investigations on glucose metabolism (e.g., ref. 287), in numerous experiments on drug metabolism (e.g., ref. 288), for studies of nitrogen retention in growth hormone deficient children [289] and for studies on the in vivo synthesis of the "essential" amino acid valine from 2-keto[^{13}C]isovalerate [290]. Stable isotope-labelled compounds

are particularly suitable for pharmacokinetic studies, as recently shown by Horning and co-workers [288, 291].

Selected ion monitoring (SIM) is today a widely used method for the quantitative and specific determination of drugs and their metabolites, steroids and biogenic amines and their metabolites. The technique has been introduced as a definite or absolute reference method in clinical chemistry, and cholesterol, triglycerides, urea, glucose, cortisol, progesterone and testosterone can now be determined in plasma or serum with high accuracy [293]. All of these methods utilize molecules labelled with ^{2}H, ^{13}C, ^{14}C or ^{15}N as internal standards. Apart from the determination of catecholamine metabolites (see p. 451), several of which are organic acids, very few other organic acids have so far been quantitated by SIM and stable isotope-labelled internal standards. It is to be expected that this measurement will be achieved in the future.

11. CONCLUSIONS

It is evident from the many results reviewed here that the profiling approach to the study of body fluids has already become a valuable method in biomedicine. It is to be expected that progress will continue rapidly, and that high-resolution separation methods (e.g., with capillary columns) will increase the potential of the approach even further. The development of advanced computer methodology opens up new possibilities, not only for the study of gross alterations, but also for the detection of more subtle changes in the profiles. Information of this type may throw new light on metabolic processes in normal and diseased states. The use of stable isotopes and profiling methods is also likely to expand in the future.

However, numerous problems remain to be solved and much information is still lacking. For example, most of the work has so far been concentrated on urine profiling, and much less on other body fluids such as serum and spinal fluid. Hardly any work has been done on tissue profiling, although it seems reasonable to assume that much information could be gained by applying advanced GC—MS to the analysis of such specimens. One can only hope that the methodology will be advanced so as to be able to handle 1—3 mg of tissue biopsies.

Apart from technical difficulties, artifacts and pitfalls, one is also concerned with the problem of identifying all of the normal peaks that occur in the various chromatograms. For example, when the organic acids in human urine are separated on glass capillary columns, less than one third of the peaks can be identified. Slowly but surely, more of these unknown peaks are likely to be identified, sometimes perhaps with surprising results.

12. SUMMARY

This review summarizes recent advances in the application of gas chromatography and mass spectrometry to the study of human diseases. Emphasis is placed upon the organic acid profiles of the various body fluids. Methods for sample work-up prior to separation and mass spectrometric analysis are reviewed, and artifacts and pitfalls are discussed. Organic acid profiles, obtained

with packed or capillary columns attached to mass spectrometers with or without computer systems, have led to the discovery of new normal metabolites, new metabolic disorders, and to new knowledge about a number of other diseases. Stable isotopes and gas chromatography—mass spectrometry are suitable for quantitative analysis of many compounds in the body fluids, and well suited for investigation of metabolic pathways.

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Journal of Chromatography, 143 (1977) 463—471
Biomedical Applications

CHROMBIO. 050

TRENNUNG UND FLUORIMETRISCHE BESTIMMUNG VON ADRENALIN UND NORADRENALIN

KOPPLUNG EINES HOCHDRUCK-FLÜSSIGKEITS-CHROMATOGRAPHEN MIT EINEM AUTOMATISCHEN ANALYSENSYSTEM*

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(Eingegangen am 28. Oktober 1976; geänderte Fassung eingegangen am 15. Januar 1977)

SUMMARY

Separation and fluorimetric determination of adrenaline and noradrenaline. Combination of a high-pressure liquid chromatograph with an automatic analysis system

The possibilities for a high-pressure liquid chromatographic analysis combined with automatic fluorimetric detection of the catecholamines adrenaline and noradrenaline are described.

The optimal conditions are given for a fast separation by ion exchange and reversed-phase chromatography, and for the sensitive fluorimetric determination of adrenaline by the trihydroxyindole technique when a high excess of noradrenaline is present.

EINLEITUNG

Für die Analyse der Katecholamine Adrenalin und Noradrenalin wird vor allem in der klinisch-chemischen Analytik die sehr empfindliche fluorimetrische Bestimmung als Trihydroxyindol-Derivate verwendet (siehe z.B. Lit. 1). Diese Methode hat jedoch den Nachteil, dass beide Amine nicht ohne gegenseitige Störungen bestimmt werden können. Diese Störungen wirken sich bei der Adrenalinbestimmung besonders dann aus, wenn Noradrenalin in einem hohen Überschuss neben sehr geringen Adrenalinmengen vorliegt, wie es in biologischem Material der Fall ist.

Die Bestimmung beider Amine erfolgt in der zur Zeit verbreitet angewendeten Routineanalytik nach der Abtrennung aus Urin oder Serum an Aluminiumoxid oder Ionenaustauschern [1]. In den Eluaten werden Adrenalin und Noradrenalin dann nebeneinander durch die Oxidation bei unterschiedlichen pH-

* Teile dieser Arbeit wurden im Rahmen eines Referates während der "Königsteiner Chromatographie-Tage für HPLC", 13—15. Oktober 1975, vorgetragen.

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Werten in zwei Arbeitsgängen bestimmt.

Mit Hilfe der Hochdruck-Flüssigkeits-Chromatographie (HPLC) sollte versucht werden, nach einer vorhergehenden Trennung diese empfindliche Reaktion der Amine in einem automatisch-fluorimetrischen Analysensystem als Detektor anzuwenden. Die Beseitigung der gegenseitigen Störungen und eine schnellere und rationellere Methode für die Routine sollten dadurch möglich werden. Erste Versuche wurden bereits von Mori [2—5] durchgeführt: Analysenzeiten von 20—45 min veranlassten uns jedoch, sowohl in der HPLC als auch für das Reaktionssystem nach Möglichkeiten für eine schnellere Methode zu suchen.

EXPERIMENTELLER TEIL

Geräte

HPLC. Hochdruckpumpe Modell 6000 A (Waters Assoc., Königstein, B.R.D.). Probeninjektionssytem Modell U 6 K; Zweistrahl-Mehrwellenlängen-UV/VIS-Photometer Modell 440 (Waters); 6-Wege-Ventil als Probenaufgabenventil mit 100-μl Schleife (Valco-Princip, bezogen von Dupont, Bad Nauheim, B.R.D.).

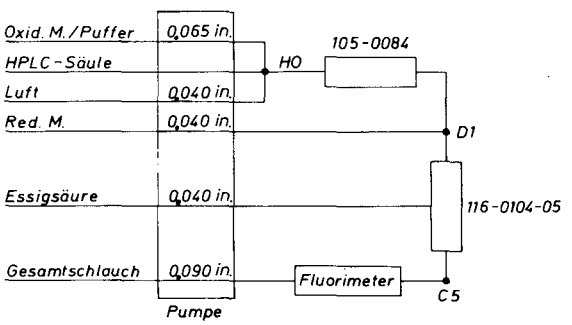

Fig. 1. Fliessschema für das Reaktionssystem. (Einzelheiten siehe Experimenteller Teil, Geräte).

Automatisches Analysensystem. Gilson Spectro/Glo Filter Fluorimeter mit Fluorescamin-Filtern; Durchflussküvette, Pyrex-Glasrohr, O.D. 6 mm, I.D. ca. 4 mm; Pumpe, Gilson Minipuls II (Gilson, Abimed, Düsseldorf, B.R.D.). Reaktionseinheit, (aus Hajoka-Ersatzteilen der Fa. Kleinfeld, Hannover, B.R.D.). Mischspiralen Kat. Nr. K 105-0084 (14 Schleifen, 3.4 mm I.D.) und K 116-0104-05 (28 Schleifen, mit Anschluss in der Mitte, 2.4 mm I.D.), Zwischen- und Verbindungsstücke H0 und D1, Entlüfter C5, Pumpenschläuche aus Polyäthylen (I.D. (in.) siehe Fig. 1), Verbindungsschlauch 1/16 in. I.D.). Die Glasteile sind untereinander bzw. mit den Schläuchen durch Schlauchstücke und "Nipples" (N5 und N6) verbunden.

Chemikalien und Lösungen

Chemikalien. Adrenalin (L-Adrenalin-bitartrat pharm. (Serva), Heidelberg, B.R.D.); Noradrenalin (L-Noradrenalin-bitartrat pharm; Serva); Isopropyl-

noradrenalin (DL-Isopropyl-arterenol hydrochlorid pharm; Serva).

Lösungen: freiens Amin, 1 mg pro/ml 0.1 *N* Salzsaüre, Verdünnungen 1:50; 2-Mercaptoäthanol, reinst (Serva). All übrigen Chemikalien mit den Reinheitsgrad "zur Analyse" (Merck, Darmstadt, B.R.D.).

Reduktionslösung. (a) 5% Mercaptoäthanol-Lösung, (b) 20% Natriumsulfitlösung, (c) 10 *N* Natronlauge. Gleiche Volumina der Lösungen a—c werden gemischt.

Pufferlösungen. Falls nicht anders angegeben, 0.5 *M* Borsäure-Lösungen, die mit Ameisensäure oder Natronlauge auf den entsprechenden pH-Wert eingestellt werden, mit einem Gehalt von $2 \cdot 10^{-3}$% Kupferacetat.

Säulenmaterialien zur HPLC. Kationenaustauscher (Sulfonsäuregruppen) Vydac-401 SA (auf inerten Glasskugeln, Teilchengrösse 30—44 μm); Kationenaustauscher (Sulfonsäuregruppen, chemisch gebunden an Kieselgel) Nucleosil 10-SA (Teilchengrösse 10 μm); Nucleosil 10-C_{18} (Octadecylgruppen chemisch gebunden an Kieselgel, Teilchengrösse 10 μm); alle Materialien von Machery, Nagel u. Co., Düren, B.R.D.).

Methodik

Aus der chemischen Umsetzung ergibt sich der Aufbau des Reaktionssystems in die Teile für die Oxidation, Isomerisierung (Alkalisierung und Reduktion) und Ansäuerung. Das automatische Analysensystem besteht aus dem eigentlichen Reaktionssystem, einer Pumpe und einem Fluorimeter mit Durchflussküvette (Fig. 1).

Als innerer Standard für die chromatographische Trennung und fluorimetrische Bestimmung wurde Isopropyl-noradrenalin eingesetzt, das in Urin und Blut nicht vorhanden ist. Die Optimierung des Reaktionssystems erfolgte im Hinblick auf folgende Anforderungen: Das in biologischen Materialien in der niedrigsten Konzentration auftretende Adrenalin sollte am empfindlichsten bestimmt werden. Die Fluoreszenzintensität sollte unter diesen Bedingungen für Noradrenalin so niedrig liegen, dass bei Mengenverhältnissen von etwa 1:5 (wie sie z.B. in einem normalen Urin vorliegen) gleich hohe Signale erhalten werden. Isopropyl-noradrenalin sollte ebenfalls bestimmbar sein. Untersucht und optimiert wurden die "chemischen" Einflüsse des Oxidations-pH-Wertes, der Art des Puffers, der Konzentration katalytisch wirksamer Kupferionen, der Alkalität und der Konzentration und Art des Reduktionsmittels für die Isomerisierung, der Säurekonzentration beim Ansäuern und der "physikalische" Einfluss der Pumpengeschwindigkeit (speed-Zahl) im System. Diese Messungen wurden ohne Verbindung mit der HPLC-Säule durch Ansaugen wässriger Lösungen der Amine mit einem Probenschlauch (0.045 in I.D., 15 sec. Saugzeit) durchgeführt.

Aus den optimalen Reaktionsbedingungen für die fluorimetrische Analyse von Adrenalin und Noradrenalin (siehe Ergebnisse und Diskussion) ergeben sich die Anforderungen an die mobile Phase für die HPLC: Mit einem Formiatpuffer pH 4 wurden die Trennmöglichkeiten sowohl durch Ionenaustausch als auch "reversed-phase"-Chromatographie untersucht. Die Detektion erfolgte mit einem UV-Detektor bei 280 nm.

Der Ausgang der HPLC-Säule wurde für die fluorimetrischen Analysen mit dem

automatischen Analysensystem direkt über eine Kapillare mit dem Verbindungsstück H0 des Reaktionssystems verbunden.

Für die Messungen mit dem UV-Detektor erfolgte die Probenaufgabe durch das Probeninjektionssystem. Bei der Verbindung der Hochdruckpumpe mit dem "Reaktionsdetektor" wurde das 6-Wege-Ventil mit 100 µl-Schleife eingesetzt.

ERGEBNISSE UND DISKUSSION

Die Oxidation der Katecholamine kann sowohl mit Jod als auch mit Kaliumhexacyanoferrat (III) (siehe z.B. Lit. 6) durchgeführt werden. Für diese Methoden sind bereits eine Anzahl automatisch-fluorimetrischer Analysensysteme beschrieben worden. Eigene Untersuchungen haben gezeigt [7], dass im beschriebenen Reaktionssystem mit Kaliumhexacyanoferrat (III) höhere Fluoreszenausbeuten als mit Jod erzielt werden. Auch für die Reduktion werden verschiedene Reduktionsmittel angegeben, z.B. Ascorbinsäure, Natriumsulfit, Mercaptoäthanol u.a. [6]. Bei der Verwendung von Ascorbinsäure oder einem Gemisch aus Sulfit und Mercaptoäthanol werden etwa gleich grosse Fluoreszenzausbeuten erhalten. Wegen der grösseren Stabilität wurde das Gemisch aus Sulfit und Mercaptoäthanol bevorzugt. Durch das Ansäuern nach der Isomerisierung im Alkalischen wird die Fluoreszenzausbeute nochmals erhöht [8].

Für dieses Verfahren erfolgte eine Optimierung der Reaktionsbedingungen. Die pH-Abhängigkeit der Oxidation [1] bei Anwesenheit von Kupferionen und die Abhängigkeit von der Konzentration des Oxidationsmittels (Fig. 2) ermöglicht die Einstellung des Systems auf gleiche Fluoreszenzintensitäten für Adrenalin und Noradrenalin bei einem Mengenverhältnis von z.B. 1:5. Im Hinblick auf die Auswahl der mobilen Phase für die HPLC wurde der Einfluss verschie-

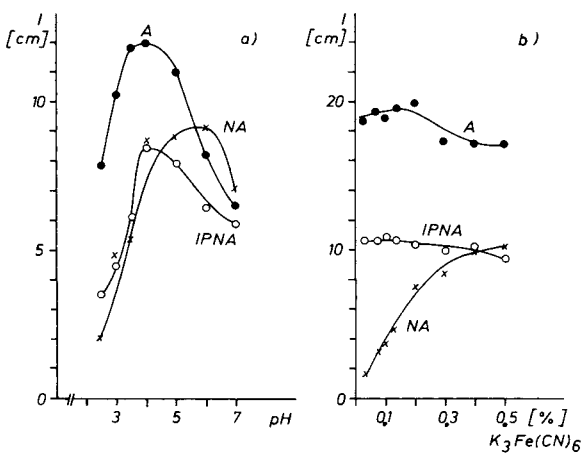

Fig. 2. Abhängigkeiten der Fluoreszenzintensitäten für Adrenalin (A), Noradrenalin (NA) und Isopropyl-noradrenalin (IPNA).
(a) vom pH-Wert der Oxidation, (b) von der Konzentration des Oxidationsmittels (pH 6).
Lösungen der Amine (200 ng/ml) in Puffer, Probenschlauch 0.045 in. I.D., Pumpengeschwindigkeit 1.8 ml/min., Spülen zwischen den Proben mit Puffer-Lösung.

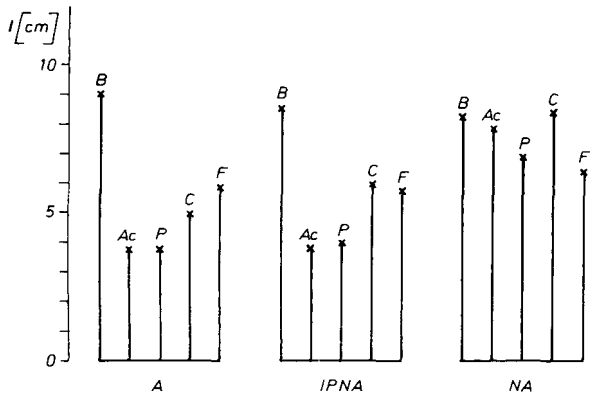

Fig. 3. Einfluss der Pufferionen auf die Fluoreszenzintensitäten (0.2 *M* Pufferlösungen, pH 6). Lösungen der Amine in Wasser, 0.4% $K_3Fe(CN)_6$ in Puffer, P = Phosphat, Ac = Acetat, F = Formiat, C = Citrat, B = Borat.

dener Pufferlösungen gleichen pH-Wertes auf die Fluoreszenzausbeute für Adrenalin, Noradrenalin und Isopropyl-noradrenalin untersucht. Fig. 3 zeigt die erhebliche Verringerung der Fluoreszenzintensität für Adrenalin in anderen Puffern als Borat.

In der Tabelle I sind die bisher beschriebenen HPLC-Trennungen für die Katecholamine zusammengestellt: Für die Trennung an Ionenaustauschern wurden vor allem Phosphatpuffer verwendet, die jedoch für anschliessende fluorimetrische Bestimmung wegen der Verringerung an Fluoreszenzausbeute wenig geeignet sind.

Mit Boratpuffern konnten jedoch an Ionenaustauschern wie Vydac 401 SA und Nucleosil 10 SA keine Trennungen erzielt werden vermutlich wegen der komplexierenden Wirkung der Borationen. Ameisensäure- und Formiatpuffer-Lösungen erwiesen sich dagegen als geeignete mobile Phasen (optimale HPLC-Bedingungen siehe Fig. 4 und 5). Die Trennung ist sowohl mit Ionenaustauschern als auch mit der "reversed-phase"-Chromatographie möglich. Die günstigste und schnellste Trennung zwischen Adrenalin und Noradrenalin wird mit der "reversed-phase"-Chromatographie erzielt (Fig. 6).

Für die Verbindung der HPLC mit der fluorimetrischen Detektion in einem automatischen Analysensystem ergibt sich die Frage nach der Verringerung der Auflösung (R) durch das Reaktionssystem. Die direkte Verbindung einer chromatographischen Säule mit einem chemischen Reaktionssystem für die photometrische Bestimmung ist bereits beschrieben worden [16,17].

Durch die Segmentierung des Flüssigkeitsstroms mit Luftblasen (AutoAnalyzer-Prinzip der Fa. Technicon) wird eine übermässige Diffusion und damit Verbreiterung der Banden verhindert. Die Veränderungen in der Auflösung durch das nachgeschaltete Reaktionssystem zeigen die Fig. 4—6 für die verschiedenen Trennmöglichkeiten.

Verändert man die Pumpengeschwindigkeit und damit die lineare Geschwindigkeit im Analysensystem, so wird eine Abhängigkeit der Auflösung von der Durchflussgeschwindigkeit erkennbar (Beispiel Fig. 7). Bei hohen Geschwin-

TABELLE I

HPLC-TRENNUNGEN DER KATECHOLAMINE (LITERATURÜBERSICHT)
o.A. ohne Angabe.

Trennmaterial	Säulenabmessungen	Mobile Phase	Druck (p.s.i.)	Durchflussrate (ml/min)	Analysenzeit für Adrenalin und Noradrenalin (min)	Literatur
Zipax SCX	1 m × 2.1 mm I.D.	0.1 *M* Na-Acetat in 0.02 *M* Essigsäure pH 5.3 (40°)	1000	1.0	6	2
	1 m × 2.1 mm I.D.	0.05 *M* Na-Acetat in 0.01 *M* Essigsäure (40°)	1200	1.4	15	3
	1 m × 2.1 mm I.D.	0.075 *M* NaH_2PO_4	570	0.5	15	4
	1 m × 2.1 mm I.D.	0.15 *M* NaH_2PO_4	550	0.4	15	5
	1 m × 2.1 mm I.D.	0.05—0.45 *M* NaH_2PO_4 (0.04 *M*/min)	ca. 570	ca. 0.4	15	9
	1 m × 2.1 mm I.D. 50 cm × 2 mm I.D.	0.1 *M* Perchlorsäure	250—400	0.3—0.5	ca. 5—6	0
Vydac Kationenaustauscher	50 cm × 2 mm I.D.	0.01 *M* Schwefelsäure—0.04 *M* Na-Sulfat	o.A.	0.40	5	11
Bondapak CX/Corasil	61 cm × 2 mm	10.5 g Citronensäure—2.1 ml Essigsäure—4.8 g NaOH—8.2 g Na-Acetat ad 2 l, pH 5.1 (0.2 *m* Na^+)	o.A.	2	3	12
Partisil-10 SCX	25 cm × 4.6 mm	0.5 *M* $NH_4H_2PO_4$ pH 4.36	275	0.76	15	13
DuPont SCX	8 ft. × 3 mm	0.2 *N* Ammoniumphosphat, pH 7.0	1200	1.0	18	14
ODS/TMS-Kieselgel (6 µm)	125 mm × 5 mm I.D.	Acetonitril—Wasser—konz. Schwefelsäure (10:90:0.3)	1100	o.A.	5	15

digkeiten ist der Verlust an Auflösung am gerinsten. Die Erhöhung der Durchflussgeschwindigkeit hat jedoch gleichzeitig einen Einfluss auf die Umsetzung der Katecholamine.

Mit Erhöhung der Pumpengeschwindigkeit werden (ohne Verbindung zur HPLC—Säule) bei gleichbleibender Saugzeit steigende Volumina an Katecholamin-Lösungen in das System aufgenommen. Wie Fig. 8 zeigt, erniedrigt sich jedoch die Fluoreszenzausbeute für Noradrenalin und Isopropyl-noradrenalin mit steigender speed-Zahl, für Adrenalin steigt sie dagegen linear an. Die Reaktionszeiten sind demnach für Noradrenalin und Isopropylnoradrenalin zu gering.

Die besonderen Vorteile einer Verbindung von HPLC mit einem automatisch-fluorimetrischen Analysensystem liegen in der schnellen, getrennten und empfindlichen Bestimmung von Adrenalin und Noradrenalin. Trotz einer Verringerung der Auflösung ist eine quantitative Analyse beider Amine auch bei

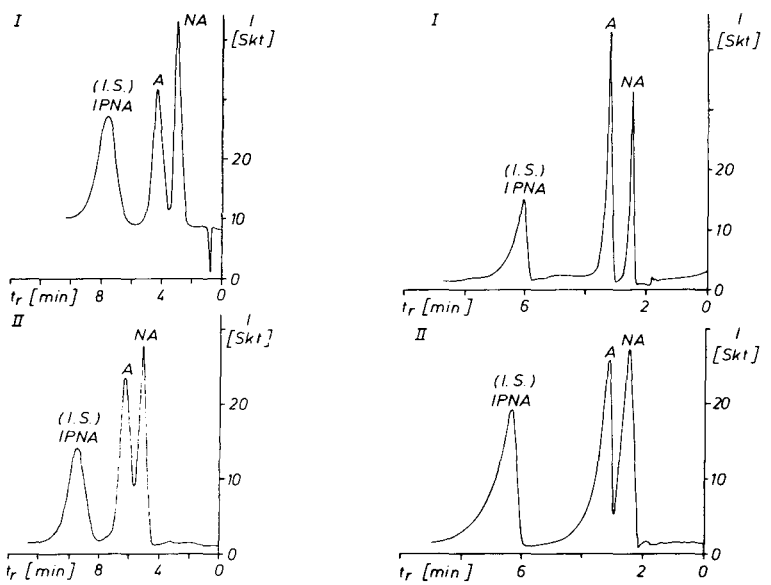

Fig. 4. HPLC-Trennung von Adrenalin (A) und Noradrenalin (NA), Ionenaustausch (IPNA = Isopropyl-noradrenalin). Säule, 500 × 2.1 mm I.D.; Packungsmaterial; Vydac 401-SA, Trokken gepackt; Mobile Phase, 0.5 *M* Ameisensäure; Durchfluss, 0.6 ml/min; Druck, 35 bar; Temperatur, 22°; Dosiervolumen 100 µl (100 ng A, 500 ng NA, 500 ng IPNA für II).
(I) UV-Detektor: 280 nm; (II) automatisches Analysensystem: Fluorimeter 1/100 der maximalen Empfindlichkeit; Pumpe, speed (7.2 ml/min); 0.1% $K_3Fe(CN)_6$—$2 \cdot 10^{-3}$% Cu-Acetat—0.5 *M* Formimat—0.2 *M* Borat-Puffer (pH 4)—6 *N* Essigsäure.

Fig. 5. HPLC-Trennung von Adrenalin (A) und Noradrenalin (NA), Ionenaustausch (IPNA = Isopropyl-noradrenalin). Säule, Fertigsäule (Macherey, Nagel & Co), 300 × 4 mm I.D.; Packungsmaterial: Nucleosil 10-SA; mobile Phase; 1*M* Natriumformiat-Puffer pH 4; Durchflussrate, 1.8 ml/min; Druck, 140 bar; Temperatur, 22°; Dosiervolumen, 100 µl (= 100 ng A, 500 ng Na, 200 ng IPNA für II).
(I) UV-Detektor: 280 nm, (II) automatisches Analysensystem: Fluorimeter 1/100 der maximalen Empfindlichkeit, Pumpe; speed (9.6 ml/min); 0.04% $K_3Fe(CN)_6$ —0.2 *M* Boratpuffer (pH 4)—$2 \cdot 10^{-3}$% Cu-Acetat—6 *N* Essigsäure.

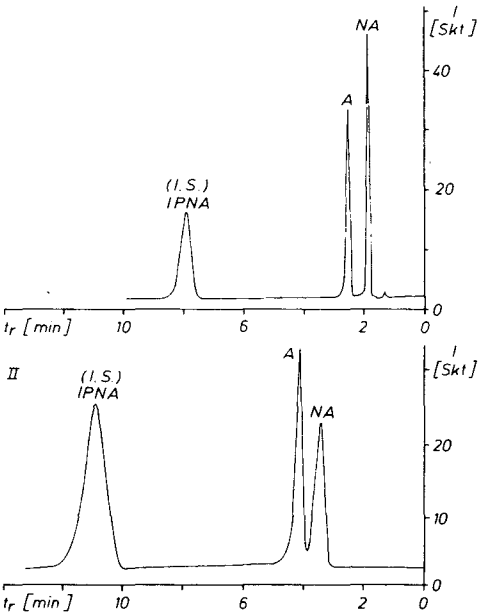

Fig. 6. HPLC-Trennung von Adrenalin (A) und Noradrenalin (NA), "reversed-phase" (IPNA = Isopropyl-noradrenalin). Säule, Fertigsäule (Macherey, Nagel & Co.), 200 × 4 mm I.D.; Packungsmaterial. Nucleosil 10-C_{18}; mobile Phase, 1 *M* Natriumformiat-Puffer pH 4; Durchflussrate, 2ml/min; Druck, 140 bar; Temperatur, 22°; Dosiervolumen, 100 µl (= 100 ng A, 500 ng NA, 500 ng IPNA für II).
(I) UV-Detektor: 280 nm, (II) automatisches Analysensystem, Fluorimeter 1/200 der maximalen Empfindlichkeit; Pumpe, speed 16.3 ml/min; 0.04% $K_3Fe(CN)_6$ –0.2 *M* Boratpuffer (pH 4)—$2 \cdot 10^{-3}$ % Cu-Acetat—6 *N* Essigsäure.

einem vielfachen Uberschuss an Noradrenalin neben Adrenalin durch die Variationsmöglichkeiten im Reaktionssystem möglich. Die Bedingungen (pH-Wert der Oxidation, Art des Puffers, Konzentration des Oxidationsmittels) können so gewählt werden, dass etwa gleich grosse Signale erhalten werden.

ZUSAMMENFASSUNG

Es werden die Möglichkeiten für eine hochdruck-flüssigkeits-chromatographische Analyse der Katecholamine Adrenalin und Noradrenalin in Verbindung mit einem automatisch-fluorimetrischen Analysensystem als Detektor beschrieben.

Die optimalen Bedingungen für eine schnelle Trennung durch Ionenaustausch und "reversed-phase"-Chromatographie sowie die empfindliche fluorimetrische Bestimmung von Adrenalin neben einem hohen Überschuss an Noradrenalin nach der Trihydroxyindol-Methode werden angegeben.

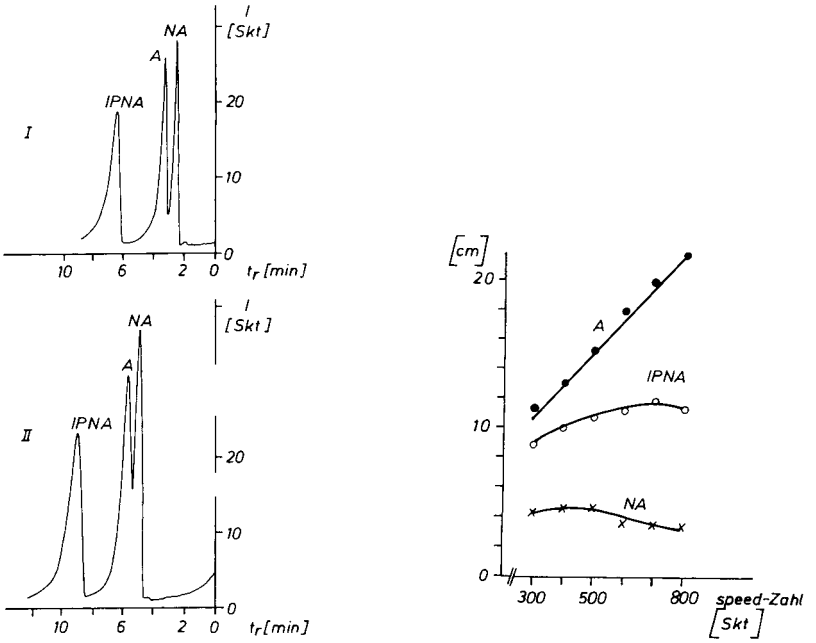

Fig. 7. Abhängigkeit der A—NA-Trennung von der Pumpengeschwindigkeit (speed-Zahl) des automatischen Analysensystems.
Bedingungen wie Fig. 5, (I) speed 14.2 ml/min, (II) speed 18.4 ml/min.

Fig. 8. Abhängigkeit der Fluoreszenzintensitäten von der Pumpengeschwindigkeit (speed-Zahl) des automatischen Analysensystems, Probenschlauch 0.045 in. I.D. (anstelle HPLC-Anschluss) 0.2 *M* Boratpuffer (pH 6)—$2 \cdot 10^{-3}$% Cu-Acetat—0.1% $K_3Fe(CN)_6$—10 *N* Essigsäure, Lösungen der Amine in Wasser, X , NA; ○, IPNA; ●, A. Umrechnung von der speed-Zahl (Skt) in die Durchflussrate (ml/min): Skt mit dem Faktor $3 \cdot 10^{-3}$ für Probenschlauch 0.045 in. I.D. multiplizieren.

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Journal of Chromatography, 143 (1977) 473—490
Biomedical Applications

CHROMBIO. 056

METHODS FOR THE RAPID SEPARATION AND ESTIMATION OF THE MAJOR LIPIDS OF ARTERIES AND OTHER TISSUES BY THIN-LAYER CHROMATOGRAPHY ON SMALL PLATES FOLLOWED BY MICRO-CHEMICAL ASSAYS

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(First received December 1st, 1976; revised manuscript received February 15th, 1977)

SUMMARY

Methods are described for the rapid separation of the major individual phospholipids and neutral lipids of tissues by thin-layer chromatography on small glass plates (75 × 75 mm), and for the specific microchemical estimation of separated lipids and for determination of fatty acid composition and radioactivity. The overall method, involving tissues extraction, thin-layer chromatographic separation and assay has been evaluated using pure standards and biological samples and gives good reproducibility and almost complete recovery of lipids.

INTRODUCTION

There is a need in many studies of tissue lipids for rapid separation and subsequent estimation of small amounts of material. This has been particularly important in our work on atherosclerosis, involving analytical and metabolic studies of individual atherosclerotic lesions from experimental animals and also organ and cell cultures. With these applications in mind, we have developed a set of methods for separation of the major individual phospholipids and neutral lipids by 2- and 3-dimensional thin-layer chromatography (TLC) on small glass plates. Separations may be made in less than 30 min and subsequent specific micro-chemical assays permit measurement of microgram amounts of each lipid, as would be obtained from a typical arterial sample of approximately 10 mg of wet tissue. The overall method has been shown to give good recovery and reproducibility for representative phospholipids and neutral lipids. It has given highly consistent separations, in our hands on over 2,000 tissue samples over a period of 4 years.

Micro thin-layer chromatography

TLC [1] is probably now the most frequently used method for the separation of lipids prior to estimation, determination of radioactivity or analysis of their constituent fatty acids. A disadvantage of commonly used 20 × 20 cm glass plates is that they may take about 1 h to run in one dimension and when two-dimensional separations are used to improve resolution, for example for phospholipid separation, the overall time for chromatography of one sample may be some hours. Much more rapid separation may be achieved by the use of smaller plates.

TLC, with plates the size of microscope slides, was first used in the early studies of Izmailov and Shraiber [2] and by Meinhard and Hall [3] and Kirchner et al. [4] which are reviewed by Mangold [5]. Since then they have found increasing use for a number of compounds, for example amino acids [6,7], peptides such as pentagastrin [8], tocopherols [9] and lipids [10—21]. Indeed for separation of lipids, Hofmann [11] recommended the technique in preference to large plates on account of its rapidity, convenience, economy and sensitivity. Despite this suggestion the small plates are still not widely used for lipid analysis. This may be partly due to the belief that resolution is compromised by small size and by the high speed of chromatography. On the contrary, the speed of migration of solvents ensures that there is minimal spreading of the spot by diffusion, giving good resolution. Furthermore, the small area occupied by the spot produces a useful increase in the sensitivity of detection, because of the higher concentration of substance per unit area. A more serious objection has been the lack of development of sufficiently sensitive methods of assay of separated substances.

When our studies were begun, Van Gent [14] had described separation of neutral lipids on small plates, and their estimation by charring, followed by scanning densitometry in situ. In extensive preliminary investigations, we found that estimation by charring either in situ or in the test tube [22—24] did not give sufficiently precise results. The charring was dependent upon the constituent fatty acids of the lipids and also upon the presence of small amounts of impurities. A similar finding has been reported for the ultrasensitive fluorimetric methods using rhodamine [18]. In view of such potential disadvantages we abandoned non-specific charring methods and developed published microchemical techniques for the tiny quantities of lipids separated on the small TLC plates.

This paper reports the details of the methods which we have developed for TLC separation and analysis and an evaluation of the recovery and reproducibility for major lipid classes.

MATERIALS AND METHODS

Reagents and glassware

The methods described are very sensitive and therefore, every precaution must be taken to ensure that glassware is clean and reagents pure. Sample vials and test tubes for assays were washed in concentrated sulphuric acid after use and reserved specially for these procedures. On no account should plastic stoppered tubes or bottles be used for samples or reagents; plasticizers are extracted by lipid solvents and interfere with chromatographic separations and analyses.

Specially made glass stoppered vials (45 mm × 5 mm I.D., vol. 2 ml, with C10 stoppers, from Camlab Glass, Cambridge, Great Britain) were used for samples of extracted lipids. Chromatography tanks were circular specimen jars or beakers with loose-fitting lids. All reagents used were AnalaR from BDH (Poole, Great Britain) or Hopkin & Williams (Chadwell Heath, Great Britain). Chloroform, methanol and petroleum ether (b.p. 40—60°) were further purified by redistillation. Diethyl ether was run through a short column of alumina (100 mesh, for chromatography, from BDH) immediately before use in order to remove peroxides. Silica gel was Camag Type DO without binder (Camag, 4132 Muttenz, Switzerland).

Pure lipids for standardisation of chromatograms were obtained as follows: Lyso-lecithin, sphingomyelin, lecithin were from Koch Light (Colnbrook, Great Britain); lyso-phosphatidyl ethanolamine was a kind gift from Dr. R.M.C. Dawson; cholesterol, cholesteryl oleate, oleic acid, glyceryl trioleate were from Sigma (London, Great Britain).

Radioactive lipid precursors for radio-labelling of lipids, to aid identification on chromatograms, were obtained from Radiochemical Centre (Amersham, Great Britain). Radioactivity was measured using a Nuclear Chicago Mk. II scintillation counter.

Preparation of silica gel for phospholipid separation

Separation of phospholipids on TLC may be improved by the addition of various substances to silica gel. In this method we have used ammonium sulphate [11,25] as suggested by Kaulen [26] for the improved resolution of phosphatidyl inositol from phosphatidyl serine. The silica gel is impregnated as follows: 200 g of silica gel are slurried in 2 l of 0.2% w/v aqueous ammonium sulphate solution and centrifuged at 60 *g* for 5 sec and the supernatant containing finings is decanted off. The silica gel is then dried by repeating the slurrying process and centrifuging at 60 *g* for 5 min three times with 500 ml of AnalaR methanol and once with 500 ml of AnalaR chloroform.

Preparation of silica gel for neutral lipid separation

The separation of neutral lipids is improved and made more reproducible by the addition of sodium carbonate to the silica gel to provide a stable, basic pH of the adsorbent: 200 g of silica gel are slurried with 2 l of 0.015 *M* (0.16% w/v) aqueous sodium carbonate solution, then allowed to stand for at least 2 h and the supernatant containing finings is decanted off. The silica gel is then dried by slurrying with methanol and chloroform as described for phospholipid plates.

Preparation of plates

Glass plates (75 × 75 mm; Hoslab, London, Great Britain) are wiped with absolute ethanol. The appropriate silica gel is slurried in chloroform and the plates coated with 0.25 mm layers using a spreader and template (Quickfit & Quartz, Stone, Great Britain). The plates are allowed to dry in air for 10 min, before being washed by running in chloroform—methanol (2 : 1). The plates are allowed to dry and stored in a dust-free environment. Immediately before use the plates are activated by heating on a hot plate at 100° for 2 min.

Extraction of tissue lipids

Tissue lipids are extracted by a modification of the Folch procedure [27,28]. The majority of lipids are removed from tissue in a "first" extraction with chloroform methanol (2:1) at room temperature for 1 h and the residual lipid which is mainly free fatty acid, in a "second" extraction at 40° for 1 h. For tissues containing phosphatidyl inositol phosphates it is necessary to use an acidified solvent such as chloroform—methanol—1 *N* HCl (100 : 50 : 2). This is best done in the second extraction, after most of the lipids have already been extracted, because this minimises the artifactual trans-methylation of complex lipids to give methyl ester of their fatty acids [29].

In the experiments reported here both extractants were chloroform—methanol (2:1). The tissue (volume V) is disrupted, if necessary by freeze pressing [30] and placed in a weighed glass fibre filter disc (Whatman GF/A). The filter disc is previously extracted with chloroform—methanol (2:1) for 1 h at 40°, in order to remove any lipid contaminants, dried in air and stored in a desiccator over silica gel. The tissue on the filter disc is then extracted with 20 volumes (20 V) chloroform—methanol (2:1) for 1 h at room temperature; the methanol must be added separately, first. The extract is then transferred to a tap funnel, the tap of which has been previously moistened with methanol to prevent leakage of solvent. The filter disc with tissue is extracted with a second 20 volumes (20 V) of chloroform—methanol (2:1) for 1 h at 40°. The filter disc is then squeezed gently with forceps and washed with 20 volumes (20 V) chloroform—methanol (2:1) and squeezed gently again. The filter disc is then dried and re-weighed, giving the weight of dry defatted tissue. The dry residue is subsequently analysed for DNA.

The chloroform—methanol extracts are then "Folch-washed" with 0.2 volumes (0.2 × 60 V) of saline (0.9% w/v NaCl, pH 7.4). The chloroform lower phase and aqueous upper phase are separated by standing at 4° overnight or by centrifugation and the chloroform phase removed. The aqueous phase is then extracted with 40 volumes chloroform—methanol—saline (86 : 14 : 1) in order to retain fatty acid. The chloroform phases are then pooled and evaporated to dryness in a rotary evaporator in vacuo at 40°. The evaporator is always vented to nitrogen. The sample is then transferred to a small glass stoppered vial with chloroform—methanol—saline (86 : 14 : 1), and the solvent evaporated to dryness in a stream of nitrogen. This process is greatly facilitated by a special apparatus which allows evaporation of solvent from a number of samples simultaneously. These are available commercially, but we use one made in the laboratory. It consists of two separable modules of perspex. The lower module has a solid base-plate and an upper support-plate which is drilled to accept the sample vials. The upper module also has two plates. The lower holds twelve 19-gauge hyperdermic needles exactly above the middle of each sample vial. Each needle is connected to a brass gas manifold mounted on the top by means of a short length of PVC tube. Each tube passes through a hole in the upper plate where it may be restricted by a thumb screw tapped into the plastic, thus permitting independent control of the gas flow on each needle. Samples are stored in benzene at —20° until required.

Pipetting of samples onto TLC plates

Before the sample is spotted onto the plates, two lines are drawn across the silica gel, 1 cm from the top and right-hand edges to act as "stop-lines" for the solvents. The benzene is then evaporated from the sample in a stream of nitrogen, and the sample redissolved in a known volume of chloroform–methanol (2 : 1) containing ammonium hydroxide (1 ml of 0.880 sp.gr. ammonium hydroxide is added to 400 ml of solvent). A known volume of sample is measured by holding the sample in a syringe, usually 100 μl total capacity (Type 710; Hamilton Micromesure, Bonaduz, Switzerland). The sample vial is washed out with a further portion of chloroform–methanol (2 : 1) containing ammonium hydroxide, which is held in a second syringe. Samples are quantitatively transferred to plates by ejecting the required volume from the main and wash syringes with press button dispensers (Type PB 600, Hamilton Micromesure).

During pipetting of samples, the plate is held in a holder (Fig. 1) which allows a stream of nitrogen to be blown over the surface, thus aiding evaporation of the solvent and preventing oxidation of the lipids.

Two-dimensional separation of phospholipids (Fig. 2)

Chromatography tanks are lined with filter paper saturated with chromatography solvent; the papers should be changed after running about 5 plates. The solvents used are: for the first dimension, chloroform–methanol–acetic acid–water (55 : 35 : 3 : 2); and for the second dimension, chloroform–acetone–methanol–acetic acid–water (45 : 16 : 15 : 11 : 6).

The plates are run in the first dimension until the solvent reaches the stop

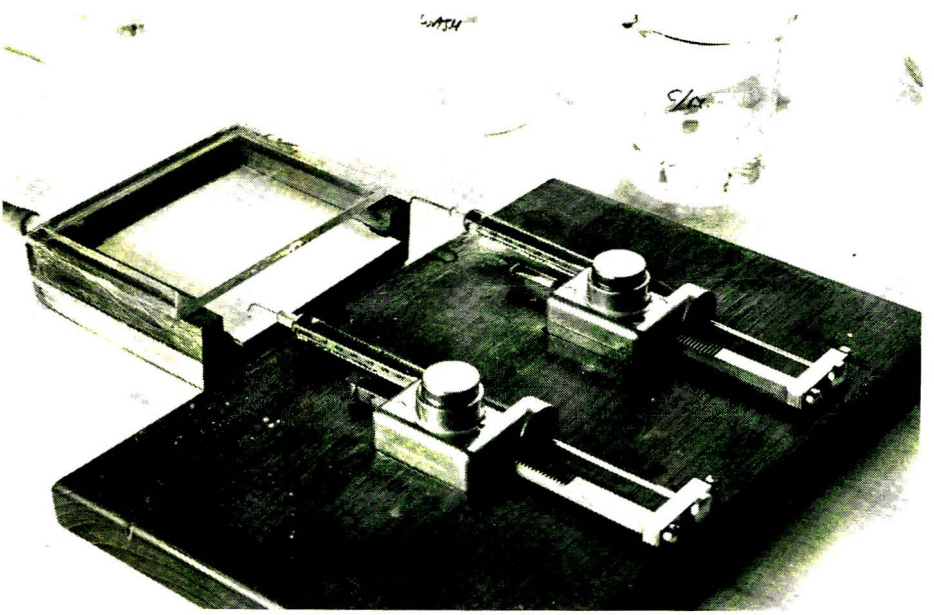

Fig. 1. On the left, a holder for a TLC plate which allows a stream of nitrogen to be passed over the plate during application of the sample, and, on the right, Hamilton syringes with dispensers for the "main" sample and "wash".

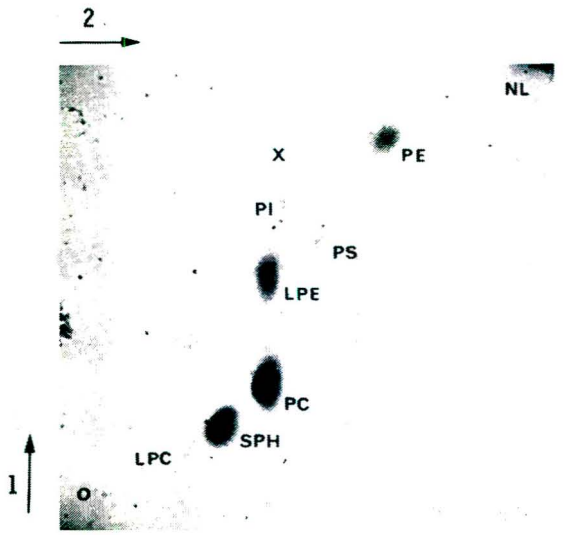

Fig. 2. Two-dimensional separation of phospholipids. See text for details. NL = neutral lipids; PE = phosphatidyl ethanolamine; X = unknown; PI = phosphatidyl inositol; PS = phosphatidyl serine; LPE = lysophosphatidyl ethanolamine; PC = lecithin; SPH = sphingomyelin; LPC = lyso-lecithin; O = origin.

line (ca. 12 min), then dried in a stream of nitrogen in a closed box for ca. 20 min. They are then run in the second dimension for an overall time of 20 min; this includes a period of ca. 10 min of "over-run" after the solvent has reached the stop line.

Two-dimensional separation of neutral lipids (Fig. 3)

Neutral lipid separations are done in unlined tanks. The solvents used are: for the first dimension, petroleum ether—diethyl ether—acetic acid (40 : 60 : 0.1); for the second dimension, petroleum ether—ethyl acetate—acetic acid (95 : 2 : 2).

Solvents are run to 1 cm from the edge of the plate in each dimension. In each case this takes about 5 min. Plates are dried as above for ca. 1 min.

"Three-dimensional" separation of neutral lipids (Fig. 4)

Occasionally it is expedient to use a "3-dimensional" separation of neutral lipids in order to improve the resolution of cholesteryl esters from methyl esters and hydrocarbons, especially when a sample containing a large concentration of these compounds is chromatographed. The third solvent is run in the opposite direction to the first as illustrated in Fig. 4. Chromatography is done in unlined tanks. The solvents used are: for the first dimension, petroleum ether—diethyl ether—acetic acid (35 : 55 : 0.1); for the second dimension, petroleum ether—ethyl acetate (90 : 4); for the third dimension, petroleum ether—ethyl acetate (90 : 2). As with the 2-dimensional separation, the solvents are run to 1 cm from the edge of the plate (ca. 5 min) for each dimension, and the plates are dried between solvents.

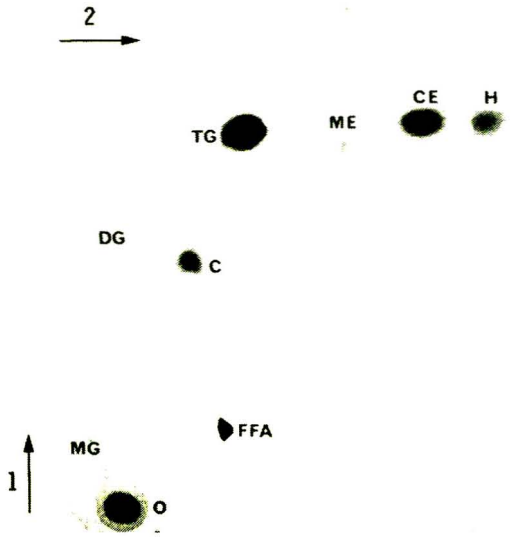

Fig. 3. Two-dimensional separation of neutral lipids. See text for details. H = hydrocarbons; CE = cholesteryl esters; ME = methyl esters; TG = triglycerides; DG = diglycerides; C = cholesterol; FFA = free fatty acids; MG = monoglycerides; O = phospholipids.

Visualisation

Charring. Because the plates do not contain any binder they do not stand up very well to spraying with aqueous reagents. They are best charred therefore, following vapour deposition of acid [31]. A plate is held in steam for 30 sec and then placed in a jar containing sulphuryl chloride vapour for 1 min. It is then heated at 180° on a hot plate for 5 min. This technique may be used prior to estimation of phospholipids by assay of phosphorus, but when measurement of neutral lipids or determination of fatty acid composition or radioactivity is required, the following non-destructive method must be used.

Iodine vapour. Plates are stood in iodine vapour for about 1 min at room temperature. The areas of lipids revealed fade rapidly on removal from the iodine vapour and should be outlined immediately with a needle.

Fluorescence. When lipids are to be analysed for fatty acid composition by gas—liquid chromatography (GLC), iodine vapour cannot be used, because it complexes with double bonds of unsaturated fatty acids. In this case the lipids are revealed by impregnating the plates with a fluorescent dye, 2,5-di-(5-*tert.*-butyl-2-benzoxazolyl)-thiophene (BBOT), which is normally used in liquid scintillation counting [32]. For both phospholipid and neutral lipid plates, BBOT (Ciba-Geigy, Duxford, Great Britain) is dissolved in the chromatography solvent (10 mg per 100 ml). After chromatography the plate is placed in a transparent plastic box under an atmosphere of nitrogen and viewed under UV light (3650 Å).

Photographic recording of separation

Charred and iodine stained plates are recorded by photography in transmitted light, using a small light box containing two 5-W fluorescent tubes. Plates

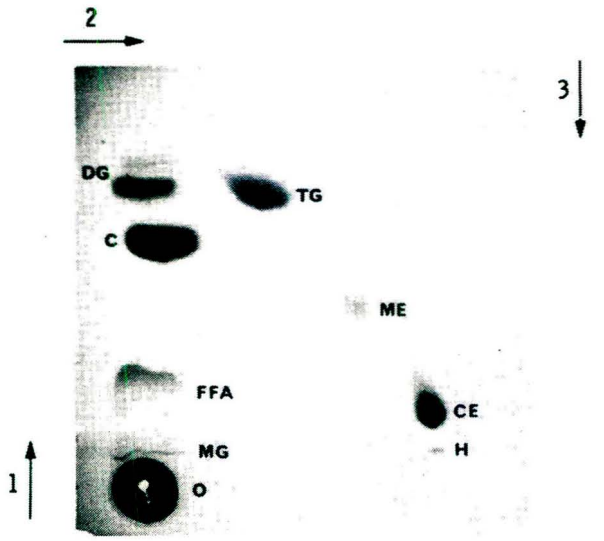

Fig. 4. "Three-dimensional" separation of neutral lipids. See text for details. Spot identification as in Fig. 3.

impregnated with BBOT are recorded by photography under UV light using a Wratten 6B lens filter.

Assays

Where assays are to be performed on samples separated by TLC, the appropriate area of the plate is scraped dry into a test tube by means of a small piece of razor-blade held in artery forceps.

Phosphorus assay. This assay is based on the malachite green method of Itaya and Ui [33]. The tubes used for this assay are 5 ml with C14 glass stoppers. They are reserved exclusively for this sensitive method. Glass distilled water is used throughout. To each tube is added 0.2 ml 60% perchloric acid and the material digested at 170—180° for 1 h. The tubes are cooled and 1.0 ml water added to each. With small spots from the plate 0.1 ml 60% perchloric acid and 0.5 ml water may be used to provide increased sensitivity, but for larger spots such as lecithin, the larger volumes must be used to ensure complete digestion of the phospholipid. The tubes are vortexed and then centrifuged at 1000 *g* for 5 min. 0.5 ml of the supernatant is sampled using an Eppendorff pipette, 1.5 ml of colour reagent (see below) is added and the tube shaken. The extinction at 660 nm is read against a reagent blank between 5 and 30 min later. A standard curve is prepared using a solution of potassium dihydrogen phosphate in the range of 0.05—1.2 μg phosphorus (1.6—38.8 nmoles).

The colour reagent is prepared as follows: one volume of ammonium molybdate (4.2% w/v in 5 *N* HCl) is mixed with 3 volumes of 0.2% (w/v) malachite green in water. After 30 min the mixture is filtered and kept at room temperature. It may be kept for 3 weeks.

Fluorimetric assay of cholesterol and cholesteryl esters. This method is based on that of Bondjers and Björkerud [34]. Lipid is extracted from the silica gel

with 2 lots of 1 ml of chloroform. The extracts are pooled, evaporated to dryness and 0.5 ml chloroform and 1.5 ml of freshly prepared reaction mixture (see below) are added. The tubes are stoppered and heated at 60° for 30 min then cooled immediately in crushed ice. The sample is measured at room temperature in a fluorimeter at an excitation wavelength of 528 nm, fluorescence wavelength of 565 nm between 40 and 80 min after heating. A standard curve of cholesterol or cholesteryl oleate is prepared in the range of 0.1—10 μg cholesterol (0.26—25.9 nmoles).

The reaction mixture is prepared as follows: a stock solution of zinc chloride is made by heating 4 g of zinc chloride (dried over phosphorus pentoxide) in 15 ml of glacial acetic acid at 80° for 2 h. This stock solution will last for 2 weeks at room temperature provided it is kept dry. A working solution is made immediately before use by adding 0.5 ml of stock solution to 10 ml of AnalaR acetyl chloride.

Glyceride assays. The glycerides are assayed using the autoanalyser method of Leon et al. [35] following elution from the silica gel: To each tube is added 1.0 ml *tert.*-butanol—isopropanol—water (50 : 50 : 4). The tubes are vortexed, stood for 1 h, vortexed again and then centrifuged at 1000 *g* for 5 min. The supernatant is decanted into a stoppered autoanalyser cup. A standard curve is prepared with diolein or triolein in the range of 5—60 μg triolein (5.7—67.8 nmoles).

Free fatty acid assay. This is based on a novel autoanalyser method of Bowyer et al. [36]. To each tube is added 1.2 ml of di-*n*-butyl ether and 1.0 ml of copper reagent. The tubes are vortexed for 2 min and centrifuged at 1000 *g* for 5 min. 1.0 ml of the upper phase is taken, 1.0 ml of colour reagent added and the colour allowed to develop for 15 min. The extinction is read at 540 nm. A standard curve is prepared using oleic acid in the range 1—6 μg (3.9—23.4 nmoles).

The copper reagent contains 0.1 *M* aqueous cupric acetate—water—glycerol—pyrrolidine (10 : 45 : 45 : 2). The colour reagent is a saturated solution of diphenyl carbazide in 0.5% (v/v) acetic acid in *n*-butanol, which is filtered before use.

Measurement of radioactivity. Silica gel from the plates is placed directly into the counter vials and 10 ml of scintillator fluid added. The solution which is based on that described by Patterson and Greene [37] contains: 1000 ml toluene; 500 ml Triton X-100, reagent grade; 6 g 2,5-diphenyloxazole (PPO), Packard, scintillation grade; 0.3 g 1,4-bis-(5-phenyloxazolyl-2)-benzene (POPOP), Packard, scintillation grade; 150 ml distilled water.

Preparation of methyl esters of fatty acids for GLC and estimation of fatty acid concentrations by an internal standard method. This is based on the method of Bowyer et al. [38]. Following chromatography, separated lipids are located by fluorescence using BBOT under nitrogen (see above). The required fractions are scraped into tubes and 1 ml of transmethylation reagent added. The tubes are stoppered and heated at 80° for 1 h except for sphingomyelin which is heated for 16 h. The tubes are then cooled, the solution transferred with 1 ml of distilled water to a tap funnel and extracted with 2 portions of 10 ml petroleum ether. The petroleum extracts are then combined, washed with 10 ml water and dried over ca. 0.5 g of anhydrous sodium bicarbonate for

5 min. The extract is then decanted off into a clean tube and evaporated to a small volume in a stream of nitrogen. This is then transferred to a new disposable glass sample vial and evaporated to dryness. The sample is dissolved in 1 μl of carbon disulphide and injected into the chromatograph (Pye Series 104, with column of 10% polyethylene glycol adipate on Celite, run isothermally at 190°; carrier gas,nitrogen; detector, hydrogen flame ionisation).

Extreme care must be taken to use ultra-clean glassware and redistilled solvents throughout. A blank of silica gel from a plate is run through the whole procedure before any samples are prepared to ensure that all of the reagents and glassware are clean.

The methylating solution is prepared specially in order to reduce contamination as follows: AnalaR methanol is redistilled before use from one pellet of sodium hydroxide using a redistillation apparatus closed with a drying tube containing calcium chloride. Concentrated sulphuric acid (3 ml) is then added to 100 ml of the redistilled methanol. The mixture is then heated at 70° for 1 h and extracted with 50 ml of petroleum ether and the extract discarded.

Where quantitation of the fatty acids of lipid fractions is required, an internal standard of margaric acid (C17:0) is added at a convenient stage [39]. In the estimation of free fatty acid the standard is added to the tissue before extraction; for other lipids it is included in the transmethylation step and it is assumed that the percentage conversion of the standard to methyl esters is the same as the conversion of the fatty acids of the complex lipids. A convenient weight of margaric acid is 10 μg for the lipids from a sample of 0.2 ml plasma or 50 mg of arterial tissue. This is added as a solution (100 μg/ml) of margaric acid in chloroform.

The weights of the separated fatty acids are determined by comparing the peak area for each fatty acid with the area of the added internal standard.

RESULTS AND DISCUSSION

Separation of phospholipids

A typical separation of phospholipids from atherosclerotic rabbit aorta is shown in Fig. 2. Such resolution has been consistently obtained in over 2000 separations and is only compromised if the plates are not properly activated or the sample is not clean, being contaminated by inorganic ions. Thus, plates should not be left in a humid environment, nor should they be heated for more than 1 min at 100° as this causes the ammonium sulphate to break down.

During preliminary investigations of optimum conditions of chromatography, the addition of materials to the silica gel to improve resolution of the phospholipids was investigated. Various substances had been suggested, for example magnesium silicate and magnesium acetate [40], sodium acetate [41,42], borax [43,44], sodium oxalate [45], and ammonium ions as ammonium sulphate or ammonium nitrate [11,25,26]. The use of the appropriate concentrations of ammonium sulphate as suggested by Kaulen [26], gave consistently good separation as shown here. The improved resolution apparently occurs because the bound hydrated ammonium ion increases the strength of hydrogen bonding to the stationary phase. Mobility of the acid phospholipids is also altered, because of the formation of their ammonium salts. A similar effect is

achieved if ammonium hydroxide is added to the chromatography solvent [25].

The separated phospholipids have been identified by co-chromatography with pure standards. In addition, the lyso-phosphatidyl ethanolamine (LPE) spot was isolated and kindly analysed by Dr. R.M.C. Dawson by hydrolysis and separation of the constituent bases [46]. Glyceryl-phosphoryl ethanolamine was the only base found, confirming the identity as LPE.

The identity of the phosphatidyl choline, phosphatidyl serine, phosphatidyl inositol and phosphatidyl ethanolamine (respectively PC, PS, PI and PE) spots was also confirmed by separation of radiolabelled lipids. These were synthesised from radiolabelled choline, serine, inositol or ethanolamine by incubation with minced arterial tissue in Krebs—Ringer bicarbonate buffer (pH 7.4) for 1 h. In each case 85% of the radioactivity was found in the expected spot. No label was found in lyso-phospholipids. Residual activity was associated with the origin of the plate, but its identity was not established.

Pure standards of phosphatidic acid and cardiolipid have also been chromatographed in this system and migrate near to the solvent front. One minor component of arterial lipids (X) remains unidentified.

In our experience, the method described for the separation of phospholipids gives much better separation than the single dimension separations, which have been described for the resolution of PC, PI, PS [47—51]. We have also found it to be as good as many of the 2-dimensional systems on large plates [52—58], but having the very important advantage of high speed of separation, good sensitivity and economy of materials. The only other published method [18] for the 2-dimensional separation of phospholipids using small plates, failed to separate PI from PS.

Assay of phospholipids

In preliminary investigations, it was found that the techniques of analysis of phosphorus [59—61] which have been widely used for phospholipids separated on large TLC plates were not sufficiently sensitive for the small amounts of material from small plates. On the other hand, the method of Itaya and Ui [33] using malachite green had sufficient sensitivity except where a spot was barely visible following charring (less than 1 μg of lipid). Other methods of similar sensitivity using malachite green have recently been published [62,63]. An even more sensitive fluorescence method using rhodamine G [18] might allow the measurement of phospholipid in even the smallest spots. That technique has the disadvantage, however, that the lipid must be eluted from the silica gel, a time consuming and potentially inaccurate technique and that a standard curve must be prepared with a pure standard for each lipid class.

The method of Itaya and Ui [33] was modified slightly in our procedure. Originally it required the addition of Tween 20 to stabilise the reaction product. We found that the Tween absorbed strongly at 660 nm, the wavelength used to measure the extinction of the reaction product. Thus, small errors in pipetting the Tween lead to large errors in the results. Its omission did not lead to rapid fading of the colour by precipitation of complexes, as suggested by Itaya and Ui, and the colour was quite stable at room temperature up to 30 min after addition of the malachite green reagent.

Using the techniques of charring on the plate and digestion of lipid in the

presence of silica gel we found no interference with the assay. This is in contrast to some reports concerning analysis by the phosphomolybdate complex methods, where the presence of silica gel may alter the pH [64] or lead to the formation of a silico-molybdate complex [65]. In our technique the very small amount of silica gel produced no effect.

In some samples, when there was a large difference in the concentration between the smallest and largest components, it was necessary to sample only a portion of the digest of the larger spots. This was simply achieved by the use of a different size Eppendorff pipette and did not affect the precision. With a little experience it was easy to judge from the intensity of charring on the plate, whether the whole sample or only a portion would be required for analysis.

In order to assess the overall recovery of phospholipids after TLC, 6 samples each of lyso-lecithin (LPC), PC and PE were assayed directly and 6 of each following TLC. The 6 samples were chosen to form a series of increasing amount within the usual range of the assay and the best straight line through the points computed by least squares. The mean recoveries for the chromatographed samples were: LPC, 98.5%; PC, 100.6%; PE, 95.0%.

Separation of neutral lipids

Typical separations of neutral lipids in 2 and 3 dimensions are shown in Figs. 3 and 4. Good resolutions in both systems have been consistently obtained. Impregnation of the silica gel with sodium carbonate markedly improved the reproducibility of separation of the free fatty acids (FFA); this was presumably due to stabilisation of the pH of the layer.

"Three-dimensional" separation was useful when a sample, such as atherosclerotic arterial tissue, contained a high concentration of free cholesterol and cholesteryl esters. The separated neutral lipids have been identified by cochromatography with pure standards.

Assay of neutral lipids

The specific chemical methods of analysis had sufficient sensitivity for analysis of the amounts of materials encountered in most samples.

The fluorimetric analysis for cholesterol and cholesteryl esters [34] was sufficiently sensitive for measurement even of the low concentrations of cholesteryl esters in undiseased arterial tissue.

The method was also tested for interference by di and triglycerides (DG, TG) and squalene; none was found. The recoveries after TLC for 6 samples each of free cholesterol (CHOL) and cholesteryl oleate (CE) in the usual range of assay, were: CHOL, 98.7%; CE, 94.8%.

The fluorimetric analysis of glycerides [35] by autoanalyser was always sufficiently sensitive for TG estimation, but in general the amount of DG was too small (<5 μg) to be measured. The recovery for 6 samples of TG was 97.5%.

Although the concentration of FFA in arterial tissue is small, the amount present in relation to the other major neutral lipids of a sample, could be measured by the novel method of Bowyer et al. [36]. Like other methods [66,67], this technique is based on the extraction of copper soaps of fatty acids into organic solvent, followed by estimation of copper. It is novel,

however, in two respects. Firstly, the di-*n*-butyl ether used as organic solvent forms an upper phase in the biphasic mixture and thus allows a cleaner and easier separation from the aqueous copper reagent. Secondly, the sensitivity of assay of copper is increased by the use of diphenyl carbazide. The recovery for 6 samples of FFA (oleic acid) was 93.6%.

General methods of analysis of phospholipids and neutral lipids separated by TLC

In the past few years many people have attempted to estimate materials which have been separated by TLC, directly on the plates, either by charring, followed by densitometry with a scanning spectrophotometer or by staining with a fluorescent dye, followed by scanning with a fluorimeter. It has been suggested that these methods have the advantage of speed over chemical analyses whilst providing good reproducibility. We have also investigated charring in situ using sulphuric acid-sodium dichromate sprays and vapour phase acid impregnation [31], followed by densitometry. In the first place small streaks of lipid were chromatographed in 1 dimension as suggested by Van Gent [14] and scanned using the Joyce Loebel scanning densitometer. When separated components were the same width as standards, good reproducibility was obtained. When, however, chromatography was not perfect because of a poor plate or poor sample application and hence the streaks were not uniform, the results were more variable. This obviously arises because the density of charred materials within the spectrophotometer window depends upon the width of the streak. In order to overcome this problem and to permit scanning of spots on 2-dimensional separation, we developed [68] a flying spot television scanner, connected to a PDP-7 computer which produced a digitised image of a plate. By the use of object recognition programs [69], it was possible almost instantaneously to present a table of integrated optical densities and hence amounts of each material on the plate. This approach thus overcame the difficulties inherent in 2-dimensional scanning. Similar techniques using a flying spot micro-densitometer and batch computation on an IBM 360 of the digitised image have been described [8].

Despite the undoubted improvement provided by this approach over conventional scanning, it has been our experience that the methods are less reproducible than chemical assays. Furthermore, the charring and fluorescence depend upon the fatty acid composition of the separated lipids, and for the most precise work a standard of the same composition as that of the unknown is required, a most improbable practicality. The difficulty of establishing reliable and stable standards to which assays done anywhere in the world can be related on an absolute scale is almost insurmountable. We have thus preferred to go back to precise chemical assays which can be reproduced anywhere. In addition. the method does not require specialised and expensive apparatus.

Measurement of radioactive samples

Various methods for measurement of radioactivity of lipids separated on TLC have been described. These include elution and direct counting of adsorbent either in scintillation cocktail or as suspension in Cabosil. The

direct method in scintillator is to be preferred from the point of view of simplicity and also because no loss of material is incurred in elution. There is a potential disadvantage, however, that if lipid remains bound to the silica gel and is not dissolved in scintillator, self-absorbtion losses of activity will occur, especially with weak β-emitting isotopes such as ^{3}H. In preliminary experiments, we investigated various scintillation mixtures and found that whereas non-polar lipids such as cholesteryl esters could be counted without self-absorbtion losses in a scintillator containing only toluene, a polar scintillator containing water was required for counting the polar phospholipids. The most effective and economical system was the heterogeneous system of Patterson and Greene [37] with the addition of 10% water. A similar system using a commercial scintillator mixture, Aquasol, with 10% water has been described by Webb and Mettrick [70].

In order to test for complete elution of representative lipids into the scintillator, samples of [7-^{3}H]cholesteryl oleate, [9,10-^{3}H]palmitic acid and 1-palmitoyl, 2-[9,10,12,13-^{3}H]linoleyl-glyceryl-phosphorylcholine, i.e. [^{3}H]-lecithin were counted, after TLC, in the toluene, Triton X-100 scintillator with and without water. The samples were shaken and the count rate determined for a period of 30 min. The results, summarised in Fig. 5, show that in scintillator without water, for lecithin and to a lesser extent for palmitic acid, the count rate falls as the silica gel sediments. On the other hand, in the scintillator containing water, although the absolute count rate is lower, it does not fall as the silica gel sediments, because the lipids are in solution. When it is required to measure counting efficiencies, either External Standard Channels Ratio (ECR) or Sample Channels Ratio (SCR) may be used, because complete elution of lipid into solution produces a homogeneous sample [71]. In the determination of efficiencies by ECR using counters such as the Nuclear Chicago, in which the external standard is positioned underneath the vial, it has been shown that the presence of more than 50 mg of silica gel on the bottom of the vial may lead to erroneous results [72]. In the method described we have shown that the small amount of silica gel introduced into the vial, even with the largest sample spot, is too small (<10 mg) to cause any interference.

Elimination of losses of lipid during the Folch extraction procedure and transfer to TLC

In view of the small amount of material which may be measured by this method and the number of stages and transfers during extraction, it was decided to investigate the recovery of lipid through the extraction procedures. Any stages at which losses or variability occurred were discovered by sequential investigations and methods were devised to overcome problems.

Firstly, an important reason for low recovery was found to be incomplete transfer of lipids through the various pieces of glassware used. Transfer from the small sample vial was improved by the use of two Hamilton syringes (one containing the sample, the other a wash of the sample vial). An unexpected loss was also found in transfer of the sample from the flasks used for evaporation of solvents. Originally chloroform—methanol (2:1) was used. Although quantitative transfer of relatively non-polar lipids such as cholesterol and cholesteryl esters was achieved, there was incomplete recovery of the more polar phopholip-

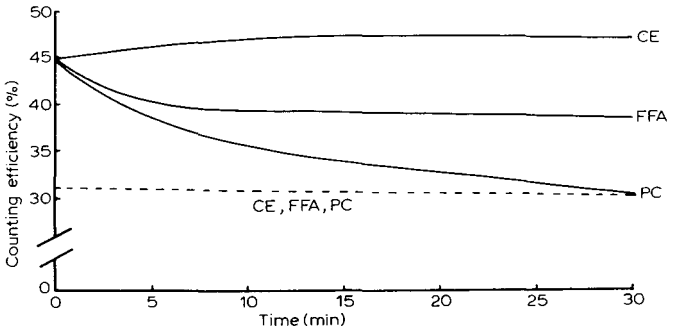

Fig. 5. Graph showing counting efficiency of [^{3}H]cholesteryl oleate (CE), [^{3}H]palmitic acid (FFA) and [^{3}H]lecithin (PC) in toluene—Triton X-100 scintillator, without (—) or with (— — —) 10% water.

ids such as LPC (80.0%) and PC (80.8%). This problem was overcome by the use of chloroform—methanol—saline (86 : 14 : 1). Lipids were dissolved in chloroform—methanol (2 : 1) and samples assayed directly. The solvent was evaporated to dryness and the sample transferred to small vials with three washes of either chloroform—methanol (2 : 1) or chloroform—methanol—saline (86 : 14 : 1). The samples were then re-assayed. Only the latter solvent gave complete transfer of polar lipids. It is probable that some of the polar phospholipids are bound to the glass by virtue of their surface active properties unless a wet solvent such as chloroform—methanol—saline (86 : 14 : 1) is used for transfers.

Secondly, careful analysis also showed that loss of extract could occur on the glass fibre filter disc. This was overcome by the procedure of squeezing the disc with forceps, washing with a further portion of solvent and then squeezing the disc again.

Thirdly, we considered the effectiveness of the extraction procedure. Although the method of Folch et al. [27,28] is well established, certain steps require careful attention or incomplete and variable extraction occurs. We have found that it is important during addition of the first extraction solvent, that the methanol be added before the chloroform. If a mixture of solvents is used, putty-like globules are formed and the lipid is only partially extracted. When methanol is added first, recovery of lipid is complete. Table I summarises the results of an experiment demonstrating this effect. Twelve 0.2-ml samples of plasma from a Cynomolgus monkey were extracted as described in the methods section, using either chloroform—methanol (2 : 1) mixture for the first solvent or methanol and chloroform sequentially. Six further samples were extracted with isopropanol as for the autoanalyser estimation [35] of cholesterol and triglyceride. The extracts were analysed for cholesterol as described [34]. When chloroform—methanol (2 : 1) was used, the recovery of cholesterol was only 56% compared with the isopropanol extract, but when methanol and chloroform were added in sequence, the recovery was 96%.

In the Folch procedure it is also important to minimise the loss of polar compounds, such as lyso-phospholipids and free fatty acids into the wash phase. In our procedure this was achieved by re-extracting the upper aqueous wash

phase with chloroform—methanol—saline (86 : 14 : 1). The use of a saline wash acidified with 0.01 *N* HCl as recommended by Bjerve et al. [73] for preventing loss of lyso-phosphatides was also investigated. This produced however, an insignificant improvement in lyso-lecithin recovery. It also had the serious disadvantage of causing hydrolysis of plasmalogens during an overnight wash even at 4° and was, therefore, not used.

The recovery of lipid through the whole procedure was checked using free cholesterol, cholesteryl oleate, triolein, lyso-lecithin, lecithin and phosphatidyl ethanolamine in separate experiments. For each lipid, standards containing 6 points in the working range of the assay were set up and the samples assayed directly. Similar amounts were then run through the whole procedure involving extraction, filtration and "Folch-washing" and then assayed. Recoveries were as follows: cholesterol, 95.0; cholesteryl oleate, 102.2; triolein, 95.7; oleic acid, 95.4; lyso-lecithin, 92.0; lecithin, 97.2; phosphatidyl ethanolamine, 88.6%.

Precision of the method for biological samples

In order to measure the precision of the methods, 6 samples of human plasma were analysed. Table II shows the results and the coefficient of variation

TABLE I

RECOVERY OF CHOLESTEROL FROM PLASMA AFTER EXTRACTION WITH DIFFERENT SOLVENTS

	Isopropanol	Methanol and chloroform in sequence	Chloroform—methanol (2 : 1)
Concentration (mg/100 ml)	385.3	367.0	198.6
± S.D.	± 14.60	± 14.09	± 9.06
Coefficient of variation (%)	3.79	3.84	4.56
Recovery compared with isopropanol extract (%)		95.9	55.7

TABLE II

REPRODUCIBILITY FOR REPLICATE ANALYSIS OF THE MAJOR LIPIDS OF HUMAN PLASMA

Concentration m*M* ± % coefficient of variation (% CV). Abbreviations as in Figs. 2 and 3.

	LPC	SPH	PC	FFA	CHOL	CE	TG
One lipid extract separated by TLC and analysed 6 times							
Mean	0.140	0.369	0.640	0.446	0.683	1.815	0.616
± % CV	9.09	3.44	8.76	9.01	9.13	5.59	3.44
6 samples of plasma extracted, separated by TLC and analysed							
Mean	0.161	0.365	0.660	0.438	0.661	1.788	0.580
± % CV	12.42	4.66	8.33	4.80	9.53	6.94	5.35

for the major lipids. In each case the errors are of the same order as commonly associated with pipetting.

ACKNOWLEDGEMENTS

We wish to thank Dr. R.M.C. Dawson for analysing and identifying LPE and for pointing out the hydrolysis of plasmalogens by acidified solvents. We are also grateful to Mrs. L.M. Wright for secretarial assistance and to Mr. P. Curtis for preparation of photographs and figures, and to the Tobacco Research Council of Great Britain, Nattermann, Köln, G.F.R. and May & Baker, Dagenham, Great Britain for financial support.

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Journal of Chromatography, 143 (1977) 491—497
Biomedical Applications

CHROMBIO. 057

QUANTITATIVE ANALYSIS OF TRICYCLIC ANTIDEPRESSANTS IN SERUM FROM PSYCHIATRIC PATIENTS

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(Received December 3rd, 1976)

SUMMARY

A method for the quantitative analysis of tricyclic antidepressants in the serum of psychiatric patients is described. The method can be used for determining amitriptyline, nortriptyline, imipramine, demethyllimipramine, clomipramine, demethylclomipramine, trimipramine and protriptyline. The method consists in a series of extraction steps followed by gas chromatography with a flame-ionization detector. The drugs are determined in their native state. The internal standard method is used for the quantitation.

INTRODUCTION

In psychiatric clinics and hospitals there is an increasing demand for determinations of concentrations of tricyclic antidepressants in plasma or serum. Various methods for determining such concentrations are available but, with few exceptions [1,2], for only one or two drugs. As most psychiatric units use several antidepressants, a single method that is capable of determining as many antidepressants as possible as well as their main metabolites would be useful.

The method proposed here can be used for determining the concentrations of amitriptyline, nortriptyline, imipramine, demethylimipramine, clomipramine, demethylclomipramine, trimipramine and protriptyline in serum. The method consists in extraction from serum, followed by sepration and quantitation of the unchanged drug by gas chromatography employing a flameionization detector and an internal standard.

MATERIALS AND METHODS

Sampling routines

Venous blood samples were drawn from fasting patients in the morning before the first dose of medicine of the day, approximately 12 h after the last dose on the day before. A condition for determination of the serum level was that the patient had received the same daily dose of antidepressive drug for at least 1 week.

Reagents

All reagents were of analytical grade, except *n*-hexane, which was of pesticide grade (Fisher). All aqueous solutions were prepared with water re-distilled in an all-glass apparatus. Sodium hydroxide solution (2 *M*) was washed with diethyl ether and with *n*-hexane. The antidepressive drugs used as reference compounds were generously provided by the manufacturers.

Glassware

The centrifuge tubes used in the first two extraction steps were new and were used only once. Before use they were cleaned by rinsing with hot tap-water and then with distilled water in a dish-washing machine. The small glass-stoppered tubes used in the third extraction step were used only for these analyses and were rinsed with ethanol—*n*-heptane (1:10) and with formic acid—water (1:10) between each run. The small conical test-tubes in the final step were of disposable type made from new Pasteur pipettes that had first been rinsed with 99% ethanol.

Internal standard

Any of the tricyclic drugs could be used as an internal standard in the determination of another tricyclic drug. For each series of determinations a suitable internal standard was chosen from those tricyclic drugs which were not to be included in that series. Later it was found that cyproheptadine could serve as an internal standard for all of the drugs, and this substance was then generally used. A 0.1 m*M* solution of the internal standard in 99% ethanol was prepared and 2.00 nmole were added to each sample.

Extraction procedure

Mixing was performed either by slowly rotating the tubes at 20 rpm or by agitating them on a Whirlimixer. The phases were separated by centrifugation for 10 min at 1000 *g*. Transfers were made with the aid of disposable Pasteur pipettes.

Each sample of 4 ml of serum was pipetted into a polyethene stoppered centrifuge tube and the internal standard was added. The samples were then made alkaline by addition of 0.2 ml of 2 *M* NaOH and extracted with 7 ml of *n*-hexane—isoamyl alcohol (100:3) by rotation for 15 min. After separation of the phases, the hexane phase was transferred into another polyethene stoppered centrifuge tube and extracted with 2 ml of 0.1 *M* HCl, also by rotation for 15 min. The hydrochloric acid phase was transferred into a smaller glass-stoppered tube, made alkaline with 0.2 ml of 2 *M* NaOH and extracted with

0.5 ml of *n*-hexane—methyl isobutyl ketone (100:3) by agitating for 30 sec. The organic extract was transferred into a small conical test-tube and 20 μl of formic acid—methanol—water (1:5:5) were added. The tube was agitated for 60 sec and then allowed to stand for 5 min for the phases to separate, whereupon the organic phase was evaporated to dryness in a KOH-containing vacuum desiccator, which was evacuated with a water suction pump. The dry residue was dissolved in 7 μl of *n*-heptane—toluene—isoamyl alcohol—diethylamine (80:20:1.5:1) by sonication, and 2 μl were injected into the gas chromatograph.

Gas chromatography

The gas chromatograph was a Perkin-Elmer F 11 instrument equipped with a flame-ionization detector and 2 m × 2 mm I.D. silanized glass column packed with Carbowax 20M (1.4%) and KOH (1.4%) on Gas-Chrom Q, 60—80 mesh. The column temperature was 200° and the carrier gas was nitrogen at a pressure of 70 kPa, giving a flow-rate of 35 ml/min. The gas was purified by passing it through an oxygen filter.

Quantitation

Standard serum samples containing known amounts of the substances to be determined were included in each set of determinations. The concentrations in the test samples were then calculated from the peak heights. Duplicate analyses were run as a routine.

RESULTS

This method has been used for routine clinical determinations of amitriptyline and nortriptyline, of imipramine and demethylimipramine, and of clomipramine and demethylclomipramine. It can also be used for measuring trimipramine and protriptyline. The relative retention times of the drugs are listed in Table I, and Fig. 1 shows gas chromatograms of serum extracts from patients.

There were linear relationships between the serum concentration of each drug and the peak-height ratio between the drug and the internal standard within the concentration ranges found in patients. This was tested by adding known amounts of the drugs to blank serum samples and then analyzing the samples.

To test the yield of the extraction procedure, known amounts of the drugs were added to blank serum samples and the extraction procedure was performed without the internal standard, which was not added until just before the injection into the gas chromatograph (Table II).

The precision of the method was calculated from duplicate values obtained from routine analyses of amitriptyline, nortriptyline, imipramine, demethylimipramine, clomipramine and demethylclomipramine (Table III). The precision of the determination of trimipramine and protriptyline was calculated by repeatedly analyzing pooled serum samples containing these drugs (Table IV).

Most other psychoactive drugs did not interfere in the determinations. Non-

TABLE I

RELATIVE RETENTION TIMES OF TRICYCLIC ANTIDEPRESSANTS

The retention times were calculated relative to cyproheptatidine, which had an absolute retention time of 5 min.

Drug	Relative retention time
Trimipramine	0.44
Amitriptyline	0.46
Imipramine	0.56
Nortriptyline	0.68
Demethylimipramine	0.84
Protriptyline	0.90
Clomipramine	1.10
Demethylclomipramine	1.74

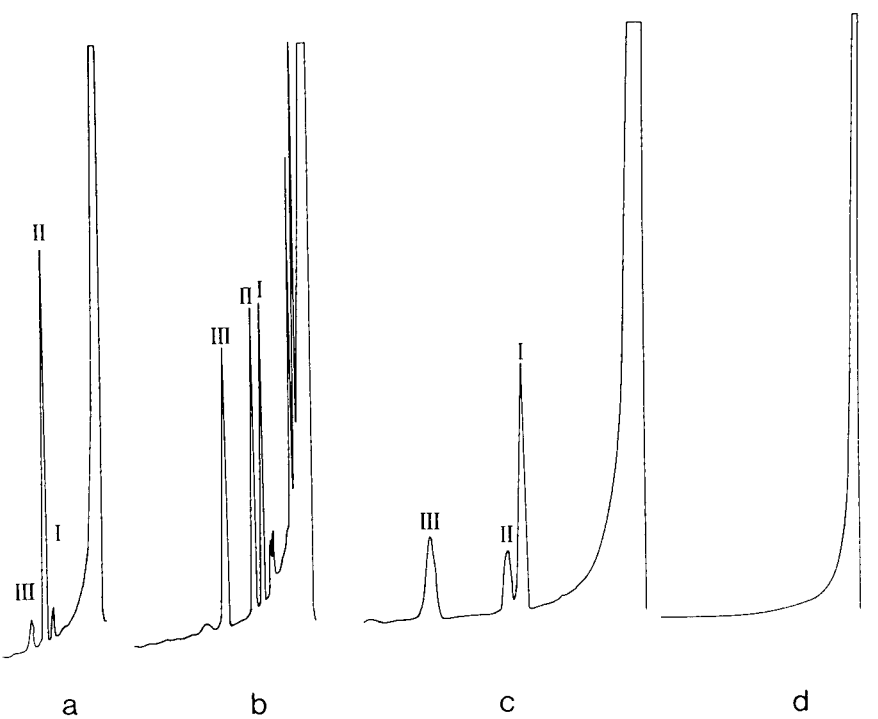

Fig. 1. Gas chromatograms of serum extracts from patients. (a) Patient treated with 30 mg of amitriptyline per day. I = Amitriptyline (0.04 μM); II = imipramine (internal standard); III = nortriptyline (0.08 μM). (b) Patient treated with 150 mg of imipramine per day. I = Amitriptyline (internal standard); II = imipramine (0.34 μM); III = demethylimipramine (0.55 μM). (c) Patient treated with 75 mg of clomipramine per day. I = Cyproheptadine (internal standard); II = clomipramine (0.16 μM); III = demethylclomipramine (0.48 μM). (d) Patient not treated with any tricyclic antidepressant.

TABLE II

PERCENTAGE YIELD OF THE EXTRACTION PROCEDURE

Drug	Concentration (μM)		
	1	0.2	0.1
Amitriptyline	84		86
Nortriptyline	79	79	
Imipramine	92		90
Demethylimipramine	81	71	
Clomipramine	71		71
Demethylclomipramine	65	59	
Trimipramine	75		75
Protriptyline	85	94	

basic drugs were separated by the extraction procedure, while basic drugs, such as opiates, benzodiazepines, butyrophenones and carbamazepine, as well as most phenothiazines, had longer retention times in the gas chromatographic system. Interfering drugs were alimemazin and promethazine, which were not completely separated from demethylimipramine, and promazine, which was not separated completely from clomipramine.

The serum concentrations of all of the drugs found in patients were of the same magnitude and ranged between 0.05 and 1.5 μM. The daily dose of antidepressant varied between 30 and 225 mg. As has been shown repeatedly before, there was a considerable inter-individual variation of the serum concentration in patients treated with an identical dose. Thus, in a group of 20 pa-

TABLE III

PRECISION OF THE METHOD CALCULATED FROM DUPLICATE VALUES

The coefficient of variation was calculated from the following equations:

$$S = \sqrt{\frac{\Sigma(x_{i1} \times x_{i2})^2}{2n}} ; \qquad V = \frac{S \times 100}{x}$$

where V = coefficient of variation as percentage of the mean, S = standard deviation, x_{i1} and x_{i2} = duplicate analysis of the same sample, x = mean of all analysis values and n = number of samples.

Drug	Coefficient of variation (% of the mean)	n
Amitriptyline	6.6	74
Nortriptyline	9.4	83
Imipramine	10.7	23
Demethylimipramine	9.1	21
Clomipramine	7.8	31
Demethylclomipramine	13.2	31

TABLE IV

PRECISION OF THE METHOD CALCULATED FROM REPEATED ANALYSES OF THE SAME SAMPLE

Drug	Concentration (μM)	Coefficient of variation (%)	n
Trimipramine	0.1	10.5	9
	1.0	4.8	9
Protriptyline	0.1	11.8	9

tients given 150 mg of amitriptyline a day in three equal doses, the serum concentration of amitriptyline varied between 0.06 and 0.60 μM and the nortriptyline concentration between 0.1 and 1.25 μM. The ratio between the amount of demethylated metabolite and that of the parent substance also showed a considerable inter-individual variation between 0.44 and 7.5, with a mean of 2.37.

DISCUSSION

The proposed method can be used for the quantitative analysis of most tricyclic antidepressive drugs in serum (all but one of those listed in the Swedish Pharmacopeia) and some demethylated metabolites. The method is simple enough for routine clinical use. The substances are analysed in their unchanged state and no preparation of derivatives was found to be necessary. Also, the type of gas chromatograph used is fairly simple and inexpensive, with an ordinary flame-ionization detector. Its sensitivity is sufficient for the determination of serum levels in patients and even levels far below those which can be regarded as therapeutically optimal. The method can also be used for kinetic studies. With the aid of mass fragmentography [1], it is also possible to determine all tricyclic antidepressants, but such a sophisticated and expensive instrument is hardly suitable for routine clinical analyses.

A general gas chromatographic method employing a nitrogen detector for the determination of plasma levels of these drugs has recently been described [2], but its sensitivity is essentially the same as that of our method. The former method was exemplified with plasma determinations of imipramine only.

One difficulty in this type of analysis is that the sample extracts may contain substances that are eluted from the gas chromatograph together with the drugs to be determined. Such interfering substances may be present in serum, but the samples may also have been contaminated during the extraction procedure. Owing to the high sensitivity of the method, even trace amounts of impurities, which may also be present in chemicals of good quality, may interfere in the analyses. Another source of contamination is the rinsing procedures, in which glassware in poor condition may become contaminated. To mitigate the risk of contamination, the extraction procedure had to be made more elaborate than is usual for this type of analysis. In addition, it is essential to adhere to very strict standards in the preparation of solvents and handling of glassware. The

routines described here have given satisfactory results, but the procedures may, of course, be modified according to the conditions in different laboratories and give equally good results.

Patients treated with an antidepressant very often also receive other psychoactive drugs, mostly sedatives and tranquillizers. As most psychoactive drugs are fairly closely related chemically, there is always a considerable risk that one drug may interfere in the determination of another. In the proposed method, this risk is small as most other psychoactive drugs are either separated by the extraction or have clearly different retention times in the gas chromatographic system.

ACKNOWLEDGEMENTS

This study was supported by a grant from Swedish Medical Research Council, No. B77-21X-03871-04A.

The authors are particularly grateful to Mrs. Ann-Britt Wassgren and Mrs. Susanna Nyiredy, who contributed substantially to the development of the method.

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Journal of Chromatography, 143 (1977) 499—512
Biomedical Applications

CHROMBIO. 054

DETERMINATION OF AMITRIPTYLINE AND SOME OF ITS METABOLITES IN BLOOD BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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(Received January 10th, 1977)

SUMMARY

Conditions for the determination of amitriptyline and some of its metabolites in serum on a reversed-phase material (C-8) by high-pressure liquid chromatography with UV detection at 254 nm were systematically investigated. The separation of tricyclic antidepressants is best carried out on a phase system consisting of C-8 bonded-phase material as the stationary phase and water—methanol—dichloromethane—propylamine as the mobile phase.

The precision and detection limit of the method and the extraction efficiency were established. A chromatogram of a serum extract from a patient treated with amitriptyline is shown. Serum levels of amitriptyline and its four main metabolites (nortriptyline, desmethylnortriptyline, *trans*-10-hydroxy-amitriptyline and *trans*-10-hydroxy-nortriptyline) in a patient receiving 150 mg of amitriptyline daily, are reported.

INTRODUCTION

Tricyclic antidepressants have been used for many years in order to treat psychiatric patients suffering from depression and amitriptyline, nortriptyline and protriptyline are the drugs most commonly prescribed for this purpose. In a number of papers [1—3], the relationship between the plasma concentration of tricyclic antidepressants and their clinical effect on depressive symptoms is discussed. Some workers maintain that there is no significant correlation between plasma concentrations and the improvement of depressive symptoms [1], while others found that the treatment of depression with nortriptyline is effective only in the concentration range 50—150 ng/ml in plasma [2,3].

It is known that tricyclic antidepressants are metabolized in the body [4], but present methods for the analysis of antidepressants, such as UV spectrometry [5,6], fluorimetry [7—9], thin-layer chromatography [10—12] and gas chromatography [11—17], are restricted mainly to the drugs themselves and cannot be applied to their metabolites. A more specific determination of antidepressants would be useful for metabolites with antidepressive properties (e.g., nortriptyline from amitriptyline) and to discriminate poor biological

availability from fast metabolic degradation (e.g., hydroxylation) in cases of low blood levels. Analytical procedures that permit the detection and quantitation of metabolites as well as the drug itself provide more reliable information about the concentration of active antidepressive compounds in body fluids and their clinical effect, more insight into the rate of the biotransformation of these drugs in the body, which can differ greatly individually, and the possibility of carrying out a more extensive pharmokinetic study.

High-pressure liquid chromatography (HPLC) has been shown to be a very powerful technique in biomedical analysis [18—22]. A few papers on the separation of some tricyclic antidepressants have been published, but metabolites were not included in these studies [23,24]. In this paper, the separation and determination of amitriptyline and some of its most important metabolites in plasma, using a reversed-phase system, by HPLC with UV detection is described.

EXPERIMENTAL

Apparatus

The liquid chromatographic system was constructed from a high-pressure reciprocating membrane pump (Orlita DMP 1515, Giesen, G.F.R.); a flow-through manometer acting as a pulse damper; a UV detector (Waters 440); an injection valve (Valco CV-6-UHP$_a$ or Rheodyne 70-10); a potentiometric recorder; and an electronic integrator (Autolab System I, Spectra Physics). The eluent reservoir was placed in a thermostated water-bath (25°).

In all experiments, stainless-steel 316 precision-bore columns with an I.D. of 3 mm and of different length were used. To connect the column to the detector and injection valve, terminators of modified Swagelok reducing unions assembled with 0.3-mm capillary tubing were used, guaranteeing minimal external peak broadening (Fig. 1). In order to prevent contamination of the analytical column by impurities in the eluent, a stainless-steel column (length 200 mm, I.D. 6 mm) was installed in front of the injection valve.

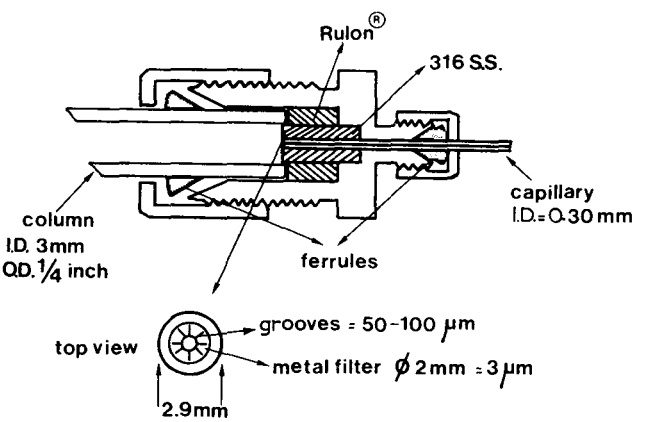


Fig. 1. Schematic representation of a column terminator.

Materials

All solvents were of analytical reagent grade and, with the exception of hexane, were used without further pre-treatment. The column materials used were commercially available C-8 (RP 8, mean particle size 5 μm; Merck, Darmstadt, G.F.R.) and C-18 (Nucleosil 10) modified silica. The antidepressants and metabolites were a gift from the St. Joris Gasthuis (Delft, The Netherlands). Their structures and the abbreviations used henceforth are given in Table I.

Procedures

Chromatography. The separation columns were packed by a pressurized balanced-slurry method [25] (the slurry liquid consisted of a mixture of chloroform and tetrabromoethane of specific gravity 1.82) and washed successively with 100 ml of acetone and 100 ml of eluent. The pre-column was packed by a dry-packing technique.

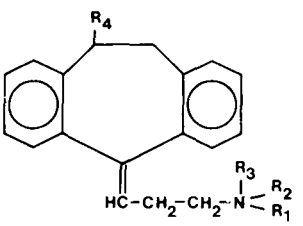
The capacity ratios (k'_i) of the solutes were calculated from their retention times and that of an unretained compound (4-aminotoluenesulphonic acid). The selectivity factors (r_{ji}) of pairs of compounds were expressed as the ratio of their capacity ratios.

The theoretical plate height of a compound was determined from its retention time and half the peak width at 0.6 of the peak height.

All solutions were prepared by mixing weighed amounts of solvents. The samples were dissolved in the eluent and injected by a valve, with a 25-μl sample loop, into the top of the column. Owing to the alkaline properties of the mobile phase, some of the column material at the top of the column becomes degraded after a few days, with the result that the column efficiency decreases.

TABLE I

STRUCTURES AND ABBREVIATIONS OF AMITRIPTYLINE AND ITS METABOLITES



Compound	Abbreviation	R_1	R_2	R_3	R_4
Amitriptyline	Ami	CH_3	CH_3	—	—
cis-10-Hydroxyamitriptyline	10-OH-Ami-C	CH_3	CH_3	—	OH
trans-10-Hydroxyamitriptyline	10-OH-Ami-T	CH_3	CH_3	—	OH
Amitriptyline N-oxide	N-ox	CH_3	CH_3	O	—
Nortriptyline	Nor	CH_3	H	—	—
cis-10-Hydroxynortriptyline	10-OH-Nor-C	CH_3	H	—	OH
trans-10-Hydroxynortriptyline	10-OH-Nor-T	CH_3	H	—	OH
Desmethylnortriptyline	Des	H	H	—	—

Removal of 3 mm of column packing and its replacement with fresh material usually restores the column efficiency completely. In all our experiments the top of the column was checked daily in order to ensure optimal performance.

Extraction. All of the glassware used for the extraction was treated successively with a solution of detergent, dilute nitric acid, ultrasonicated in acetone (10 min), washed with ethanol and finally washed with dichloromethane and dried at 50°. It was found to be necessary to use freshly distilled hexane for each extraction. The full extraction scheme for amitriptyline and some of its metabolites from plasma is outlined in Fig. 2.

The background in the chromatograms resulting from some sera can be improved by back-extraction using the following procedure. The collected hexane layers are shaken with 2 ml of 1 *M* hydrochloric acid, which extracts all of the antidepressants into the aqueous phase. The hexane layer is discarded and the aqueous phase neutralized, and the aqueous solution is then treated as the serum as outlined above.

RESULTS AND DISCUSSION

The choice of the optimal chromatographic and extraction conditions for the analysis of a mixture of compounds present at very low concentrations in a limited amount of sample by HPLC is based on a compromise between resolution and dilution on the one hand and between extraction yield and selectivity on the other. Large selectivity factors, medium retardation, minimal dispersion and short narrow-bore columns combined with a selective extraction procedure are the favourable conditions for obtaining sufficient resolution on one side and a low detection limit and precision on the other.

In order to determine the optimal chromatographic conditions for the analysis of amitriptyline and its metabolites by HPLC, a number of experiments were carried out. Initially, a C-18 bonded-phase material (Nucleosil 10 C-18) was tested as the stationary phase with water—organic solvent mixtures as the mobile phase. The effects of the type and amount of organic solvent added to the aqueous phase and the pH of the eluent on k'_i, r_{ji} and the theoretical plate

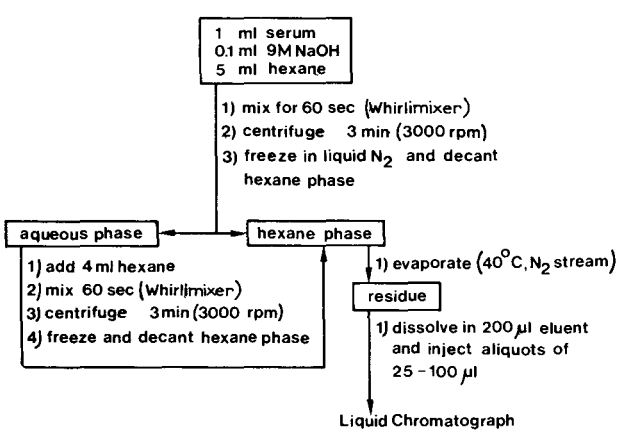


Fig. 2. Extraction scheme for amitriptyline and some of its metabolites.

height and peak form were investigated. From these primary experiments, it could be concluded that:

(i) the column efficiency of the C-18 bonded-phase material is far from ideal (tailing peaks) and is not suitable for trace analysis;

(ii) the capacity ratios and selectivity factors can be adjusted by the amount of organic solvent added to the mobile phase (methanol or acetonitrile);

(iii) the capacity ratios and elution sequence change irregularly with the pH of the mobile phase;

(iv) in acidic media the peak shape of the more retained compounds is asymmetrical;

(v) in alkaline media the peak shape of all substances is more symmetrical and favourable selectivity factors can be obtained.

To improve the column efficiency, a C-8 bonded-phase material (RP 8) with a mean particle size of 5 μm was chosen, and the possibilities of adjusting the retardation and selectivity as well as the optimal column efficiency were investigated in more detail.

Column efficiency

On the basis of the earlier results obtained with C-18 material and by means of some repeated experiments on C-8 (such as the effect of pH on k_i' and peak shape), it was decided that for precise quantitative analysis the elution of the substances as the free bases (i.e., using an alkaline mobile phase) was far more favourable with respect to column efficiency and peak shape. Fig. 3 shows a plot of the theoretical plate height, H, versus the mobile phase velocity, u, for two compounds (Nor, $k' = 9.52$; N-ox, $k' = 2.50$) on C-8 silica as the stationary phase and water—methanol, containing 1% (v/v) of propylamine, as the mobile phase. The curves are rather flat, indicating rapid mass transfer, in contrast to the results obtained on the C-18 bonded-phase material. Owing to the small particle size, the convective mixing is very favourable. The larger plate height of N-ox compared with that of Nor must be attributed to the larger contribution of the external peak broadening, mainly caused by the detector, to the overall dispersion of this compound (the external variance was found to be 325 μl^2).

Fig. 3 demonstrates that, with C-8 bonded-phase material, highly efficient narrow-bore columns can be packed that are suitable for the determination of amitriptyline and its metabolites at low concentrations.

Composition of the mobile phase

In order to optimize the composition of the mobile phase with respect to retardation and selectivity, the effects of methanol content and the type and amount of the base added to the mobile phase and the addition of dichloromethane to the water—methanol mixtures on the capacity ratios and selectivity factors were investigated.

Influence of methanol

Fig. 4 shows the effect of the methanol content of the mobile phase on k_i' and r_{ji}. An increase in the methanol content decreases the capacity ratios, changes the elution sequence and lowers the selectivity factors. A medium methanol content (ca. 60%, v/v) seems to be an appropriate choice with respect to selectivity. Such a phase system, however, is less favourable with

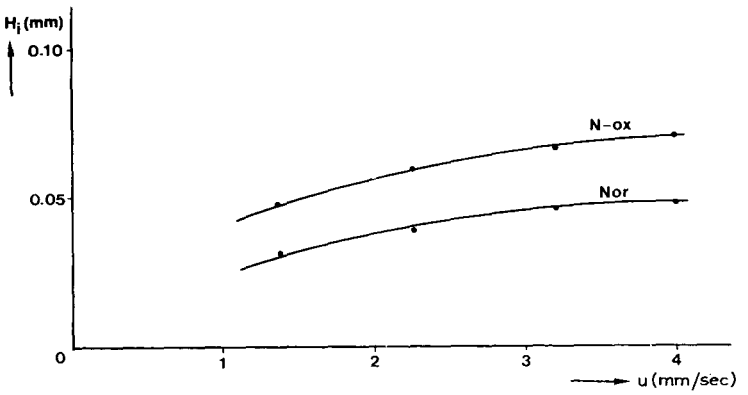


Fig. 3. H versus u curve for nortriptyline and amitriptyline N-oxide on a reversed-phase column. Stationary phase: C-8 bonded silica (RP 8). Mobile phase: water—methanol—dichloromethane (13:8:3, v/v) + 1% (v/v) of propylamine.

respect to the separation time and detection limit. The separation time of a mixture is determined by the solute pair with the smallest selectivity factor ($r_{j,i}$) and the capacity ratio of the most retained compound, and is expressed by

$$t_{R\,\text{min.}} = \frac{1}{(r_{j,i}-1)^2} \cdot R^2 \left(\frac{k'_i+1}{k'_i}\right)^2 (k'_{\text{max.}}+1)\,\frac{H}{u} \qquad (1)$$

where

$r_{j,i}$ = the selectivity factor of the solute pair that is the most difficult to separate, i.e., $\frac{1}{r_{ji}-1} \cdot \frac{k'_i+1}{k'_i}$ is minimal;

R = resolution;

k'_i = capacity ratio of compound i;

$k'_{\text{max.}}$ = capacity ratio of the most retained compound;

H_i = theoretical plate height of compound i;

u = linear fluid velocity.

The maximum concentration of a solute in the mobile phase at the end of the column ($<c_i^m>_{\text{max.}}$) as function of the amount injected, Q_i, is expressed by [26]

$$<c_i^m>_{\text{max.}} = \frac{Q_i}{\sqrt{2\pi}\,\epsilon_m A(1+k'_i)\,(H_i L)^{1/2}} \qquad (2)$$

where

ϵ_m = porosity of the mobile phase;

A = cross-sectional area of the column;

L = column length.

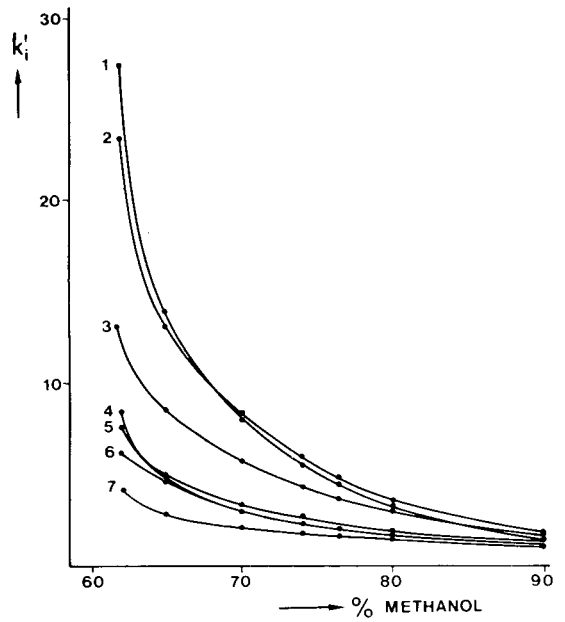


Fig. 4. Influence of the methanol content of the mobile phase on the capacity ratio (k_i'). Stationary phase: C-8 bonded silica. Mobile phase: water—methanol + 1% (v/v) of propylamine. Curves: 1 = amitriptyline; 2 = nortriptyline; 3 = desmethylnortriptyline; 4 = *trans*-10-hydroxyamitriptyline; 5 = *trans*-10-hydroxynortriptyline; 6 = *cis*-10-hydroxynortriptyline; 7 = amitriptyline N-oxide.

According to eqns. 1 and 2, large capacity ratios result in long separation times and poor detection limits.

Influence of dichloromethane

In an attempt to decrease the capacity ratios and to maintain the sufficiently large selectivity factors obtained with a medium methanol content, a more apolar organic solvent (dichloromethane) was added to a water—methanol—propylamine mixture used as the mobile phase.

The effect of the amount of dichloromethane added to the mobile phase on k_i' and r_{ji} is shown in Fig. 5. As could be expected, owing to its stronger elution strength in reversed-phase chromatography, the capacity ratios decrease with increasing dichloromethane content. At the saturation point (ca. 18.2% of dichloromethane), a small increase in k_i' occurs, which can be explained by a "spontaneous" loading effect as described earlier for straight-phase adsorption systems [27] (i.e., the solid support is loaded with the co-existing less polar phase).

On the addition of dichloromethane, the selectivity factors also decrease. Compared with the water—methanol system (Fig. 3), however, more favourable selectivity factors are obtained. This result indicates that the addition of a less polar third component such as dichloromethane or diethyl ether to water—methanol mixtures, as a type of modifier, is a useful means of adjusting retention and selectivity in reversed-phase chromatography.

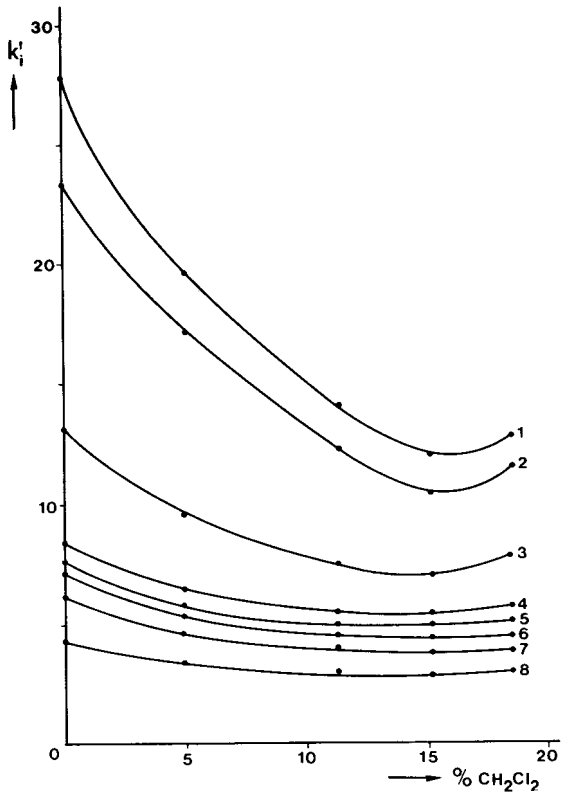


Fig. 5. Influence of the dichloromethane content of the mobile phase on the capacity ratio. Stationary phase: C-8 bonded silica. Mobile phase: water—methanol (62:38, v/v) + dichloromethane + 1% (v/v) of propylamine. Curves: 1 = amitriptyline; 2 = nortriptyline; 3 = desmethylnortriptyline; 4 = *trans*-10-hydroxyamitriptyline; 5 = *trans*-10-hydroxynortriptyline; 6 = *cis*-10-hydroxyamitriptyline; 7 = *cis*-10-hydroxynortriptyline; 8 = amitriptyline N-oxide.

Influence of the type of base

The influence of the type of base on the retention, selectivity and column efficiency was investigated by dissolving different types of bases in the mobile phase (fixed concentration) and by measuring the capacity ratios of the components (Fig. 6). Almost no difference in behaviour can be noticed between ethylamine and propylamine. The capacity ratios are smaller with hexylamine, probably owing to a stronger competition with the substances for occupation of adsorption sites, as would be expected for the hydrophobic hexalyamine.

With ammonia as the base, a drastic increase in the capacity ratios and a completely different elution sequence occur. This anomalous behaviour shows that the more hydrophobic bases also act as a modifier. According to Fig. 6, ammonia is by far the best choice with respect to selectivity but, especially for the most retained compounds, the peak shapes are less symmetrical. Propylamine seems to be a good compromise, because on the one hand the peak symmetry is reasonable while on the other the elution sequence is favourable for the determination of the metabolites, which are usually present at lower concentrations than that of the main drug.

Influence of propylamine concentration

The addition of propylamine to the mobile phase improves the column efficiency and peak shape considerably, but also affects the capacity ratio and selectivity factors, as is shown in Fig. 7. It can be seen that the amount of propylamine significantly influences the capacity ratio, selectivity factors and elution sequence. The capacity ratios decrease sharply with increasing amount of propylamine and then tend to become constant. According to Fig.7, a small content of propylamine (ca. 0.2—0.6%) seems to be favourable for the separation of these substances. Unfortunately, the column efficiency and peak shape at low propylamine contents is poor. A content of 1% seems to be a good choice.

The final choice of the mobile phase composition and column length for the determination of amitriptyline and some of its metabolites is a column length of 125 mm and an eluent composition of water—methanol—dichloromethane (8:13:3, v/v) containing 1% (v/v) of propylamine. The efficiency of this system in the separation of amitriptyline and its metabolites is illustrated by Fig. 8, which shows the separation of amitriptyline and five of its metabolites in about 10 min. On the column used, some pairs of metabolites (10-OH-Nor-T/10-OH-Ami-C and 10-OH-Nor-C/N-ox) are not well resolved owing to the limited number of theoretical plates. In practice, however, the metabolites 10-OH-Ami-C, 10-OH-Nor-C and N-ox are usually less important and are present in plasma at such low concentrations that no disturbance of the determination of the other metabolites occurs. If one is particularly interested in these metabolites, longer columns have to be used or the phase system has to be modified (e.g., by using ammonia instead of propylamine). In both instances one has to accept larger capacity ratios and higher detection limits.

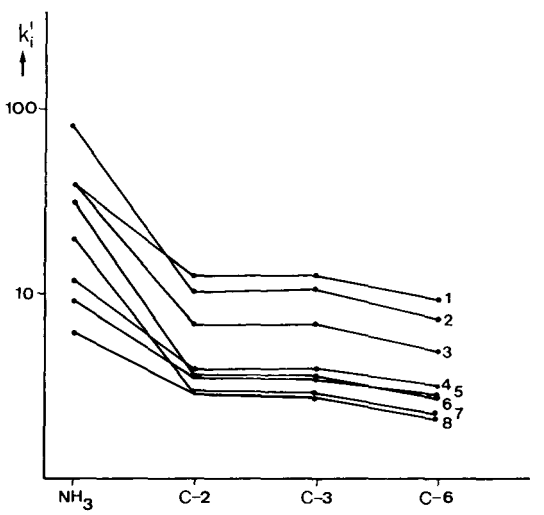


Fig. 6. Influence of the type of base added to the mobile phase on the capacity ratio. Stationary phase: C-8 bonded silica. Mobile phase: water—methanol—dichloromethane (13:8:3, v/v + 0.12 *M* of the base. Base: C-2 = ethylamine; C-3 = propylamine; C-6 = hexylamine; NH_3 = ammonia. Curves as in Fig. 5.

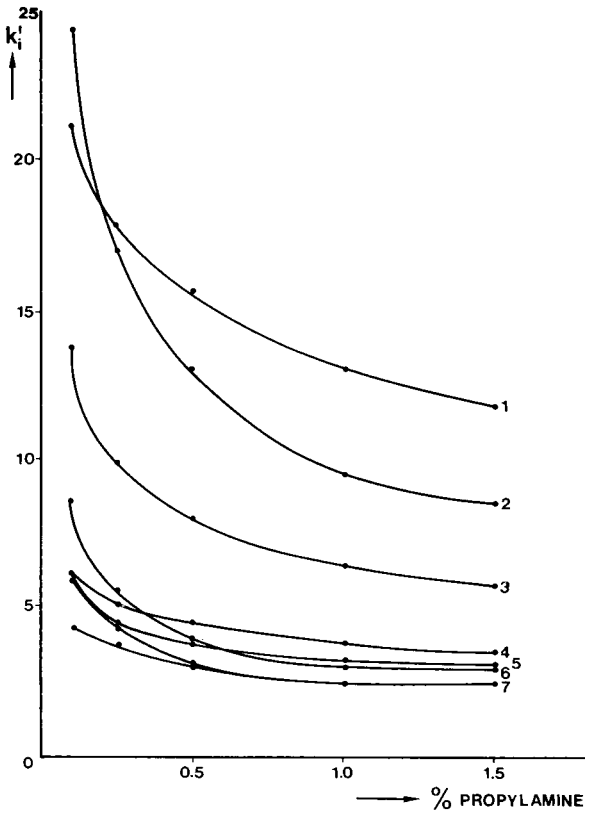


Fig. 7. Influence of the amount of propylamine on the capacity ratio. Stationary phase: C-8 bonded silica. Mobile phase: water—methanol—dichloromethane (13:8:3, v/v) + propylamine. Curves as in Fig. 4.

Quantitative aspects of the method

Precision and linearity. The precision of the determination of amitriptyline and its metabolites by HPLC and the linear range were determined by injection of a constant volume (25 μl) of solutions of the solutes at different concentrations (20—4000 ng/ml) and peak-area measurements. The correlation coefficient of the linear regression of peak area versus amount of amitriptyline injected was found to be 0.9999, indicating a high degree of linearity. The relative standard deviation was about 0.6% for 4000 ng/ml and 15% for 20 ng/ml (i.e., 100 and 0.5 ng injected, respectively). The sensitivity of the whole system, defined as the slope of the peak area versus amount of amitriptyline injected and calculated by linear regression, was found to be 1425 IU/ng (1 IU = 1 μV · sec).

The standard deviation of the baseline noise, measured during the same period of time as the peak integral, was determined to be 150 IU, corresponding to 0.1 ng of amitriptyline. The detection limit of amitriptyline for a signal-to-noise ratio of 3 is about 0.3 ng. For all metabolites the detection limit falls within the range 0.3—0.6 ng.

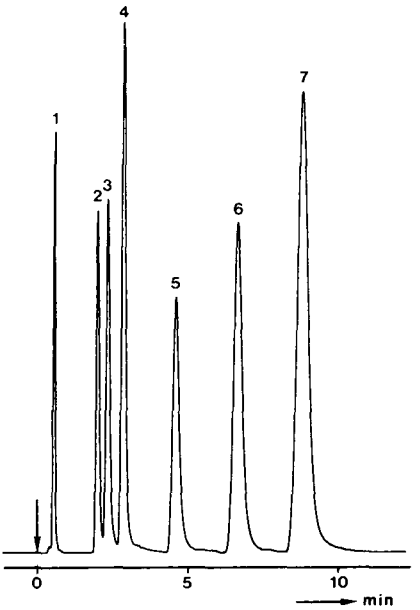


Fig. 8. Separation of a test mixture of amitriptyline and five of its metabolites. Stationary phase: bonded silica; column length, 125 mm. Mobile phase: water—methanol—dichloromethane (13:8:3, v/v) + 1% of porpylamine. Peaks: 1 = unretained; 2 = *cis*-10-hydroxynortriptyline; 3 = *trans*-10-hydroxynortriptyline; 4 = *trans*-10-hydroxyamitriptyline; 5 = desmethylnortriptyline; 6 = nortriptyline; 7 = amitriptyline.

Protriptyline, with a k'_i value between those of Nor and Des, can be used as an internal standard.

Recovery and reproducibility of the extraction. The recovery and reproducibility of the extraction procedure were determined by HPLC and by extraction of known amounts of amitriptyline and some metabolites added in different amounts of distilled water and blank serum. In order to check that no interferring substances are co-extracted from water and serum, some blank extractions were carried out. As shown in Fig. 9, clean extracts were obtained, showing little interference even at the most sensitive detector attenuation (0.005 absorbance units full-scale). It was found that under the chosen extraction conditions, the most polar metabolite (N-ox) was not extracted. The recoveries of amitriptyline and the polar metabolite 10-OH-Nor-T from both water and serum were found to be 98% and 79%, respectively. The recoveries of the other metabolites (except N-ox) were between these two values.

The reproducibility of the extractions was about 4% at 500 ng/ml (n = 3) and 7% at 80 ng/ml (n = 3).

Fig. 10 shows the separation of amitriptyline and four of its most important metabolites (about 150 ng of each) added to 1 ml of blank serum and extracted as described above.

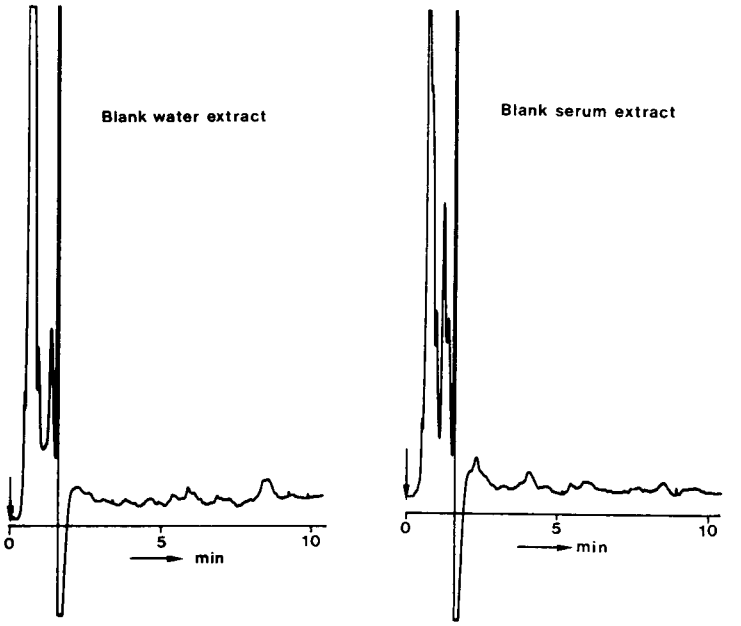


Fig. 9. Background of blank water and serum extracts. Conditions as in Fig. 8. Detector attenuation, 0.005 absorbance units full-scale. Injection volume, 50 μl.

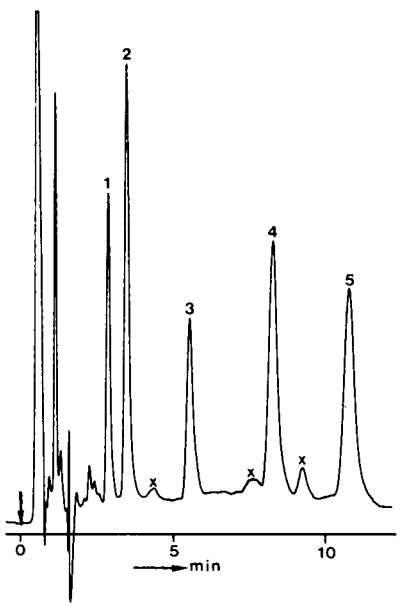


Fig. 10. Analysis of amitriptyline and four metabolites (ca. 150 ng of each) extracted from a spiked serum. Conditions as in Fig. 8. Peaks: 1= *trans*-10-hydroxynortriptyline; 2= *trans*-10-hydroxyamitriptyline; 3= desmethylnortriptyline; 4= nortriptyline; 5= amitriptyline; x= unknown compounds.

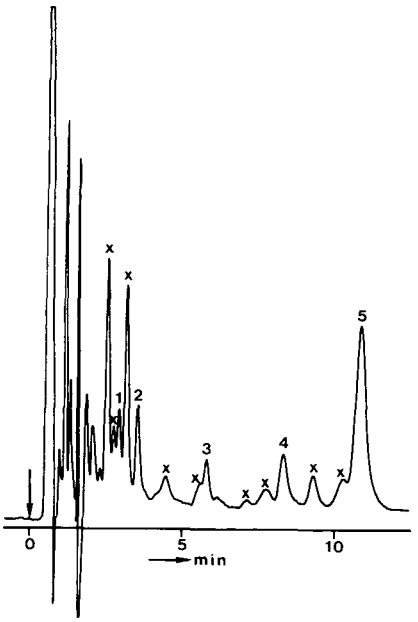


Fig. 11. Analysis of a serum extract from a patient after a daily oral administration of 150 mg of amitriptyline for more than 2 weeks. Conditions as in Fig. 8. Peaks as in Fig. 10.

Determination of amitriptyline and its metabolites in serum

The procedure described above was applied to the determination of amitriptyline and some of its metabolites in a sample of serum from a psychiatric patient supposed to receive only one of the tricyclic drugs.

Fig. 11 shows the chromatogram of a serum extract of a patient who had been receiving 150 mg of amitriptyline daily for more than 2 weeks. The large number of peaks possibly indicate either co-medication or the residues from such treatment. At least five peaks could be identified positively: Ami, Nor, Des, 10-OH-Ami-T and 10-OH-Nor-T. The serum levels found for this patient were: Ami 181; Nor 35; Des 25; 10-OH-Ami-T 16; and 10-OH-Nor-T 84 ng/ml.

CONCLUSION

The studies described show that reversed-phase adsorption chromatography is very suitable for the determination of a drug and its more polar metabolites in blood at very low concentrations. Future work will be devoted to the determination of the very polar metabolites of amitriptyline and to the determination of antidepressants in the presence of co-medicaments.

ACKNOWLEDGEMENT

The authors thank Dr. R.G. Muusze for his stimulating and valuable discussions during the preparation of the manuscript.

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Journal of Chromatography, 143 (1977) 513—518
Biomedical Applications

CHROMBIO. 061

Note

Procedure for the determination of 4-hydroxy-3-methoxyphenylethyleneglycol in urine by gas chromatography with flame-ionization detector

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(Received November 29th, 1976)

Measurements of urinary 4-hydroxy-3-methoxyphenylethyleneglycol (HMPG) are of great importance in clinical studies of psychiatric disorders [1, 2] and in the diagnosis of catecholamine-secreting tumours [3]. Recent papers have reported elaborate methods for the determination of HMPG either by gas—liquid chromatography with electron-capture detection (GC—ECD) [4] or by mass fragmentography (MF) [5].

We have devised a selective procedure for the routine determination of urinary HMPG by GC with a flame-ionization detector (GC—FID). This detector is more generally used than the ECD in clinical laboratories. Kahane et al. [6] performed Sharman's [7] preliminary acetylation of HMPG in alkaline solution and finally determined the HMPG by GC—FID of the triacetate derivative. In comparison with this method, we increased the specificity and the rapidity of the HMPG determination.

We previously introduced the use of boronic acids as specific reagents for HMPG bearing a diol group and analysed HMPG as the trimethylsilyl (TMS)—boronate derivative [8]; we then adapted the method described by Sharman [7] to obtain the 4′-acetyl-HMPG—boronates (Ac-HMPG—boronates) available for the MF determination of HMPG [9].

A brief outline of the use of Ac-HMPG—boronates in the analysis of urinary HMPG by GC—FID was given in a communication on the possible clinical applications [10]. However, a more extensive description and discussion of the procedure is necessary, which is the purpose of this paper.

EXPERIMENTAL

Reagents

Bis(4-hydroxy-3-methoxyphenylethyleneglycol) piperazine salt was purchased from Regis, Morton Grove, Ill., U.S.A.; Suc d'*Helix pomatia* containing 10^5 units (Fishman) of β-glucuronidase and 10^6 units (Roy) of aryl sulphatase per millilitre was obtained from Industrie Biologique Française, Genevilliers, France, methaneboronic acid [$MeB(OH)_2$] from Applied Science Labs., State College, Pa., U.S.A., *n*-butaneboronic acid [$BuB(OH)_2$] from Serva, Heidelberg, G.F.R. and 2,2-dimethoxypropane (DMP) from J.T. Baker, Deventer, The Netherlands; all other solvents and inorganic compounds were obtained from Carlo Erba, Milan, Italy.

Apparatus

A Carlo Erba dual-column gas chromatograph (Fractovap Model GV) equipped with a flame-ionization detector was used. The U-shaped glass column (2 m × 2.5 mm I.D.) was packed with 3% OV-101 on 100—200 mesh Gas-Chrom Q (Applied Science Labs.). The injector and detector temperatures were 200° and 260°, respectively. The column temperature was programmed from 150° to 240° at 15°/min and maintained at 240° for 3 min. The carrier gas (nitrogen), hydrogen and air flow-rates were 40, 35 and 390 ml/min, respectively.

Combined GC—mass spectrometry was carried out on a Finnigan Model 3100 instrument with the same gas chromatographic conditions as mentioned above and with an electron energy of 70 eV.

Procedure

The urine sample (15 ml) from the 24-h pool (stored at - 18°) was incubated overnight at 37° with Suc d'*Helix pomatia* (0.3 ml) after adjustment of the pH to 6.5 with dilute HCl or NaOH. The sample was acidified to pH 1 with 6 *N* HCl, saturated with NaCl and extracted with three 40-ml portions of ethyl acetate; the combined organic phases were dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure. The residue was dissolved in water (3 ml), treated with acetic anhydride (0.3 ml) and enough powdered $NaHCO_3$ was added to saturate the reaction mixture. The resulting solution was extracted with two 10-ml volumes of dichloromethane; the combined organic phases were dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure in a water-bath at 40°. The residue was dissolved in a solution of phenanthrene, which was used as the internal standard, (50 μg/ml) in DMP (0.2 ml). Finally, 1 μl of this solution was injected together with 0.5 μl of $MeB(OH)_2$ or $BuB(OH)_2$ solution in DMP (3 mg/ml) into the gas chromatograph.

Quantitative analysis

Peak areas were calculated as peak height times the width at half-height. Calibration graphs were obtained with standard aliquots (5, 10, 20, 30 and 40 μg) of pure Ac-HMPG, prepared according to Sharman [7]. The peak areas of HMPG derivatives were corrected against that of phenanthrene (10 μg). All of

the test runs on aqueous solutions were carried out both with $MeB(OH)_2$ and with $BuB(OH)_2$ and the final results calculated as the mean of those obtained from the two different reagents.

Recovery

The recoveries were calculated from a comparison of the experimental and theoretical corrected areas for the five different standard amounts (5, 10, 20, 30 and 40 μg) and the mean value was taken.

In order to ascertain the percentage recovery of Ac-HMPG from aqueous solution into dichloromethane, equal aliquots (3 ml) of $NaHCO_3$ saturated solution containing standard amounts (5—40 μg) of Ac-HMPG were extracted and treated according to the described procedure.

To determine the yield of acetylation in the aqueous phase, the above-mentioned recoveries were compared with those obtained from standard amounts (5—40 μg) of pure HMPG in water (3 ml) treated with acetic anhydride, $NaHCO_3$ and then extracted according to the described method.

To check the influence of urine on the recovery throughout the whole procedure, standard aliquots (5—40 μg) of HMPG were added to urine samples from the same pool. Their recoveries were compared with those obtained from the aqueous solutions containing similar amounts of HMPG treated according to the procedure described above.

A blank run with enzyme preparation was carried out in order to check its content of HMPG.

RESULTS

The calibration graphs for Ac-HMPG—methaneboronate (Ac-HMPG—MeB) and Ac-HMPG—butaneboronate (Ac-HMPG—BuB) were linear in the experimented range (5—40 μg) and identical with those for the derivatives obtained by reaction with boronic acids in DMP before injection into the gas chromatograph. The relative responses against phenanthrene were 0.91 ± 0.06 and 0.97 ± 0.03 (mean ± S.D.) (n = 25) for the methyl and butyl derivative, respectively.

Calibration graphs calculated with the peak heights were also linear but with a slightly higher standard deviation.

Fig. 1 shows typical gas chromatograms of the two derivatives obtained from urine samples taken through the described procedure. As the Ac-HMPG—BuB peak appeared to be better separated from neighbouring peaks than the Ac-HMPG—MeB peak, the analyses of biological samples were performed on the butyl derivative. Nevertheless, during test runs with aqueous solutions, the methyl derivative was also used because of its lower retention time relative to the internal standard (0.90 ± 0.05) (n = 50) compared with that of the butyl derivative (1.42 ± 0.13) (n = 50).

The identities of the two derivatives were checked by ensuring that the mass spectra of authentic Ac-HMPG—boronates were identical with those obtained from urine samples. The mass spectra have been reported elsewhere [9].

The recovery of Ac-HMPG was 89.2 ± 5.4 % (n = 10) from the extraction into dichloromethane. The yield of acetylation was 97 ± 2.5 % (n = 10). By

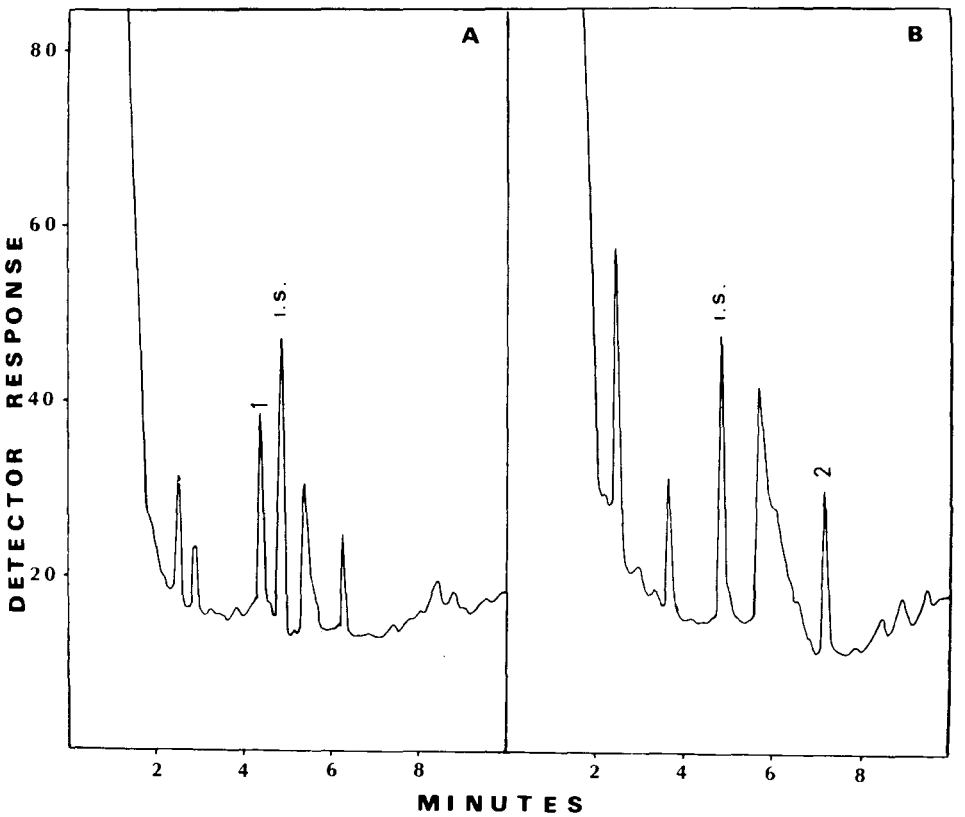

Fig. 1. Typical gas chromatograms of urinary extracts subjected to the complete procedure, using $MeB(OH)_2$ (A) or $BuB(OH)_2$ (B); 1 μl of each sample, containing 0.05 μg of phenanthrene (internal standard), was injected. 1 = Ac-HMPG—MeB, 2 = Ac-HMPG—BuB.

using the whole procedure, the recovery of pure HMPG added to water was 52.1 ± 4.1 % (n = 40).

By adding pure HMPG to urine samples, no significant differences were detected in its recoveries with respect to those obtained from water.

No detectable peak with a retention time the same as that of HMPG was observed when an enzyme preparation was treated according to the described procedure. The amount of HMPG released from the same urine sample was not increased by using a volume of enzyme preparation greater than 0.3 ml. The precipitation of proteins after hydrolysis performed with acetone in preliminary experiments showed no advantages and its use was discontinued.

The precision of the method was tested by evaluating the percentage difference between duplicate samples of urine; the result was 8.15 ± 7.43 % (mean ± S.D.) (n = 20).

The contents of urine samples (15 ml) and the calculated total output of HMPG in 24-h pools ranged between 6.5 and 37.8 μg and between 650 and 1530 μg per 24 h, respectively.

DISCUSSION

In order to make the specificity of an analytical GC method as high as possible, the structural features of the biological compound of interest must be considered when selecting a technique for its separation and the most suitable volatile derivative. In this connection, aqueous phase acetylation and reaction with boronic acids are together very selective for HMPG as it bears both phenolic and glycolic moieties.

With regard to the preliminary treatment of the samples, we used extraction with ethyl acetate from acidified urine, as discussed earlier [11]. In this step, basic compounds were removed from organic extracts. Later, after acetylation in alkaline solution according to the method introduced by Sharman [7], the sample was purified from acidic compounds by extraction into dichloromethane. The final mixture therefore contained only neutral metabolites.

Sharman's GC—ECD procedure was reviewed by Bond [12], who separated HMPG from urine on an anion-exchange resin (AG1-X4) and by O'Keeffe and Brooksbank [13] who purified Ac-HMPG by thin-layer chromatography.

In our work, AG1-X4 resin did not give satisfactory results because, with the larger volumes of urine that we used we did not obtain reproducible chromatographic conditions. On the other hand, Ac-HMPG extracted with dichloromethane did not, in our case, require preliminary purification before gas chromatographic analysis as it did in O'Keeffe's method.

In spite of the lack of sensitivity of the FID itself, the treatment with boronic acids yields derivatives that give a chromatographic profile with fewer and better separated peaks. Hence preliminary purification was not necessary when the urine samples were analyzed. This is due to the specificity of the reagents, which enable part of the sensitivity lost by using an FID instead of an ECD to be regained. In fact, they facilitate accurate quantitative analysis by allowing the GC—FID apparatus to be used successfully at its maximum sensitivity.

Unlike our previous experiments with HMPG—boronates, we introduced the improvement that the reaction with boronic acids can be carried out by direct injection of the reagents into the gas chromatograph.

In conclusion, we can state that the procedure described here is rapid, specific and applicable to the routine analysis of urinary HMPG in clinical studies.

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Journal of Chromatography, 143 (1977) 519—521
Biomedical Applications

CHROMBIO. 052

Note

Purification of alanine aminotransferase from human serum on a cycloserine-derivatized agarose

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(Received January 20th, 1977)

In a previous paper [1] we described an approach designed to find a simple affinity chromatographic method for purifying alanine aminotransferase (E.C. 2.6.1.2) with gels replaced with inhibitors of the enzyme. Some of the derivatized agaroses separated bovine albumin from commercial pig-heart alanine aminotransferase (AlaAT) reasonably well.

The purpose of the present work was to investigate the applicability of the technique to the purification of crude AlaAT. A cycloserine derivative of agarose was used because it is easy to prepare and because its properties have been previously studied most thoroughly, although the gel was not the best of those tested with respect to separation of AlaAT from albumin [1]. The results demonstrate that AlaAT can be highly purified from crude sources in a single run. It is suggested that the large and serrated activity peak obtained is due to partly resolved sub-forms of the enzyme.

MATERIALS

Sepharose 4B 200, D-cycloserine and pig-heart AlaAT were purchased from Sigma (St. Louis, Mo., U.S.A.). Cyanogen bromide was obtained from Eastman-Kodak (Rochester, N.Y., U.S.A.). The other reagents were of commercial and analytical grade. Two samples of human serum were obtained from the Central Hospital of the University of Turku and were stored at −20°.

METHODS

D-Cycloserine was coupled to the cyanogen bromide-activated Sepharose (0.1 g of cyanogen bromide per millilitre of the gel) at pH 9.5 [2]. After washing, the derivatized gel was poured into a glass column of 12 mm I.D. The gel (settled volume about 75 ml) was equilibrated at 8° with 100 ml of 0.1 *M* sodium chloride solution containing 0.025 *M* sodium phosphate buffer, pH 6.0. The serum sample (0.5 ml) was applied to the gel and the elution was carried out at 8° with the above buffer solution at an elution rate of about 35 ml/h. The fractions (1.85 ml) were collected with an ISCO Model 820 fraction collector. Between runs, the gel was washed with 50 ml of 1 *M* sodium chloride solution before the equilibration.

The AlaAT activity was measured as the change in absorbance of NADH [1] and aspartate aminotransferase (AspAT) activity with the 2,4-diphenylhydrazine method [3]. The absorbance at 280 nm indicates the protein concentration.

RESULTS

Fig. 1 shows the elution curves of two human sera (I and II) chromatographed on the cycloserine-derivatized agarose. As can be estimated (see AlaAT I or II and the protein), several hundred-fold purification was achieved.

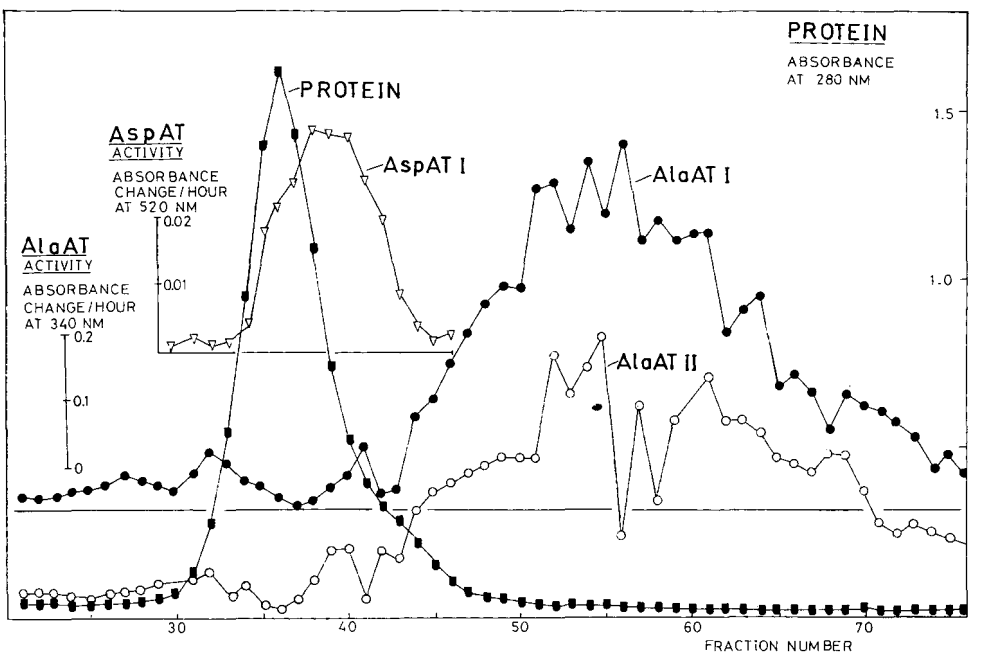

Fig. 1. Chromatography of two samples (I and II) of human serum on a cycloserine-derivatized Sepharose 4B gel. Samples I and II had aspartate aminotransferase activities 10 and 5 times the normal value, respectively. The fraction volume was 1.85 ml.

The recovery of AlaAT and serum protein was 95±5%. Commercial pig-heart AlaAT containing 5 mg of bovine serum albumin gave a sharper activity curve (maximal activity in the 54th fraction) than serum AlaAT. The behaviour of *Escherichia coli* crude protein on chromatography was similar to that of serum protein. As shown in Fig. 1, AspAT (I) formed one sharp peak that separated from the bulk of protein only slightly.

The activity of AlaAT in Fig. 1 increased smoothly between fractions 40 and 50, then saw-toothed activity patterns appeared in both curves. The serrated shape is due to the chromatographic process; the maximal difference between points of duplicate determinations was only 0.03 absorbance unit. The presence of reducing or oxidizing agents in the sera, which could disturb the measurement of AlaAT activity, was not excluded, but their retardation into the fractions of AlaAT seems improbable.

DISCUSSION

The results suggest that cycloserine-derivatized agarose is suitable for the purification of AlaAT from sources other than serum also. If a better resolution from the bulk of the protein is desired, aminooxyalanine bound to aminoethylagarose can presumably be used [1]. The advantages of the affinity chromatographic method, other than speed and simplicity, lie in the fact that the fractions contain all naturally existing forms of AlaAT.

The properties of cytosolic and mitochondrial AlaAT are different in several mammals [4, 5]. The fact that samples I and II had high AspAT activities (see Fig. 1) indicates that they contained some AlaAT originating from damaged cells and thus both cytosolic and mitochondrial enzymes. The effect of mercaptoethanol on the chromatography of rat-liver AlaAT on Sephadex G-200 suggests a subunit structure [6]. An insect cytoplasmic AlaAT exists as both a monomer and a tetramer, whereas the mitochondrial enzyme exists mainly as a dimer [7]. Consideration of these results in the present work suggests that the sera contained multiple forms of the enzyme. Because partially purified pig-heart AlaAT gave a sharper peak than crude AlaAT from serum, it is possible that the large serrated shape of the activity curve in Fig. 1 is due to partly resolved multiple forms of the enzyme. This fact might have clinical use in indicating of cell damage.

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Journal of Chromatography, 143 (1977) 522—526
Biomedical Applications

CHROMBIO. 053

Note

Behaviour of the pertechnetate ion in humans

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Ever since the availability on a large scale [1] of technetium-99 and its application in science and technology [2], followed by the increasing use of technetium-99m compounds in nuclear medicine [1—5], there has been great interest in the biological fate of this element in animals and humans and many workers [1, 3—10] have studied the problem. Studies have been concentrated mainly on the behaviour of the pertechnetate-99m ion, the most stable species of the metal, now in routine use in brain and thyroid imaging. For this purpose, the techniques of whole-body scanning [1], autoradiography [5], electrophoresis [6, 7] and chromatography [7] have been employed, and the last two techniques have been used to study the metabolism of pertechnetate-99m in the thyroid gland. The results obtained, however, have led to contradictory conclusions. Thus, Harper and co-workers [6, 10] found that, unlike iodide, pertechnetate is not organically bound in the thyroid, while electrophoretic and chromatographic evidence has indicated some organic binding of technetium-99m in rat thyroid [7, 8]. After entering the body, pertechnetate-99m ion is localized in the stomach and the salivary and thyroid glands [10]. Technetium-99m is excreted very rapidly in the urine, and urinary excretion has been shown to be the main path of technetium elimination for the first 3 days after administration [1, 10]. Hence the study of the composition of technetium excreted in urine should give an indication of whether or not pertechnetate-99m ion is organically bound during its passage through the body. The total activity being excreted, at different intervals, in urinary and faecal excreta has been measured

in order to establish the retention of technetium in the body but the excreta samples have not so far been analyzed to determine the technetium composition. As the half-life of technetium-99m is only 6 h, the excretion of this isotope can be taken to be mainly in urine. We therefore analyzed samples of urine collected from 37 patients admitted for thyroid or brain imaging and the results are reported here.

EXPERIMENTAL

Sodium pertechnetate-99m was eluted in saline from a Stercow TM 99m generator, supplied by Philips-Duphar, Petten, The Netherlands. Before injection, the radiochemical purity of the pertechnetate-99m ion was controlled by paper chromatography in physiological saline solution on Whatman 3MM paper strips (20 × 3 cm) with a chromatographic run of 15 cm. The chromatographic development was carried out by the ascending technique at room temperature (20°). Under these conditions, the pertechnetate-99m ion migrates with an R_F value of 0.68.

The total urine eliminated from the 37 patients during the post-injection period of 48 h was collected and examined. There were 25 euthyroid (of whom one was obese), 8 hypothyroid and 2 hyperthyroid patients of both sexes in the age range from 18 to 70 years. Two patients were injected with sodium pertechnetate-99m for brain scanning. The chemical form of technetium in the urine and in the blood serum of the patients was examined by paper chromatography and by low-voltage paper electrophoresis. The urine was stored at room temperature and was analyzed when fresh and when aged for different periods.

Paper electrophoresis was carried out at 400 V and 6—10 mA for 1 h on Whatman 3MM paper strips (30 × 3 cm) sandwiched between two thin glass plates.

After the run, the chromatograms and electropherograms were dried under a hair dryer and cut into 5-mm wide pieces. The activity in each piece of paper was counted with an automatic γ-counting well system (Model 4230, Nuclear-Chicago, Des Plaines, Ill., U.S.A.).

RESULTS AND DISCUSSION

The concentration of technetium-99m in the urine varied with the post-injection time, increasing rapidly to a maximum and then falling more slowly. The rate of urinary elimination of the nuclide for two patients is shown in Fig. 1. The trends of the curves are similar in the two examples, but for one the technetium-99m concentration in the urine reached a maximum 1 h after injection while for the other it took 3 h. The time required to give maximal activity in the urine from the other 35 patients was between these two limits of 1 and 3 h. This variation in the rate of elimination of technetium-99m was found to be due to the difference in the urine secretion rate, which was higher for the former patient than for the latter. A similar difference in the technetium-99m excretion has been reported by other workers [6]. These results

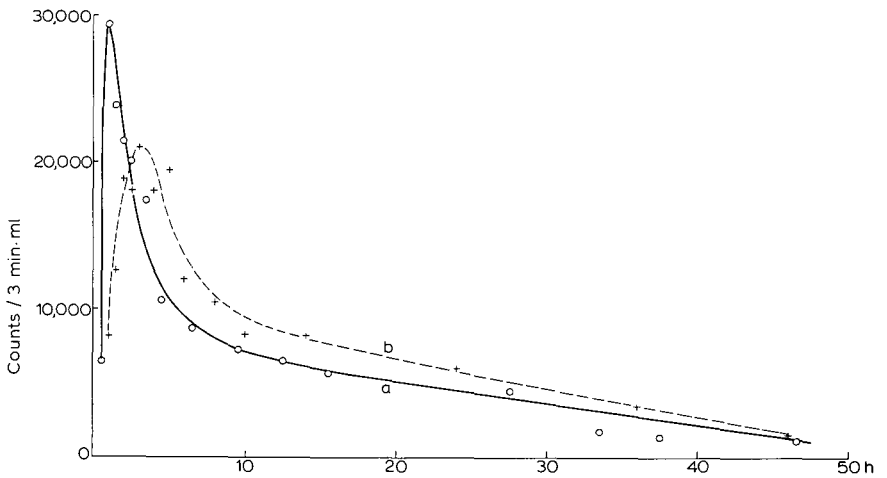

Fig. 1. Elimination, with time, of technetium-99m in the urine of (a) a patient with a higher urinary secretion (ca. 2.5 l/day) and (b) a patient with a lower urinary secretion (ca. 1.5 l/day) injected with sodium pertechnetate-99m in physiological saline.

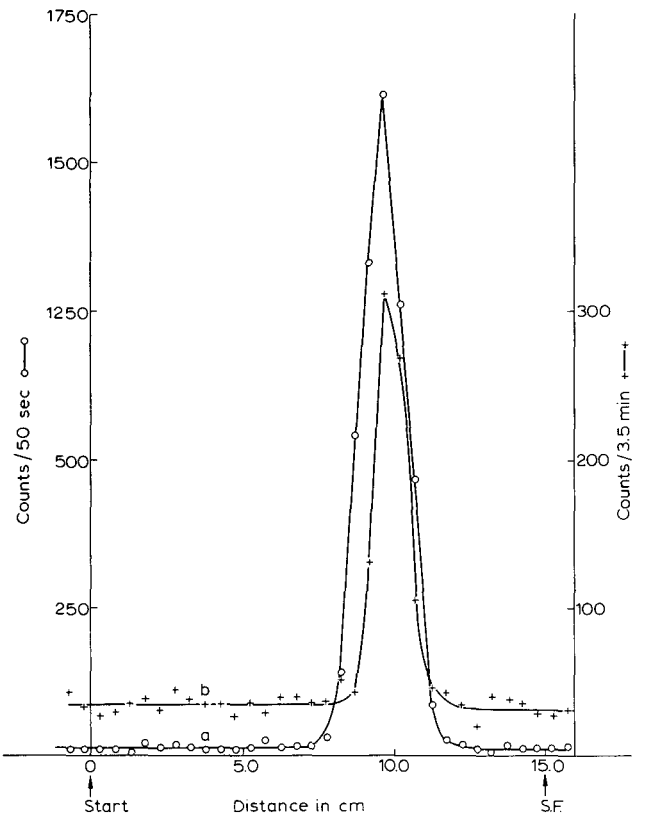

Fig. 2. Radiochromatogram (HCl-washed Whatman 3MM paper; mobile phase, physiological saline; temperature, 20°) of (a) injected sodium pertechnetate-99m in physiological saline and (b) technetium-99m in the urine of a hypothyroid patient (euthyroid patients' urine gives a similar radiochromatogram). S.F. = Solvent front.

suggest that after a scanning has been performed, the patient should drink a large volume of water in order to ensure the rapid elimination of the nuclide from the body in the urine.

Chromatographic and electrophoretic examinations of urine samples at different times after the injection of the radionuclide showed (Fig. 2) that the pertechnetate-99m ion is excreted unchanged in the urine of euthyroid and hypothyroid patients. The urine of patients admitted for brain scanning also contained only pertechnetate-99m ion. This result confirms the findings of Harper and co-workers [6, 10] that pertechnetate-99m is trapped in the organs of the body but is not organically bound. In hyperthyroid and obese patients, on the other hand, two other species of technetium-99m, one at the point of application and the other at an R_F value of 0.87, were also observed (Fig. 3). The chromatogram of blood serum of the hyperthyroid patients taken within 2 min after injection of the pertechnetate-99m ion also showed a similar technetium-99m distribution. These observations suggest that in hyperthyroid and obese patients pertechnetate-99m ion is partially metabolized when it comes in contact with the blood. In vitro studies with pertechnetate-99m in the blood and urine of these patients, however, did not show any modification in its

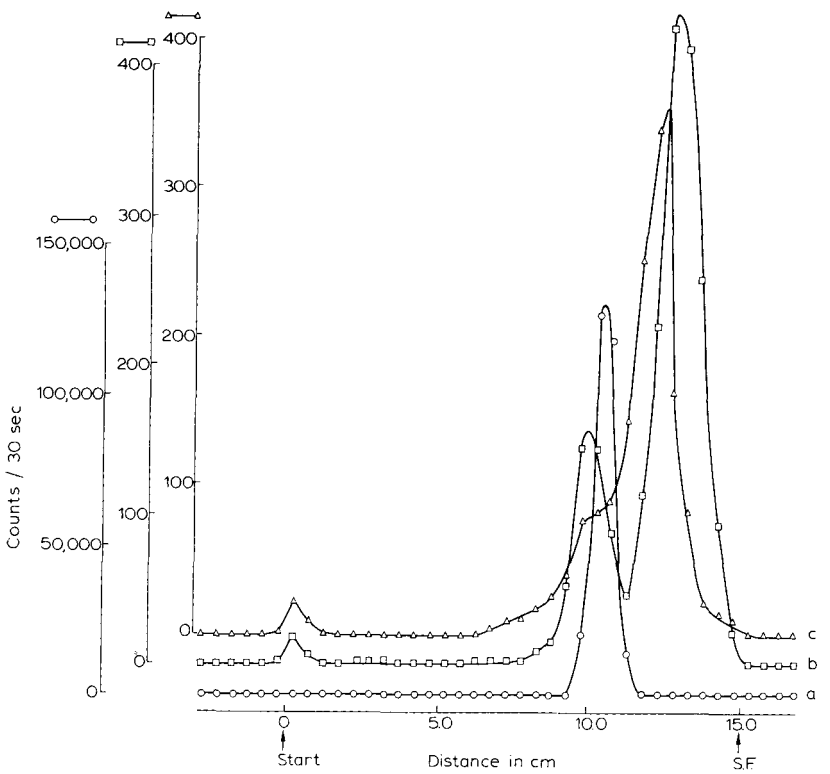

Fig. 3. Radiochromatogram (HCl-washed Whatman 3MM paper, mobile phase physiological saline, temperature 20°) of (a) injected sodium pertechnetate-99m in physiological saline, (b) technetium-99m in the urine of a euthyroid obese patient and (c) technetium-99m in the urine of a hyperthyroid patient. S.F. = Solvent front.

chemical form. We are continuing the studies with hyperthyroid and obese patients in order to obtain more data on the metabolization of pertechnetate-99m by studying the nature of these two technetium-99 species observed in chromatograms and electropherograms of urine and blood samples. Socolow and Ingbar [7], and Papadopoulos et al. [8] have also observed the metabolization of pertechnetate-99m in the thyroid gland of the rat, but the product was not characterized.

The results so far obtained on the chromatographic behaviour of the various species of technetium-99 [11, 12] and technetium-99m in solution [13] suggest that the species with $R_F \approx 0.87$ is technetium(IV)-99m and that at the point of application is organically bound technetium-99m [7].

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Journal of Chromatography, 143(1977) 527—529
Biomedical Applications

CHROMBIO. 062

Note

Rapid gas—liquid chromatographic estimation of doxapram in plasma

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(Received February 11th, 1977)

Doxapram (1-ethyl-4-(2-morpholinoethyl)-3,3-diphenyl-2-pyrrolidinone) is a respiratory stimulant which has been in clinical use for about ten years. Very little is known of its pharmacokinetics in man. Previously described methods for its estimation include high-pressure ion-exchange and thin-layer chromatography for urine [1] and UV absorption after oxidation to benzophenone for plasma [2].

We have developed a simple and sensitive gas—liquid chromatographic method for the direct estimation of doxapram in plasma. By the use of a nitrogen sensitive flame ionisation detector, only a single stage extraction is necessary and interference from the solvent front and extraneous peaks is minimal.

EXPERIMENTAL

One millilitre of 0.2 *M* borate buffer pH 9.5 was added to 2 ml of plasma in a 15-ml round-bottomed centrifuge tube and extracted with 5 ml of redistilled dichloromethane containing 0.5 μg/ml of naftidrofuryl oxalate as the internal standard. A plasma standard containing 1 μg/ml of doxapram hydrochloride was run with each set of unknown samples. After centrifugation the aqueous layer was removed by aspiration and the organic layer evaporated at 55° in a stream of air. The residue was dissolved in 20 μl of ethanol using a vortex mixer and 3-μl aliquots were injected into the gas chromatograph (Hewlett-Packard Model 5750 with 15160B nitrogen detector). The column was glass (4 ft. × 0.25 in. O.D.) packed with 1% OV-17 on Gas-Chrom Q, 80—100 mesh. The carrier gas (helium), hydrogen and air flow-rates were 60, 28 and 180 ml/min

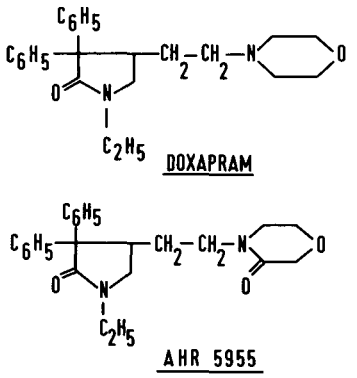

Fig. 1. Structural formulas of doxapram (1-ethyl-4-(2-morpholinoethyl)-3,3-diphenyl-2-pyrrolidinone) and AHR 5955 (1-ethyl-4-[2-(morpholin-2-one)-ethyl]-3,3-diphenyl-2-pyrrolidinone).

and the injection port, column oven and detector temperatures were 320°, 265° and 375°, respectively. The rubidium bromide crystal of the nitrogen detector was adjusted to give the maximum ionisation current.

RESULTS AND DISCUSSION

Under these conditions, the retention times of internal standard and doxapram were 2.3 min and 3.6 min, respectively, and the limit of detection was about 0.01 μg/ml. The calibration graph obtained by plotting the peak-height ratios of doxapram to naftidrofuryl versus plasma concentration of doxapram was linear up to 5 μg/ml and passed through the origin. The mean coefficient of variation for replicate analyses of doxapram added to plasma over the concentration range of 1—5 μg/ml was 2.3%. None of the known metabolites of doxapram interfered with the assay, but AHR 5955 (Fig. 1) gave a symmetrical peak and could be estimated simultaneously by temperature programming from 265° to 290° at 30°/min after an initial delay of 4 min. The calibration graph for this metabolite was also linear over the range 0.25—5 μg/ml and the coefficient of variation of replicate analyses was about 8%. Chromatograms of extracts of blank plasma and plasma obtained from a patient receiving an infusion of doxapram (4.2 mg/min) are shown in Fig. 2.

ACKNOWLEDGEMENTS

Financial assistance and samples of doxapram and metabolites were provided by Dr. C.J. Jones, A.H. Robins Company Limited, Horsham, Great Britain. Naftidrofuryl was obtained from Lipha (U.K.), West Drayton, Great Britain. We gratefully acknowledge the technical assistance provided by Mr. I. King.

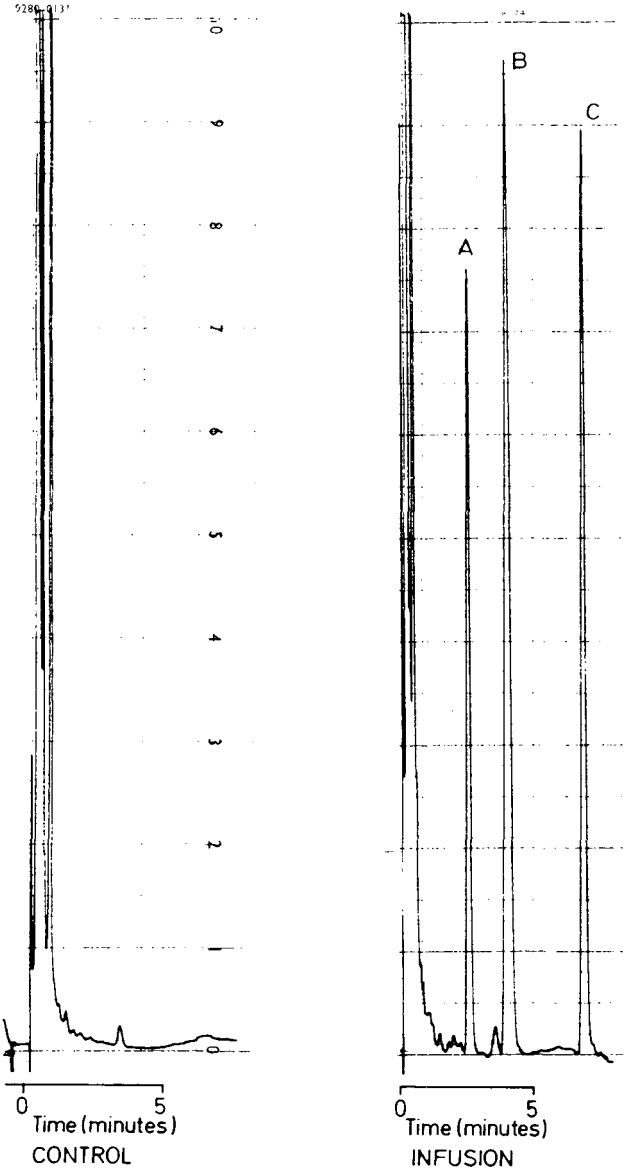

Fig. 2. Gas chromatogram of plasma extracts before, and following an intravenous infusion of doxapram (4.2 mg/min). A, Internal standard; B, doxapram (1.2 μg/ml); C, AHR 5955 (1.25 μg/ml).

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Journal of Chromatography, 143 (1977) 530—534
Biomedical Applications

CHROMBIO. 064

Note

Determination of flunitrazepam in body fluids by means of high-performance liquid chromatography

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Flunitrazepam, a benzodiazepine tranquillizer, is used in anesthesia and in intensive care units in hospitals. Owing to its very strong action, only small doses (0.010—0.030 mg/kg) can be administered to man, so the plasma concentration is to be expected very low. De Silva and co-workers [1, 2] reported effective plasma concentrations of 1—10 ng/ml, measured by means of gas chromatography with electron-capture detection.

The compound is extensively metabolized by N-demethylation to N-desmethylflunitrazepam and by reduction to its amino derivative (aminoflunitrazepam). Both metabolites may be present in blood and excreted in a conjugated form or as the unbound compound in the urine.

For the study of the pharmacokinetics of the drug and of effect—plasma concentration relationships, and for plasma monitoring during or following anesthesia and surgery, a method should be available that requires only a relatively short time for extraction and analysis and that permits the detection of plasma concentrations as low as 1 ng/ml.

In this paper, the determination of flunitrazepam and its metabolites by means of high-performance liquid chromatography is described.

EXPERIMENTAL

Apparatus

A Spectra Physics 3500B high-performance liquid chromatograph was used. The column, 25 cm × 3 mm I.D., was packed with Spherisorb S-W10 (particle size 10 μm) obtained from Chrompack (Middelburg, The Netherlands). An injection loop of 100 μl was used. Detection was effected at 230 nm. The solvents were *n*-hexane + 5% of ethanol or *n*-hexane + 3% of ethanol, and the solvent flow-rate was 2 ml/min.

Drugs

Flunitrazepam, desmethylflunitrazepam (Ro 05-4435/000) and aminoflunitrazepam (Ro 20-1815/601) were obtained from Hoffmann-La Roche (Mijdrecht, The Netherlands) by the courtesy of Dr. J. Kuitert.

Subjects and animals

Patients undergoing surgery were administered flunitrazepam intravenously in a dose of 0.010 mg/kg. Blood samples were taken at regular time intervals.

Labrador dogs (Central Animal Laboratory, Nijmegen) were administered 0.10 mg/kg of flunitrazepam intravenously. Blood and urine samples were collected frequently.

Extraction

To 1 ml of plasma (pH 7.4) are added 10 μl of internal standard (diazepam, 5 ng/μl) and 2 ml of fresly distilled *n*-hexane, and the mixture is mixed on a Vortex mixer. The hexane layer is removed and the plasma is extracted a second time with 2 ml of *n*-hexane. The combined hexane layers are evaporated to dryness and the residue is dissolved in 200 μl of *n*-hexane, 100 μl of the resulting solution being injected into the chromatograph.

Before each series of determinations a calibration graph is constructed. The recovery of the extraction is 75 ± 3%.

RESULTS

Flunitrazepam was well separated from its metabolites and related benzodiazepines (Table I and Fig. 1). After extraction and separation, no measurable amounts of the metabolites desmethylflunitrazepam and aminoflunitrazepam could be found in the plasma of dog and man after one single intravenous injection.

Flunitrazepam shows a biphasic elimination in the dog with a half-life of 15 min for the α-phase and 150 min for the β-phase. The volume of distribution of the β-phase is calculated to be 5 l/kg. A dose of 0.12 mg/kg (2.5 mg i.v.) in dogs results in an apparent maximum plasma concentration for the β-phase ($C_0\beta$) of 20 ng/ml. In man, following a dose of 0.010 mg/kg, the $C_0\beta$ value in plasma is 10 ng/ml.

A typical chromatogram of flunitrazepam, obtained after extraction of a plasma sample from a dog, is shown in Fig. 2. Peaks I and II are not observed in plasma from man. The relative retention times of these peaks are I=0.64

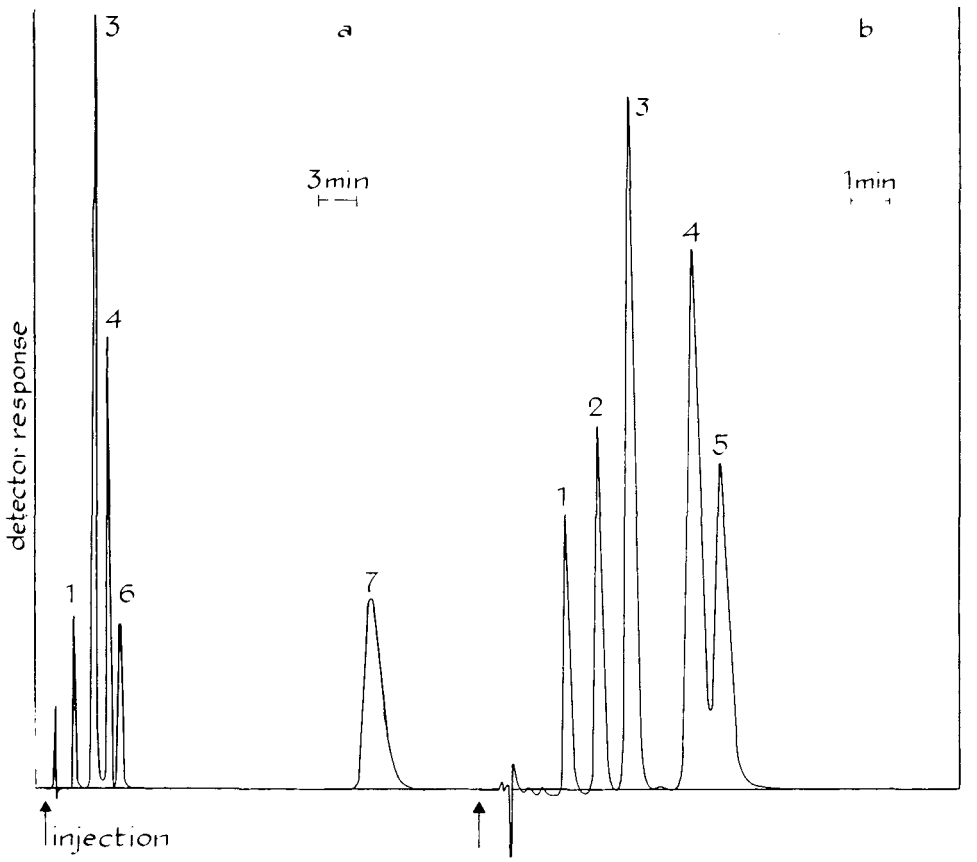

Fig. 1. Chromatogram of diazepam (1), desmethyldiazepam (2), flunitrazepam (3), nitrazepam (4), clonazepam (5), desmethylflunitrazepam (6) and aminoflunitrazepam (7). Column, Spherisorb S-W10; flow-rate, 2 ml/min; pressure, 34 atm. Eluent: (a) *n*-hexane—5% ethanol; (b) *n*-hexane—3% ethanol.

TABLE I

RELATIVE RETENTION TIMES OF FLUNITRAZEPAM AND VARIOUS BENZODIAZEPINES

Compound	Relative retention time
I*	0.64
diazepam	1.00
desmethyldiazepam	1.39
II*	1.42
flunitrazepam	1.66
nitrazepam	2.38
desmethylflunitrazepam	2.50
clonazepam	2.78
aminoflunitrazepam	10.83

* Peak in Fig. 2.

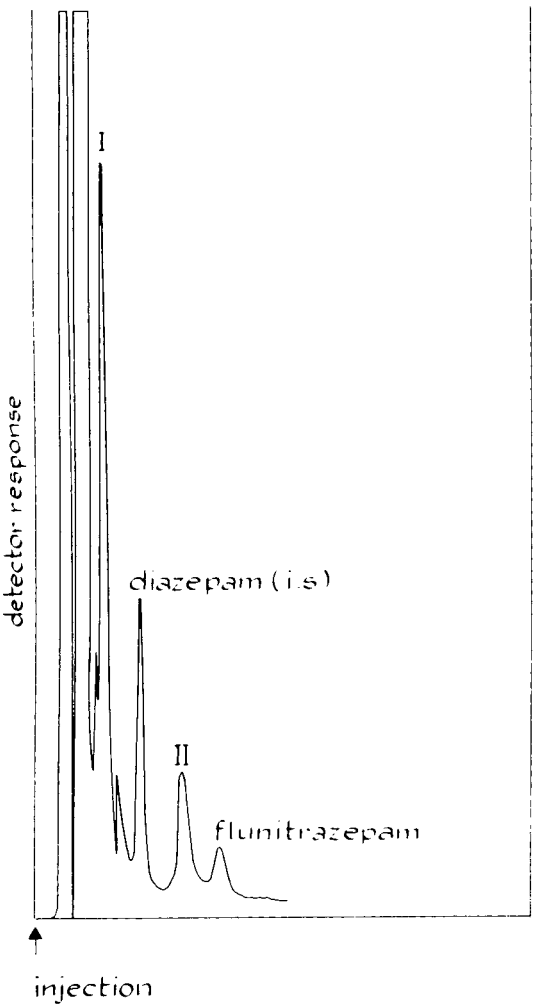

Fig. 2. Chromatogram of flunitrazepam and diazepam (internal standard) after extraction of a plasma sample from a dog. Peaks I and II do not appear in plasma from man. Column, Spherisorb S-W10; flow-rate, 2 ml/min; eluent, *n*-hexane—3% ethanol.

and II = 1.42 relative to diazepam = 1.00. Peak II almost coincides with desmethyldiazepam (Table I).

DISCUSSION

The method described permits the determination of plasma concentrations of flunitrazepam as los as 1 ng/ml, and is therefore comparable with that of De Silva and co-workers [1, 2]. The sensitivity of the method (10—1 ng/ml) is sufficient for most pharmacokinetic studies and routine determinations of plasma concentrations associated with anesthesia.

The column of Spherisorb S-W10 permits the separation of various benzodiazepines that may be used as co-medicants or for the induction of anesthesia,

e.g., diazepam (Valium), nitrazepam (Mogadon) and clonazepam (Rivotril). Two of the metabolites of diazepam, oxazepam (Seresta) and oxydiazepam (Temazepam), are not eluted from this column. In those instances, in routine determinations of flunitrazepam when it is not known if co-medication has been applied, a second extraction without internal standard must be made in order to avoid erroneous results.

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Journal of Chromatography, 143 (1977) 535—539
Biomedical Applications

CHROMBIO. 079

Note

Separation and measurement of tricyclic antidepressant drugs in plasma by high-performance liquid chromatography

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(Received April 7th, 1977)

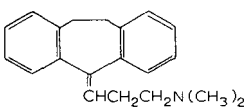
Amitriptyline is one of the most widely used drugs for the treatment of mental depression. The drug is extensively metabolised by animals and man, mainly by 10 (11)-hydroxylation and N-demethylation [1]. One of its metabolites, nortrityline, formed by mono-N-demethylation, also contributes towards the pharmalogical activity and is itself marketed as a therapeutic agent. Studies in patients have indicated that therapeutic effects are better correlated with plasma concentrations of both amitriptyline and nortriptyline [2]. A number of methods have been reported for the measurement of amitriptyline and/or nortriptyline in plasma using various analytical techniques. These include gas chromatography (GC) with specific nitrogen [3—5] flame ionisation [6—8] or electron capture [9] detectors; GC—mass spectrometry in the selected ion monitoring mode [10]; double-radioisotope derivative dilution analysis [11] and radioimmunoassay [12].

High-performance liquid chromatography (HPLC) is proving to be increasingly useful for the routine assay of drugs and their metabolites in biological fluids with sensitivity and specificity equivalent to or superior to corresponding GC methods. The separation of tricyclic antidepressant drugs, including amitriptyline and nortriptyline, by HPLC has been reported [13] using ion-pair partition and adsorption chromatography and a study on the quantitative measurement of these components by HPLC using a silica gel column has been published [14]. The latter method has recently been adapted to the measurement of the drugs in plasma but an unknown metabolite gave some interference with nortriptyline [15].

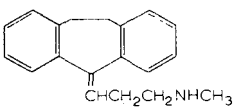
The method described here has been developed for the routine analysis of amitriptyline and/or nortriptyline in plasma.

MATERIALS AND METHODS

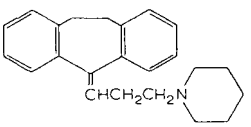
Authentic samples of amitriptyline, nortriptyline, desmethylnortriptyline, 10(11)-hydroxyamitriptyline, 10(11)-hydroxynortriptyline and the internal standard, a piperidine analogue of amitriptyline, were synthesised in these laboratories and used as the hydrochlorides. Standard aqueous solutions of these compounds were stored at 4°. All organic solvents were AnalaR grade.



Amitriptyline



Nortriptyline



Internal standard

HPLC conditions

The HPLC analyses were performed on a Pye Unicam Model 20 LC with a fixed wavelength ultraviolet (UV) detector operating at 254 nm and at a sensitivity of 0.02 a.u.f.s. The Column was a Partisil-10 ODS (25 cm × 4.6 mm I.D.) (Whatman, Maidstone, Great Britain). This column contains a packing material consisting of a C_{18} hydrocarbon bonded to microparticulate silica gel for reversed-phase chromatography. The mobile phase used was 50% (v/v) acetonitrile in potassium dihydrogenphosphate (0.6%, w/v) adjusted to pH 3 with phosphoric acid, at a flow-rate of 2 ml/min.

Calibration and extraction procedure

The linear response of the detector was established by constructing calibration curves using standard solutions. Standard solutions containing 40 to 400 ng of both amitriptyline and nortriptyline and 200 ng of the internal standard were injected directly into the chromatograph and peak height ratios measured. The recovery of the internal standard was established by comparing the peak heights after direct injection of a standard solution with those obtained from plasma extracts.

Calibration curves were constructed by adding to blank plasma samples (4 ml), 50 ng/ml of the internal standard and 10, 20, 50 and 100 ng/ml of both amitriptyline and nortriptyline. The plasma samples were made alkaline by adding 0.1 *M*-sodium hydroxide (0.5 ml) and then shaken for 10 min with freshly distilled diethyl ether (15 ml). After centrifugation the ether layer was removed and shaken with 1 *M*-sulphuric acid (2 ml) for 10 min. The mixture was centrifuged, the ether layer discarded and the acid layer neutralised and adjusted to about pH 9.5 with 4 *M* sodium hydroxide in an ice-bath. The mixture was shaken for 10 min with freshly distilled diethyl ether (15 ml), the ether layer was removed and evaporated to dryness under a stream of nitrogen. The residue was dissolved in methanol (25 μl) containing a trace of hydrochloric acid and 20 μl of this solution was injected into the liquid chromatograph.

RESULTS AND DISCUSSION

The chromatogram in Fig. 1 shows the separation of amitriptyline, nortriptyline and the internal standard, the three compounds having retention times of 4.8, 3.9 and 5.9 min, respectively. Under these chromatographic conditions some other possible interfering metabolites, desmethylnortriptyline, 10(11)-hydroxyamitriptyline and 10(11)-hydroxynortriptyline were eluted before nortriptyline and all had retention times of less than 3 min.

A chromatogram of blank plasma extract is shown in Fig. 2a. Most of the endogenous UV-absorbing plasma constituents occurred at retention times less than 3 min and did not interfere with the analysis. It was, however, necessary to incorporate back extraction into acid in the extraction procedure in order

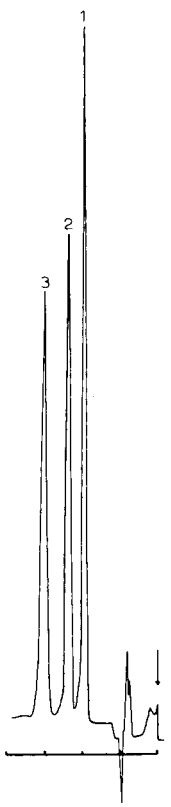


Fig. 1. HPLC separation of standards, nortriptyline, (1), amitriptyline (2) and internal standard (3). Conditions: Column 25 cm × 4.6 mm Partisil-10 ODS; eluent, 50% (v/v) acetonitrile in potassium dihydrogenphosphate (0.6%, w/v) adjusted to pH 3 with phosphoric acid; flow-rate, 2 ml/min.

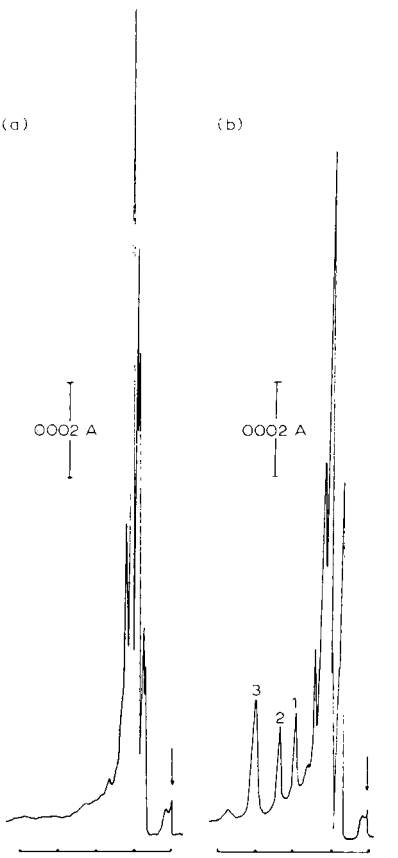


Fig. 2. Chromatograms of plasma extracts. (a) Blank plasma. (b) Plasma containing 20 ng/ml of nortriptyline (1) and amitriptyline (2) with 50 ng/ml of internal standard (3). Conditions as for Fig. 1.

to remove background interferences. A chromatogram of an extract from plasma containing 20 ng/ml of amitriptyline and nortriptyline is shown in Fig. 2b. The mean recovery of the internal standard from plasma was 94% and the mean relative recoveries of amitriptyline and nortriptyline were 99.8% (n = 8) and 90.8% (n = 8) respectively, calculated by comparing peak height ratios of plasma extracts and standards injected directly.

Calibration curves for both amitriptyline and nortriptyline constructed by plotting peak height ratios relative to internal standard showed a linear relationship over the concentration range 10 to 100 ng/ml. Over this concentration range nortriptyline and amitriptyline could be measured with a precision of ± 4.5% (n = 8) and ± 4.7% (n = 8) respectively.

This method provides an assay for both amitriptyline and nortriptyline with a sensitivity of about 5 ng/ml, comparable to all previously reported methods, and with an analysis time of less than 10 min. Most of the published GC methods report a detection limit of 10—20 ng/ml and one method giving a detection limit of 2 ng/ml involves measurement of total amitriptyline and nortriptyline as a common derivative [8]. Although a better absolute limit of detection can be obtained using GC detectors, comparable sensitivity is achieved during HPLC by the injection and analysis of larger amounts of plasma extracts. Due to the selectivity of the UV detector and the rapid elution of the mainly hydrophilic endogenous plasma components which have a low affinity for the lipophilic stationary phase, there is little background interference.

Subsequent investigations using a variable wavelength UV detector monitoring at 242 nm, corresponding to a $\lambda_{max.}$ for amitriptyline and nortriptyline, have shown that a sensitivity of 2 ng/ml could be easily achieved. This represents a sensitivity superior to most of the presently available methods. Steady-state concentrations of amitriptyline and nortriptyline in patients have been reported to be in the range 10—400 ng/ml [1, 3, 7] and the method reported here provides a reliable routine method for analysis of these components in plasma from patients.

ACKNOWLEDGEMENT

The autors are grateful to Dr. W.J. Price of Pye Unicam Ltd., Cambridge, Great Britain for his help and interest.

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1 A. T. James and A. J. P. Martin, *Biochem. J.*, 50 (1952) 679.
2 L. R. Snyder, *Principles of Adsorption Chromatography*, Marcel Dekker, New York, 1968, p. 201.
3 H. C. S. Wood and R. Wrigglesworth, in S. Coffey (Editor), *Rodd's Chemistry of Carbon Compounds, Vol. IV, Heterocyclic Compounds*, Part B, Elsevier, Amsterdam, Oxford, New York, 2nd ed., 1977, Ch. 11, p. 201.
4 E. C. Horning, J.-P. Thenot and M. G. Horning, in A. P. De Leenheer and R. R. Roncucci (Editors), *Proc. 1st Int. Symp. Quantitative Mass Spectrometry in Life Sciences, Ghent, June 16–18, 1976*, Elsevier, Amsterdam, Oxford, New York, 1977, p. 1.

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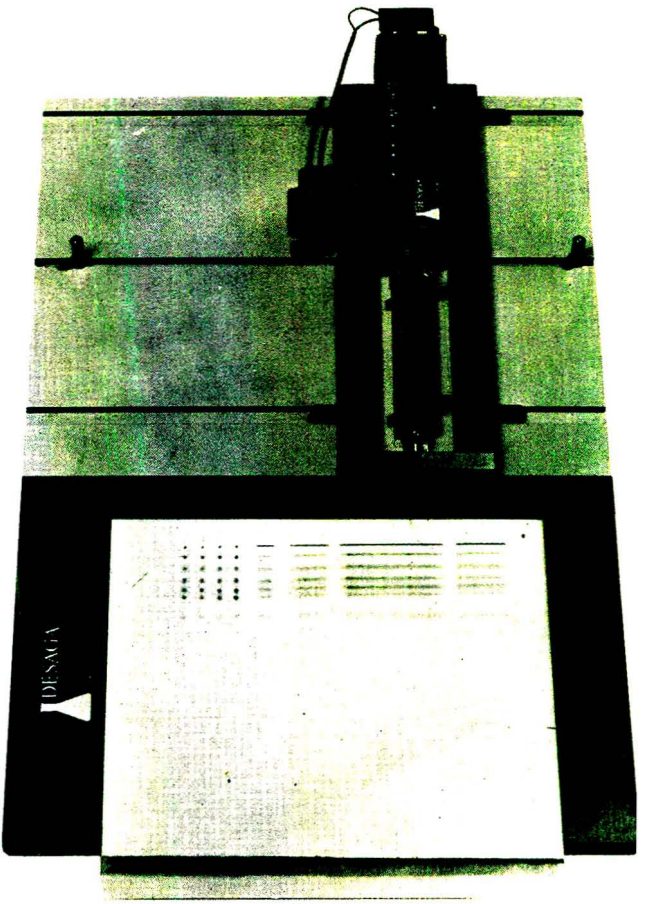

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