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PUBLICATION SCHEDULE FOR 1977

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

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Journal of Chromatography	130 131	132/1 132/2 132/3	133/1 133/2	134/1 134/2	135/1 135/2	136/1 136/2 136/3	137/1 137/2	138/1 138/2	139/1 139/2	140/1 140/2 140/3	142 144/1	144/2 144/3
Biomedical Applications	143/1		143/2		143/3		143/4		143/5		143/6	
Chromatographic Reviews				141/1				141/2				141/3

Scope. The *Journal of Chromatography* publishes papers on all aspects of chromatography, electrophoresis and related methods. Contributions consist mainly of research papers dealing with chromatographic theory, instrumental development and their applications. The section *Biomedical Applications*, which is under separate editorship, deals with the following aspects: developments in and applications of chromatographic and electrophoretic techniques related to clinical diagnosis (including the publication of normal values); screening and profiling procedures with special reference to metabolic disorders; results from basic medical research with direct consequences in clinical practice; combinations of chromatographic and electrophoretic methods with other physicochemical techniques such as mass spectrometry. In *Chromatographic Reviews*, reviews on all aspects of chromatography, electrophoresis and related methods are published.

Submission of Papers. Papers in English, French and German may be submitted, if possible in three copies. Manuscripts should be submitted to:
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Reviews are invited or proposed by letter to the Editors and will appear in *Chromatographic Reviews* or *Biomedical Applications*. An outline of the proposed review should first be forwarded to the Editors for preliminary discussion prior to preparation.

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For further information, see page 3 of cover.

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VOL. 143, NO. 1 JOURNAL OF CHROMATOGRAPHY JANUARY 1, 1977
(Biomedical Applications)

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Book Review

Chromatography of Steroids

by E. HEFTMANN

JOURNAL OF CHROMATOGRAPHY LIBRARY, Vol. 8

1976. about 225 pages
US $34.75/Dfl. 90.00
ISBN 0-444-41441-X

Written by one of the pioneers in the field, this is the only complete and up-to-date book on steroid chromatography currently available.
Although some theory is included, it is mainly a laboratory handbook, arranged according to the steroids analyzed as well as according to the methods used. The subject index, along with extensive tables of *R* values and a list of requisite chemicals, permits the reader to select appropriate procedures, many of which are described in detail. The bibliography covers 1200 of the most important articles published on this subject since 1964.

CONTENTS: 1. Introduction. 2. Liquid column chromatography. 3. Paper and thin-layer chromatography. 4. Gas chromatography. 5. Relations between structure and chromatographic mobility. 6. Sterols. 7. Bile acids and alcohols. 8. Estrogens. 9. Androstane derivatives. 10. Pregnane derivatives. 11. Corticosteroids. 12. Miscellaneous steroid hormones. 13. Vitamins D. 14. Molting hormones. 15. Steroid sapogenins and alkaloids. 16. Cardenolides and bufadienolides. Subject index.

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1939 E

Isotachophoresis

Theory, Instrumentation and Applications

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

JOURNAL OF CHROMATOGRAPHY LIBRARY, Vol. 6

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

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

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

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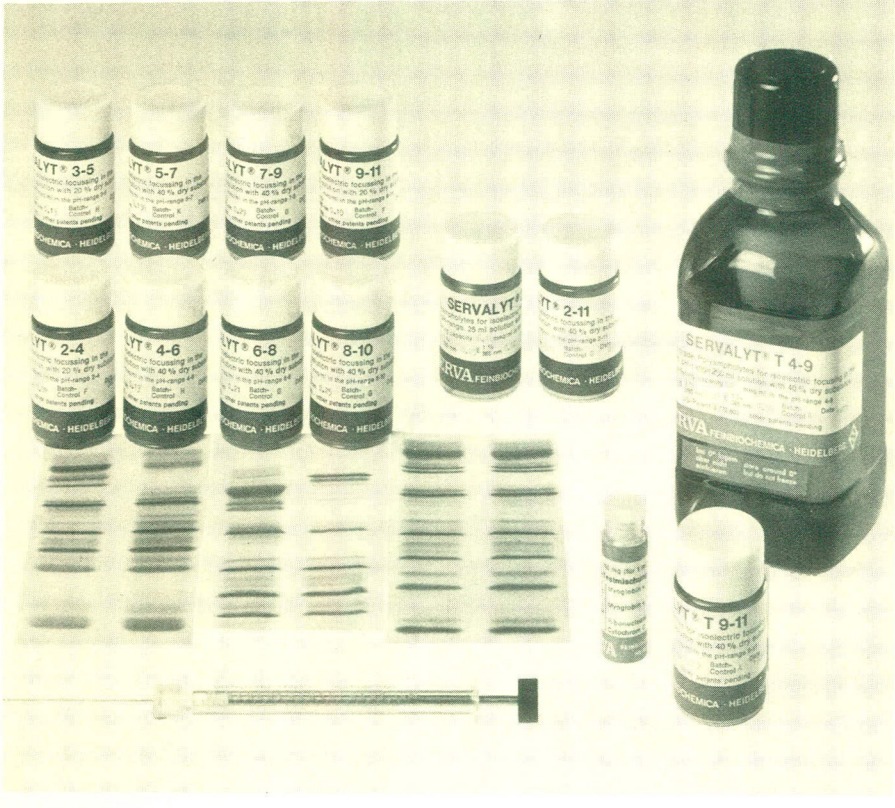

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JOURNAL OF CHROMATOGRAPHY

VOL. 143 (1977)

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BIOMEDICAL APPLICATIONS

VOL. 1 1977

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Journal of Chromatography, 143 (1977) 1—2
Biomedical Applications

Editorial

The birth of a new journal is always preceded by intensive enquiries during which even obvious reasons for its establishment are carefully judged. The present publication was certainly not an exception in this respect, although it is really not a journal in its own right but rather a specialized section of a journal that already exists. The main theme that required discussion in this instance was whether chromatographic and/or electrophoretic papers oriented towards clinical chemistry should be published preferentially according to the problem being solved, that is, in a medical journal, or according to the method being exploited, that is, in an analytical journal. There are obviously reasons both for and against each of these alternatives, but it appeared to us that the reasons supporting the latter alternative prevail: chromatographic methods are nowadays firmly established in clinical chemistry laboratories, where they are used for solving highly specialized problems. The demands placed upon these techniques are beyond the limits of routine service work as they involve small amounts in analysis and utilize complex instrumentation, which requires a specialist in the field. Thus, specialization according to the method being used is to be preferred to specialization according to the problem being solved. The fact that identical procedures are applied to such diverse areas as the determination of steroid levels in plasma, drug levels in various fluids or highly specialized problems in basic medical research, are strongly in favour of our decision.

We believe that this specialized section will be welcomed by the numerous specialists working with chromatography and related techniques in the field of clinical chemistry, as hopefully they will find most of the papers relating to their interests in a single journal and will be saved from time-consuming searches for such papers scattered in various other journals, where they may even escape their attention. The journal will not be large and will certainly offer the possibility to all clinical chemists of using these separation methods in their own work and of obtaining inspiration from this rapidly expanding area.

The journal will publish papers dealing with the following aspects: developments in and applications of chromatographic and electrophoretic techniques related to clinical diagnosis (including the publication of normal values); screening and profiling procedures with special reference to metabolic disorders; results from basic medical research with direct consequences in clinical practice; combination of chromatographic and electrophoretic methods with other physico-chemical techniques such as mass spectrometry.

Further, the journal will encourage contributions that give new information concerning the composition of tissues and body fluids. Manuscripts dealing with drug and metabolite levels, with special reference to blood, urine and other

body fluids, particularly when related to therapy, will be welcome. Also acceptable are papers describing new chromatographic and electrophoretic techniques devoted to clinical toxicology, and the determination of toxic levels of commonly used pharmaceuticals, but excluding toxicological studies per se. Chromatographic and electrophoretic studies on environmental hazards will be considered for publication, provided that they are directly related to clinical analysis.

KAREL MACEK

Journal of Chromatography, 143 (1977) 3—30
Biomedical Applications

CHROMBIO.023

REVIEW

ROUTINE CHROMATOGRAPHY OF SIMPLE LIPIDS AND THEIR CONSTITUENTS

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(Received August 17th, 1976)

CONTENTS

1. INTRODUCTION

Application of chromatographic methods to the separation of lipid mixtures has revealed a progressive increase in the number of subfractions with increasing power of resolution of the method. By a combination of complementary chromatographic techniques, it has been possible to obtain pure lipid classes and frequently pure molecular species within a lipid class. Not all of these separation techniques, however, are subject to routine utilization and many require specialized equipment. Since there exists a rapidly rising general interest in the detailed molecular structure and metabolism of various natural lipid classes, a brief summary is presented of those chromatographic methods which in the experience of several lipid research laboratories have reached a routine level of application. It is suggested that this complement of chromatographic methods serves as a guide to the quality of lipid analyses to be expected in general biochemical or clinical studies where lipids are analyzed as part of an overall experimental protocol.

2. PREPARATION OF LIPID EXTRACTS

The selection of the analytical sample and the method of lipid extraction are as important as the methods of lipid separation in the evaluation of the final results. The most efficient and carefully executed analyses cannot retrieve the data lost by poor sample selection and/or non-representative lipid extraction. Furthermore, the more efficient methods of resolution of lipid classes and molecular species require high quality samples that are free of non-lipid material.

A. Initial isolation

There are two basic routines which yield essentially quantitative extraction of the major lipid classes, when applied to homogenates of whole tissue or tissue subfractions. The most popular extraction method is that described by Folch et al. [1] which employs a chloroform—methanol (2:1) mixture in a solvent to tissue ratio of 20:1. This method gives excellent recoveries for neutral lipids and the diacylglycerophospholipids and sphingolipids. Lysophospholipids are

only partly recovered, and the more polar acidic phospholipids may be lost during backwashing with salt solutions and water.

The second most popular extraction method is that proposed by Bligh and Dyer [2], which effects a single phase solubilization of the lipids using a chloroform—methanol (1:1) mixture in a ratio of 4:1. The eventual partitioning of the extracts between chloroform and water results in losses of the more polar acidic phospholipids and lysophospholipids, as already noted for the Folch et al. [1] procedure. The advantages and disadvantages of these methods of lipid extraction have been discussed in great detail by Nelson [3] who has proposed improvements relating to the purification of the initial extracts. Bjerve et al. [4] have shown that complete extractions of the lysophospholipids from aqueous systems may be obtained by means of 1-butanol. Schmid et al. [5] have suggested that for the extraction of free fatty acids and neutral lipids, the chloroform—methanol—water system be replaced with benzene—methanol or toluene—ethanol solvent systems.

B. Purification of extracts

After filtration of the initial lipid extract some 25—75% of the total mass of the extract may represent non-lipid contaminants. These must be removed by purification. The crude lipid extract obtained as the final product of the evaporation of the solvent from a chloroform—methanol extract can be freed of essentially all non-lipid material by column chromatography on dextran gel columns [6, 7]. For this purpose Sephadex G-25 (Pharmacia) is packed in a column as a slurry in methanol—water (1:1) and weighted down with a layer of clean sand to prevent the gel from floating in solvent mixtures containing chloroform. The packing is washed with the entire sequence of solvent mixtures used during the column purification of the lipid extract. The sample is applied in chloroform—methanol (19:1), saturated with water (5 ml/l), which also elutes hydrocarbons and all lipids except gangliosides and conjugated bile salts. For a 10-g Sephadex column about 170 ml of solvent is required to complete the elution. The gangliosides are recovered with chloroform—methanol (9:1) (5 vol.) plus acetic acid (1 vol.). The column can be regenerated by washing it with methanol—water (1:1). This technique of lipid extract purification has been critically reviewed [3]. A batch method of purification of lipid extracts has been described by Williams and Merrilees [8].

C. Sample protection

Since most common lipids contain fatty acids with one or more double bonds, care must be taken to avoid autoxidation of the sample at any time during the manipulation and storage. This can be minimized by working with oxygen-free solvents and by performing all manipulations under a nitrogen atmosphere [9]. In addition, an antioxidant such as 2,6-di-*tert*.-butyl-*p*-cresol (BHT) or a similar compound may be added to the extracting solvents, which effectively prevents oxidative degradation of unsaturated lipids at a concentration of less than 0.005%. Furthermore, this antioxidant may be easily removed at various stages of the experiment by chromatographic means [7].

Before extraction the sample must be protected against the action of degradative enzymes. It should be noted that enzymatic hydrolysis may be significant even at −20° when stored for prolonged periods of time [10].

Finally the sample must be protected from contamination with lipids or other substances in solvents, reagents and on equipment. This can be guarded against by avoiding the use of solvents and reagents that leave lipid residues upon evaporation of appropriate volumes of the solvent and by washing all equipment with pure organic solvents before use.

Purified lipid extracts may be stored in tightly closed vials at low temperatures (−20° or lower) in the presence of inert solvents and inert atmosphere, for short periods of time.

3. SEPARATION OF LIPID CLASSES

There are several well established and reliable routines for the separation of individual lipid classes. A choice between column and thin-layer chromatography is usually made on the basis of sample size. Since the final separations of the individual lipid classes depend upon a careful adjustment of polarity of the eluting solvents, it is best to bring about a general group separation of lipids first and then follow it up with a complete resolution of individual chemical classes.

A. Neutral lipids

The neutral lipids are isolated on the basis of polarity by means of adsorption chromatography and include free fatty acids when present.

a. Initial isolation

A generally applicable strategy is to isolate the non-polar lipids as a mixture by adsorption column chromatography if time and quantity of material permits. This can be effectively accomplished by means of silicic acid columns [11, 12]. The non-polar lipids are recovered as a mixture in chloroform, while the polar lipids are retained on the column. Alternatively, the neutral lipids may be recovered as a group from TEAE-cellulose columns by elution with 5 volumes of chloroform, while the polar phospholipids are retained on the column. Detailed outlines of this method are available [12, 13].

Small amounts of neutral lipids may be isolated as a group by TLC using any of the solvent systems that separate the polar phospholipids and carry the neutral lipids to the solvent front [14, 15]. In all instances, the neutral lipids may be recovered from the scrapings of the silica gel by elution with chloroform.

b. Separation of neutral lipid classes

The most effective resolutions of individual neutral lipid classes are obtained by TLC. There are several excellent solvent systems that yield pure fractions for hydrocarbons, steryl esters, triacylglycerols, free fatty acids, diacylglycerols, free sterols and monoacylglycerols. When the TLC separations are performed in the presence of boric acid, it is possible to recover both *sn*-1,2(2,3)- and *X*-1,3-diacylglycerols as well as *X*-1- and 2-monoacylglycerols as separate neutral

lipid classes free of isomerization [16]. The monoacylglycerols frequently remain at or near the origin and require rechromatography with more polar solvents [17]. Further resolution may be required also for the *X*-1,2-diacylglycerols, which overlap with free cholesterol under these conditions. Any of these separations can be readily performed in quantities that are sufficient for subsequent GLC examination of the individual molecular species and the separation and identification of the component fatty acids.

Effective resolution of the neutral lipid classes may also be obtained by direct GLC on short columns prepared with non-polar liquid phases [18]. For this purpose the free fatty acids are converted into the trimethyl(silyl) (TMS) esters, and the free sterols and mono- and diacylglycerols as well as any free ceramides into the TMS ethers. The various neutral lipid classes including triacylglycerols and steryl esters are then eluted from the column in order of increasing molecular weight by means of temperature programming. There is also a separation within each chemical class of lipids according to the total carbon number. The method is not suitable for a quantitative isolation of the neutral lipid classes on a routine basis.

B. Polar lipids

The polar lipids contain a much more heterogeneous population of functional groups than the neutral lipids and may be subjected to lipid class resolutions that exploit these differences in their structure. By this means it is possible to effect a complete separation of the major lipid classes as well as to isolate many minor components in enriched form and in sufficient amounts for further analysis.

a. Initial isolation

The polar lipids may be recovered in toto or in small groups of related classes by chromatography on anion-exchange cellulose columns. Rouser et al. [12,13] have described the practical aspects of both the DEAE- and TEAE-cellulose column operation for this purpose. Following the initial displacement of the neutral lipids with chloroform, chloroform—methanol (9:1) (8 column volumes) will elute the choline phospholipids (phosphatidylcholine, lysophosphatidylcholine, and sphingomyelin) as well as cerebrosides and glycosyldiacylglycerols; chloroform—methanol (2:1) (9 volumes) will elute the ceramide polyhexosides; chloroform—methanol (2:1) containing 1% glacial acetic acid (8 volumes) will elute phosphatidylethanolamine, dimethylphosphatidylethanolamine, lysophosphatidylethanolamine as well as free fatty acids; while glacial acetic acid (8 volumes) will elute phosphatidylserine. The residual acidic phospholipids (phosphatidic acid, phosphatidylglycerols, cerebroside sulfates, sulfolipids, and phosphatidylinositols) are eluted with chloroform—methanol (4:1) containing 0.1 *M* potassium acetate and 20 ml/l of 28% ammonia (10 volumes). Although the various phospholipids or their subgroups may be further purified by ion-exchange columns, it is usually more efficient to complete the isolation of the fractions along with the lipid class separation by means of TLC.

Alternatively, the polar lipids are recovered from adsorption columns as a residual group of lipids following the elution of the neutral or non-polar lipids [11—13]. Glycosphingolipids can be removed with acetone and/or mixtures of

ethyl acetate and acetone [12, 13]. A 10—50% methanol in chloroform gives satisfactory recovery of the less polar glycerophospholipid classes, while elution with pure methanol will bring off all but the most polar lipids from a silicic acid [11—13]. Similar methods of elution may be used to recover the glycerophospholipids from the origin of a TLC plate following prior displacement of the neutral and the less polar glycolipids [12, 13]. Saito and Hakomori [19] have described a quantitative method for the isolation of total glycosphingolipids from crude lipid extracts without contamination from other lipid classes. The method consists of acetylation of total lipid extracts and separation of acetylated glycolipids from non-glycolipids on a Florisil column, followed by deacetylation of glycolipids in chloroform—methanol—sodium methoxide. Elution with 1,2-dichloroethane—acetone (1:1) yielded ceramides, cerebrosides, lactosylceramides, ceramide trihexosides, globosides, ceramide pentahexosides, ceramide octasaccharides, hematosides, monosialogangliosides, disialosylhematosides, and trisialogangliosides, while 1,2-dichloroethane—methanol—water (2:8:1) yielded cardiolipin, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, sphingomyelin, and phosphatidic acid.

b. Separation of phospholipid classes

The conditions of isolation of the major phospholipid classes from the more common natural mixtures have been described in detail by Skipski and Barclay [20] and by Rouser et al. [13]. The TLC systems provide the most reliable separations for routine work. An excellent system [21, 22] for the isolation of phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidylcholine, sphingomyelin and lysophosphatidylcholine of animal tissues consists of chloroform—methanol—acetic acid—water (25:15:4:2). The system is limited by the overlapping of phosphatidylglycerol and phosphatidylethanolamine as well as by the occasional tailing of phosphatidylserine and phosphatidylinositol. The acidic phospholipids, phosphatidylglycerol, cardiolipin and phosphatidic acid may be resolved from phosphatidylethanolamine in chloroform—methanol—acetic acid—water (80:13:8:0.3) following a prior development of the plate in acetone—light petroleum (1:3) [20].

Another excellent system [23, 24] for the resolution of the major glycerophospholipids is chloroform—methanol—water (65:25:4). In this system an essentially complete overlap is realized for phosphatidylserine, phosphatidylinositol and sphingomyelin, which run below phosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine, but above the lysoglycerophospholipids, all of which are well resolved. Cardiolipin emerges at or near the solvent front and may overlap with any neutral lipids which may be present. The separation of phosphatidylserine and phosphatidylinositol may be effected by rechromatography [6, 12] in chloroform—methanol—28% ammonia (65:35:5), or in chloroform—acetone—methanol—acetic acid—water (5:2:1:1:0.5) [6, 12,25].

The major plasma phospholipids may be effectively resolved [7] using one-dimensional TLC in chloroform—methanol—aqueous ammonia (65:25:5).

Rouser and coworkers [12, 13] and Simon and Rouser [26] have described several two-dimensional TLC systems that separate both acidic and neutral phospholipid classes. For general separation of glycerophospholipids a solvent system of high resolving power is chloroform—methanol—aqueous ammonia (65:35:5) for the first dimension and chloroform—acetone—methanol—acetic

acid—water (5:2:1:1:0.5) for the second dimension. Comparable results may be obtained without the acetone in the second solvent [27].

Another solvent system of comparable resolving power employs chloroform—methanol—aqueous ammonia (65:25:5) in the first dimension and chloroform—acetone—methanol—acetic acid—water (3:4:1:1:0.5) in the second dimension. However, phosphatidylglycerol and phosphatidylethanolamine frequently overlap or cochromatograph in these systems. An improved two-dimensional separation of the glycerophospholipid classes from a total lipid extract of most tissues has been described by Poorthuis et al. [28]. The solvent system for the first dimension is chloroform—methanol—water—concentrated ammonia (70:30:3:2) and for the second dimension chloroform—methanol—water (65:35:5). In comparison with the patterns obtained in the systems of Rouser et al. [12, 13] the positions of phosphatidylinositol and phosphatidylserine are reversed and phosphatidylglycerol is more distinctly separated from phosphatidylethanolamine. Both of these differences are due to addition to the silica gel of boric acid, which forms complexes with compounds containing vicinal hydroxyl groups [16] and retards their mobility. The alkyl and 1-alkenyl ether derivatives of the glycerophospholipids cannot readily be resolved from the corresponding acyl analogues [29] and must be left in the mixture until a fractionation stage is reached where it is appropriate to release the acyl glycerols.

c. Separation of glycolipid classes

The diacylglycerol mono- and digalactosides may be resolved by one-dimensional TLC using benzene—acetone—water (30:91:8) [30] or chloroform—methanol—ammonia—water (60:35:5:2) [31] as the developing solvents. A mixture of chloroform—methanol—water (65:35:8) has yielded a separate fraction for glycerophosphoryldiglucosyldiacylglycerol [32]. Excellent separations of galactosyldiacylglycerols from mixtures with glycerophospholipids have been obtained by two-dimensional TLC using chloroform—methanol—7 *N* ammonia (65:30:4) in the first direction and chloroform—methanol—acetic acid—water (170:25:25:6) in the second direction [33, 34].

The simple ceramide mono- and oligosaccharides can be separated using the solvent systems developed by Svennerholm and Svennerholm [35], Vance and Sweeley [36], and Skipski et al. [37]. Vance and Sweeley [36] have separated plasma glycosyl ceramides into cerebrosides, dihexosyl ceramides, trihexosyl ceramides and globosides, using chloroform—methanol—water (100:42:6) as the developing solvent. The sphingolipids are separated primarily according to the number of monohexosyl units per molecule. Skipski et al. [37] recommend an initial run with acetone—pyridine—chloroform—water (40:60:5:4) to separate neutral lipids and glycolipids, with the phospholipids remaining at origin of the TLC plate. A second development with a non-polar solvent made up of diethyl ether—pyridine—ethanol—2 *N* ammonia (65:30:8:2) is used to wash away the neutral lipids, while the glycolipids remain more or less stationary. A third run with diethyl ether—acetic acid (100:3) is finally made to wash away the free fatty acids. Under these conditions, the neutral lipids migrate just ahead of the ceramide monohexosides and all phospholipids move more slowly than a ceramide tetrahexoside. The more polar glycolipids occupy the same area of the TLC plate as the phospholipids unless the latter have been removed prior to TLC. An effective way of removing phospholipids from glycosphingolipids has

been described by Saito and Hakomori [19]. The more complex neutral diacylglycerol and ceramide oligosaccharides containing five to eight monosaccharide units may be resolved by TLC after conversion into the fully acetylated form [38]

Glucosyl and galactosyl ceramides which run together on plain silica gel can be resolved on borate impregnated TLC plates [39,40]. The sulfatides, galactosylceramide sulfates, run with similar mobility on TLC as the ceramide di- and trisaccharides in many solvent systems commonly employed for the neutral glycolipids, unless they have been resolved by chromatography on the ion-exchange celluloses [7].

The sialic acid-containing sphingoglycolipids, the gangliosides, are commonly resolved by one-dimensional TLC using propanol—water (7:3) as the developing solvent [41—43]. Complete resolution of the various classes of gangliosides is obtained provided these lipids have been first resolved from other lipids by Sephadex column chromatography [7]. These separations are based on the number of sialic acid residues within each oligosaccharide subclass.

Horning et al. [44] and Samuelsson and Samuelsson [45] have demonstrated that the ceramide monohexosides can be recovered from conventional GLC columns containing non-polar packings when chromatographed as the TMS ethers or the heptafluorobutyl esters. By means of similar columns Auling et al. [46] and Tulloch et al. [47] were able to resolve the mono- and dihexosyl diacylglycerols as the TMS ethers. The mono- and dihexosides of both diacylglycerols and ceramides have been resolved by Kuksis [48] and Williams et al. [49] in the form of the TMS ethers, acetates and methyl ethers. Using a short GLC column of the type commonly employed in triacylglycerol analyses, it was possible [48] to elute also the TMS ethers of the tri- and tetrahexoside ceramides. The latter separations, however, gave evidence of decomposition of the solutes and could not be recommended for routine applications. The GLC separations of the intact glycosides also allow a resolution of the molecular species of these lipids as discussed in Section 4F.

The chromatography of phospholipid and glycolipid classes on Whatman SG-81 silica gel loaded paper has been reviewed by Wuthier [50] while Witting [51] has summarized the more recent methods applicable to glycolipids as a class.

4. SEPARATION OF MOLECULAR SPECIES

The most effective and most widely applicable methods of fractionation of molecular species of intact lipid classes utilize differences in degree of unsaturation and in molecular weight of the fatty acid chains of the lipid molecules. The most complete resolutions are obtained by those methods of separation which allow a systematic exploitation of both of these differences.

A. Steryl esters

The steryl esters of various degrees of unsaturation of the component fatty acids may be resolved by argentation TLC. Morris [52] used a double development starting with diethyl ether, which resolved the tetraenes, pentaenes and hexaenes, and ending with diethyl ether—hexane (1:4), which allowed the sepa-

ration of the monoenes, dienes and trienes. The system did not allow the separation of saturated and unsaturated sterols esterified to the same fatty acid. A complete identification of the steryl esters in the various subfractions may be obtained following saponification and a GLC analysis of the component sterols, as described in Section 5D.

Tichy and Dencker [53] and Alling et al. [54] have described routine methods for the separation of serum cholesterol esters by TLC on plain silica gel using multiple developments in *n*-heptane—toluene (80:20) or (65:25), respectively. Separate fractions are obtained for saturated, monoenoic, dienoic, trienoic and tetraenoic, pentaenoic and hexaenoic fatty acid esters. The total steryl ester mixture as well as any of the subfractions derived by argention TLC may be resolved according to molecular weight or carbon number by direct GLC on short columns containing non-polar liquid phases [55, 56].

When more than one type of sterol is present two or more peaks may be obtained for each fatty acid ester [56]. For the resolution of steryl esters with short and long chain saturated fatty acids (C_2—C_{18}) as well as certain unsaturated acids (erucic, oleic, linoleic and linolenic), Kaufmann et al. [57] have utilized hydrophobic layers of paraffin impregnated silica gel, with ethyl methyl ketone—acetonitrile (7:3) as the solvent system. Despite the excellence of the resolution, this method is much inferior to the ease and simplicity of the direct GLC separation of the steryl esters according to molecular weight differences [55, 56, 58].

B. Acylglycerols

The acylglycerols are most effectively resolved by argentation TLC and GLC methods. Complete separations of the molecular species of the triacylglycerols, however, cannot be obtained even by a combination of the two methods, while the molecular species of the monoacylglycerols can be resolved essentially completely by either one of the methods. The completeness of resolution of the diacylglycerols depends on the molecular weight of the species, the lower-molecular-weight homologues being resolved more completely.

a. Triacylglycerols

Argentation TLC of triacylglycerols has given the most useful resolution of the molecular species comprised of mono-, di- and triethylenic fatty acids of uniform chain length of 18 carbons. A fat containing saturated, oleic, linoleic and linolenic acids could contain triacylglycerols with 0 to 9 double bonds. It has proved experimentally feasible [59] to resolve the triacylglycerols into the following classes of unsaturation in order of decreasing rate of migration: 000 > 100 > 200 > 111 > 210 > 211 > 220 > 300 > 221 > 310 > 222 > 311 > 320 > 321 > 322 > 330 > 331 > 332 > 333, where 0 to 3 represent the content of double bonds per molecule of fatty acid. These correlations apply to *cis*-methylene-interrupted polyethylenic acids of the same chain length.

Triacylglycerols containing both short and long chain fatty acids must first be resolved into triacylglycerol subclasses of short, medium and long chain length prior to effective argentation TLC [60, 61]. The position of the acyl group in the triacylglycerol molecule influences the resolution since 1-oleoyl-

2,3-distearoylglycerol is retained longer than 2-oleoyl-1,3-distearoylglycerol [62]. The triacylglycerols containing ethylenic fatty acids with a *trans*-configuration of the double bond migrate faster than those with a *cis*-configuration [59,63].

The triacylglycerols are effectively resolved in hexane—diethyl ether (70:30) when applied to silica gel containing 5—10% silver nitrate [64]. Alternative solvents for the resolution of triacylglycerols of the common fatty acids are mixtures of chloroform—methanol (99:1 to 94:6) [65—67]. A rechromatography of the polyunsaturated triacylglycerol fractions is performed by redeveloping the plate in the more polar solvent system one or more times [67]. The triacylglycerols are recovered from the silver nitrate plates by elution with 10% methanol in diethyl ether.

The GLC resolution of intact triacylglycerols is most easily accomplished with molecules containing short and medium chain length fatty acids, whose molecular weights do not exceed that of tristearoylglycerol [15,68,69]. It is also applicable to triacylglycerols composed of long chain fatty acids exclusively, but these separations require specially optimized columns [15,70] and cannot be utilized routinely at the present time. About 50—60 cm is an optimum length of the column for most triacylglycerol separations for which the losses of the higher molecular weight materials are not too serious [71,72]. A listing of currently available phases for triacylglycerol GLC has been compiled [72].

Triacylglycerols of uniform molecular weight and degree of unsaturation may be isolated by preparative GLC provided a preliminary resolution by argentation TLC has been carried out [73—75]. Owing to the critical nature of the separation conditions, it has been necessary to make repeated collections from essentially analytical columns by means of semi-automatic operation of fraction collectors in conjunction with stream splitters. The collected triacylglycerol peaks may be subjected to partial degradation and positional analysis of fatty acids for complete determination of the molecular structure of the component molecular species, as described in Section 5A.

For the purpose of further resolution of the component triacylglycerols the various subfractions collected from argentation TLC may be submitted to a reversed-phase partition TLC [76]. Although not as simple as direct GLC, this method has yielded some of the most complete resolutions of natural triacylglycerol mixtures. Excellent separations of C_9—C_{56} triacylglycerols according to acyl carbon number have been obtained by Lindqvist et al. [77] using hydroxyalkoxypropyl-Sephadex columns for liquid—liquid chromatography. Complete baseline resolutions were obtained with sample loads as high as 0.5 g/cm^2 of column. The reversed-phase partition TLC as well as the reversed-phase column partition chromatographic systems, however, are not as yet sufficiently reproducible for routine application in a general purpose laboratory.

Trisubstituted acylglycerols containing ether linkages may be separated with relative ease from triacylglycerols by adsorption TLC [78,79]. The relative order of increasing migration in hexane—diethyl ether (95:5 or 90:10) is triacylglycerols, alkyldiacylglycerols, alk-1-enyldiacylglycerols, dialkylacylglycerols and trialkylglycerols. These alkylacylglycerols may be subjected to argentation TLC under the general conditions of triacylglycerol resolution. Likewise they can be effectively resolved by high-temperature GLC under the

conditions employed for the separation of triacylglycerols of comparable molecular weight [72].

b. Diacylglycerols

The fractionation of diacylglycerols by argentation TLC allows separations of molecular species containing 0 to 12 double bonds [80]. A conversion into the acetates helps to prevent isomerization as well as reduces tailing of the components during chromatography. Many positional isomers are also separated. Thus the 1,2-dioleoyl-*sn*-glycerol moves ahead of the 1-palmitoyl-2-linoleoyl-*sn*-glycerol acetate. Likewise, the acetate of 1-oleoyl-2-linoleoyl-*sn*-glycerol moves ahead of the 1-palmitoyl 2-linolenoyl acetate and the acetate of 1-oleoyl-2-arachidonoyl-*sn*-glycerol moves ahead of the acetates containing one saturated and one pentaunsaturated fatty acid. Similar separations are well established among the triacylglycerols [59]. Comparable resolutions of the diacylglycerols have been obtained by argentation TLC of their *tert.*-butyldimethylsilyl ethers [81]. The latter compounds have the advantage of stability to moisture. Dyatlovitskaya et al. [82] have employed the trityl derivatives of diacylglycerols for argentation TLC, while Van Golde et al. [83] have employed free diacylglycerols with equal success.

The argentation TLC usually does not yield pure molecular species and other methods are required to complete the separations. For this purpose GLC methods are best suited [84, 85] as they provide effective resolutions based on molecular weight. Complete separations of diacylglycerols as the acetates or the silyl ethers may be obtained according to both molecular weight and degree of unsaturation on certain polar liquid phases [86], but these methods are still at an experimental stage. The identity of the various diacylglycerol fractions obtained by argentation TLC is confirmed by determination of the fatty acid composition as described in Section 5A. Similar separations are obtained with the appropriate derivatives of the 1,3-diacyl-*sn*-glycerols, which exhibit slightly higher R_F values on TLC and slightly longer retention times on GLC than the corresponding *sn*-1,2- or 2,3-diacylglycerols [87].

The diacylglycerols may be readily resolved from the corresponding alkyl acyl and alk-1-enylacylglycerols by adsorption TLC [80]. Furthermore, both alkylacyl- and alk-1-enylacylglycerols can be readily resolved according to degree of unsaturation by argentation TLC [79, 80] as well as according to molecular weight and degree of unsaturation by GLC [72] on non-polar and polar columns, respectively.

c. Monoacylglycerols

Mixtures of monoacylglycerols may be resolved according to degree of unsaturation by argentation TLC [62, 88]. Preparation of the diacetates prior to resolution avoids isomerization of the monoacylglycerols and facilitates their subsequent analysis by GLC. The monoacylglycerol acetates require a relatively polar solvent system, hexane—diethyl ether (60:40). The monoacylglycerols may be resolved according to the number of double bonds and the position of the fatty acid residue when chromatographed on silica gel impregnated with both silver nitrate and boric acid [89]. The monoacylglycerols are recovered from the silica gel by elution with 20% methanol in diethyl ether [64].

The *tert.*-butyldimethylsilyl ethers of the monoacylglycerols may be resolved by argentation TLC using solvent systems similar to those employed for the separation of the acetates [81].

Mixtures of monoacylglycerols may be resolved by GLC on the basis of molecular weight using non-polar columns and on the basis of molecular weight and degree of unsaturation using polar columns [90]. For this purpose the acetates of monoacylglycerols would appear to be better suited, because of their greater stability on polar liquid phases.

The above TLC and GLC systems also permit the resolution of the acyl-, alkyl- and alk-1-enylglycerols of corresponding carbon number [90].

C. Ceramides

Efficient separations of molecular species of either free ceramides or ceramides released from natural sphingolipids by enzymic [80] or chemical degradation [91] are obtained by ordinary silica gel, or silica gel impregnated with borate or arsenate [92]. The separations are dependent upon the number, position and the stereochemistry of the hydroxyl groups in the ceramide molecules. Borate and arsenate have little effect on the ceramide mobility as determined by the hydroxyl groups of the fatty acid, but borate retards ceramides containing trihydroxysphingosines very strongly, while arsenate has the opposite effect. Borate also retards the ceramides containing an ethylenic double bond at C_4 of the sphingosine base. The effect of chain length on the separation of ceramides on silica gel G is minimal, but compounds with increasing chain length travel faster.

The separations of ceramides on the basis of the number of double bonds are best performed by argentation TLC with the ceramide acetates [92,93].

Further separation based on molecular weight and to a lesser extent on other features may be obtained by GLC.

The GLC separation of ceramides is best accomplished on non-polar columns using the TMS derivatives [18,93]. Prior to GLC the ceramides are resolved as the acetates according to degree of unsaturation, the major chain lengths and the content of hydroxyl groups by argentation TLC. The free ceramides of plasma yield two fractions on argentation TLC, the faster moving one of which corresponds to N-stearoylsphingosine diacetate and the slower one to N-nervonylsphingosine diacetate. After mild methanolysis the ceramides are silylated and examined by GLC on 1% OV-1 columns. Under these conditions the faster moving TLC fraction is resolved into eleven components. Samuelsson and Samuelsson [93] have identified the major peaks by mass spectrometry as the C_{16}—C_{24} fatty acid derivatives of sphingosine. The mass spectra indicated that each GLC peak also contained ceramides with sphinganine and hexadecasphing-4-enine as long chain bases. The ceramides with the latter long chain base contained a fatty acid with two more carbon atoms than the sphingosine ceramides of the same GLC fractions. The slower TLC fraction contained all the same fractions as isolated in the faster TLC band in addition to two major components, one of which was N-docosenoylsphingosine and the other the N-tetracosenoylsphingosine.

The ceramides from the plasma sphingomyelins give four fractions on

argentation TLC of the acetates. On GLC of the TMS ethers two to eleven components may be distinguished in each of the four fractions. The fastest moving TLC fraction was shown by Samuelsson and Samuelsson [93] to contain mainly sphingosine and hexadecasphing-4-enine combined with saturated fatty acids, whereas the second fastest moving TLC fraction consisted primarily of the same long-chain bases combined with monounsaturated fatty acids (mainly N-tetracosenoylsphingosine). The second slowest TLC fraction was a mixture made up of ceramides with sphinga-4,14-dienine as base and saturated fatty acids or sphingosine combined with monounsaturated fatty acids. The slowest moving TLC fraction consisted mainly of two components, N-docosenoylsphinga-4,14-dienine and N-tetracosenoylsphinga-4,14-dienine.

Horning et al. [44] have successfully recovered both TMS ethers and heptafluorobutyryl esters of 2-hydroxy fatty acid ceramides from natural sources from relatively long columns containing 1% SE-30 packing. The GLC analysis of ceramides in the form of permethylated derivatives has been described by Huang [94], who has used short columns with non-polar packings and temperature programming.

D. Glycerophospholipids

Intact glycerophospholipids may be resolved into individual molecular species or small groups thereof by both argentation TLC and reversed-phase partition TLC. The most complete separations are obtained by a sequential combination of the two techniques. In several instances a preparation of a suitable derivative greatly enhances the resolution of the molecular species of a given phospholipid class.

a. Phosphatidylcholines

The separation of intact phosphatidylcholines according to degree of unsaturation may be obtained by argentation TLC [95]. An effective solvent is chloroform—methanol—water (60:30:5). This allows the resolution of monoenes, dienes, tetraenes and hexaenes when applied to rat liver phosphatidylcholines. The individual subfractions are recovered from the silica gel by elution with chloroform—methanol—acetic acid—water (50:39:1:10) in a 90—95% yield. Many other laboratories have now employed this procedure with satisfactory results [96—98].

There is minimal resolution of the phosphatidylcholine species according to the fatty acid structures making up the various unsaturation classes, such as that noted for triacyl- and diacylglycerols.

Each of the fractions obtained on the basis of unsaturation by argentation TLC may contain two or more subfractions, which may be resolved according to molecular weight differences by reversed-phase TLC [99]. This separation is effected by rechromatography of the silver nitrate subfractions on calcium sulfate-free silica gel impregnated with undecane and using methanol—water (9:1). By this means the monoenes, dienes, tetraenes and hexaenes of the rat liver phosphatidylcholines may be resolved into their palmitoyl and stearoyl species. However, further separations may be obtained in those instances where more complex mixtures of homologues are present. Many workers have now

reproduced these fractionations with remarkable success [100,101]. The reversed-phase TLC plates may be easily prepared with commercially available hydrophobic silica gel.

b. Phosphatidylethanolamines

The separation of the phosphatidylethanolamines according to the degree of unsaturation of the molecules may be obtained under the general conditions of separation of the phosphatidylcholines using chloroform—methanol—water (55:35:7) as the developing solvent [95]. These separations also have been reproduced in other laboratories with comparable results [101,102].

The reversed-phase TLC of the various unsaturation classes of the phosphatidylethanolamines has been les effective [95].

Masking of the polar groups in phosphatidylethanolamine by O-methylation and N-dinitrophenylation allows a more complete resolution of the molecular species by both argentation TLC and by reversed-phase partition [103]. These derivatives also allow a complete resolution of the alkenylacyl-, alkylacyl- and diacylglycerophosphorylethanolamines on plain silica gel [104] using multiple developments with hexane—chloroform. The order of migration is the same as with diacylglycerol acetates: alkenylacyl > alkylacyl > diacyl. The separations based on the number of double bonds per molecule of these phosphatidylethanolamine subclasses were obtained [105] with the solvent system chloroform—methanol (98:2). Comparable resolution of the molecular species of phosphatidylethanolamine are obtained [106] following N-acetylation and O-methylation when the silver nitrate plates are developed in chloroform—methanol—water (80:15:2) and chloroform—methanol (97:3). These derivatives are more easily prepared and do not leave non-volatile reaction by-products. Work with the N-trifluoroacetyl derivatives of phosphatidylethanolamines [107] has proved that O-methylation is not necessary for effective separation of these derivatives according to the degree of unsaturation of the molecules.

The subfractionation of the dinitrophenylated phosphatidylethanolamines according to molecular weight by counter-current distribution has been extensively utilized by Trewhella and Collins [108] and Collins and Trewhella [109] as well as Shamgar and Collins [110]. Although the latter separations are highly reproducible and subject to routine application, the counter-current distribution equipment may require an experienced operator.

c. Phosphatidylinositols

The phosphatidylinositols may be separated according to degree of unsaturation of the component fatty acids by argentation TLC under the general conditions employed for the analysis of phosphatidylcholines provided the solvent system is properly adjusted. Using chloroform—methanol—water (65:35:5) as the developing solvent, Holub and Kuksis [111,112] obtained four fractions of different number of ethylenic bonds per molecule for the phosphatidylinositols of rat liver.

Luthra and Sheltaway [113] have modified the phosphatidylinositol molecules by periodate oxidation and diazomethylation or acetylation and diazomethylation prior to argentation TLC. Solvent mixtures containing acetone and distilled chloroform were found most suitable for the resolution of the monoenoic, dienoic, trienoic and tetraenoic species.

d. Phosphatidylserines

It has not been possible to separate the phosphatidylserines by argentation TLC of the original compounds. N-trifluoroacetylation, however, has allowed such separations. A development of an ordinary argentation TLC plate with chloroform—methanol—water (65:25:4) has given separate bands for the monoenes, dienes, trienes, tetraenes and hexaenes of phosphatidylserine of rat liver, as well as the appropriate unsaturation classes of the phosphatidylserines of the brain and red blood cells of various animal species [114]. N-Dinitrophenylation and O-methylation [103] has previously allowed a counter-current resolution of the major molecular species of phosphatidylserine.

e. Phosphatidylglycerols

Haverkate and Van Deenen [115] resolved phosphatidylglycerols of spinach leaves into two subfractions on argentation TLC, using chloroform—ethanol—water (65:30:3.5). The slower moving fraction contained three to four double bonds per molecule, while the faster one contained zero to two double bonds per molecule. The 1-linolenoyl-2-Δ^3-*trans*-hexadecenoyl phosphatidylglycerol accounted for about one half of all phosphatidylglycerols of spinach leaves.

The diphosphatidylglycerol (cardiolipin) may be subfractionated [116] by argentation TLC using chloroform—methanol—water (80:20:1) as the developing solvent. Separations are obtained for molecular species containing four linoleic acid residues and species containing two linoleic acid residues along with saturated and monounsaturated fatty acids. The molecular species of cardiolipin may also be assessed [117] by subfractionating the diacylglycerols obtained by acetolysis. These data, however, are more difficult to interpret.

f. Phosphatidic acids

Naturally occurring phosphatidic acids [118] and phosphatidic acids derived from glycerophospholipids by enzymic or chemical degradation [119, 120] allow extensive resolution by TLC techniques, when analyzed as the dimethyl esters. TLC on plain silica gel allows the separation [120] of the alkenylacyl, alkylacyl and diacyl derivatives using solvent systems similar to those employed for the resolution of the corresponding disubstituted glycerols. Argentation TLC in chloroform—methanol—water (90:10:1) gives subfractions according to the number and position of double bonds in these molecules [118, 120, 121]. Individual molecular species of phosphatidic acid dimethyl esters may be resolved [119] by reversed-phase TLC using acetonitrile—acetone—water (8:1:1) and silica gel layers made hydrophobic with tetradecane.

E. Sphingomyelins

Sphingomyelins frequently yield a double spot on plain silica gel TLC [122, 123]. This is apparently due to a segregation of the molecular species with long-chain fatty acids (C_{22}—C_{26}), which migrate faster, and with short-chain fatty acids (C_{16}—C_{18}), which migrate more slowly. Svennerholm and Svennerholm [35] showed that this separation could also result from the presence of α-hydroxy fatty acids in the ceramide moieties of the sphingolipid. In each instance the slower migrating component contained the hydroxy fatty acids while the faster migrating component did not.

The small differences in the unsaturation of the various ceramide moieties of the sphingomyelins are best exploited by argentation TLC of the ceramides or ceramide acetates derived from them by acetolysis or enzyme degradation [80, 93, 124] (see Section 4B).

F. Glycolipids

Only the simplest glycolipid classes have thus far been separated into molecular species. Nichols and Moorhouse [125] have resolved the monogalactosyldiacylglycerols according to the degree of unsaturation of the fatty acids by argentation TLC using chloroform—methanol—water (60:21:4) as solvent. Five major fractions were isolated corresponding to groups of molecular species containing 1 to 5 double bonds per molecule. A comparable resolution has been obtained for the molecules species of the mono- and digalactosyldiacylglycerols of the broad bean [126]. Since these diacylglycerols were made up largely of 18:3 fatty acids (80—90%), argentation TLC gave strong bands for hexaenes and minor bands for pentaenes, tetraenes, trienes and dienes. Each of the fractions gave a major peak for the component containing two C_{18} and a minor peak for the component containing a C_{16} and C_{18} fatty acid when examined intact on GLC.

The various glycosyl ceramides may be resolved on plain silica gel according to chain length and the number of hydroxyl functions [127] as already noted for free ceramides and sphingomyelins. The simple ceramide glycosides are subject to a limited resolution by argentation TLC because of the absence of large differences in the degree of unsaturation of the different molecular species. Direct GLC allows the separation of the ceramide glycosides according to the carbon number of the nitrogenous base and the component fatty acid [126]. A GLC examination of the intact rat brain cerebrosides in the form of the TMS ethers gave comparable elution patterns for the normal chain and the 2-hydroxy fatty acid derivatives. The major peaks corresponded to cerebrosides with lignoceric and cerebronic acids in the normal and 2-hydroxy acid fractions, respectively. Minor peaks due to acids of 18—23 carbon atoms were also present, which was in agreement with previous results based on fatty acid analyses. A direct GLC of the lactosylceramides from rat bone marrow [126] also gave evidence for a resolution of molecular species. The major peaks represented the 16 and 18 carbon fatty acid derivatives.

The molecules of the more complex glycosphingolipids do not yield readily to the resolution of molecular species within an intact oligosaccharide type. Some progress in this area has been made by high-performance liquid chromatography [128, 129], but these techniques are not yet available for routine application.

5. SEPARATION OF LIPID CONSTITUENTS

The ultimate purpose of the separation of the lipid classes and molecular species is their identification and quantitation. The determination of the lipid components of the various lipid classes and molecular species serves to confirm the identification derived by chromatographic methods as well as provides means of their quantitative measurement.

A. Fatty acids

The fatty acid composition of a lipid class or a molecular species is determined after the fraction has been purified, usually by TLC. The fatty acid analyses may be made on the total molecule or on some part of it derived by controlled enzymic or chemical degradation. The lipid sample is methylated with sulfuric acid—methanol, boron trifluoride—methanol, or hydrochloric acid—methanol reagent [3, 130]. The tubes are closed and heated at 90° for a minimum of 2 h. After cooling to room temperature, the fatty acid methyl esters are extracted with several washes of hexane, which may contain 0.01% BHT [7]. Samples containing material other than fatty acid methyl esters may require purification which may be accomplished on small columns of silicic acid using 1% diethyl ether in hexane to elute the methyl esters and the antioxidant and a subsequent wash with methanol to recover any sterols, sphingosine and glyceryl ethers, if present. Alternatively the methyl esters may be purified by TLC using pure benzene as the developing solvent, which also allows the removal of the BHT antioxidant as a separate band [7].

Samples of fatty acid methyl esters are routinely analyzed by GLC using polar columns, which separate the acids according to chain lengths and total number of double bonds, as well as the position of the double bonds. When working with fatty acids from well characterized sources, the separation of the esters on any one of a number of polyester columns may be sufficient to identify all but the minor components on the basis of the retention times or equivalent chain length [130—132]. The conventional polyester columns, however, do not allow the separation of the geometric isomers of the unsaturated fatty acids or the closer positional isomers. Some of these separations can be obtained by means of capillary columns [132]. Despite much progress in the utilization of capillary columns these columns are not yet available for routine application [133—135]. On the other hand, the availability of the polar cyanopropylsiloxane phases, which are capable of effective separation of the geometric isomers of fatty acids using conventional columns [136, 137], may reduce the need for capillary columns to some extent.

In any event, whenever sample size permits, it is helpful to subject the fatty acid methyl esters to argentation TLC and rechromatograph the fractions by GLC [14, 131, 138].

On the basis of the known order of migration of the fatty esters in the TLC and GLC systems it is usually possible to reduce the probable identities of any unknown peaks to a single or a few related components. The combined argentation TLC—GLC approach is much more effective than the comparisons of retention times or equivalent chain length values from two or more different GLC columns where it may be difficult or impossible to decide which peak is which in a changing pattern of the elution profile. Ultimately the unknown fatty acids may be identified by GC—MS and chemical synthesis, which are outside the scope of routine methodology.

B. Carbohydrates

The carbohydrate components of the glycolipids are routinely liberated by

cleavage of the glycosidic bonds with methanolic hydrochloric acid, resulting in the quantitative formation of O-methyl glycosides, although other methods may also be employed [139]. The O-methyl glycosides are resolved by GLC as the TMS ethers [140], N-trifluoroacetyl esters [141] or acetates [142]. Owing to the presence of an anomeric carbon in the sugar molecules, the O-methyl glycosides yield up to four separate peaks for each monosaccharide in the GLC elution pattern obtained with most derivatives on most liquid phases. An exception is provided by the single peaks recorded for each O-methyl glycoside on Apiezon L columns when run as the acetates. Dawson [139] has shown that L-fucose, D-galactose, D-glucose, D-mannitol (internal standard), N-acetylgalactosamine, N-acetylglucosamine, and N-acetylneuraminic acid give single, completely resolved peaks on an 8% Apiezon L column when temperature programmed from 170 to 220° at 2°/min.

Another method that allows the recording of single peaks for each sugar component in the mixture requires the liberation of the sugar moieties in the free form which are then converted to the alditols. The sugar alcohols are analyzed as the TMS ethers or acetates to yield single peaks for each component when analyzed on either polar or non-polar columns. The most complete resolutions of these derivatives are obtained on polar columns [143]. However, the apparent advantages of obtaining single peaks for each sugar is counterbalanced by the increased complexity in preparing alditol acetates from glycolipids and the apparent inability to estimate sialic acids in the free form.

In the past few years the technique of permethylation of glycolipids followed by GLC and mass spectrometric analyses has greatly increased the understanding of the structure of these compounds. In one of the more successful of these procedures [143] the free hydroxyl groups in the oligosaccharide are methylated in dimethylsulfoxide, methylsulfinyl carbanion and methyl iodide. Following isolation, the glycolipid is subjected to formolysis and hydrolysis, and a reduction with sodium borohydride. The residue is acetylated and the resulting partially methylated partially acetylated sugar alcohols are examined by GLC. Darvill et al. [144] have developed a mixed phase (0.3% OV-275—0.4% XF-1150) column packing which allows the complete separation of the methylated alditol acetates without resorting to multicolumn systems usually employed for complete resolution of these derivatives. Although this technique of structural analysis of oligosaccharides is now extensively used, it cannot be recommended for routine application outside a specialized research laboratory.

C. Nitrogenous bases

The short-chain nitrogenous bases may be released by hydrolysis with 6 *N* hydrochloric acid for 3 h at 100°. Free choline, dimethylethanolamine, monomethylethanolamine and ethanolamine may be separated by TLC using methanol—conc. hydrochloric acid (95:5) or *n*-butanol—methanol—conc. hydrochloric acid—water (50:50:10:10) as the developing solvents. The bases [145, 146] may be recovered from the TLC plates with a mixture of methanol—acetic acid—water (39:1:10). The free nitrogenous bases may be resolved by GLC on non-polar columns containing sodium hydroxide [145], from which they emerge in the order dimethylamine, monomethylamine, ethanolamine,

Choline is not recovered and there is some tailing of all the peaks. Choline may be estimated by GLC following a removal of one of the methyl groups by the Jenden reaction [147,148]. The resulting dimethylethanolamine would overlap with the dimethylethanolamine already present in the sample. Improved separations of the methylated ethanolamines and ethanolamine may be obtained by GLC of their acetates on non-polar columns.

The long-chain bases of ceramides, cerebrosides, sphingomyelins and gangliosides constitute complex mixture of difficultly soluble components which are sensitive to degradation and rearrangement. Heating with conc. hydrochloric acid—methanol—water (3:29:4) for 18 h at 78° has given satisfactory yields of the long-chain bases from most of these compounds [149]. The bases are recovered from an alkaline solution by extraction with chloroform. After drying in vacuo over phosphorus pentoxide the bases are converted to TMS ethers and subjected to GLC. The bases are separated according to chain length, degree of unsaturation and the number of hydroxyl groups present. Carter and Gaver [150] and Karlsson [151] have presented extensive tabulations of the retention times and equivalent chain length values for the TMS ethers of long-chain bases obtained in their laboratories.

D. Sterols

The most convenient methods of resolution of the sterols found in lipids are TLC [152,153] and GLC [152,154]. Argentation TLC allows the separation of saturated and monounsaturated sterols after a development in chloroform [155]. In the same system complete resolution is obtained [156] also for cholesterol—allocholesterol, cholesterol—desmosterol, and other sterol pairs. More recently effective separation of closely related sterols has been achieved by argentation TLC using chloroform—acetone (9:1) [156] or chloroform—dimethylketone (95:5) at 4° [157] as developing solvents. The TLC separation of many other sterol mixtures has been reviewed by Lisboa [153]. The simple sterols are routinely resolved according to molecular weight and the overall shape of the molecule by GLC on both polar and non-polar packings using conventional columns [152, 154]. A combination of argentation TLC and GLC methods insures the greatest success in routine analyses [152, 156—158].

E. Partial hydrolysis products

Chromatography of the partial hydrolysis products of a phospholipid or a glycolipid yields information about the structure of the molecular species or lipid class [159]. The lipid-soluble components derived from enzymic or chemical degradation of the lipid molecules are analyzed using the methods described for the various neutral and simple polar lipid classes, such as the mono- and diacylglycerols, phosphatidic acids, ceramides and lysophospholipids. Completely or partially water-soluble products are obtained by mild alkaline hydrolysis of the lipids, which selectively removes the O-fatty acyl groups leaving the N-fatty acyl, alkyl and alk-1-enyl groups intact. The water-soluble products such as the glycerolphosphate esters of the nitrogenous bases can be separated

by paper chromatography, paper chromatography and ionophoresis, or ion-exchange chromatography [9,159]. TLC has proved to be well suited for the separation of the partial hydrolysis products of plasmalogens [159].

Ohashi and Yamakawa [160] have described a GLC method for the analysis of the oligosaccharides released from glycosphingolipids by ozonolysis and alkaline cleavage. With short columns containing non-polar packings and temperature programming (110—350°) excellent separations were obtained for the TMS glycitols of mono-, di-, tri-, tetra- and pentasaccharides.

6. SPECIFIC APPLICATIONS IN BIOMEDICAL RESEARCH AND CLINICAL CHEMISTRY

Chromatographic methods of lipid analyses are extensively utilized in biomedical research but only a few of them have become established routines in clinical chemistry. In the following section reference has been made to selected applications of lipid chromatographic methods in analyses of clinical material in both routine and research laboratories.

A. Total lipid profiles

TLC has been extensively utilized in the determination of plasma lipid profiles in various clinical conditions [161]. TLC using densitometry and other techniques of charred spot measurement offer rapid micromethods for quantitative analysis of lipid classes [162]. A review of literature on this subject reveals that different procedures are used in virtually every laboratory engaged in this work. Recent improvements in the method relate to charring of the lipids following removal of the gel from the plate [163] and incorporating the charring reagent in the silica gel [164]. Under these conditions the results for serum free cholesterol, cholesterol esters and triglycerides compare favourably to the results obtained with the conventional Sperry-Web and Van Handel-Zilversmit methods for cholesterol and triglyceride determination, respectively. Extensive use of TLC techniques in the separation and quantitation of the phospholipids of red blood cells has been made by Nelson [7] who has also reviewed the subject [3]. Applications of TLC in analyses of complex lipids in health and disease have been discussed by Witting [51].

Along with determination of plasma lipid patterns by TLC may be mentioned the profiling of plasma lipids achieved by GLC. Kuksis et al. [165] and Horning et al. [44] have demonstrated that glycerophospholipids can be analyzed directly by pyrolysis GLC. For routine application, however, these techniques are inadequate. A modified procedure involving dephosphorylation of phospholipids by phospholipase C prior to GLC, however, is satisfactory [18]. The latter technique has been employed for a preliminary examination of plasma lipid profiles of a limited number of normolipemic and hyperlipemic subjects, as well as for assessing the effect of diet and hypolipemic drugs on plasma lipid levels and profiles [166]. The method has been recently automated and the calculations facilitated by computer programming [167]. By this means several thousand plasma samples from normo-

lipemic and hyperlipemic subjects have been examined in a study of the prevalence of hyperlipemia in a free living urban population [168]. In addition to providing quantitative estimates for free and esterified cholesterol, triacylglycerols and the glycerophospholipids and sphingomyelins, the method also gives quantitative values for plasma free fatty acids [169] and any plant sterols [170] that may be present.

High-temperature GLC has been evaluated [171] as a technique to monitor changes in composition of intact triacylglycerols of human serum lipoproteins after ingestion of oils containing fatty acids of widely differing chain lengths. The technique proved useful in this type of study since it measured the appearance of triacylglycerols of specific molecular weight. Random or non-random distributions of fatty acids in serum lipoproteins could be detected after ingestion of the oils. With simple fatty acid analysis, only changes in the proportions of fatty acids in triacylglycerols can be measured.

B. Lipid class ratios

When properly recorded the total lipid profiles provide both absolute quantities and characteristic ratios of the different lipid classes which are of clinical interest. In many instances, however, it is convenient to determine the ratios of specific lipid classes without obtaining a total lipid profile. Thus the ratio of free to esterified or total cholesterol can be readily obtained by quantitating the corresponding fractions from neutral lipid TLC [162, 163]. This ratio is of interest in familial lecithin:cholesterol acyltransferase deficiency [172, 173]. TLC can also provide a ratio for free cholesterol and total phosphatidylcholine, which is of interest in assessing the stability of certain abnormal plasma lipoproteins, such as the LPX component appearing in cholestasis [174] and during Intralipid infusion [175]. A lysolecithin index defined as the ratio of log lysolecithin phosphorus to total phosphorus has been computed to characterize uremia patients [176].

Determination of the ratio of phosphatidylcholine (lecithin) and sphingomyelin (L/S) provides means for assessing the development of the fetus in pregnancy and yield reliable information about the pulmonary maturity. Glueck et al. [177] have observed that the concentration of phosphatidylcholine in amniotic fluid increases to approximately four times that of sphingomyelin at 35 weeks and in subsequent weeks continues to rise, while sphingomyelin declines. Glueck et al. [177] separated the phospholipids in chloroform—methanol—water (65:25:4) and charred the spots with sulfuric acid. Many modifications of the original methods have been suggested since, including the development of the plate and the quantitation of the spots [178, 179]. Others have proposed the quantitation of the L/S ratio from GLC analysis of the component palmitic acid moieties [180]. The need for standardization of the Glueck test has also been pointed out [181].

C. Separation and quantitation of lipid constituents

The separation and quantitation of lipid constituents by TLC is also a well established routine in many clinical laboratories. For this purpose, use may be

made of most of the systems employed for determination of neutral lipids. These analyses are performed either for a more detailed assessment of the structure of complex lipids or for quantitation of the lipid classes separated on TLC plates, as an alternative to charring.

There have been several successful applications of GLC in the determination of cholesterol in clinical materials [182, 183]. These methods have employed relatively large samples of plasma or tissue and generally have not provided the sensitivity obtained by spectrometric methods. However, MacGee et al. [184] have reported a practical micromethod using 50 μl of plasma for the determination of total cholesterol by GLC. The authors have revised the original method to utilize 5 to 20 μl of plasma [185] with the same accuracy and precision as reported for the 50 μl samples. At this sample level the GLC methods become fully competitive with spectrometric methods in sensitivity and surpass them in specificity. This latter method has been recently adopted for the analysis of cholesterol in the high density plasma lipoprotein fraction (corresponding to 50 μl of plasma) to complement the GLC analyses for total cholesterol [186].

Comparative studies of plasma cholesterol concentrations by GLC, colorimetric and enzymatic methods have given essentially identical results [187].

Bjorkhem et al. [188] have described a highly sensitive and accurate reference method for estimation of total cholesterol in serum. A fixed amount of [2,2,3,4-D_4] cholesterol is added to a fixed amount of serum (usually corresponding to 10 μl). After saponification and extraction with hexane, the amount of unlabeled cholesterol is determined from the ratio between recordings at *m/e* 384 and *m/e* 389 obtained after analyses with a mass spectrometer equipped with a multiple ion detector. The method can be used for determination of cholesterol down to a level of 10 pmoles (4 ng).

The GLC analysis of fatty acids is an important and well established routine in most clinical laboratories. It permits to establish the existence of normal fatty acid profiles in specific clinical samples, as well as allows the recognition of any unusual fatty acid components. Of specific interest is the identification in serum of methylbranched fatty acids in Refsum's disease [189], and of fecal hydroxy fatty acids in sprue [190]. Other important applications relate to the assessment of the adequacy of essential fatty acid levels in infants, and in adults with massive dissection of the small bowel [191]. For this purpose the triene/tetraene ratio, 20:3ω9/20:4ω6, provides an adequate analytical measurement [192].

The utilization of conventional GLC columns in the analysis of fatty acids in biomedical research and clinical chemistry, however, presents a number of difficulties, which arise from the temperature limitations imposed by high-bleed liquid phases and the relatively low resolution. As a result most conventional analyses of fatty acids are lacking in estimates of long-chain components as well as of geometrical isomers of unsaturates. Lin et al. [133] have developed a new gas chromatographic method for a simultaneous analysis of long-chain fatty acids, α-tocopherol and cholesterol. This method has been applied to the analysis of plasma free fatty acids [193] and of the acids of plasma phosphatidylcholines and cholesteryl esters [134] in stroke patients and in normal young adults. The analysis of fatty acids of plasma lipids with

special emphasis on the geometric isomers of the unsaturated fatty acids has been reported by Jaeger et al. [135].

There has been a gradual increase in the utilization of GC—MS systems for separation and identification of lipid compounds in clinical chemistry, and many clinical laboratories now have access to such facilities. Lawson [194] has reviewed the scope of mass spectrometry in clinical chemistry and instrumentation, as well as of structure identification of physiological compounds including lipids. A recent review of the biomedical applications of mass spectrometry and GC—MS in the analysis of lipids has been prepared by Burlingame et al. [195].

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8. SUMMARY

This review summarizes the basic chromatographic routines commonly employed in lipid research laboratories in the analysis of the lipid mixtures normally isolated from natural sources. Emphasis is placed upon a systematic application of complementary chromatographic techniques as a means of ensuring maximum resolution and complete identification of lipid classes and molecular species. Many lipid samples, however, are simple enough to be analyzed completely by means of one or a few of the analytical sequences discussed. Regardless of the chromatographic routine selected, the analysis should be preceded by an effective isolation of the lipid sample free of contamination and in the absence of decomposition. Both aspects of sample handling are considered in the early part of the discussion.

The bibliography has been selected to call attention to the most recent comprehensive coverage of each subject from which the original references, if other, can be located. Hopefully this survey will show that for many purposes adequate analyses of known lipids can be obtained with conventional equipment of thin-layer and gas chromatography.

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INVESTIGATION OF THE METABOLIC PATTERN IN MAPLE SYRUP URINE DISEASE BY MEANS OF GLASS CAPILLARY GAS CHROMATOGRAPHY AND MASS SPECTROMETRY

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SUMMARY

Urine and serum from patients with maple syrup urine disease (MSUD) have been examined quantitatively and qualitatively using glass capillary gas chromatography in combination with mass spectrometry. During clinical episodes, patients with this disease were found to excrete increased amounts of the following metabolites in addition to the previously recognized branched-chain 2-keto and 2-hydroxy acids, lactate and 3-hydroxybutyrate: 2-hydroxybutyrate, 2-hydroxyisobutyrate, 3-hydroxyisovalerate, 3-hydroxyisobutyrate and 2-methyl-3-hydroxybutyrate. Most of the latter compounds seem to accompany ketoacidosis and lactic acidosis. The capillary column also separated the D- and L-forms of 2-keto-3-methylvalerate, and both isomers were, in contrast to earlier assumptions, present in the MSUD patients. The results clearly demonstrate that new information on the metabolic situation in well known disorders may be obtained by exploiting the high resolving power of capillary columns.

INTRODUCTION

Maple syrup urine disease (MSUD) has been the subject of numerous reports and review articles (see, e.g., refs. 1—5). The disease, which may have at least four distinguishable forms, depending inter alia on the residual enzyme activities [3, 6], leads to the accumulation and increased excretion in the urine of the branched-chain 2-keto and 2-hydroxy acids originating from leucine, isoleu-

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cine and valine. Urine from patients with MSUD normally also contains a considerable amount of 3-hydroxybutyrate, acetoacetate and lactate. Recent results have shown that ketoacidosis in general is accompanied by increased excretion of 3-hydroxyisovaleric acid [7], 3-hydroxyisobutyric acid, 2-methyl-3-hydroxybutyric acid [8] in addition to the dicarboxylic acids adipic and suberic acid [9]. Similarly, there is a correlation between the excretion of lactic and 2-hydroxybutyric acids [10]. It is thus evident that MSUD patients who also have ketoacidosis and lactic acidosis may excrete increased amounts of many different organic acids in their urine. The presence of these chemically similar carboxylic compounds makes the exact quantitative measurement of the MSUD branched-chain keto and hydroxy acids difficult. Gas chromatography (GC) on packed columns and combined gas chromatography—mass spectrometry (GC—MS) have recently been used for the study of these acids in MSUD patients [6,11,12].

However, even with the use of these methods, one may run into difficulties because the packed GC columns do not separate all of the acids that accumulate in the patients. Thus, on packed GC columns, several of the closely related keto and hydroxy acids have a tendency to be co-chromatographed. For instance, it is extremely difficult to separate the D-form of 2-keto-3-methylvaleric acid from 2-ketoisocaproic acid on any type of packed column.

The development of highly efficient glass capillary columns with 80,000—100,000 theoretical plates, combined with a mass spectrometer for absolute identification, opens up the possibility of separating and identifying all of the organic acids that occur in MSUD patients who are also suffering from ketoacidosis and lactic acidosis. In this paper, we describe the use of this technique in the re-investigation of the metabolic pattern in blood and urine from such patients before and after dietary treatment.

MATERIALS

The reference compounds DL-lactic, pyruvic, DL-3-hydroxybutyric, DL-2-hydroxyisovaleric, malonic, DL-2-hydroxy-*n*-valeric, methylmalonic, 2-ketoisovaleric, acetoacetic, L-2-hydroxy-3-methylvaleric, L-2-hydroxyisocaproic, DL -2-keto-3-methylvaleric and 2-ketoisocaproic acids (usually the sodium salts) were obtained from Sigma (St. Louis, Mo., U.S.A.). 2-Hydroxyisobutyric acid was obtained from Koch-Light (Colnbrook, Great Britain) and 2-ketobutyric acid from Fluka (Buchs, Switzerland). All acids were used without further purification. 2-Hydroxybutyric acid was synthesized from 2-ketobutyric acid by reduction with sodium borohydride (Fluka).

Sulphosalicylic acid, used for protein precipitation, was obtained from BDH (Poole, Great Britain). The extraction solvent (ethyl acetate) was obtained from Merck (Darmstadt, G.F.R.) and the reagents used for the preparation of derivatives [bis(trimethylsilyl)trifluoroacetamide (BSTFA), trimethylchlorosilane and hydroxylamine hydrochloride] were purchased from Pierce (Rockford, Ill., U.S.A.). The glass capillary column for gas chromatography (25 m × 0.25 mm) was obtained from LKB (Stockholm, Sweden) and was coated with SE-30. Packed GC columns were also used and contained 10% OV-17 on Gas-Chrom Q, or 8% BDS on Chromosorb W. The solid support and stationary phases were obtained from Applied Science Labs. (State College, Pa., U.S.A.).

METHODS

Preparation of derivatives for gas chromatography

All keto, hydroxy and other organic acids were determined by GC of their corresponding trimethylsilyl and/or trimethylsilyl oxime derivatives. A modified method based on the procedure described by Sternowsky et al. [13] was used. To a urine sample or deproteinized serum sample (2 ml) was added malonic acid (0.1 mg) as internal standard. The mixture was acidified using 6 *N* hydrochloric acid. Saturated sodium chloride solution (2 ml) was added before extraction with ethyl acetate (three 5-ml volumes), and the organic phases were combined and evaporated to dryness in a rotary evaporator. The residue was dissolved in pyridine (50 μl) containing hydroxylamine hydrochloride (20 mg/ml). The mixture was kept at room temperature for 30 min, before addition of BSTFA (50 μl, containing 10%, v/v, of trimethylchlorosilane). After a further 30 min at room temperature, an aliquot was injected into the gas chromatograph. Aqueous mixtures of reference compounds were treated in an identical manner for comparison.

Gas chromatography

A Varian Model 2100 gas chromatograph, equipped with a hydrogen flame-ionization detector, was used. The instrument was fitted with the glass capillary column or packed columns as described above. The gas chromatograph was operated under the following conditions: injector temperature, 250°; detector temperature, 250°; column temperature, programmed from 80° to 110° at a rate of 0.5°/min. A splitting ratio of 1:12 was used and the gas flow-rate through the capillary column was adjusted to 1.2 ml/min. The number of theoretical plates was approximately 85,000.

Gas chromatography—mass spectrometry

In the combined GC—MS, one instrument consisted of a Varian 1440 gas chromatograph, a molecular separator of the glass-frit type (kept at 230°) and a single-focusing mass spectrometer, Type CH7 (Varian-MAT), normally operated with an ionization energy of 70 eV. The gas chromatograph was alternatively equipped with three different packed columns (2 m × 1/4 in. O.D.) filled with 10% OV-17 on Chromosorb Q, 8% BDS on Chromosorb W or 20% SE-30 on Chromosorb Q. Helium was used as the carrier gas (30 ml/min). Parts of this investigation were also carried out using a Varian 112 mass spectrometer fitted with a glass capillary column (SE-30, 25 m × 0.25 mm; LKB) connected directly to the ion source. Both GC—MS instruments were connected on-line to a computer system (Spectro System 100 MS; Varian-MAT).

Amino acid analyses

The free amino acid levels in urine and serum (deproteinized with sulphosalicylic acid, 40 mg/ml) were determined on a JEOL JLC-6AH Automatic Analyzer, fitted with an electronic integrator (JEOL). Lithium buffers were used.

Identification and quantitative analyses of the organic acids

The identities of the organic acids in the biological specimens were deter-

mined by comparison of the GC retention times and MS data with those of authentic reference compounds. The amount of an acid excreted in the urine was always related to the creatinine content (determined in a Technicon Auto-Analyzer). Correction factors, including both the degree of extraction and the relative responses in the flame-ionization detector, were determined by treating known amounts of the different organic acids and the internal standard (malonic acid) in the same way as the patient samples.

Patients

Samples were obtained from three patients with MSUD. One patient had MSUD of the persistent type (patient 1), the other two had intermittent types. Of these, patient 3 had the least severe clinical form. This 6-month-old girl was diagnosed for the first time during the present work. All patients were treated with a regular MSUD diet, which was administered and adjusted according to the serum leucine, valine and isoleucine levels.

RESULTS

Fig. 1 (bottom) shows the capillary gas chromatogram of a standard mixture of most of the organic acids expected to occur in patients suffering from MSUD combined with ketoacidosis and lactic acidosis. The hatched peaks refer to solvents, excess of reagents and reagent by-products. All acids were eluted as single GC peaks, except acetoacetic, 2-ketoisovaleric and 2-keto-3-methylvaleric acids. With the last acid two peaks were obtained owing to the separation of the L- and D-isomers. It should be noted that the D-2-keto-3-methylvaleric acid was well separated from 2-ketoisocaproic acid using the capillary column. The separation of these two compounds cannot be achieved using packed columns.

Fig. 1 (top and middle) shows the gas chromatograms of the urinary organic acids of patient 3 before and after dietary treatment. It can be seen that most of the abnormal carboxylic acids (except 2-hydroxyisovaleric acid) had disappeared after 2 days on the special diet.

Table I shows the amounts of organic acids in the urine samples of patients 1, 2 and 3 and the serum of patient 3. The values were calculated from the capillary GC data. It can be seen that both D- and L-2-keto-3-methylvaleric acid were present in nearly equal amounts and 2-hydroxyisovaleric acid was the dominating MSUD hydroxy acid, in agreement with earlier reports [6, 11, 12]. Also, 2-hydroxy-3-methylvaleric acid was present to a significant extent, whereas 2-hydroxyisocaproic acid occurred in much smaller amounts (Fig. 1 and Table I). When the ratios between the branched-chain keto acids and the hydroxy acids were calculated (Table I, last column), it was found that these values are entirely different for each pair of corresponding acids (see also ref. 6). It is also noteworthy that many organic acids known to be associated with ketoacidosis and lactic acidosis (2-hydroxybutyric, 3-hydroxyisobutyric, 2-methyl-3-hydroxybutyric and 3-hydroxyisovaleric acid) were present. In addition, the patients excreted significant amounts of 2-hydroxyisobutyric acid, which disappeared on dietary treatment (Fig. 1 and Table I). The most persistent metabolite, which was still excreted after 2 days on an MSUD diet, was 2-hydroxyisovaleric acid.

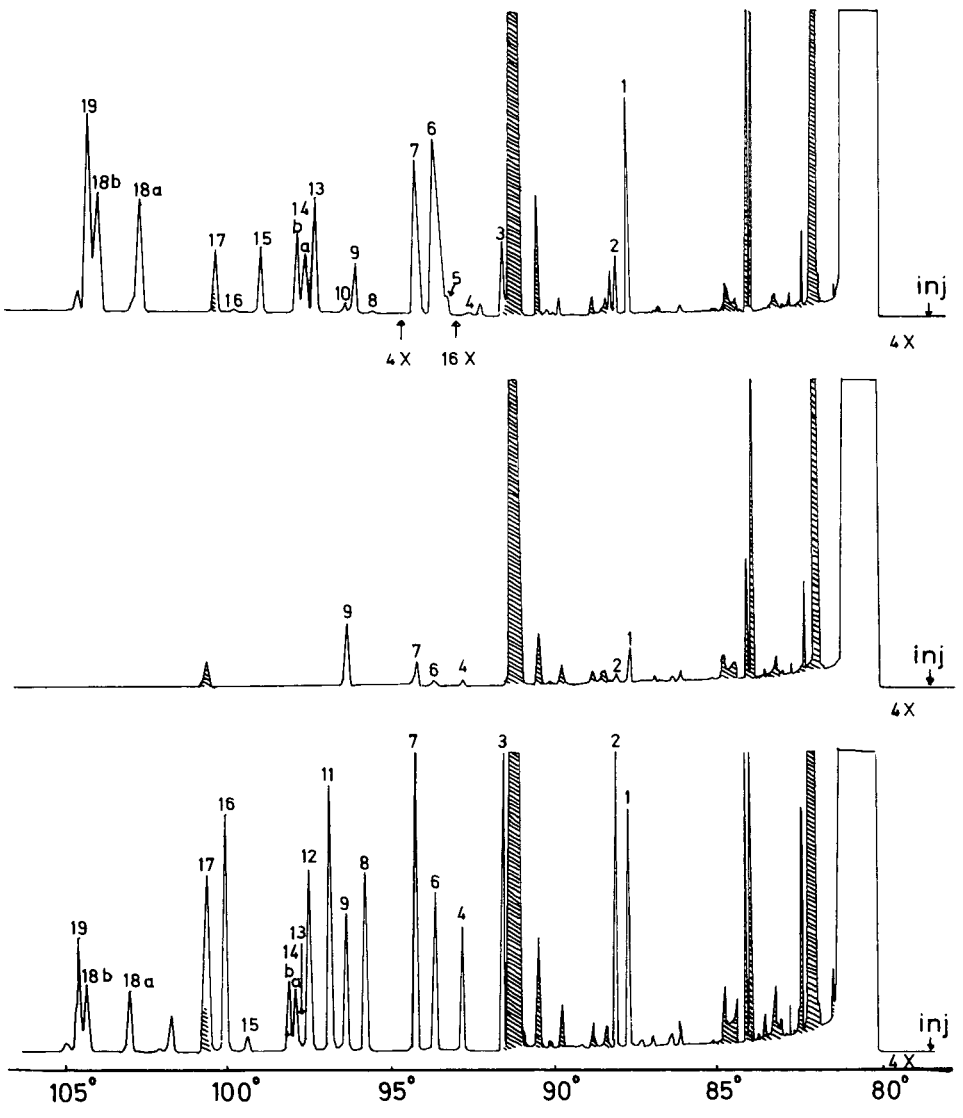

Fig. 1. Gas chromatographic separation of keto and hydroxy acids in the urine of a patient with maple syrup urine disease. Top chromatogram, patient 3 before dietary treatment; middle chromatogram, patient 3 after 2 days on diet; bottom chromatogram, a mixture of reference compounds. An SE-30 glass capillary column (25 m × 0.25 mm) was used. Peaks: 1= lactic acid; 2 = 2-hydroxyisobutyric acid; 3 = 2-hydroxybutyric acid; 4 = pyruvic acid; 5 = 3-hydroxyisobutyric acid; 6 = 3-hydroxybutyric acid; 7 = 2-hydroxyisovaleric acid; 8 = 2-ketobutyric acid; 9 = malonic acid (internal standard); 10 = 2-methyl-3-hydroxybutyric acid; 11 = 2-hydroxy-*n*-valeric acid; 12 = methylmalonic acid; 13 = 3-hydroxyisovaleric acid; 14a and 14b = 2-ketoisovaleric acid; 15 = acetoacetic acid; 16 = 2-hydroxyisocaproic acid; 17 = 2-hydroxy-3-methylvaleric acid; 18a = L-2-keto-3-methylvaleric acid; 18b = D-2-keto-3-methylvaleric acid; 19 = 2-ketoisocaproic acid.

TABLE I

ORGANIC ACIDS IN THE URINE AND SERUM OF PATIENTS WITH MSUD

Organic acid	Urine value (mg/g creatinine) before treatment		Patient 3, female, 6 months					Average ratio of keto acid to hydroxy acid in the urine of the 3 patients before treatment
			Urine value (mg/g creatinine)			Serum value (mg/l)		
	Patient 1, male, 20 days	Patient 2, female, 2 years	Before treatment	1st day after start of diet	2nd day after start of diet	Before treatment	2nd day after start of diet	
Valine metabolites								
2-Hydroxyisovalerate	3144	3810	875	131	40	149	88	
2-Ketoisovalerate	816	753	330	Not detectable	Not detectable	13	Not detectable	0.26
Isoleucine metabolites								
2-Hydroxy-3-methylvalerate	420	438	70	Not detectable	Not detectable	5	Not detectable	
L-2-Keto-3-methylvalerate	1373	1275	279	Trace	Trace	19	4	6.66
D-2-Keto-3-methylvalerate	1200	1600	287	Trace	Trace	20	3	
Leucine metabolites								
2-Hydroxyisocaproate	91	27	3	Not detectable	Not detectable	Trace	Not detectable	
2-Ketoisocaproate	5100	612	496	Trace	Trace	90	11	45.5
Lactate	489	756	173	69	53	374	157	
Pyruvate	69	80	13	41	33	6	17	
2-Hydroxybutyrate	58	1366	65	14	Not detectable	15	8	
2-Hydroxyisobutyrate	204	44	38	14	13	60	19	
Acetoacetate	41	1051	170	28	Not detectable	19	Not detectable	
3-Hydroxybutyrate	3464	24900	2059	262	13	90	65	
3-Hydroxyisovalerate	107	181	122	14	Not detectable	6	Not detectable	
3-Hydroxyisobutyrate	120	150	110	11	Not detectable	Trace	Not detectable	
2-Methyl-3-hydroxybutyrate	40	50	20	Trace	Not detectable	Trace	Not detectable	

Numerous serum amino acid analyses were performed on the patients during the observation period of several months. Before treatment, the valine level was about twice, leucine about ten times and isoleucine six times the normal level. The alanine level, on the other hand, was reduced, being only one quarter of the normal value. Dietary treatment normalized the amino acid pattern.

DISCUSSION

Although MSUD is a disorder that has been known and studied for many years, the cause of the clinical symptoms still remains unclear. Recent theories suggest that the accumulated branched-chain keto acids, particularly 2-ketoisocaproic acid, competitively inhibit pyruvate decarboxylase in the brain, thereby causing damage [14, 15]. Quantitative analyses of the keto acids are of interest in examining this hypothesis and it is also of interest to study the accumulation and excretion of other organic acids. Various forms of chromatography, e.g., paper, thin-layer and, more recently, gas chromatography on packed columns [6, 11, 12], have been used for this purpose. Because the resolving power of these methods was not sufficient to separate and quantitate all of the closely similar metabolites that were likely to occur in MSUD patients, we have adopted highly efficient glass capillary columns, combined with mass spectrometry for identification purposes. The results showed that as many as twelve different metabolites in addition to the branched-chain keto acids accumulated in MSUD patients during clinical episodes. Most of the former metabolites were related to the ketoacidosis and lactic acidosis accompanying the clinical condition. The role of these new metabolites in the disease and the clinical picture is not known. It is not unlikely, however, that some of them may be toxic to the brain cells, for instance through enzyme inhibition, and that these metabolites also may contribute to the clinical symptoms.

In this context, the observation that some MSUD patients are clinically ill for at least 1 day after they stop excreting the traditional keto acids should be mentioned. The explanation might be that some of the metabolites other than the branched-chain keto acids have a longer half-life in the brain cells and thus prolong the clinical condition.

Apart from demonstrating that MSUD patients excrete elevated amounts of at least 15 different organic acids, it is of interest that both the D- and L-forms of 2-keto-3-methylvaleric acid accumulated in nearly equal amounts. Although the presence of both isomers has been postulated and can be explained in terms of in vivo transformation via enolization [16], previous methods, including GC, have failed to detect the D-form. The reason for this failure may be that on packed columns the D-compound is co-chromatographed with 2-ketoisocaproic acid. The amount of the latter is thereby determined with an erroneously high value, and this keto acid may therefore not necessarily be the major MSUD metabolite excreted, as is usually assumed [4]. Thus, two of the three patients excreted more of L- + D-2-keto-3-methylvaleric acid and much more (2—5 times) 2-hydroxyisovaleric acid than 2-ketoisocaproic acid (Table I).

The large amount of 2-hydroxyisovaleric acid in MSUD patients and the large differences in the ratio of keto acid to hydroxy acid (Table I), which have previously been observed by other workers [6] and are confirmed in this study,

may warrant some discussion. Most likely the hydroxy acids are formed enzymatically from their corresponding keto acids by the action of one or more dehydrogenases. One enzyme to be considered is lactate dehydrogenase (LDH), which is known to be of broad specificity. However, it is unlikely that this particular enzyme plays a significant role in the above conversion as the branched-chain keto acids that occur in MSUD are very poor substrates for LDH (unpublished results). Other dehydrogenases must therefore be considered. The entirely different ratios of, e.g., the ketoisovaleric—hydroxyisovaleric acid pair and of the ketoisocaproic—hydroxyisocaproic acid pair may indicate that not only one, but several, dehydrogenases are involved. Alterations in the NADH:NAD ratio cannot explain these findings as the redox situation within the same cells of a patient must necessarily be identical whether one is concerned with the reduction of 2-ketoisocaproic acid or of 2-ketoisovaleric acid. Further enzyme investigations are required, however, in order to give an adequate solution to the large variations in the proportions of keto to hydroxy acid, and factors such as isoenzymes, K_m values and product inhibition must also be considered.

The presence of a hitherto unrecognized metabolite in connection with MSUD, 2-hydroxyisobutyric acid, was demonstrated in all three patients. The metabolic origin and fate of this compound are not known, although one might speculate that it stems from α-oxidation of branched-chain fatty acids.

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QUANTITATIVE GAS CHROMATOGRAPHY AND SINGLE-ION DETECTION OF ALIPHATIC α-KETO ACIDS FROM URINE AS THEIR O-TRIMETHYLSILYLQUINOXALINOL DERIVATIVES

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SUMMARY

A method is described for the quantitative determination of aliphatic α-keto acids in urine after derivatization with *o*-phenylenediamine and bis(trimethylsilyl)trifluoroacetamide. α-Ketovaleric acid and α-ketocaprylic acid are used as internal standards.

The chemical yield is 80—100%. At physiological concentrations, the coefficient of variation after repeated derivatizations is 4% for pyruvic acid and 14% for α-ketoglutaric acid.

With mass spectrometric single-ion detection at m/e = 217, 232 and 245, the biologically interesting aliphatic α-keto acids can be determined at very low levels in biological fluids.

INTRODUCTION

In a previous paper [1], we reported on the gas chromatography (GC) of eleven O-trimethylsilylquinoxalinols (TMS-quinoxalinols) which were synthesized from α-keto acids, *o*-phenylenediamine and bis(trimethylsilyl)trifluoroacetamide (BSTFA). On silicone phases, these derivatives have favourable chromatographic properties [1,2]. In addition, as a useful prerequisite to single-ion detection, their mass spectra have many abundant fragments in common with each other [3].

Owing to its inherent technical difficulties, the quantitative GC of α-keto acids in maple syrup urine disease, for example, has only rarely been reported [4—10]. It therefore seemed worthwile to develop a quantitative quinoxalinol procedure that could be reliably applied to the analysis of small urine samples

*Part of the methods described here were developed during medical thesis work of K.M. and H.-U.M.

of patients with hereditary ketoacidurias [11,12]. In this paper we report on our efforts to achieve that aim.

MATERIALS AND METHODS

The GC studies were carried out with a Hewlett-Packard Model 7611A gas chromatograph, equipped with a flame-ionization detector (FID) and a Model 7128A dual-pen recorder (1 and 10 mV full scale) from the same manufacturer. U-shaped glass columns, 180 cm × 3 mm I.D., were used. High-purity nitrogen at a flow-rate of 60 ml/min was used as the carrier gas.

A Finnigan Model 3000 quadrupole mass spectrometer was coupled to a Varian Model 1400 gas chromatograph by a heated glass jet Boehlke-type separator and a thermoconstant metal transfer line. The gas chromatograph was equipped with a spiral glass column, 240 cm × 1.5 mm I.D. High-purity helium was used as the carrier gas at 20 ml/min. The mass spectrometer was run at 70 eV with ion energies between 4 and 8 V and a multiplier voltage of 1.5—2.0 kV. A Servogor Model RE 511 recorder (Metrawatt) with multiple spans was used.

A Sartorius Model 4401 microbalance was used for weighing the keto acid standards.

The carrier gases were obtained from Messer Griesheim (Düsseldorf, G.F.R.). Dexsil 300 GC, 3% on 100—120 mesh Supelcoport, was obtained from Supelco (Bellefonte, Pa., U.S.A.), OV-1, 3% on 100—120 mesh Gas-Chrom Q from Applied Science Labs. (State College, Pa., U.S.A.) through Serva (Heidelberg, G.F.R.) and BSTFA, pyridine (silylation grade) and reaction vials with PTFE-lined screw-caps from Pierce Eurochemie (Rotterdam, The Netherlands). Sodium pyruvate was obtained from Boehringer Mannheim (Tutzing, G.F.R.), α-ketoglutaric acid from E. Merck (Darmstadt, G.F.R.) and the remaining keto acids from Sigma (St. Louis, Mo., U.S.A.). All other chemicals and solvents were obtained from Merck.

For the quantitative derivatization of α-keto acids to the corresponding quinoxalinols, we modified the method of Mowbray and Ottaway [13]. The procedure is as follows: 10 ml of urine are mixed with 500 μl each of acetic acid and toluene. The aliphatic urinary keto acids are then stable for about 5 days without refrigeration. To 2 ml of the acidified urine, 50 μl of internal standard solution [20 m*M* α-ketovaleric acid (KVA) and α-ketocaprylic acid (KCA)] in water plus acetic acid are added and well mixed. Then 0.5 ml of urine plus internal standard are heated for 1 h at 70° with 0.5 ml of 4 *N* hydrochloric acid and 1 ml of a 1% solution of *o*-phenylenediamine in 2 *N* hydrochloric acid. The incubation is carried out in vials with PTFE-lined screw-caps using a Reacti-Therm Heating Module (Pierce Eurochemie).

The samples are then saturated with solid ammonium sulphate and extracted twice with 5 ml of chloroform in vials as described by Mamer et al. [14]. Vigorous shaking for 1 min each time is performed with a VirTis Model Whirlmix shaker. The extract is dried for 2 h over anhydrous sodium sulphate and then filtered. After evaporation to dryness in a Heidolph-Elektro Model VV1 rotary evaporator at room temperature, the residue is taken up in 50 μl of pyridine and incubated for 30 min at 70° after addition of 50 μl of BSTFA. GC

is performed with a two-step linear temperature programme from 70° to 160° at 2°/min, then from 160° to 200° at 4°/min to purge the column. The injection port temperature is 200° and the FID 250°. About 1 μl of sample is injected, which corresponds to 5 μl of urine.

The molar response factors [15] are determined in normal urine samples in the following way. One sample is derivatized without any additional keto acids, and to a second sample of 2 ml only the internal standard solution is added. Five more samples of 2 ml each receive from 0.3 to 3 μmole of the natural α-keto acids in addition to a constant amount of about 1 μmole of internal standard.

Quantitation is made by peak height measurements. From these measurements, a straight line is obtained when the ratios of the peak heights of natural and internal standard keto acids are plotted against the amounts of natural keto acids added. The linear regression coefficient, B, was calculated on a Wang Laboratories Model 450 programmable desk-top calculator. The molar response factor (RF) is then obtained as

$$RF_1 = \frac{\text{Internal standard added } (\mu\text{mole})}{B}$$

The value of the correlation coefficient, r, reflects the quality of the analytical technique.

The molar chemical yield, A, of quinoxalinoles in the derivatization of keto acids can be calculated from the molar response factor, RF_2, of pure added quinoxalinoles (recrystallized from ethanol—water) versus the internal standard keto acids:

$$A(\%) = [RF_1/RF_2]100$$

With the molar response factor RF_1, differential chemical and extraction yields are fully taken into account.

RESULTS

Flame-ionization detection

The chromatograms of the derivatives of pyruvic acid, ketoglutaric acid and the internal standards KVA and KCA are shown in Fig. 1. On Dexsil 300, palmitic acid and carboxyethylquinoxalinol (from α-ketoglutaric acid) are well separated, whereas they are eluted as a single peak on OV-1. Palmitic acid may be excreted in considerable amounts. Therefore, chromatographic separation should be achieved in quantitative work with an FID.

Branched chain α-keto acids are determined on OV-1 because n-propylquinoxalinol (from KVA) and isobutyl-1-quinoxalinol (from α-keto-β-methylvaleric acid) are well separated on this phase (Fig. 2) but not on Dexsil 300 [1]. Hexylquinoxalinol (from KCA) overlaps with an unknown organic acid on OV-1 (methylene unit = 19, 23). However, the effect on quantitative determinations is negligible in most instances.

In Table I the molar response factors (RF_1) and the chemical yields for six aliphatic α-keto acids are given. In our method of determination of RF_1 we obtained correlation coefficients between 0.965 and 0.999 (mean 0.992). The

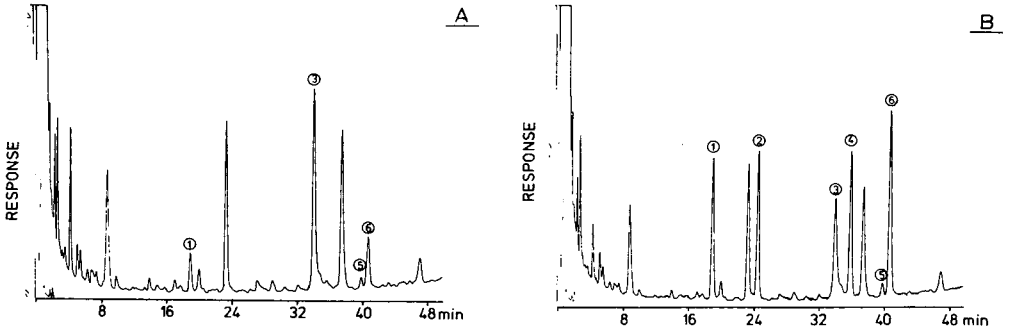

Fig. 1. Urinary extract of a normal male control. (A) No keto acids added; (B) Keto acids are added to urine: pyruvate 0.90; ketovaleric acid 1.04; ketocaprylic acid 1.03; ketoglutaric acid 1.33 μmole/ml. Methods of derivatization and extraction as described in the text. Conditions of chromatography: 3% Desxil 300 GC; 50—160° at 2°/min; range 10^2; attenuation 2. The numbered peaks are the following trimethylsilylated compounds: 1, methylquinoxalinol (from pyruvic acid); 2, propylquinoxalinol (from α-ketovaleric acid); 3, hippuric acid; 4, hexylquinoxalinol (from α-ketocaprylic acid); 5, palmitic acid; 6, carboxyethylquinoxalinol (from α-ketoglutaric acid).

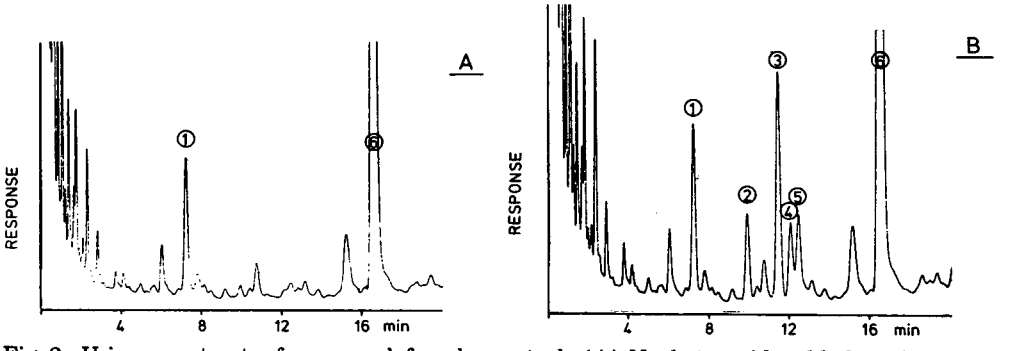

Fig. 2. Urinary extract of a normal female control. (A) No keto acids added; endogenous pyruvic acid (1) at a concentration of 356 μM; (B) keto acids added to give the following concentrations: 2, α-ketoisovaleric acid, 172 μM; 3, α-ketovaleric acid, 507 μM; 4, α-keto-β-methyl-*n*-valeric acid, 152 μM; 5, α-ketoisocaproic acid, 152 μM; 6, TMS-hippuric acid. Methods of derivatization and extraction as described in the text. Conditions of chromatography: 3% OV-1; 70—160° at 2°/min; range 10^2; attenuation 4.

recovery from extraction is about 50% for hexylquinoxalinol and about 30% for carboxyethylquinoxalinol. On repeated (n=6) derivatization and extraction of endogenous pyruvic and α-ketoglutaric acid from seven urines, the mean coefficients of variation were 4 and 14% for pyruvic and α-ketoglutaric acid, respectively.

In 11 urines from normal adults we determined the concentrations of pyruvic and α-ketoglutaric acids as 2.6 ± 0.8 mg-% and 4.8 ± 1.4 mg-%, respectively. The quantitative determination with either of the two internal standards agreed satisfactorily. On Dexsil 300, correlation coefficients of 0.995 and 0.997 were found for pyruvic and α-ketoglutaric acid, respectively (n = 21).

The GC samples could be stored for at least 2 weeks at room temperature without detectable (less than 5%) loss of the quinoxalinols with a carboxyalkyl side-chain. The other quinoxalinols are even more stable.

TABLE I

MOLAR RESPONSE FACTORS (RF_1) OF ALIPHATIC α-KETO ACIDS IN FLAME-IONIZATION DETECTION WITH α-KETOVALERIC (KVA) AND α-KETOCAPRYLIC (KCA) ACIDS AS INTERNAL STANDARDS

For each keto acid, five experiments were performed.

Keto acid	RF_1 with KVA	RF_1 with KCA	Molar chemical yield (%)	GC column
Pyruvic	0.932 ± 0.058	0.894 ± 0.075	93 ± 11	Dexsil 300
α-Ketoisovaleric	0.845 ± 0.060	0.890 ± 0.077	81 ± 10	OV-1
α-Keto-β-methyl-*n*-valeric	0.850 ± 0.092	0.901 ± 0.166	N.D.*	OV-1
α-Ketoisocaproic	1.01 ± 0.077	1.09 ± 0.142	94 ± 12	OV-1
α-Ketoglutaric	0.781 ± 0.056	0.755 ± 0.065	115 ± 6	Dexsil 300
α-Ketoadipic	0.784 ± 0.139	0.784 ± 0.164	93 ± 4	Dexsil 300

*N.D. = not determined.

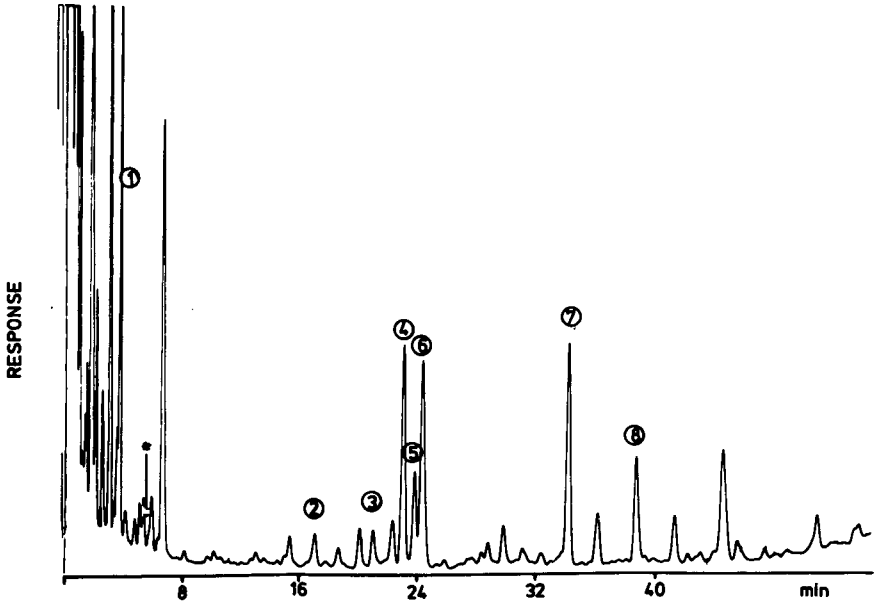

Fig. 3. Urinary extract of a 3-week-old Turkish girl (Tav.) with maple syrup urine disease. The urine was kept frozen from April 10th, 1974, to February, 1976. Methods of derivatization and extraction as described in the text. Conditions of chromatography: 3% OV-1; 50—60° at 1°/min, 60—160° at 2°/min then 160—200° at 4°/min; range 10^2; attenuation 2. Peaks: 1, α-hydroxyisovaleric acid (by methylene units); 2, methylquinoxalinol (from pyruvic acid); 3, isopropylquinoxalinol (from α-ketoisovaleric acid); 4, *n*-propylquinoxalinol (from α-keto-*n*-valeric acid, internal standard); 5, isobutyl-1-quinoxalinol (from α-keto-β-methyl-*n*-valeric acid); 6, isobutyl-2-quinoxalinol (from α-ketoisocaproic acid); 7, hexylquinoxalinol (from α-ketocaprylic acid, internal standard); 8, palmitic acid plus carboxyethylquinoxalinol (from α-ketoglutaric acid). * = Position of α-hydroxy-β-methyl-*n*-valeric acid and α-hydroxyisocaproic acid. The following concentrations (μM) were measured (The 24-h output in milligrams is given in parentheses): pyruvic acid, 69.0 (0.79); α-ketoglutaric acid, 187.3 (3.56) (both on Dexsil 300); α-ketoisovaleric acid, 103.1 (1.56); α-keto-β-methyl-*n*-valeric acid, 248.2 (4.20); α-ketoisocaproic acid, 476.0 (8.05).

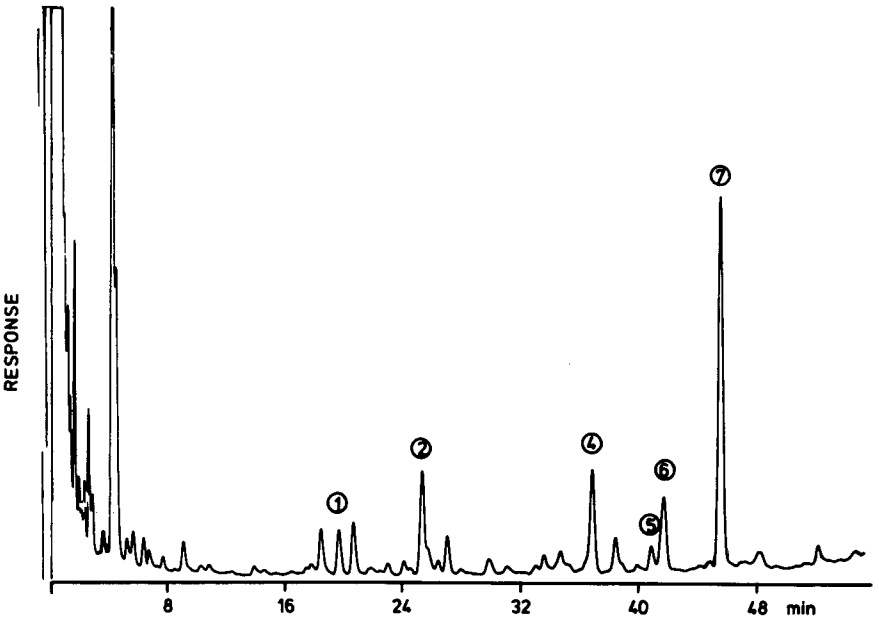

Fig. 4. Urinary extract of a patient with α-ketoadipic aciduria (S.G., described in ref. 17). The urine was kept frozen from September 11th, 1974, to February, 1976. See legend to Fig. 1 for explanation; range 10^2; attenuation 4. 7, Carboxypropylquinoxalinol (from α-ketoadipic acid). The following concentrations (μM) were measured (The 24-h output in milligrams is given in parentheses): pyruvic acid, 273.9 (3.13); α-ketoglutaric acid, 438.8 (8.33); α-ketoadipic acid, 2423 (50.39). The high concentration of α-ketoglutaric acid reported in ref. 17 could not be confirmed. The values for α-ketoadipic acid agree fairly well.

In Figs. 3 and 4, it is shown that our method can be applied, for example, to the study of maple syrup urine disease [16] and α-ketoadipic aciduria [17]. The GC of urine from a patient (T.) with pyruvic acid dehydrogenase deficiency was dominated by a large peak (6.0 mM) of O-TMS-methylquinoxalinol.

In a survey of ketoaciduria among patients with severe mental defects [18], we found a 14-year-old incontinent girl (A.Mü. 190262) with 106.7 μmole of pyruvic acid per mmole of creatinine and 262.5 μmole of ketoglutaric acid per mmole of creatinine, which are about 5 and 10 times the normal levels, respectively. The urine culture yielded various or no bacteria [19, 20].

We checked the method further on urine samples from two normal persons who were given orally 200 mg of L-leucine plus 400 mg of glucose per kilogram of body weight. No excretion of α-ketoisocaproic acid above 12 nmole/min could be detected within 10 h. The excretion rate of pyruvic and ketoglutaric acid (about 100 and 150 nmole/min, respectively) was never depressed [8].

Mass spectrometric single-ion detection

With an FID, urinary keto acids can conveniently be quantitated down to a concentration of about 100—150 μM. At lower concentrations, other organic acids interfere in the analysis, as shown in Fig. 5A. *n*-Propyl- and isobutyl-2-quinoxalinols were added to urine to give a final concentration of 10 μM. Quantitation is no longer possible with an FID. Single-ion detection [21, 22] in prin-

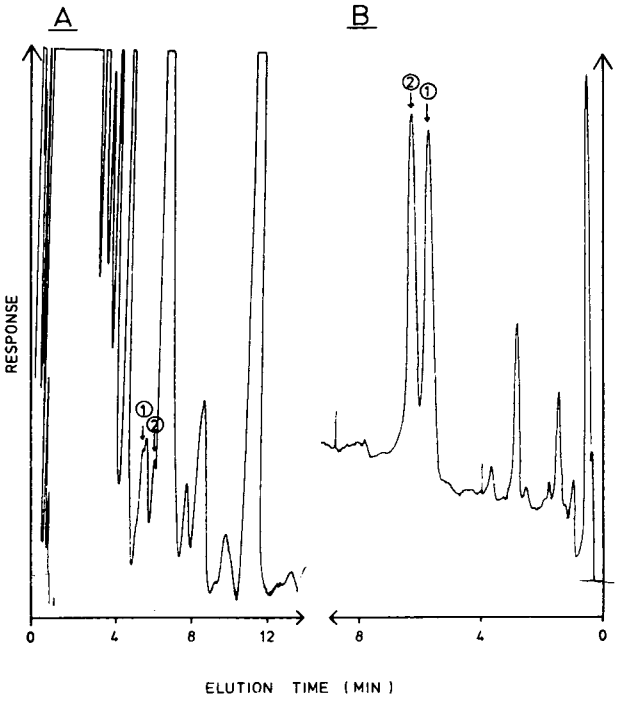

Fig. 5. Comparison of FID and single-ion detection of quinoxalinol standards in urine. To 5 ml urine 10 μl of a 5 m*M* standard solution of *n*-propyl- and isobutyl-2-quinoxalinol were added. After acidification, the urine was extracted three times with 4 ml of ethyl acetate and diethyl ether. Final sample volume, 250 μl. (A) Chromatography of 1 μl on a 180 cm × 3 mm I.D. Dexsil 300 GC column; 120° isothermal; FID; range 10^2; attenuation 4. (B) Chromatography of 1 μl on a 240 cm × 2 mm I.D. Dexsil 300 GC column; 180° isothermal; single-ion detection at *m/e* = 232; electron energy 70 eV; ion energy 6.0 V; recorder span 1 V. Peaks: 1, O-TMS-*n*-propylquinoxalinol; 2, O-TMS-isobutyl-2-quinoxalinol; 1 μl of final sample contained about 200 pmole of each compound from 20 μl of urine, assuming complete extraction.

ciple solves this problem, as shown in Fig. 5B. The mass spectrometer was focused at an *m/e* ratio of 232. Both quinoxalinols have their base peak at this *m/e* ratio owing to a McLafferty-type rearrangement [3]. The other urinary constituents were not detected because they have no major fragments in their mass spectra at *m/e* = 232. Thus, the quinoxalinols were easily determined at these very low concentrations.

In Fig. 6 it is shown that a linear and almost proportional response is obtained for *n*-propyl- and isobutyl-2-quinoxalinol when amounts from 30 to 800 pmole are injected. The molar response factor in 11 determinations carried out on the same day was 1.28 ± 0.02 (coefficient of variation: 1.5%).

In Table II the molar response factors of standard quinoxalinols in single-ion detection are given for three *m/e* ratios. (Interpretation of the mass spectra is the subject of a forthcoming paper [3].) In order to obtain the response factors in single-ion detection when keto acids are analyzed, the corresponding values in Tables I and II have to be multiplied: pyruvic acid, for example, measured at

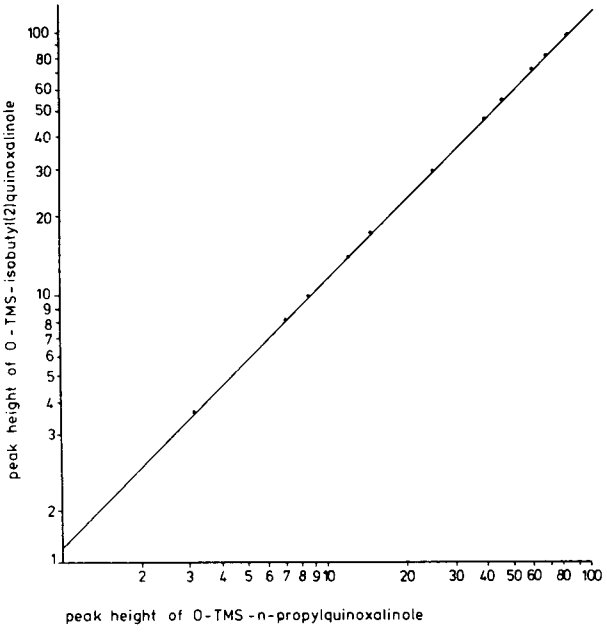

Fig. 6. Single-ion detection of O-TMS-*n*-propyl- and isobutyl-2-quinoxalinol standards at *m/e* = 232. From 30 to 800 pmole of each compound were injected on to 3% Dexsil 300 at 180°. Electron energy 70 eV; ion energy 6.0 V; recorder span 0.5—5 V.

TABLE II

MOLAR RESPONSE FACTORS (RF) OF O-TMS-QUINOXALINOL STANDARD COMPOUNDS IN SINGLE-ION DETECTION WITH O-TMS-HEXYLQUINOXALINOL AS INTERNAL STANDARD

Similar data are to be obtained with propylquinoxalinol as internal standard (cf., Table III). The conditions of operation were: 240 cm × 2 mm I.D. glass column; 100—230° at 4°/min; electron energy 70 eV; ion energy 4.0 V; recorder span 1 V; 0.5—1 nmole of each compound per injection yielded peak heights of up to 25 cm.

Substituent in position 3 in quinoxalinol	Parent α-keto acid	RF		
		m/e = 217	*m/e* = 232	*m/e* = 245
Methyl	Pyruvic	2.37	0.17	0
Ethyl	Ketobutyric	0.40	0.14	0
Isopropyl	Ketoisovaleric	0.25	0.06	3.30
Isobutyl-1	Keto-β-methylvaleric	0.33	0.43	0.42
Isobutyl-2	Ketoisocaproic	1.05	0.95	0
2-(Methylthio)ethyl	Keto-γ-(methylthio)butyric	0.11	0.04	3.90
Carboxyethyl	Ketoglutaric	0.20	0	8.66

m/e = 217 with α-ketocaprylic acid as the internal standard would give a molar response factor of 0.894 × 2.37 = 2.12. The response factors in Table II are directly dependent on the percentage portion of the total ion current that is present in these fragments ($\%\Sigma_{35}$). These values are given in Table III.

TABLE III

PERCENTAGE PORTION OF THE TOTAL ION CURRENT FROM m/e = 35 TO M^++2 ($\%\Sigma_{35}$) FOUND IN THE THREE MAJOR FRAGMENTS OF O-TMS-QUINOXALINOLS AT 70 eV

Substituent in position 3	m/e		
	217	232	245
Methyl	32.6	4.1	0
Ethyl	5.1	3.1	0
Isopropyl	2.6	0.9	11.3
Propyl	9.8	19.5	7.8
Isobutyl-1	2.4	5.2	0.8
Isobutyl-2	11.9	18.3	<0.1
Hexyl	7.2	12.6	2.2
2-(Methylthio)ethyl	0.8	0.4	11.3
Carboxyethyl	0.7	0	11.2
Carboxypropyl	4.3	4.6	18.6

The data in Tables II and III were collected at different times, and a close examination reveals that they are not entirely compatible and can serve only as a guide. Because of day-to-day variations in performance of the mass spectrometer for single-ion detection, standards must be run every day. Re-focusing of the fragments had to be carried out every 3—4 h. The good results of determination of isobutyl-2-quinoxalinol with *n*-propylquinoxalinol as the internal standard (Figs. 5B and 6) could not be duplicated with hexylquinoxalinol. This internal standard was eluted 6 min after isobutyl-2-quinoxalinol, leaving the system enough time to change. Fluctuations in the beam current seem to be the major source of this analytical error.

The sensitivity of the single-ion detection method could not be increased further by lowering the electron energy. The increase in the percentage portion of the fragments at m/e 217, 232 and 245 is more than compensated for by the decrease in the total ion current.

DISCUSSION

Disorders that are characterized by the accumulation of α-keto acids in the body fluids are phenylketonuria [23], maple syrup urine disease [4—10, 16], pyruvic acid dehydrogenase deficiency [24—26], α-ketoadipic aciduria [17, 27], ketotic hypoglycaemia with dwarfism and congenital cataract [28], methionine malabsorption syndrome [29], hereditary tyrosinemia [30] and cystinosis [31]. The association of most of these disorders with severe mental deficiency deserves much interest and justifies efforts to improve techniques for the analysis of α-keto acids.

The GC of α-keto acids has been reported for the free acids [32] as well as for the methyl esters of the free acids [6, 33], methoximes [34, 48] and 2,4-dinitrophenylhydrazones [35]. Furthermore, trimethylsilyl esters of the free acids [35], the oximes [8, 9, 36, 37], methoximes [38—40], ethoximes [40]

and benzoximes [39, 40] have been used in GC. This large number of analytical methods indicates the inherent technical difficulties.

The O-TMS-quinoxalinol method described here and elsewhere [1, 2, 41—43] offers some useful advantages which make it worthy of consideration: (1) the derivatives have a low volatility; (2) multiple derivatives do not occur; (3) derivatives of isomeric α-keto acids are completely separated; (4) complete separation from the corresponding α-hydroxy acids is accomplished; (5) the chemical yield is close to 100%; (6) the derivatives are stable for several weeks; (7) chromatographic losses are negligible even at $3 \cdot 10^{-11}$ mole per injection; (8) with only three fragments at relatively high mass numbers (m/e = 217, 232 and 245), all α-keto acids can be measured by combined GC—MS.

The method described here also offers advantages over the original quantitative O-TMS-quinoxalinol method [41—43]: (1) we take 2 ml of urine instead of 50 ml for addition of the internal standard, and only 0.5 ml or even 0.1 ml is used for derivatization; (2) use of α-keto acids instead of decyl cyanides as internal standards gives a greater specificity to the assay of natural α-keto acids; (3) extraction with chloroform instead of ethyl acetate leaves most of the by-products in the aqueous phase. The extraction recovery of quinoxalinols with chloroform is not quantitative, but this does not have much influence on the quantitative determination of keto acids because the internal standards belong to the same chemical class. The response factors indicate that all steps in the procedure, including extraction, are reproducible fairly well.

Only two α-keto acids cannot be measured with the quinoxalinol method: (1) oxaloacetic acid decarboxylates to yield pyruvic acid [1, 42, 44]; also the quinoxalinol is unstable [13, 44]; (2) *o*-hydroxyphenylpyruvic acid at low pH forms a δ-lactone which does not react with *o*-phenylenediamine [45].

Our quantitative results with single-ion detection were not as good as expected. More modern instrumentation (ours dates from 1969) with stable electronic parts [46] might not pose such problems. We have no experience yet with the use of a selective nitrogen-sensitive FID as described by Hoffman and co-workers [41—43]. High selectivity will also eventually be obtained with electron-capture detection of fluoroquinoxalinoles: it has been reported [13] that 1,2-diamino-4-fluorobenzene reacts with α-keto acids to the same extent as *o*-phenylenediamine. 1,2-Diamino-3,4,5,6-tetrafluorobenzene can also be used [47].

In urine extracts from four untreated adult patients with phenylketonuria [18] we consistently found a large peak of benzylquinoxalinol (from β-phenylpyruvic acid) together with a similarly large peak of phenyllactic acid. Our quantitative GC—FID results on aromatic keto acids will be published later. Our further work is aimed at extending the quinoxalinol method to the GC study of α-keto acids in blood [44] and tissues.

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Biomedical Applications

CHROMBIO.002

TRYPTOPHAN AND KYNURENINE DETERMINATION IN UNTREATED URINE BY REVERSED-PHASE HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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SUMMARY

A fast and sensitive method for the analysis of tryptophan and some of its metabolites is discussed. A reversed-phase chromatographic system with water mobile phase can separate tryptophan, N-formalkynurenine, kynurenine and 3-hydroxykynurenine in less than 15 min at a flow-rate of 1 ml/min. The application of the method to the analysis of tryptophan and kynurenine in untreated urine of a patient loaded with tryptophan is described. The ease and speed of analysis makes the method very attractive for clinical purposes. Among other things, it was found that tryptophan in untreated urine degrades with time, even if the sample is frozen at -11°.

INTRODUCTION

Important clinical information can be obtained from the analysis of various amino acids and their related metabolites in serum and urine. With the advent of high-performance liquid chromatographic (HPLC) systems, the opportunity exists for quick and reliable assays with minimum work up and minimum analysis times. An example of the application of HPLC to a biologically important system is the analysis of uric acid as described by Pachla and Kissenger [1].

The measurement of urinary tryptophan and its metabolites has considerable clinical importance. Abnormally large amounts of kynurenine and 3-hy-

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droxykynurenine are excreted in most patients with hemoblastic diseases such as myeloid or lymphoid leukemia, Hodgkin's disease, and multiple myeloma [2], as well as in many patients with bladder cancer [3]. Elevated tryptophan with low kynurenine following tryptophan loading has been reported in cases of congenital tryptophanuria with dwarfism [4] and Hartnup's disease [5]. Increased excretion of kynurenine, 3-hydroxykynurenine, kynurenic acid, and xanthurenic acid has even been reported in patients with infantile spasm due to pyridoxine deficiency, pyridoxine dependency, or abnormalities of kynureninase [6].

It is thus desirable to develop a fast and simple method of analyzing tryptophan and some of its metabolites in urine. Rable [7] has demonstrated the separation of a synthetic mixture of some tryptophan metabolites on a Pellidon column. Some metabolites have been determined by ion-exchange systems [8—11], but most analyses have been accomplished by thin-layer chromatography [11—15]. Several spectroscopic methods were also utilized in the analysis of tryptophan metabolites [10, 16, 17]. These papers represent only a fraction of the published work which can be found in the literature.

Considerable interest has recently been focused on the tryptophan—kynurenine—nicotinic acid pathway. The importance and scope of this metabolic pathway has been aptly discussed in a recent review by Allegri and De Antoni [18]. The present work describes a quick and highly sensitivity assay for urinary tryptophan, kynurenine, N′-formylkynurenine, and 3-hydroxykynurenine. The utilization of this method in the quantitative determination of the first two compounds in normal and loaded urine will be demonstrated.

EXPERIMENTAL

Liquid chromatograph

The liquid chromatograph consisted of a Waters Assoc. 6000A pumping system, and a Waters Assoc. 440 dual wavelength absorbance detector, which was capable of monitoring 254 nm and 280 nm simultaneously. The detector was set at 0.5 a.u.f.s. Injections were made via a Waters Assoc. Model U6K injector. Data were collected on a Laboratory Data Control dual-pen chart recorder. The columns were a 25 cm × 4.6 mm I.D. Partisil 10 column, and a 25 cm × 4.6 mm I.D. Partisil ODS column (Whatman, Clifton, N.J., U.S.A.). The solid support size was 10 μm. All chromatograms were obtained at ambient temperature.

Reagents and chemicals

The mobile phase was distilled and deionized water. Solutes were obtained from various sources. Urine specimens were collected from a normal adult male before, and at two-hour intervals after receiving a tryptophan load of 100 mg/kg body weight (5.66 g in the present study). Samples were frozen at $-11°$ until use. Standard mixtures were made by dissolving either 0.5 or 1 mg/ml of mobile phase. Five or ten microliters of untreated urine were injected onto the various columns.

Data treatment

The amounts of tryptophan and kynurenine in the urine were determined from peak heights which were compared with those of known standards. The determined weights are believed to be accurate to ±5% since the columns were not thermostatted.

RESULTS AND DISCUSSION

Several systems for the separation of tryptophan and its metabolites were tested before a suitable one for real urine samples was selected. For example, a Partisil 10 column, conditioned to heptane using the series of solvents recommended by Scott and Kucera [19], and a mobile phase consisting of 1% acetic acid—1% sodium chloride—water gave good separations of synthetic standard mixtures. Unfortunately, this and other attempted normal phase systems proved to be unsuitable for the analysis of real samples owing to interfering substances from urine.

Owing to the polar nature of the solutes, it became apparent that a reversed-phase column would be more advantageous. The initial studies with an ODS (C_{18}

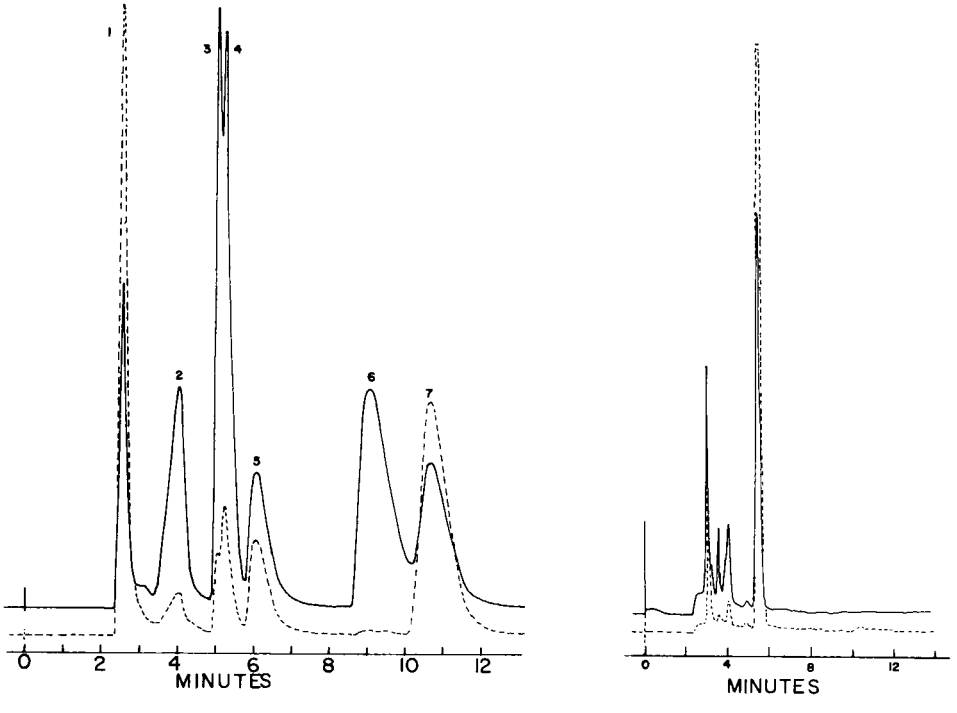

Fig. 1. Separation of tryptophan metabolites and several other compounds of interest. Column: Partisil ODS 25 cm × 4.6 mm I.D. Mobile phase, water ; flow-rate, 1 ml/min; temperature, 26° ; sensitivity, 0.5 a.u.f.s. ———, 254 nm; — — —, 280 nm. 1 = Orotic acid (3 μg); 2 = N-formylkynurenine (3 μg); 3 = uracil (1 μg); 4 = uridine (3 μg); 5 = 3-hydroxykynurenine (3 μg); 6 = kynurenine (5 μg); 7 = tryptophan (5 μg).

Fig. 2. Control urine profile, 5-μl sample. Conditions, see Fig. 1. ———, 254 nm; — — —, 280 nm.

bonded to the silica gel support) column, indicated that such a column could give better and more reproducible separations. Fig. 1 shows the separation of a synthetic mixture of four tryptophan metabolites and orotic acid, uracil and uridine on an ODS column with distilled deionized water as mobile phase. (The orotic acid, uracil and uridine were present as a part of another study not related to tryptophan metabolism.) The total analysis time is about 12 min.

Fig. 2 shows a chromatogram of control urine injected directly without any prior work up. At 0.5 a.u.f.s. no tryptophan or kynurenine could be seen. Fig. 3 shows a profile of a urine sample collected 2 h after the patient received a tryptophan load. Peaks for kynurenine and tryptophan are clearly evident. Fig. 4 shows the chromatogram of a urine specimen taken 4 h after the load. The kynurenine and tryptophan levels have increased.

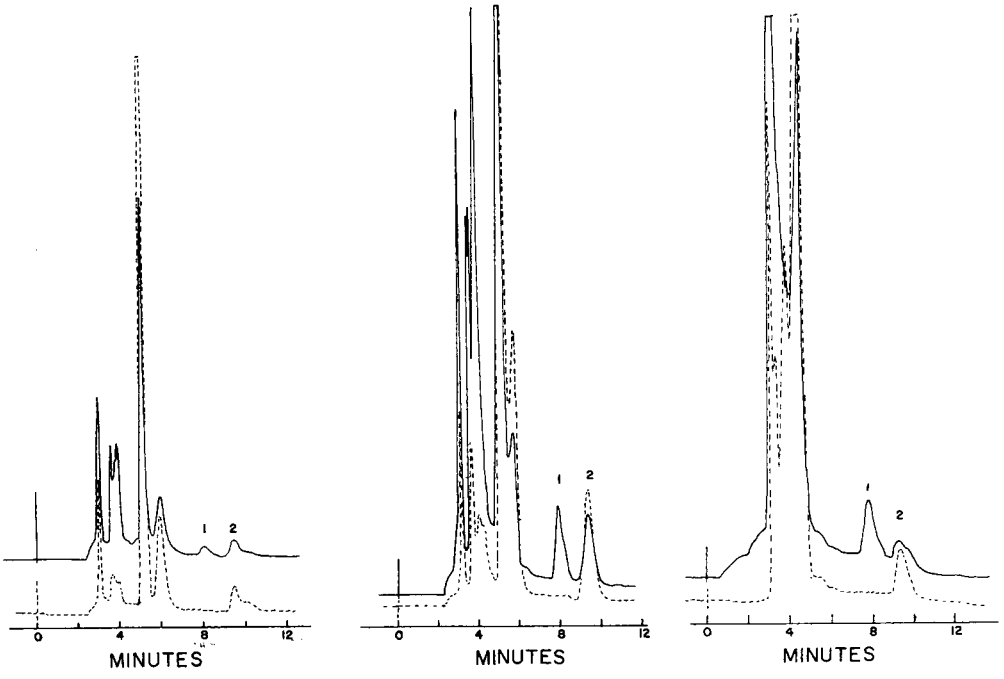

Fig. 3. Urine profile of a 2-h sample, 5-μl injection. Conditions, see Fig. 1. ———, 254 nm; — — —, 280 nm. 1 = Kynurenine; 2 = tryptophan.

Fig. 4. Urine profile of a 4-h sample, 10-μl injection. Conditions, see Fig. 1. ———, 254 nm; — — —, 280 nm. 1 = Kynurenine; 2 = tryptophan.

Fig. 5. Urine profile of a 4-h sample, 1 week old (frozen at —11°). Conditions, see Fig. 1. ———, 254 nm; — — —, 280 nm. 1 = Kynurenine; 2 = tryptophan.

The identification of these two compounds was confirmed by spiking the urine samples with pure standards. Position and relative intensity of the absorbances at 254 nm and 280 nm matched those obtained with standard mixtures.

The general pattern of the urine changed drastically upon administration of the tryptophan load. A number of additional peaks are present in Figs. 3 and 4. No identification of these was attempted; however, most likely some of these are tryptophan metabolites which were not characterized in this study. The

complex elution pattern around 4 min did not allow measurements of N-formylkynurenine. The very small shoulder appearing slightly after 6 min in Fig. 4 is thought to be 3-hydroxykynurenine. This identification, however, is only tentative at this point.

All the above mentioned chromatograms were run one day after the administration of the tryptophan load. Fig. 5 shows a chromatogram of the 4-h sample after being stored frozen (at $-11°$) for one week. A drastic change in the urine chromatographic profile is evident along with a drop in tryptophan and kynurenine levels.

The standard mixture was rerun in order to determine if the observed changes were due to deterioration of, or variations in, the system's performance. The standards showed, for all practical purposes, no significant variations in column performance. The initial samples that we analyzed were collected 2—4 h after the tryptophan loading, and delivered to our laboratory on the following day. However, urine samples were collected every 2 h for 12 h, and the whole set of specimens was frozen at $-11°$. Our laboratory received these samples a week after administration of the load. As shown in Table I there is a drastic difference in the amounts of tryptophan and kynurenine in the day old samples and in the week old ones. These data indicate that analysis should be carried out as quickly as possible. Even freezing immediately after urine collection has not assured constant levels of tryptophan and its metabolites with passing time. Such freezing and long term standing is frequently common in the clinical laboratory. Although this point was not investigated carefully, it seems that freezing at dry ice temperature will arrest the destruction of tryptophan. The tryptophan degradation was observed in all the urine samples independent of their sources.

Table I seems to indicate that the maximum amount of kynurenine in the urine occurs several hours after the maximum amount of tryptophan is observed. More work is needed to quantitate precisely the amounts of these compounds in urine as a function of time immediately after a tryptophan load, and as a function of elapsed time from administration of the load.

The lower limit of detection, at present, is estimated to be about 0.05 $\mu g/\mu l$ of urine. No attempts were made to improve the sensitivity of the method.

TABLE I

TRYPTOPHAN (Trp) AND KYNURENINE (Ky) LEVELS IN URINE SAMPLES

Time after load (h)	1 day old		1 week old		Change (%)	
	$\mu g/10\ \mu l$ Ky	$\mu g/10\ \mu l$ Trp	$\mu g/10\ \mu l$ Ky	$\mu g/10\ \mu l$ Trp	Ky	Trp
Control	∿0	∿0	∿0	∿0	—	—
2	0.24	0.82	0.12	0.60	50	27
4	0.95	1.9	0.88	0.44	7	77
6	—	—	1.32	0.47	—	—
8	—	—	0.16	0.05	—	—
10	—	—	0.16	0.05	—	—
12	—	—	0.12	∿0	—	—

Based on the chromatogram of the standard mixtures, however, it is believed that a tenfold increase in the sensitivity could be attainable if needed.

The chromatographic column has been used for over 125 h with direct injections of untreated urine. No degradation in column performance has been observed. It is conceivable that, after prolonged use, urinary proteins and other macromolecules might destroy the column efficiency. In the present study, this did not present a problem.

After the compilation of this study, the column was stored for several weeks in distilled water. The column was then re-installed in the chromatograph in order to analyze urine of a mentally retarded patient. It was found that the retention characteristics of the column changed considerably. An examination of the previous history of the column revealed that it was conditioned by the manufacturer with methanol—water. Apparently some of the methanol was adsorbed on the surface of the support. Eventually, after a long time of usage with, and storage in, water, the methanol was depleted, causing a change in the retention times. However, adding about 0.5—1% methanol to the water mobile phase restored the previously observed elution pattern.

ACKNOWLEDGEMENTS

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Biomedical Applications

CHROMBIO.006

MICROCHROMATOGRAPHY OF HEMOGLOBINS

VII. DETECTION OF SOME UNCOMMON HEMOGLOBIN VARIANTS AND TWO RAPID METHODS FOR THE QUANTITATION OF Hb-A_2 IN THE PRESENCE OF Hb-C

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SUMMARY

Microchromatographic procedures on columns of DEAE- or CM-cellulose are described for the separation of the more common abnormal hemoglobins S and C from other, often uncommon, variants such as J, N, I, K-Woolwich, Hope, D, E, O, and Deer Lodge. Two procedures on conventionally sized columns of CM-cellulose permit the quantitative determination of Hb-A_2 in the presence of Hb-C in a day.

INTRODUCTION

Although certain microchromatographic procedures were devised for specific purposes such as the quantitative determination of Hb-A_2 [1] or the testing of cord blood for Hb-S and/or Hb-C [2], their utility is by no means limited to such usage. Thus, the method for the quantitative determination of Hb-A_2 is easily applicable to testing programs for hemoglobinopathies [3]. The present paper describes how minor modifications extend the applicability of these methods to the study of such fast moving hemoglobins as J and N or to the distinction of Hb-S from Hb-D and Hb-E or Hb-O from Hb-C. In addition, two rapid methods for the determination of Hb-A_2 in the presence of Hb-C on conventionally sized chromatograms are presented.

*Contribution No. 5288.

MATERIALS

Blood samples

Informed consent was obtained from individuals whose blood was examined. Specimens were obtained through the Sickle Cell Centers at Augusta and Los Angeles or had been sent to Augusta or Pasadena for further evaluation. The blood was collected in ethylene diaminetetraacetic acid or heparinized microhematocrit tubes.

Of the uncommon abnormal hemoglobins that were used in this investigation, hemoglobins N-Baltimore (β95, Lys→Glu), J-Baltimore (β16, Gly→Asp), K-Woolwich (β132, Lys→Gln), Hope (β136, Gly→Asp), I-Philadelphia (α16, Lys→Glu), and Deer Lodge (β2, His→Arg) were identified by chemical study of the aberration. Hemoglobins D-Los Angeles (β121, Glu→Gln), E (β26, Glu→Lys), and O-Arab (β121, Glu→Lys) were not so identified but had the proper electrophoretic behavior at both acid and alkaline pH and derived from the ethnic group at risk.

For most procedures, whole blood was used for the chromatograms. When hemolysates were used, they were prepared from washed cells by hemolysis with water equal to 1.5 times the packed cell volume plus 0.4 volume of carbon tetrachloride for 20 min at room temperature after which cellular debris was removed by centrifugation. In some instances, the sample was dialyzed against a large volume of the appropriate developer overnight at 4°.

Developers

The solutions for the several procedures to be described were made up with the quantities and to the molarities that are given in Table I. Some of these developers have been designated by letters or Roman numerals in other papers [1, 2, 4].

TABLE I

COMPOSITION OF DEVELOPERS*

Developer No.	Bis-tris		NaCl		Glycine		$NaH_2PO_4 \cdot H_2O$		pH
	M	g/l	*M*	g/l	*M*	g/l	*M*	g/l	
1					0.2	15			Unadjusted**
2			0.015	0.88	0.2	15			Unadjusted**
3			0.02	1.17	0.2	15			Unadjusted**
4	0.03	6.28	0.03	1.75					6.1***
5	0.03	6.28	0.12	7.01					6.1***
6	0.03	6.28	0.04	2.34					6.2***
7	0.03	6.28	0.05	2.92					6.2***
8							0.01	1.38	7.0§
9							0.01	1.38	8.0§

*All solutions contain 0.1 g KCN per liter (0.01%).
**The pH is about 7.6.
***pH adjusted with conc. HCl.
§ pH adjusted with 2 *N* NaOH.

Preparation of ion exchangers

CM-Cellulose or DEAE-cellulose as CM-52 or DE-52 (microgranular and preswollen) from Whatman (Clifton, N.J., U.S.A.) was used in all experiments.

DE-52 was prepared in developer No. 1 and equilibrated as described previously with the pH adjusted for optimal separation of Hb-A_2 and Hb-S [1]. CM-52 was prepared and equilibrated either with developer Nos. 4, 6, 7, or 8 as previously described for similar solutions [2, 4].

PROCEDURES AND RESULTS

Separation of Hb-N, Hb-J and similar hemoglobins

Procedure. A 0.5 × 8 cm column of DE-52 which has been equilibrated with developer No. 1 and adjusted in pH was poured in a Pasteur pipette. One drop of blood or undialyzed hemolysate was mixed with eight drops of water. If blood is used, hemolysis should proceed for at least 5 min before the sample is applied to the column. After the sample had been applied, development was made with developer No. 1 at a flow-rate of about 15 ml/h until the effluent equaled 5 ml. Developer No. 1 was then replaced by developer No. 3 and 15 ml were passed through the column.

Alternatively, a 0.5 × 15 cm column may be used at a flow-rate of about 10 ml/h. After development with 5 ml of developer No. 1, 60 ml of developer No. 2 was used.

Results. Development with developer No. 1 on the shorter column is the procedure for the quantitative determination of Hb-A_2 [1]. Therefore, Hb-A_2, as

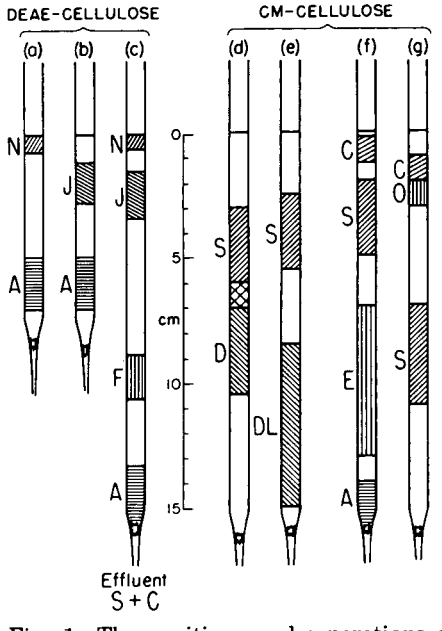

Fig. 1. The positions and separations of some common and uncommon hemoglobins on DEAE-cellulose and CM-cellulose under various conditions that are described in the text. The limits of the zones are depicted as sharp whereas in fact they are diffuse.

well as any Hb-C, passes through virtually unretarded. By the time that 5 ml of developer No. 1 has been used, Hb-S has moved to the middle of the column but any Hb-A, Hb-F, and electrophoretically fast moving hemoglobins at alkaline pH adhere to the top of the column. After development with developer No. 3 is complete, Hb-A and Hb-F are at the bottom, Hb-J in the middle and Hb-N still at the top of the column. Fig. 1a and b show the results when the method was applied to AN-Baltimore and AJ-Baltimore samples.

Experiments with other fast moving hemoglobins gave these results. Hb-K-Woolwich moved more rapidly down the microcolumn than N but more slowly than J. Hemoglobin Hope moves between A and J. On the other hand, Hb-N and Hb-I were indistinguishable. Hb-A and Hb-F did not separate.

When a complex artificial mixture of hemoglobins C, S, A, F, J, and N was applied to the longer column and developed as indicated, the hemoglobins emerged as well separated zones (Fig. 1c).

Comments. The procedures will clearly distinguish a wide variety of known or potential heterozygosities for hemoglobins. The study has included hemoglobins A, S, C, F, J, N, I, K-Woolwich, and Hope. Of the possible combinations, only that of N and I would not be identified, and Hb-F and Hb-Hope might be confused on microchromatographic evidence alone.

Samples of blood or hemolysate should be no older than three weeks or alteration products of Hb-A (that is, Hb-A_1) will interfere with identification of Hb-F or even possibly Hb-J.

Separation of Hb-S and Hb-C from electrophoretically similar hemoglobins

Procedure. A 0.5 × 15 cm column of CM-52 which has been equilibrated with developer No. 6 (or 7) was poured. The sample was prepared from 0.02 ml of blood, 0.2 ml of 0.004 *M* maleic acid, and 0.3 ml of 0.05% saponin (Calbiochem, Los Angeles, Calif., U.S.A.) in developer No. 6 (or 7). At least, 5 min was allowed for hemolysis. After the sample had been applied, the chromatogram was developed with developer No. 6 (or 7) at a flow-rate of about 10 ml/h. Developers Nos. 6 and 7 differ only in molarity of sodium chloride and were chosen in specific cases to provide a reasonable movement of the hemoglobins.

Results. When the blood of an individual with SD disease was chromatographed as above with 60 ml of developer No. 6, two zones were apparent on the column although not completely separated (Fig. 1d). The interzone was apparent but not clean.

Similarly, when blood of an S-Deer Lodge (DL) case [5] was chromatographed with 25 ml of developer No. 7, the hemoglobins occupied positions shown in Fig. 1e. On starch gel electrophoresis at pH 9, hemoglobins S and Deer Lodge produce a band that is broader in the anodal direction but distinct from Hb-S alone. This slight difference in charge is no doubt responsible for the better chromatographic separation of Hb-S and Hb-Deer Lodge as compared to that of Hb-S and Hb-D.

The separation of Hb-C and Hb-E is readily accomplished. When AC, AS, and AE samples were chromatographed on parallel columns and developed with 50 ml of developer No. 6, the final positions are shown in composite in Fig. 1f.

Hb-O-Arab can be distinguished from Hb-C but the separation is not as great as that of Hb-C and Hb-E. Thus, when 75 ml of developer No. 7 had been used

in parallel chromatograms, the results shown in composite in Fig. 1g were obtained.

Comments. The distinction between the SS and SD genotypes or between the CC and CE or CO genotypes is commonly done by citrate—agar electrophoresis [6] in which hemoglobins A, S, and C take distinctive positions but hemoglobins D and E behave like Hb-A, and Hb-O moves between the hemoglobins A and S. Microchromatography under these conditions provides more definite distinctions because Hb-D and Hb-Deer Lodge not only separate from Hb-S and, likewise, Hb-E and Hb-O-Arab from Hb-C but they do not mimic Hb-A.

The movement of hemoglobins in these developers as stressed previously [4] is very dependent upon sodium chloride concentration. The examples provided illustrate the altered movements that change in sodium chloride concentration brings about. No doubt by increase in sodium chloride concentration, Hb-C and Hb-O could be more completely separated. Because of the sensitivity to sodium chloride concentration, parallel chromatograms of known and unknown hemoglobins provide a more accurate comparison of chromatographic behavior.

Quantitative determination of Hb-A_2 in the presence of Hb-C

Procedure. Two procedures are available for this determination.

One procedure has been described [4] and uses the full gradient of sodium chloride in N,N-bis(2-hydroxymethyl)iminotris(hydroxymethyl)methane (Bis-tris) at pH 6.1 on CM-52. The chromatogram was complete in 24 h.

The second procedure is a modification of the method of Huisman [7]. CM-52 was equilibrated with developer No. 8 and poured into a column 1 × 32 cm in dimension which shrank to 30 cm in length during equilibration with the

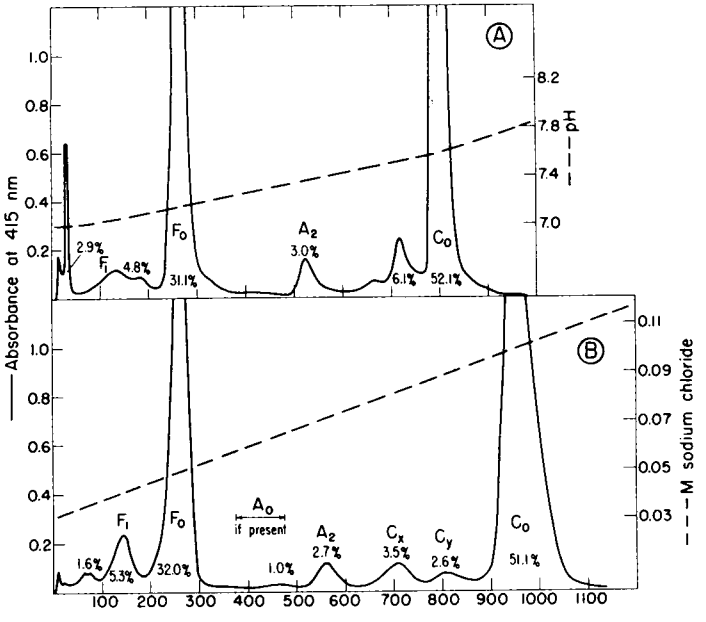

Fig. 2. The quantitative determination of Hb-A_2 in the presence of Hb-C by two methods on CM-cellulose.

same developer at 50 ml/h. A 40-mg sample which had been dialyzed against developer No. 8 was applied. Development was made with a two-vessel gradient system that contained 500 ml of developer No. 8 in the mixer and 500 ml of developer No. 9 in the second vessel. At a flow-rate of 50 ml/h, the chromatogram was complete in 20 h. The absorbance of the 5-ml fractions was read at 415 nm. The column may be used again after equilibration with 100 ml of developer No. 8.

Results. Fig. 2 depicts the application of these procedures to the hemoglobin of an individual with C-HPFH (hereditary persistence of fetal hemoglobin). The application of the Bis-tris procedure to an AC sample has been presented in Fig. 3B of ref. 4.

In order to test the validity of the new procedures, they have, in addition,

TABLE II

RESULTS OF THE APPLICATION OF SEVERAL METHODS FOR THE DETERMINATION OF Hb-A_2

Condition	Glycine micro A_2*	CM-Cellulose		DEAE-Sephadex**	Immuno***
		Bis-tris	Phosphate		
CC		4.6	4.8		4.5
C-β^+-Thal		6.8	6.8		
C-HPFH		2.7	3.0		
AC		3.7	3.3, 3.1		
AC		3.6	3.9		2.9
AC		4.0	3.9		3.0
AC		4.2	4.0		3.1
AC		3.9	4.1		
AC		3.9	4.4		
A-β-Thal	5.3		5.9		
A-β-Thal	4.9, 4.8	5.4	5.0	4.7	
AA	1.6, 1.6	2.8	2.3		
AA	2.0	2.8	2.4		
AA	2.9	3.1			
AA	2.5	4.4	2.7		
AA	2.5		3.1		
AA	2.5		3.5		
AA	2.5		2.9	3.0	
AA	2.7	4.2	2.6		2.3
AA	2.4, 2.3	3.5	2.0		2.1
AA	2.3, 2.5, 2.4, 2.6	3.6	3.3		
AA	2.5, 2.5, 2.4, 2.1	3.3	2.6		
AA	2.9	3.5	2.9		
AA	2.6	3.1	2.6		
AX§		3.1	2.6		2.9

*Procedure of ref. 1.
**Conventional chromatography [8, 9].
***Radioimmunoassay by Dr. Fred Garver [10].
§ An unidentified fast moving variant.

been applied to non-Hb-C-containing samples so that other chromatographic methods could be used for comparison [1, 8, 9]. Some samples with Hb-C where also tested by a newly developed radioimmunoassay [10]. The results are presented in Table II. Although some samples gave discrepant results for undetermined reasons, the data are generally concordant and show that the two methods here described provide means for the rapid determination of Hb-A_2 in the presence of Hb-C.

Comments. The prime consideration in the design of these procedures has been a good and rapid separation of Hb-A_2 from other hemoglobin components. The phosphate procedure has been started at a higher pH than originally [7] and hemoglobins that precede Hb-A_2 move out quickly. Hb-A will coincide with Hb-F in the phosphate method but will occupy the indicated position in the Bis-tris method. The separation of Hb-A and Hb-A_2, therefore, is markedly different in the two procedures. The fast flow-rate that is used reduces the time required to one-fourth that of the previously described chromatographic method [7].

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CHROMATOGRAPHY OF SUGARS IN BODY FLUIDS

III. STEPWISE DETECTION OF SUGARS WITH ANILINE CITRATE ON PAPER AND THIN-LAYER CHROMATOGRAMS

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SUMMARY

The paper chromatographic detection method of White and Hess for urinary aldoses using aniline citrate has been studied and modified. As the reactivity of individual sugars is determined mainly by their molecular weight and structure, our modification is based on a gradual increase in the reaction temperature (room temperature, 37 or 55 and 105°) and variations in heating time. This technique enables reaction conditions to be obtained that lead to a full display of both the transient and final characteristics of the fluorescence and colour of the individual spots in the sugar spectrum of any analyzed material.

Compared with other reagents based on aniline or other aromatic amines, aniline citrate possesses unusually wide operational flexibility. The procedure is suitable for paper and thin-layer chromatography and is especially valuable in the analysis of urinary and tissue sugar extracts for the identification of largely overlapping spots with different reaction times, the differentiation of oligosaccharides, particularly those with different linkages, and obtaining additional data for the identification of unknown metabolites.

INTRODUCTION

The neutral sugar component of normal human urine consists of wide concentrations of at least 24 aldo and keto sugars [1—6]. Under certain pathological conditions, the number of sugars and their concentrations may be even higher [7—9]

The complexity of urinary sugar components and their close structural similarity lead to overlapping of the individual spots in paper or thin-layer chromatography. Neither repeated development with the same solvent system nor two-dimensional chromatographic procedures [2, 10] work satisfactorily,

especially in the more numerous aldose fraction [5]. The situation is also aggravated by the fact that some aldoses share their positions with ketoses in any solvent system. Their interference cannot be completely overcome even by using highly specific detection reagents for aldoses such as phthalate, oxalate or citrate salts of aniline [1, 2, 10].

This paper describes a detection procedure that improves the identification of incompletely resolved sugar spots on paper and thin-layer chromatograms when complex mixtures of sugars in urine and a large variety of tissues are analyzed. The technique is based on the observation that on a chromatogram sprayed with aniline citrate as detection reagent [10] the individual or groups of spots require different reaction temperatures and times to develop optimal colour and fluorescent properties.

EXPERIMENTAL

Urinary specimens were collected from a severely injured surgical patient and a healthy individual who had been given a large dose of *d*-galactose. The patient was a 42-year-old male, semi-conscious with a fractured leg and gas-gangrene, on parenteral nutrition consisting of glucose and occasional administration of blood and its fractions. Routine examination of the urine showed no significant pathological changes. The healthy individual was a 40-year-old male. Galactose was administered after 11 h of fasting in a dose of 0.5 g per kilogram of body weight (45 g total). Urine was collected in 3-h periods prior to and after the ingestion of galactose. During the collection, the urines were refrigerated and then kept frozen until required.

Sugar standards were mostly commercial preparations. All chemicals were of ACS grade or better. Aniline was re-distilled every 2 months.

Preparation of urine for chromatography

Urines were filtered through filter-paper, pre-washed with water and de-salted by passage through ion-exchange columns [6]. For paper chromatographic, colorimetric and enzymatic analysis, the dry residues obtained after lyophilization of concentrated column effluents were re-dissolved in small amounts of water. For chromatography, the urine extracts were streaked on a 30-mm line on Whatman No. 17 paper in a portion corresponding to a 10- or 20-min aliquot of diuresis [5]. Standards of aldoses, ketoses and sugar alcohols were spotted as small circles in the amounts indicated in the legends of Figs. 1 and 3.

Chromatography and detection

Urine (Figs. 1 and 3) was chromatographed by the ascending technique in at least three solvent systems: (1) *n*-butanol—pyridine—benzene—water (5:3:1:3); (2) ethyl acetate—acetic acid—water (3:1:1); and (3) isobutanol—acetic acid—water (4:1:1) [5].

The spots on the chromatograms were detected with aniline citrate [10] in a modification described in detail under Results. Ketoses were revealed by the orcinol—trichloroacetic acid method [11], modified by replacing the spraying technique with dipping and increasing the heating temperature to 120—125°

Sugar alcohols and other sugar derivatives were located with periodate—benzidine reagent [2].

The total contents of reducing sugars, glucose and galactose in de-salted urinary preparations were determined as described elsewhere [6].

Preservation of detected chromatograms

Darkening of chromatograms treated with aniline citrate or orcinol—trichloroacetic acid can be prevented by keeping them from contact with air at a temperature below 0°. Storage in plastic folders in a deep-freezer or box of solid carbon dioxide enables the detection of aldoses to be interrupted at any stage (overnight or for periods of several days or even weeks).

RESULTS

Preparation of developed chromatograms for detection

As the chromatographed material (Figs. 1 and 3) was of biological origin and contained UV-absorbing and fluorescing spots, before staining the chromatograms were viewed under the light from two UV lamps. Of several brands tested, Mineral-Light UVS-11 (main wavelength 260 nm) and Black Ray UVL-21 (360 nm) lamps, both from Ultraviolet Products (San Gabriel, Calif., U.S.A.) [7], were the most suitable. Their light was strong enough to display the fluorescent and UV-absorbing properties satisfactorily and yet did not affect the surface of the paper even after prolonged exposure. The shape and intensity of the fluorescing and absorbing spots were marked on the back of the sheet, which was of value in checking the regularity of the solvent flow after each run and in pre-determining the locations of the expected sugar spots and fluorescent spots of non-sugar substances. After staining, most fluorescent and UV-absorbing spots disappeared entirely and only a few showed colours. UV-absorbing spots usually seen in the urine are recorded in Fig. 3.

Spraying technique

Whatman No. 1 and similar thin papers were sprayed with aniline citrate on the front. On Whatman No. 3 and thicker papers, optimal intensity of the spots was achieved by applying a gentle spray on the back so that the paper was uniformly saturated on both sides without the formation of shiny spots.

Changes in colour properties of spots during stepwise detection

The detection procedure with aniline citrate using Whatman No. 17 filter-paper was divided into five stages and is outlined in Table I. Standard sugars and urinary spots with a distinct colour and/or fluorescence in the detection stages I—III are recorded in Fig. 1, while spots evident in stages IV and V are shown in Fig. 2.

The colour development of the sugar spots in its initial stage has a number of characteristic features common to all sugars, regardless of the final colour. At first there is a weak fluorescence of an indefinite light colour, changing within 15—30 min (at room temperature) into greenish yellow, yellowish green or similar shades characteristic of the sugar or its class. The change in fluorescence is preceded by the appearance of a yellow spot, which is at first most visible in

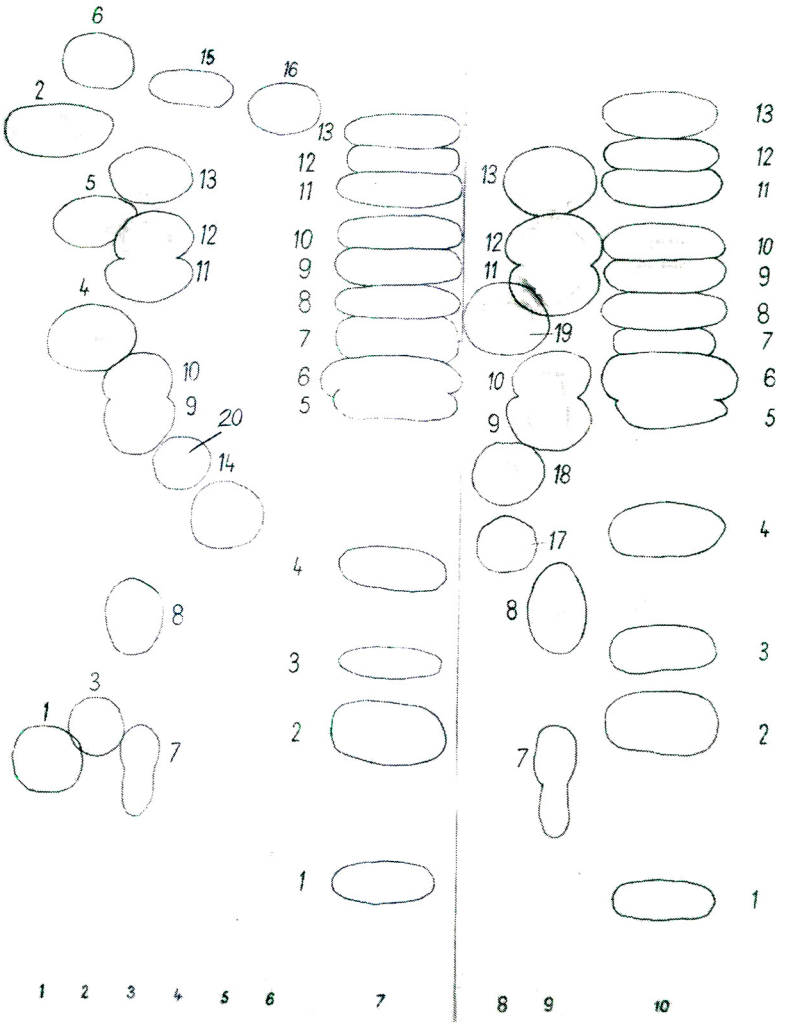

Fig. 1. Paper chromatograms of urinary sugars of a severely injured patient illustrating colour development after stage III (Table I) of the detection procedure using aniline citrate. Ascending chromatography in isobutanol—acetic acid—water (4:1:1) on Whatman No. 17 filter-paper repeated eight times [5]. Ten-minute aliquots of urine specimens collected in two successive 1-h periods were streaked on two chromatograms at positions 7 and 10. Chromatography and detection of both sheets were carried out simultaneously. Standard sugars were spotted in amounts of 100 μg or as indicated in parentheses at positions 1—6 and 8—9: 1 = melibiose (150 μg); 2 = rhamnose (200 μg); 3 = cellobiose (200 μg); 4 = gulose; 5 = lyxose; 6 = glyceraldehyde (200 μg); 7 = lactose; 8 = 3-O-D-galactosyl-D-arabinose (General Biochemicals, Chagrin Falls, Ohio, U.S.A.); 9 = galactose; 10 = glucose; 11 = arabinose; 12 = xylose; 13 = ribose; 14 = mannosamine; 15 = deoxyribose (300 μg); 16 = erythrose; 17 = galactosamine; 18 = galacturonic acid; 19 = mannose; 20 = glucuronic acid. Spots in urine samples: 1 = unknown; 2 = isomaltose + lactose; 3 = unknown; 4 = tentatively identified as glucosyl-xylose; 5 = galactose; 6 = glucose; 7 = allose; 8 = mannose; 9 = arabinose; 10 = xylose; 11 = ribose + fucose; 12 = ribulose + xylulose; 13 = unknown with yellow-orange fluorescence and colour.

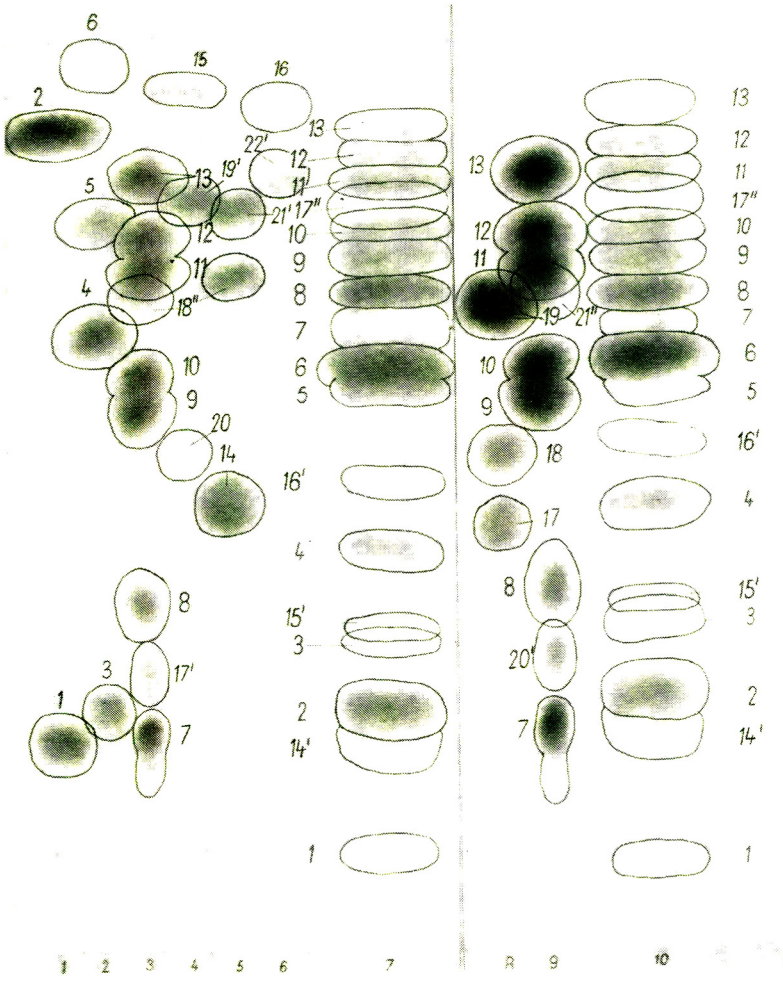

Fig. 2. Chromatograms of sugars in the urine of a severely injured patient after stage V of the detection procedure (Table I). Chromatograms are identical with those illustrated in Fig. 1. Spots recorded after stage IV are designated with numbers with one superior prime and spots recorded after stage V with two superior primes. Standard sugars at positions 1—6 and 8—9: 17′ = maltose; 18′′ = fructose; 19′ = N-acetylglucosamine; 22′ = N-acetylmannosamine; 20′ = maltose; 21′ = N-acetylgalactosamine; 21′′ = fructose. Urinary specimens at positions 7 and 10 : 14′ and 16′ = unknowns, no colour; 15′ = unknown with weak light brown colour and light fluorescence; 17′′ = N-acetylglucosamine.

a transparent light. Starting from the centre, it changes gradually into the colour characteristic of the particular sugar, which is pink to cherry red for pentoses and greenish brown or yellow for hexoses and their oligosaccharides. Subsequent heating at 55° for an appropriate time causes intensification of the characteristic fluorescence and colour. Continuation of heating or even extend-

TABLE I

SCHEME OF DETECTION PROCEDURE FOR URINARY SUGARS

Stage	Temperature (°C)	Duration
I	24—26	2 h
II	55	10 min
III	55	60 min
IV	55	60 min
V	105—110	10 min

ed exposure at room temperature at this stage leads to a loss of brilliance and darkening. The colour due to pentoses changes into purple with a brown tone and that of aldohexoses into sepia brown. Final heating at 110° turns all sugar spots into various shades of brown with little individual differences. At the same time, the initially hardly discernible light yellow tint of the background becomes gradually darker with a light bluish fluorescence absorbing light fluorescing spots.

Table II summarizes the fluorescence and colours of the sugars included in Figs. 1 and 2. The onset of the stage I occurred by the time the chromatogram was dry. However, while the paper was still damp, in its lower part some standards (Figs. 1 and 2) such as mannosamine (spot 14), glyceraldehyde (6), erythrose (16) and aldopentoses (5, 11, 12, 13), especially ribose (13), were already yellow.

The colours and fluorescence of sugar derivatives often differ markedly from those of the parent sugar. At 55°, deoxyribose (15) yields an intense lemon yellow fluorescence and colour, both very resistant to deterioration by further heating, while the ribose (13) spot is red. The greenish brown colour of glucose and galactose differs from the pink of glucuronic acid (20) and the orange of galacturonic acid (18). Both can be more easily differentiated by fluorescence, which is strong purple for glucuronic acid and its lactone but orange for galacturonic acid. N-Acetylation of amino sugars drastically changes their reactivity and their intense yellow colour changes to light brown, which is visible only at the much higher temperatures of stage V.

TABLE II

FLUORESCENCE AND COLOUR PROPERTIES OF SUGAR SPOTS RECORDED IN FIGS. 1 AND 2

Stage numbers as in Table I.

Spot No.	Sugars*	First fluorescence stage No.	Peak of fluorescence stage No.**	First colour stage No.	Peak of colour stage No.**
(A) Standards					
1	Melibiose	II	III (g-y)	II	IV (g-br or y-br)
2	Rhamnose	I	III (g-y)	II	IV (br)
3	Cellobiose	II	III (g-y)	II	IV (g-br or y-br)
4	Gulose	I	III (g-y)	II	III (g-br)
5	Lyxose	I	II (y-g)	I	II (r)

TABLE II *(continued)*

Spot No.	Sugars*	First fluorescence stage No.	Peak of fluorescence stage No.**	First colour stage No.	Peak of colour stage No.**
6	Glyceraldehyde	I	I (l.y)	I	II (b-br)
7	Lactose	II	III (g-y)	II	IV (g-br)
8	Galactosyl-arabinose	I	II (y-g)	I	III (r)
9	Galactose	I	III (g-y)	II	III (y-br)
10	Glucose	I	III (g-y)	II	III (y-br)
11	Arabinose	I	II (y-g)	I	II (r)
12	Xylose	I	II (y-g)	I	II (r)
13	Ribose	I	II (y-g)	I	II (r)
14	Mannosamine	I	II (y)	I	III (y-br)
15	Deoxyribose	I	II (y)	I	II (y)
16	Erythrose	I	II (y)	I	II (y)
17	Galactosamine	I	II (y)	I	III (y-br)
18	Galacturonic acid	I	II (w.o)	I	III (o-r)
19	Mannose	I	III (g-y)	II	III (y-br)
20	Glucuronic acid	I	III (p)	I	III (o-r)
17′	Maltose	III	IV (260nm, bl; 360nm, g-y)	IV	V (l.br)
18″	Fructose	IV	V (g-y)	IV	V (l.br)
19′	N-Acetylglucos-amine	IV	IV (y)	IV	V (l.br)
21′	N-Acetylgalac-tosamine	IV	IV (y)	IV	V (l.br)
21″	Fructose	IV	V (g-y)	IV	V (l.br)
22′	N-Acetylman-nosamine	IV	IV (y)	IV	V (l.br)
(B) Urinary sugars					
1	Unknown	I	I (g-y)	—	I-V (n.c.)
2	Isomaltose and lactose	II	III (y)	II	IV (r-br)
3	Unknown	II	III (g-y)	—	II-V (n.c.)
4	Glucosylxylose	I	II (y-g)	I	III (r)
11	Ribose and fucose	I	II (y-g) and III (g-y)	I	II (r) and III (br)
12	Ribulose and xylulose	I	II (l.y)	II	III (o-pk)
13	Unknown	II	IV (y-o)	III	IV (y-o)
14′	Unknown	IV	IV (g-y)	—	IV—V (n.c.)
15′	Unknown	IV	IV (g-y)	V	V (l.br)
16′	Unknown	IV	IV (g-y)	—	IV—V (n.c.)
17′	N-Acetylglucos-amine	IV	V (y)	V	V (l.br)

*The numbers against the names of the sugars are identical with the numbers of the spots in Figs. 1 and 2.

**Abbreviations for colours: b = beige; bl = blue; br = brown; g = green; l. = light; n.c. = no colour; o = orange; p = purple; pk = pink; r = red; y = yellow; w. = weak.

The colour and fluorescence of oligosaccharides seem to be dependent not only on the sugar component, e.g., red for galactosylarabinose (8) compared with greenish yellow or brown for lactose (7), but also by the kind of linkage. Melibiose (1), with a β-1,6- bond, gave a reddish brown colour with a yellow fluorescence, both distinctly different from the green-yellow colour and similar fluorescence of isomeric lactose (7), with a β-1,4- bond. Unlike other reducing oligosaccharides, maltose and its derivatives, with an α-1,4- bond (i.e., maltotriose, panose, etc.), reacted at considerably higher temperatures, developing a weak greenish brown colour. This drawback was compensated for by their highly sensitive and specific blue fluorescence at 260 nm and greenish yellow fluorescence at 360 nm.

Application of stepwise detection in the identification of urinary sugars

The spot of urinary glucose (6) in the final detection stage (Fig. 2, samples 7 and 10) overlapped considerably the spot of galactose (5), yet they could be distinguished in stage I or II by the yellow fluorescence and/or colour of uneven intensity, which are stronger with glucose. In Figs. 1 and 2, fucose, which is always present in the urine, shared its position with ribose (11). The latter is the fastest reacting pentose and was easily identified by the red colour and green-yellow fluorescence in stage I, while fucose first became apparent after heating for 10 min at 55°. Its fluorescence altered that of ribose and, after heating for 60 min (stage III), due to the admixture of fucose, there was a pronounced difference between the spots of standard and urinary ribose in the fluorescence and especially in the colour. After final heating at 110°, only an experienced eye could discern the presence of two sugars.

Another partially overlapping group of urinary spots included xylose (10), N-acetylglucosamine (17″), ribose and fucose (both 11, Fig. 2). The differentiation of N-acetylglucosamine (17″) was relatively easy as it started to produce fluorescence and a light brown colour when the fluorescence of all of the above-mentioned sugars was extinguished. Mannose (8) and arabinose (9) (Fig. 2) were partially overlapped by the always present fructose, which reacted non-specifically with aniline citrate. [Urinary fructose is not recorded in Figs. 1 and 2. Its position is indicated by its standard (18″) in Fig. 2.] In the urine, differentiation is facilitated by the pink colour of arabinose reacting in stage I, unlike mannose, which appears as a yellow to brown spot in stage II. During stage IV, arabinose and mannose lose their characteristics while fructose shows peak fluorescence and a light brown colour of the part of its spot that does not overlap in stage V. Apart from fructose, other ketoses such as ribulose and xylulose also react non-specifically with aniline citrate. Both share the same position in the solvent used (12, Figs. 1 and 2) and react in stages I and II. Unlike neighbouring ribose and fucose, they initially display a characteristic pale yellow colour, which in stage III turns orange-pink.

Most of the spots marked on the chromatograms as "unknown" are permanent features of the sugar spectrum, located predominantly in the disaccharide region. Spot 2 in Fig. 2 and spot 4 in Fig. 3 showed typical qualities of disaccharides, yielding peak colour and fluorescence at 55° in stage IV. Its red-brown colour differs from those of standard lactose (7) and cellobiose (3) but is similar to that of melibiose (1). In severely ill patients, the spot is usually

formed by small amounts of lactose and large amounts of isomaltose; in healthy subjects, lactose prevails. Another unknown but characteristic feature of human urine is the intense spot 4 (Figs. 1 and 2). It reacts earlier than hexosyldisaccharides but later than hexoses. In stage II it yields a pink to red colour and fluorescence. Both properties are identical with those of standard galactosylarabinose. It was tentatively identified as glucosylxylose.

Unidentified urinary spots 14′, 15′ and 16′ (Fig. 2, samples 7 and 10) appeared during stage IV in the region of disaccharides and showed a weak green-yellow fluorescence. Additional heating at 110° changed only spot 15′ to a weak light-brown colour.

Unknown spot 13 was the only one that did not share its yellow-orange colour with any standard tested. The metabolite yielded a feeble yellow fluorescence after heating for 10 min at 55° (stage II), reaching the highest intensity of fluorescence and its yellow-orange colour simultaneously at the end of stage IV. Unlike other urinary spots, both properties were unusually stable on further heating.

In addition to simple aldoses and ketoses, de-salted urine frequently contains low-molecular-weight neutral or weakly ionized fragments of glycolipids and glycoproteins and always at least 15 sugar alcohols, which on the chromatograms share positions with reducing sugars. The relative positions of some sugar derivatives and sugar alcohols are demonstrated in Fig. 3, samples 6—10.

Unidentified spots 17f, 23f and 26f (Fig. 3) could be located only after spraying with aniline citrate by their fluorescence, which is identical with or similar to that produced by standard carbohydrates.

Urinary sugar extracts prepared by ion-exchange techniques are not entirely free of non-carbohydrate compounds such as spots 10a, 16a, 20a, 24a and 26a. They are a characteristic feature of urinary sugar chromatograms and can easily be detected by their absorption at 260 nm. They have been described in detail elsewhere [6].

The chromatogram in Fig. 3 is an example of an experimental situation in which unknown metabolites may be expected. A 45-g oral dose of galactose given to a healthy person produced several changes in the urinary sugar pattern. Unidentified spot 3 (Fig. 3) is a metabolite with chromatographic properties characteristic of a disaccharide. Unknown spot 2, of a trisaccharide nature, intensified after administration of galactose. The remaining aldoses in the spectrum were excreted in decreased amounts, in contrast to spot 7, shared by glucose and galactose.

DISCUSSION

White and Hess [10] introduced the aniline citrate detection reagent into sugar chromatography. As with other similar reagents [1,2], the original procedure involved heating the sprayed, still damp chromatogram for several minutes at 100°. This treatment proved to be satisfactory to give an overall picture of the sugar spectrum when a complete separation of a simple mixture is expected or only one or few sugars are in question. On the other hand, it does not have the advantage of revealing the changing colour and fluorescence properties of individual sugars that provide a useful data for identification purposes. In

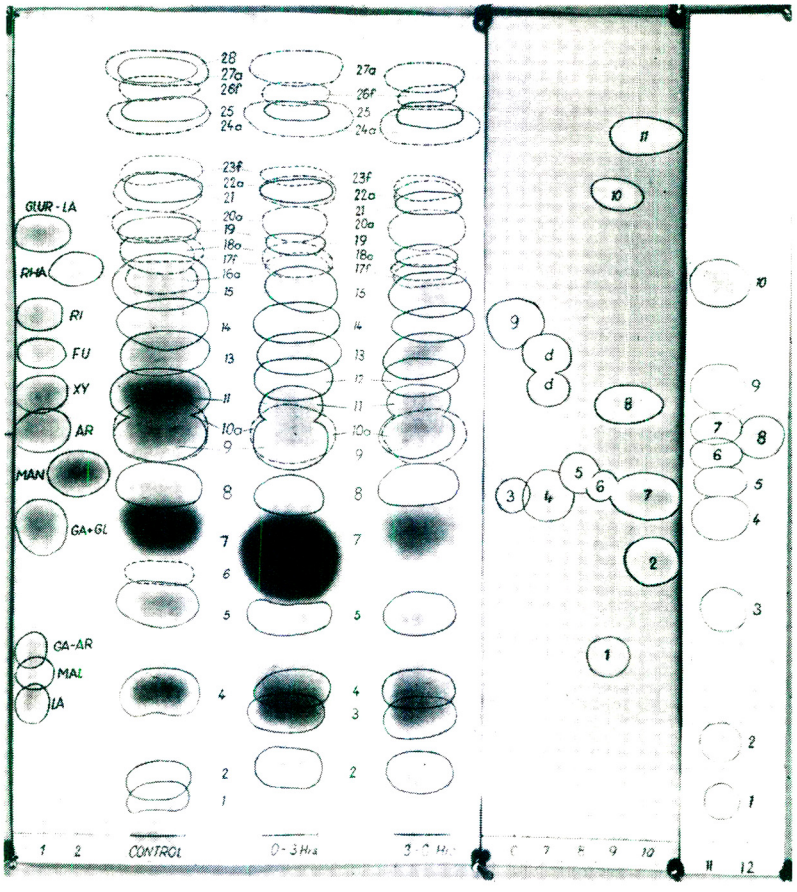

Fig. 3. Chromatogram of sugars in the urine of healthy person after a large dose of galactose. Ascending chromatography on Whatman No. 17 filter-paper in ethyl acetate—acetic acid—water (3:1:1) repeated four times [5]. Detection with aniline citrate as described in the text. Spots marked with a dashed line and "f" were apparent before or after the detection process only by their fluorescence; a dot-and-dashed line and "a" indicate UV light-absorbing spots (wavelength 260 nm) before the detection; a full line shows coloured spots, the size of which is marked by their fluorescence. Standards of aldoses were applied at positions 1 and 2 in amounts of 100—200 μg. Standards of sugar alcohols, sugar acids and their lactones were applied at positions 6—10 in amounts of 200—350 μg. Positions 11 and 12 carried standard ketoses in amounts of 100—200 μg. Control urine was collected in a 3-h period preceding administration of galactose in a dose of 45 g (0.5 g per kilogram of body weight). Samples 0—3 h and 3—6 h represent changes that occurred during the corresponding periods. All urinary samples represent a 10-min aliquot of diuresis with the following amounts of reducing substances determined by the Somogyi—Nelson method and expressed as milligrams of glucose: control, 2.0; 0—3 h, 10.26; 3—6 h, 1.13. At the same time the three urinary specimens contained 1.20, 1.53 and 0.75 mg of pure glucose, respectively, as determined by a glucose-oxidase method [6]. The 0—3-h urine sample contained 7.1 mg of galactose. Standard spots of aldoses at positions 1 and 2: LA = lactose; MAL = maltose; GA-AR = *d*-galactosylarabinose; GA = galactose; GL = glucose; MAN = mannose; AR = arabinose; XY = xylose; FU = fucose; RI = ribose; RHA = rhamnose; GLUR-LA = glucuronolactone. Standards of sugar alcohols (positions 6—10): 1 = myoinositol (200 μg); 2 = perseitol (300 μg); 3 = galactonic acid released from galactonolactone; 4 = gluconic acid released from *d*-gluconolactone (350μg); 5 = mannitol (200 μg); 6 = sorbitol (200 μg); 7 = galacticol

general, the rate of colour development of sugar spots decreases with increasing molecular weight in the following order: tetroses, pentoses, hexoses, heptoses, disaccharides and higher oligosaccharides. Within a homologous series of sugars applied in equimolar amounts, the time of colour development is roughly proportional to the relative speed of migration of the individual member (in non-phenolic solvents) and the fastest-moving sugar reacts first, e.g., in the pairs ribose—arabinose, talose—galactose, mannosamine—glucosamine the second sugar always reacts markedly later than the first. It is also apparent that other structural properties play a substantial role, e.g., the glucose spot appears later than the glucuronolactone spot, which shows up at the same time as that of ribose.

The stepwise detection procedure described above is especially suitable for Whatman No. 17 paper and a complex mixture of sugars containing some unknown substances such as those found in urine. Its timing schedule should be changed when thinner paper is used, as in general the reactions then proceed faster. Simplification of the heating and timing schedule may be advantageous when less complex or well separated mixtures are used. In this instance the colour development at room temperature may be avoided or abbreviated. In one of our alternative modified procedures employing Whatman 3MM paper, the sprayed and dried sheet is maintained at room temperature for 1 h, then heated at 37° for 30—60 min, at 55° for 60 min and finally at 110° for 10 min. On other occasions, particularly when working with di- and higher oligosaccharides, the colour development at room temperature is omitted and the dried or still damp sheet is heated at 55° for 10 or 20 min, then twice at 55° for 60 min and finally at 110° for 10 min.

The procedure is also applicable to thin-layer chromatographic plates coated with silica gel and gives even better results with microcrystalline cellulose. Sugars chromatographed on silica gel do not react at low temperatures or react

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(300 μg); 8 = arabitol (200 μg); 9 = 1,4-*d*-galactonolactone (300 μg); 10 = glycerol (200 μg); 11 = dihydroxyacetone (300 μg). Decomposition products of *d*-galactonolactone are designated by the letter "d". Standard spots of ketoses (position 11 and 12): 1 = stachyose; 2 = raffinose; 3 = sucrose; 4 = mannoheptulose; 5 = sedoheptulose; 6 = fructose; 7 and 8 = tagatose; 9 = impurities of ribulose; 10 = ribulose. Urinary spots: 1 = mixture of unknown saccharides with weak yellow-green fluorescence and light brown colour; 2 = unknown trisaccharide with green-yellow fluorescence and light brown colour; 3 = unknown disaccharide with green-yellow fluorescence and brown colour; 4 = isomaltose and lactose; 5 = tentatively identified as glucosylxylose; 6 = unknown spot apparently of non-carbohydrate nature, with a deep purple fluorescence before and after detection; 7 = glucose and galactose; 8 = mannose with trace amounts of allose; 9 = arabinose; 10 = unknown, always the strongest UV-absorbing spot on the chromatogram visible before but not after detection; 11 = xylose; 12 = N-acetylglucosamine; 13 = fucose; 14 = ribose; 15 = unknown carbohydrate with strong yellow-orange colour and fluorescence; 16 = weak UV-absorbing spot of non-carbohydrate nature; 17 = unknown sugar derivative with white-blue fluorescence; 18a = unknown UV-absorbing spot seen before detection; 19 = glucuronolactone; 20 = unknown, strong UV-absorbing spot; 21 = unknown with a pink colour developed at room temperature with yellow-green fluorescence, after stage five, light brown colour with white fluorescence; 22a = unknown UV-absorbing spot; 23f = unknown with a faint pink colour with room temperature detection, intense white-blue fluorescence after heating at 110°; 24a = unknown strong UV-absorbing spot; 25 = unknown weak orange-brown spot; 26f = unknown with a light fluorescence; 27a = unknown UV-absorbing spot of moderate intensity; 28 = unknown with yellow-orange colour.

to a negligible extent and much higher starting temperatures are required, especially in the presence of borates.

A number of reagents are available for the detection of aldoses [1, 2] but only very few methods are suitable for the detection of the complex range of urinary sugars [1, 5] or other similar mixtures, mainly because of their low specificity or sensitivity. The most widely used reagent for aldoses is aniline phthalate, introduced by Partridge [12], which is considered to be more sensitive than the previously introduced aniline oxalate. Aso et al. [13] reported that different sugars possess different optimal reaction temperatures with aniline phthalate. In our experience, however, this reagent was completely unsuitable for the stepwise detection method, requiring much higher initial heating, which markedly narrowed the range of optimal temperatures of individual sugars. In addition, the fluorescence and colour sensitivity were much more inferior than with aniline citrate reagent. Better results, but still unsatisfactory in all respects, were obtained with aniline oxalate. The popular diphenylamine—aniline—orthophosphoric acid reagent [14, 15] also proved to be unsuitable for good colour differentiation because aldoses and ketoses react simultaneously and lack fluorescence.

The procedure described, in conjunction with a good chromatographic separation technique [5] and an effective de-salting technique [6], enabled us to identify isomaltose as a regular component of human urine [8] and also other previously unknown carbohydrates [5]. In addition, it was instrumental in demonstrating the great complexity and also the remarkable similarity and regularity of the range of urinary sugars in different individuals [5].

ACKNOWLEDGEMENTS

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Biomedical Applications

CHROMBIO. 008

STUDY OF SERUM CHOLINESTERASE VARIANTS BY MEANS OF ONE- AND TWO-DIMENSIONAL ELECTROPHORESIS IN DENSITY GRADIENT POLYACRYLAMIDE

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SUMMARY

Techniques are described for the electrophoresis of the isoenzymes of cholinesterase present in human serum using density gradient polyacrylamide. Application of the method to samples taken from patients homozygous for the abnormal, fluoride-resistant and silent genes has resulted in patterns recognisably different from those of normal individuals.

It is suggested that application of density gradient electrophoresis to serum from patients shown to be sensitive to muscle relaxants of the succinyl dicholine type, and yet normal by existing biochemical criteria, may prove to be useful in identifying new inherited enzyme abnormalities.

INTRODUCTION

Considerable advances have taken place in the electrophoretic separation of the serum isoenzymes of cholinesterase (acylcholine acyl-hydrolase EC 3.1.1.8.) in the twenty years since Pinter [1] succeeded in identifying three bands of enzyme activity located in α_2—β region. Improvements in electrophoretic and staining techniques enabled the enzyme to be separated into six or seven fractions [2], while the introduction of two-dimensional electrophoresis, coupling filter paper with starch gel, resulted in four areas of activity being detected in fresh serum in addition to the non-specific aryl esterase associated with albumin [3]. In serum subjected to storage two additional species could be recognised, while in a proportion of sera a fifth major band was observed [4], which is now thought to derive from a second locus. Sera found to possess this enzyme are referred to as C5 positive.

By use of polyacrylamide Juul [5] was able to increase the number of bands

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resolved to 12, while Nagy et al. [6] showed a similar number of electrophoretically distinct isoenzymes by use of density gradient polyacrylamide.

Several explanations can be put forward to explain the large number of bands of activity which are recognized including the possibility that each is a distinct molecular species [7]. Lamotta et al. [8] suggested that they represented a number of polymers of the same protein molecule, a theory which they supported on the basis of work incorporating ultracentrifugation. Previously Hess et al. [7] had considered the possibility that one enzyme molecule might be attached to a number of different carrier proteins. Although they discounted this theory, in view of the increasing data now available on the association of cholinesterase with lipoproteins [9, 10], it is a suggestion which might usefully be reconsidered.

With the exception of the C_5 band, relatively little attention has been paid to the separation of variants of cholinesterase by electrophoretic techniques. It is the purpose of this paper to describe differences in electrophoretic patterns, achieved by one- and two-dimensional electrophoresis in gradient polyacrylamide gel, of serum taken from patients homo- and heterozygous for the abnormal and fluoride-resistant genes.

MATERIALS

Bytyrylthiocholine iodide, α-naphthyl acetate and eserine sulphate were obtained from Sigma (London, Great Britain); fast red TR salt from Raymond A. Lamb (London, Great Britain) and PAA 4/30 polyacrylamide density gradient gel slabs from Pharmacia Fine Chemicals (London, Great Britain). All other chemicals were of analytical grade.

The following two buffer solutions have been used. Electrophoresis buffer, pH 8.35: 88.7 m*M* tris (hydroxymethyl)aminomethane—81.5 m*M* boric acid—2.5 m*M* disodium EDTA. Gel casting buffer, pH 8.35: 2.7 m*M* disodium EDTA—16.2 m*M* boric acid—18.9 m*M* ammonium sulphate—3.1 m*M* sodium azide.

METHODS

Sera were typed on the basis of dibucaine and fluoride inhibition as measured by the techniques of Kalow and Genest [11], and Harris and Whittaker [12].

Electrophoresis was performed using a Pharmacia GE-4 electrophoresis apparatus. The apparatus consists of two tanks, the lower one into which the buffer is placed being connected via a centrifugal pump to the upper vessel which supports the gel slabs and rods and the electrode mountings. In addition, a cooling platten is incorporated which ensures that the temperature of the buffer is kept constant during prolonged electrophoresis.

Polyacrylamide density gradient slabs were used for single dimensional electrophoresis and for the second dimension in two-dimensional electrophoresis. These slabs had a gel concentration at the origin of 4% increasing through the slab to a final concentration of 30%. For two-dimensional electrophoresis, the first dimension was carried out in 60 mm × 2.7 mm diameter polyacrylamide gel rods consisting of 7.5% polyacrylamide cast in the appropriate buffer.

Sera under study were diluted 1:1 with 5% sucrose containing bromophenol blue marker dye, and 10 μl of this solution were layered on top of the gel rods or into the special applicator above the PAA 4/30 gel slabs, positioned in the apparatus. In the case of the gel rods, the apparatus was connected to a power source, the current set to 21 mA and the electrophoresis continued until the marker dye approached the bottom of the gel. Gels were then removed either for staining or for layering across the gradient gel slab prior to electrophoresis in the second dimension.

For the PAA 4/30 gel slabs it was found that the best separation was achieved if the manufacturer's recommendation of a short period of electrophoretic pre-equilibration was carried out prior to loading the samples or gel rod. Electrophoresis was then performed at a current of 30 mA until the marker dye had entered the gel, following which the circulating pump was turned on, the current increased to 50 mA and the electrophoresis continued for at least 15 h. After this time the gels were removed and sliced for staining.

Total esterase activity was demonstrated using the staining procedure of Stern and Lewis [13] in which the gels were incubated for 45 min in a solution of α-naphthyl acetate and fast red TR salt in 0.2 *M* phosphate buffer pH 6.0, prior to fixing in a solution of methanol—water—acetic acid (50:50:10).

Staining for cholinesterase utilised the method of Karnosky and Roots [14] in which a 4-h incubation was carried out in a solution of butyryl thiocholine in phosphate buffer pH 6.0 containing copper sulphate and potassium ferricyanide.

RESULTS

The results of one-dimensional electrophoresis of serum taken from homozygotes for the normal, abnormal, fluoride and silent genes are shown in Fig. 1, staining having been performed with α-naphthyl acetate. Normal serum usually shows ten bands of activity in addition to the band due to non-specific aryl esterase. An eleventh band occasionally appears and may result from enzyme breakdown during storage. The bands due to cholinesterase are reduced to five in the case of patients homozygous for the abnormal and fluoride-resistant genes, and to one band only in the patient homozygous for the silent gene. In all cases the most marked differences are apparent in the bands showing intermediate mobility although in the case of the E_sE_s genotype slow running bands are also absent. Two-dimensional electrophoresis of normal serum is shown in Fig. 2 when no less than 22 areas of activity can be recognized. These are reduced to 12 in serum from the individual possessing only abnormal enzyme (Fig. 3), 10 for the E_fE_f genotype (Fig. 4) and 1 in serum from the E_sE_s homozygote (Fig. 5). Serum from heterozygotes for the normal enzyme with any of the three other variants cannot be distinguished with certainty from the normal pattern, but the electropherogram obtained with serum from an E_aE_f heterozygote is shown in Fig. 6 and this has clear differences from the other electrophoretic patterns obtained.

In all illustrations the position occupied by aryl esterase is shown as revealed by staining with α-naphthyl acetate. Staining with butyryl thiocholine

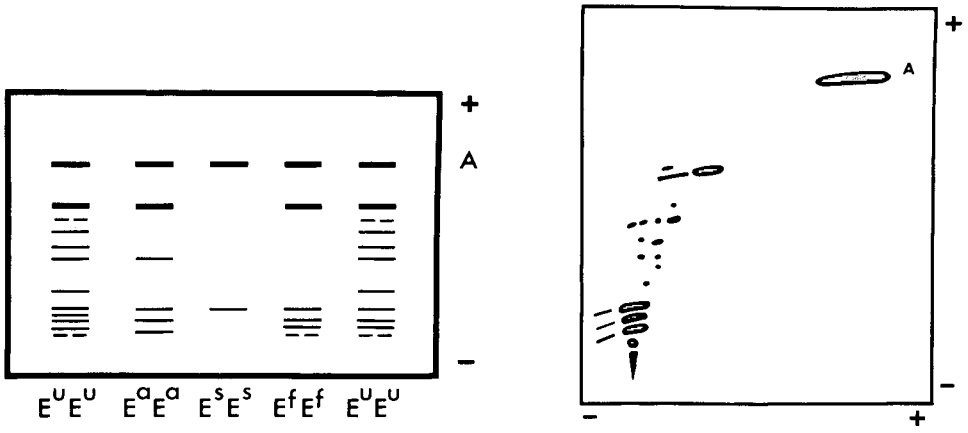

Fig. 1. One-dimensional electrophoretic patterns in gradient polyacrylamide gel of cholinesterase from individuals homozygous for the normal (E_uE_u), abnormal (E_aE_a), fluoride-resistant (E_fE_f) and silent genes (E_sE_s). Two normal sera are illustrated, one showing ten and one eleven bands. Activity due to both cholinesterase and aryl esterase is shown, band A representing the non-specific aryl esterase associated with albumin. Broken lines indicate areas of weak activity only.

Fig. 2. Two-dimensional electropherogram of cholinesterase isoenzymes from normal serum. Electrophoresis in the first dimension has incorporated constant density polyacrylamide and in the second dimension gradient gel polyacrylamide. Staining is for total esterase activity, band A representing the non-specific aryl esterase associated with albumin.

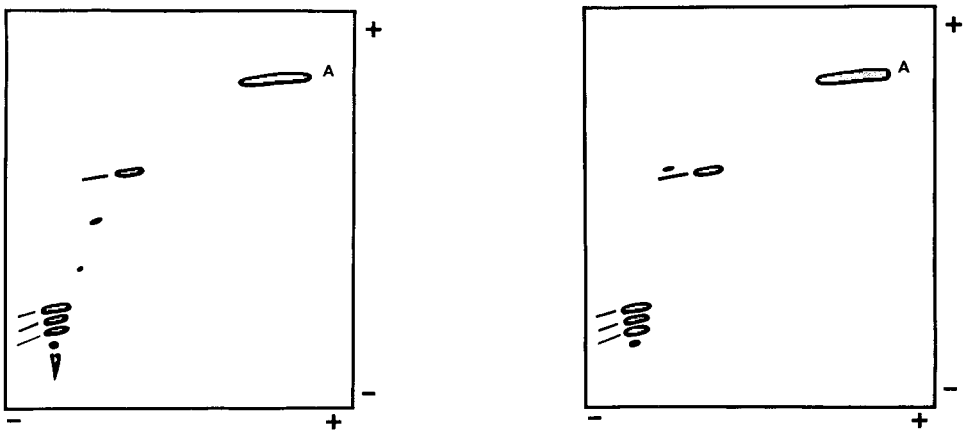

Fig. 3. Two-dimensional electropherogram of cholinesterase isoenzymes from the serum of an individual homozygous for the abnormal gene. Staining is for total esterase activity, band A representing the non-specific aryl esterase associated with albumin.

Fig. 4. Two-dimensional electropherogram of cholinesterase isoenzymes present in the serum of an individual homozygous for the fluoride-resistant gene. Staining is for total esterase activity, band A representing the non-specific aryl esterase associated with albumin.

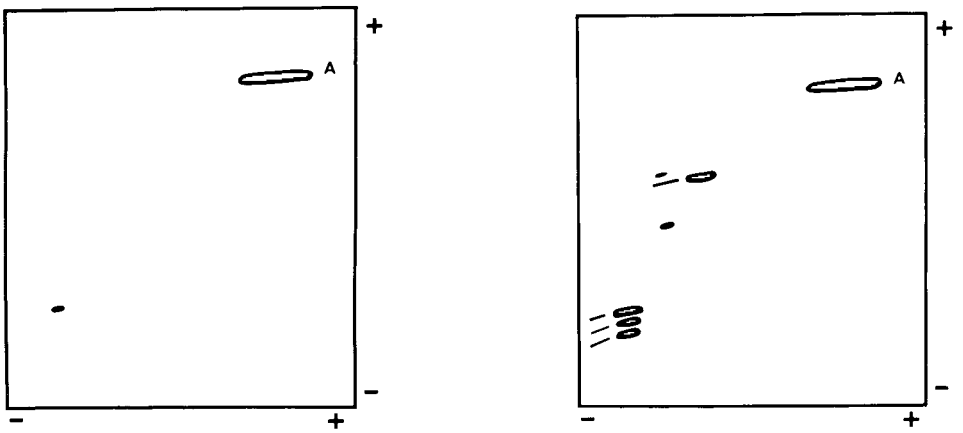

Fig. 5. Two-dimensional electropherogram of serum from an individual homozygous for the silent gene for cholinesterase. Staining is for total esterase.

Fig. 6. Two-dimensional electropherogram of serum from an individual heterozygous for the abnormal and fluoride-resistant genes for serum cholinesterase. Staining is for total esterase activity, band A representing the non-specific aryl esterase associated with albumin.

eliminates this band while incorporation of eserine sulphate into the α-naphthyl acetate results in patterns of activity indistinguishable from that given by serum from a homozygote for the silent gene, confirming the presence of a second serum enzyme with non-specific esterase activity. No alteration in electrophoretic pattern was observed following the inclusion of eserine into the staining reagent for serum from the homozygote for the silent gene, indicating in this patient, at least, a total absence of cholinesterase activity.

DISCUSSION

The biochemical identification of individuals showing abnormal sensitivity to short acting muscle relaxants of the succinyl dicholine type has until now depended entirely upon measurement of enzyme inhibition by means of dibucaine and fluoride. Unfortunately up to 50% of patients assessed as succinyl dicholine sensitive by clinical criteria have been shown to possess normal enzyme on the basis of biochemical findings. While some of these patients have doubtless been wrongly assessed clinically, in view of the many other causes of prolonged respiratory paralysis following anaesthesia [15], it is equally clear that some do not fall into this category and other abnormal biochemical variants of the enzyme undoubtedly exist. It is possible that investigations based upon the use of inhibitors may eventually resolve some of these problems, but it is equally possible that the application of different tools including electrophoresis may help to identify sensitive patients. Before this can be done, methods must be available for the separation of the different enzyme types likely to be present in serum with a high order of resolution and reproducibility in order that dif-

ferences in patterns can be recognised and interpreted. Until now techniques have not been good enough to be able to show differences between established genotypes, even less enzyme forms as yet undescribed. The technique described here enables marked differences between normal and abnormal enzyme forms to be distinguished and as a result may represent a useful tool for the study of succinyl dicholine sensitive patients.

Data at present available are insufficient to enable definite conclusions to be made concerning the interpretation of the two-dimensional electropherograms, but tentative hypotheses can be postulated.

A striking observation on all sera showing significant enzyme activity is the separation achieved during gradient polyacrylamide electrophoresis of the bands which are slowest moving during electrophoresis in the first dimension. In all cases a uniform separation has been achieved into either three of four bands. This indicates the presence of enzyme molecules having the same charge density but different molecular sizes and hence different molecular weights. In view of the uniformity of separation it is interesting to speculate that these bands indicate the presence of polymers of a basic enzyme subunit. Further information may come from experiments designed to reduce these enzymes to a single band of monomer.

Little information can yet be derived from the other areas of activity which have been revealed. However, the diffuse enzyme band shown to have slowest mobility in the second dimension in specimens from E_u and E_a homozygotes can perhaps be explained on the basis of an association of cholinesterase with lipoprotein in large molecular weight particles which have partially broken down during storage.

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Biomedical Applications

CHROMBIO. 011

MICRO METHOD FOR THE GAS CHROMATOGRAPHIC DETERMINATION OF SERUM THEOPHYLLINE UTILIZING AN ORGANIC NITROGEN SENSITIVE DETECTOR*

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SUMMARY

A gas chromatographic micro method utilizing an organic nitrogen sentitive detector for the determination of serum theophylline is described. The method incorporates 3-isobutyl-1-methylxanthine as the internal standard and involves extraction and off-column derivatization of theophylline and the internal standard to their pentyl derivatives. Using 50 μl of serum, concentrations of 1 μg/ml in serum can easily be measured. The method is linear up to 50 μg/ml and the precision of the method is 3.4% in the therapeutic range. No interferences from endogenous compounds or from drugs commonly co-administered with theophylline have been encountered.

INTRODUCTION

Theophylline, 1,3-dimethylxanthine, is a bronchodilator extensively used in the treatment of asthmatics, many of whom are pediatric patients [1]. The serum therapeutic level of theophylline has been demonstrated to be 10—20 μg/ml [2]. At higher serum concentrations, theophylline toxicity is expressed by a number of symptoms, including nausea, irritibility and convulsions; overdoses have also proven fatal [3—5]. The need for an accurate determination of serum theophylline levels is, therefore, well established. Existing spectrophotometric [6—8] and gas chromatographic methods using a flame ionization detector require fairly large amounts of serum (1—3 ml) to achieve the desired sensitivity and precision, an impracticality in pediatric cases. Also in particular

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with the spectrophotometric procedures, there is the possibility of interferences from caffeine, theobromine and other xanthines as well as barbiturates that may be co-administered.

Recently, the obvious need for a sensitive, specific assay for theophylline has produced a relatively large number of new assays utilizing gas chromatography [9—12] or high-pressure liquid chromatography [13—16].

Our gas chromatographic method utilizes an organic nitrogen-sensitive detector which has greatly enhanced sensitivity for organic nitrogen- and phosphorus-containing materials but has a decreased sensitivity as compared with a flame ionization detector for simple carbon containing materials [17].

This detector has both the sensitivity needed to determine theophylline levels in a micro sample, as well as the selectivity necessary to simplify the extraction procedure since it has decreased sensitivity to non-nitrogen- and phosphorus-containing species.

The method involves a single extraction and derivatization procedure which allows relatively fast, accurate and precise determination of serum theophylline levels. To permit easy determination by gas chromatography, the volatilities of theophylline and the internal standard, 3-isobutyl-1-methylxanthine, are enhanced by pentylation [18]. A standard curve is constructed by plotting the ratio of peak heights of derivatized theophylline to derivatized internal standard against theophylline concentration; serum unknowns are determined by reading their values off the standard curve.

EXPERIMENTAL

Reagents

Trimethylanilinium hydroxide (TMAH), 25% in methanol was prepared from trimethylphenylanilinium iodide (Eastman, Rochester, N.Y., U.S.A.) as described by Skinner et al. [19].

Theophylline was obtained from Mallinckrodt (St. Louis, Mo., U.S.A.). A stock of 500 μg/ml was prepared by dissolving 100 mg of theophylline in 200 ml of deionized water. Working standards were prepared by diluting the stock standard with deionized water to give 1, 2.5, 5, 10, 15, 25, 35 and 50 μg of theophylline per ml. N, N-Dimethylacetamide, spectro grade and the internal standard, 3-isobutyl-1-methylxanthine (>99%) were obtained from Aldrich (Milwaukee, Wisc., U.S.A.). The internal standard solution was prepared by dissolving 3-isobutyl-1-methylxanthine in 0.1 molar acetate buffer, pH 4.8. 1-Iodopentane was purchased from Eastman. All other solvents were of reagent grade.

Gas chromatography

A Perkin-Elmer dual column gas chromatograph, Model 3920, equipped with a phosphorus/nitrogen detector was used in all experiments. A glass 6 ft. × 2 mm I.D. 3% OV-17 on Gas-Chrom Q (100—120 mesh) (Applied Science Labs., State College, Pa., U.S.A.) column was found most suitable. The following gas chromatographic parameters were used: column temperature, 240° isothermal; detector temperature, 250°; injector temperature, 250°; helium flow-rate, 40 ml/min; amplifier range of 1 and electrometer attenuation setting at ×16;

recorder chart speed, 10 mm/min. The detector had a hydrogen flow of 1.5 ml/min, an air flow of 100 ml/min and course and fine current settings, to heat the bead, were 3 and 550, respectively. Under these conditions, the retention times were 1.5 min for theophylline and 1.9 min for the internal standard.

Procedure

Pipet 50 μl of serum or standard, 100 μl of acetate buffer containing the internal standard and 6 ml of dichloromethane into a Kimax 13 × 100 mm culture tube with PTFE-lined cap. Extract for 5 min on a wrist action shaker. Centrifuge for 2 min at 1000 *g* and remove the upper aqueous layer by aspiration and decant the dichloromethane layer into a clean, dry 15-ml conical centrifuge tube with PTFE-lined cap. Evaporate the dichloromethane to dryness by passing a stream of air into the tube placed in a 50° water-bath. Dissolve the dried samples in 50 μl of N,N-dimethylacetamide, add 10 μl TMAH and vortex for 1 sec to mix. Then add 10 μl 1-iodopentane, cap the tube, vortex again and incubate at room temperature for 10 min. Stop the reaction by adding 0.5 ml of cyclohexane—dichloromethane (95:5) to each tube and vortex 5 sec; a precipitate will form. Centrifuge at 1000 *g* for 2 min and transfer the cyclohexane—dichloromethane layer to a clean dry Kimax 13 × 100 mm culture tube with a glass disposable transfer pipette. Evaporate the organic solvent to dryness by passing a stream of air into the tube in a 50° water-bath. Redissolve each sample by adding 50 μl of methanol quickly capping each tube, and vortexing. Inject 1 μl of this methanol mixture into the gas chromatograph. The serum theophylline concentration is determined from a standard curve established by plotting the peak height ratios of standard to internal standard against the theophylline standard concentration.

RESULTS AND DISCUSSION

Fig. 1A illustrates a chromatogram of a serum free of theophylline without the internal standard added. Fig. 1B is the same serum spiked with theophylline and the internal standard added. Fig. 1C is the same serum with caffeine, theophylline, theobromine, internal standard and phenobarbital added. This figure illustrates the separation of common endogenous compounds which will be coextracted from serum with theophylline and the internal standard.

A typical standard curve is shown in Fig. 2. The linearity of the procedure was found to be good in the range of 0—50 μg/ml. The accuracy of the method was evaluated by recovery studies. Different amounts of theophylline were added to a theophylline free serum and the recovery calculated. Table I shows the results of the recovery study. The average recovery was 101% with a range of 97 to 106%.

The precision of the method was checked by analyzing 18 replicates of a serum sample from an asthmatic patient on chronic theophylline therapy. The mean serum concentration was found to be 14.7 μg/ml, with a standard deviation of 0.5 μg/ml and a coefficient of variation of 3.4%.

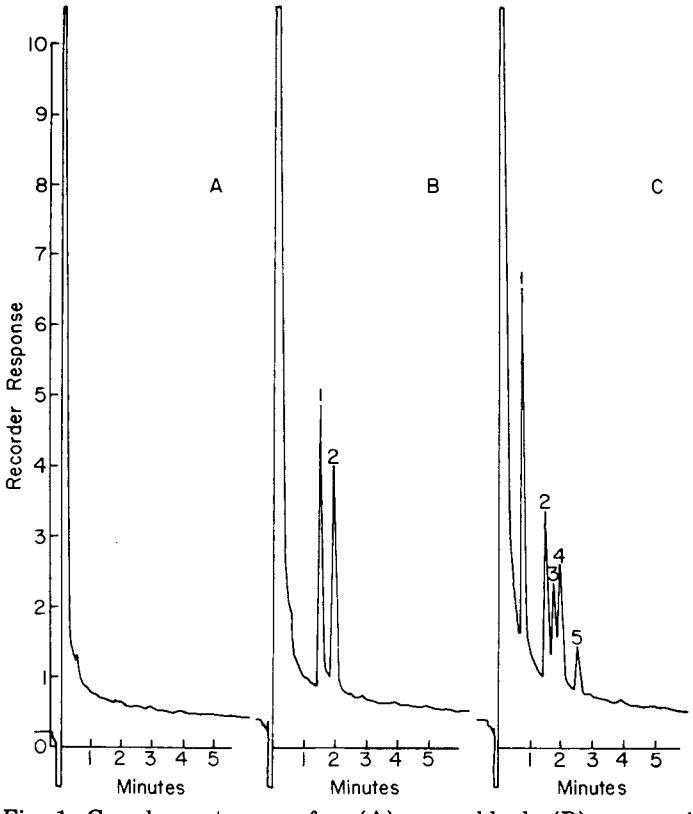

Fig. 1. Gas chromatograms for: (A) serum blank; (B) serum with theobromine (3), inter-internal standard (2); (C) serum with caffeine (1), theophylline (2), theobromide (3), internal standard (4), and phenobarbital (5) added.

TABLE I

RECOVERY OF ADDED THEOPHYLLINE FROM SERUM

Theophylline (μg/ml)		Recovery (%)
Added	Found	
5.0	5.3	106
15.0	15.0	100
25.0	25.4	102
35.0	34.0	97

Drug interferences were studied. Medications containing ephedrine, pseudoephedrine, phenobarbital, glyceryl gauiacolate, triprolidine, ampicilline, phenoxy-methylpenicillin, chlorpheniramine maleate and brompheniramine maleate, as well as pure theobromine, and caffeine were dissolved in water. Extraction, derivatization and gas chromatographic determination were performed as described. None of these compounds was found to interfere with the determination of theophylline.

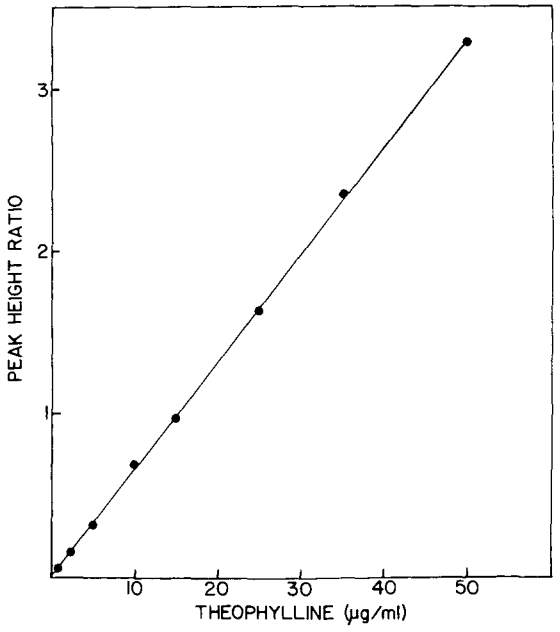

Fig. 2. Standard curve for theophylline.

Urinary metabolites of theophylline include 3-methylxanthine, 1-methyluric acid, and 1,3-dimethyluric acid [20]. In serum, however, only 3-methylxanthine has been detected in significant amounts [13]. Neither 3-methylxanthine nor uric acid was extracted under the above conditions.

With the micro method presented here, plasma or serum samples from patients receiving theophylline can be assayed rapidly and with good accuracy and precision.

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Biomedical Applications

CHROMBIO. 014

RAPID THIN-LAYER CHROMATOGRAPHIC METHOD FOR THE SIMULTANEOUS DETERMINATION OF CARBAMAZEPINE, DIPHENYLHYDANTOIN, MEPHENYTOIN, PHENOBARBITAL AND PRIMIDONE IN SERUM

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(First received March 5th, 1976; revised manuscript received June 11th, 1976)

SUMMARY

A thin-layer chromatographic method for the simultaneous determination of five anticonvulsant drugs is presented. The serum is extracted with toluene and the dried extract is dissolved in chloroform and applied on to a thin-layer chromatographic plate. After development, the plate is scanned at 215 nm without staining. The drug peaks are well defined. Most of the interfering substances that occur naturally in serum are soluble in and eliminated by the liquid front.

INTRODUCTION

The extraction procedure used in this work is the same as that used by Solow et al. [1] in their gas—liquid chromatographic (GLC) method. We have avoided the complicated staining techniques used in other quantitative thin-layer chromatographic (TLC) methods [2, 3] by measuring directly the diffuse light reflectance from the native drugs with a Zeiss chromatogram spectrophotometer at 215 nm.

EXPERIMENTAL

Apparatus

A BTL shaker (Baird & Tatlock, London, Great Britain), a Hamilton 50-μl precision syringe and a Zeiss chromatogram spectrophotometer were used.

Materials

Reagent-grade sodium dihydrogen orthophosphate, toluene, chloroform, acetone and absolute ethanol were obtained from Merck (Darmstadt, G.F.R.).

Pre-coated silica gel 60 TLC plates (without fluorescent indicator), 20 × 20 cm with a layer thickness of 0.25 mm, were also obtained from Merck.

A working standard solution of the native drugs caffeine, carbamazepine (Tegretol, donated by Ciba-Geigy, Basle, Switzerland), diphenylhydantoin, mephenytoin (Mesantoin, donated by Sandoz, Basle, Switzerland), phenobarbital and primidone (donated by Imperial Chemical Industries, Macclesfield, Great Britain) in absolute ethanol at a concentration of 25 mg per 100 ml of each drug was prepared. This solution is stable for at least 6 months at room temperature.

Procedure

A 300-μl sample of serum is placed in a 12-ml Sovirel screw-topped tube and 150 μl of 0.3 *M* NaH_2PO_4 solution and 5 ml of toluene are added. The tube is tightly closed and shaken vigorously for 3 min with a BTL shaker. The tube is then centrifuged for 10 min at 1400 *g*. A 4-ml volume of the toluene phase is transferred into a centrifuge tube, placed in a 60° water-bath and evaporated to dryness by means of a direct air stream. The residue is dissolved in 50 μl of chloroform and the solution obtained is applied with a Hamilton syringe as a very thin streak on a TLC plate at a width of 0.8 cm and along an axis 1.5 cm from the bottom. The TLC plate is placed in an unlined glass chromatography tank containing 100 ml of chloroform—acetone (85:15). Approximately 1.5 h are required for the development of the plate.

A standard curve is prepared by adding 5, 10, 20, 30 and 40 μl of the working standard solution to drug-free serum. The highest point in this curve corresponds to a drug level of 33 μg/ml in serum, which covers the therapeutic range of these drugs (samples with higher values are diluted with drug-free serum). The TLC plate is scanned at 215 nm for diffuse reflectance by means

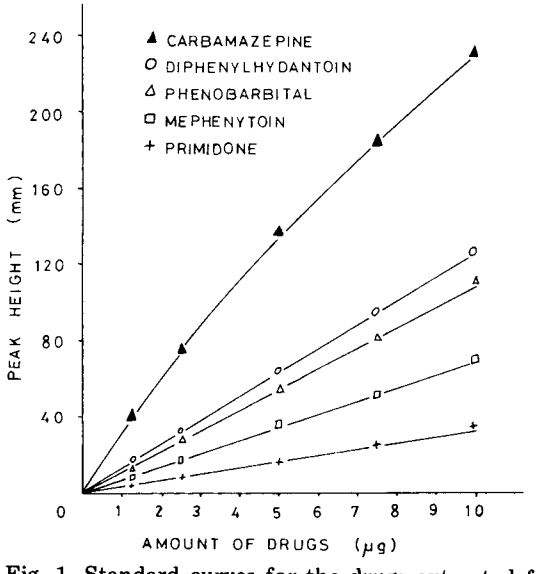

Fig. 1. Standard curves for the drugs extracted from serum, expressed as peak height versus amount.

of a Zeiss chromatogram-spectrophotometer. The result obtained from the standard curve (Fig. 1) is multiplied by 3.33 in order to calculate the concentration in microlitres per millilitre of serum.

RESULTS AND DISCUSSION

One of the advantages of our TLC method compared with most GLC methods is that derivatization is unnecessary. Some GLC assays have been described that do not require derivatization [4, 5], but in these assays diphenylhydantoin and phenobarbital give asymmetric peaks and a decrease in sensitivity. It has also been demonstrated that unmethylated diphenylhydantoin tends to be adsorbed on the support [6]. Another advantage of the TLC method over GLC methods is the possibility of scanning the spots on the TLC plate directly in the UV range in order to obtain the absorption spectrum and then comparing this pattern with known absorption spectra for positive identification. In the TLC method, time is employed more efficiently than in GLC methods: there is approximately 1.5 h of free time during the development of a TLC plate, compared with 20 min of waiting for a GLC run with only four of our drugs [7]. Our method has proved to be faster with a greater volume of tests, even when the time of application (1.5 min) and scanning (1.5 min) per test is taken into consideration.

The chromatographic separation shown in Fig. 2 was carried out at room temperature (20—25°). It was found that when the tank was not lined with paper, the time for chromatography increased from 60 to 90 min, but the separation pattern of the drugs was improved.

As shown in Fig. 3, the wavelength of 215 nm selected does not correspond to the absorption maxima of drugs tested. This wavelength was chosen because below 215 nm the high attenuation gave an unsteady baseline and in the range 270—300 nm, where caffeine and carbamazepine have their maximal absorption, the other drugs exhibit no absorption. Serum extracts from many of our patients showed a peak at R_F=0.22, which was found to be caffeine and was therefore included in our working standard (Fig. 2).

In our acid extraction method we used a dilution technique which maintains a constant buffered system by retaining the total serum volume. We found this to be necessary when diluting serum containing carbamazepine, which is usually extracted from an alkaline medium, in order to maintain a serum buffered system comparable with that employed for the standard curve. This precaution is not necessary when diluting the other drugs, but is used to lend uniformity to our procedure.

In over 1 year of using the method daily, we have observed few interferences One interferent, however, is ethylphenacemide, which has the same R_F value as diphenylhydantoin. These substances can be differentiated by using toluene—acetone (80:20). Another interferent is the antibiotic Bactrim, which is composed of sulfamethoxazol and trimethoprim; in this instance sulfamethoxazol has the same R_F value as carbamazepine. Differentiation between these two substances was accomplished with an alkaline extraction procedure. In cases of patients taking mephenytoin, demethylated mephenytoin [8] has the same R_F value as carbamazepine. This presents no problem, however, as

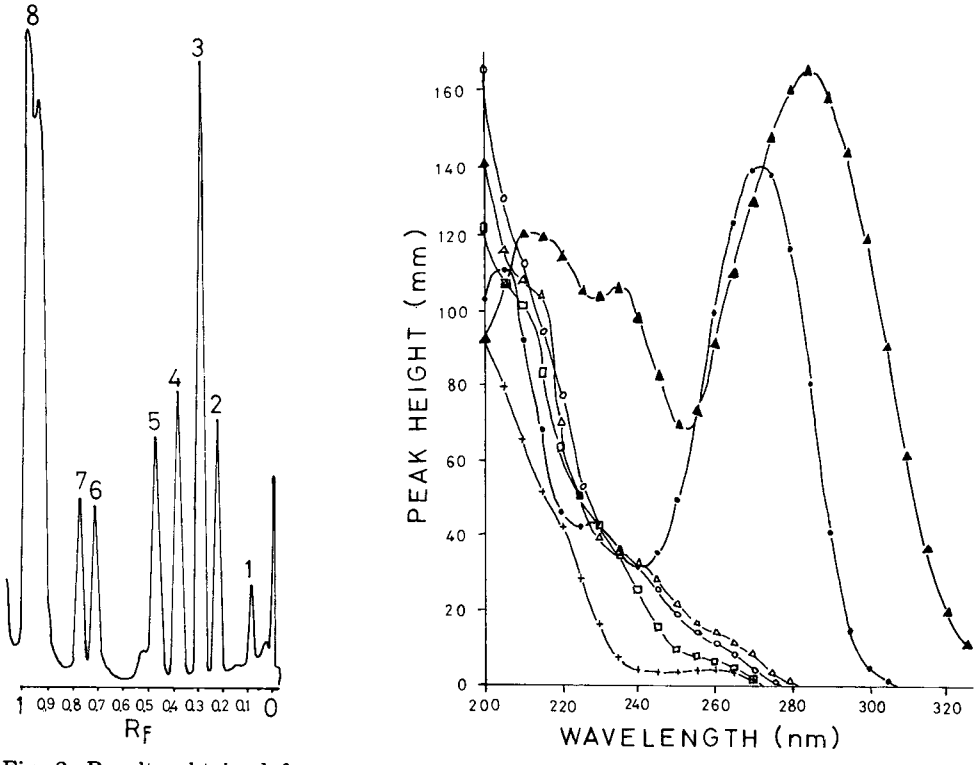

Fig. 2. Results obtained from a scan at 215 nm of a TLC plate after the separation of a serum containing 16.5 μg/ml each of primidone (1), caffeine (2), carbamazepine (3), diphenylhydantoin (4), phenobarbital (5) and mephenytoin (6). Peak 7 is an unidentified serum peak and peak 8 is the solution front of lipids.

Fig. 3. Ultraviolet absorption spectrum obtained by scanning a TLC plate containing 2.5 μg each of caffeine (•) and carbamazepine (▲) and 5.0 μg each of diphenylhydantoin (○), mephenytoin (□), phenobarbital (△) and primidone (+), which were applied directly on to the plate, chromatographically separated and measured in situ by means of a Zeiss chromatogram-spectrophotometer.

these two substances are not been taken together. As the metabolite of mephenytoin exhibits the same UV absorption curve as mephenytoin, it is easily distinguished from carbamazepine.

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

The reproducibility indicated in Table I is better than that given by Simon et al.'s TLC method [9], with which 30 samples of diphenylhydantoin gave a mean value of 23.0 μg/ml with a standard deviation of ± 2.07 μg/ml, which gives a coefficient of variation of 9.0%. On comparison with the GLC method of Grimmer et al. [10], which gave a coefficient of variation of 4.5% for diphenylhydantoin, and the Emit® (Palo Alto, Calif., U.S.A.) system, which

TABLE I

RECOVERY OF DRUGS FROM SERUM AND REPRODUCIBILITY OF THE METHOD

Drug	Recovery (%)	Reproducibility (30 samples)	
		Mean ± S.D. (μg/ml)	C.V. (%)
Carbamazepine	99.0	12.6 ± 0.5	4.3
Diphenylhydantoin	90.2	11.9 ± 0.6	4.7
Mephenytoin	79.3	13.7 ± 0.8	5.9
Phenobarbital	86.0	13.0 ± 0.8	6.0
Primidone	51.0	13.3 ± 0.7	5.2

gave a coefficient of variation with phenobarbital as high as 15.0%, we conclude that our quantitative TLC method is precise and rapid and our results are well within the accepted limits of deviation.

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Biomedical Applications

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THIN-LAYER CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF DIGITOXIN IN HUMAN SERUM

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SUMMARY

A specific assay for therapy control purposes and the toxicology of digitoxin is described. Digitoxin is isolated from serum (2 ml) by means of a single extraction with chloroform. Part of the organic phase is evaporated to dryness at 40° in a stream of dry air and the residue is dissolved in a small volume of chloroform. Conventional thin-layer chromatography (TLC) is used for the separation of digitoxin and its metabolites and digoxin. Fluorescence of the spots can be generated by treatment with hydrogen chloride vapour under the influence of a quartz-halogen lamp. Interpolation between two reference standards gives the concentration in the sample. Linearity is observed between 2 and 20 ng. Extensive recovery studies in the therapeutic range of 10—50 ng/nl have been performed. The results showed an overall recovery of 99.1% with a standard deviation of 11.2%. The sensitivity is 1—2 ng of digitoxin when standards are applied on a conventional TLC plate with a small diameter. The time needed for one analysis is about 4½ h, the real time of analysis being 2½ h; in serial studies, 20—24 determinations could be made daily. Whereas in the descriptions of most methods it is not mentioned whether digitoxin metabolites are co-determined, the present assay separates the digitoxin completely from the other compounds in serum, and thus enables the total fate of digitoxin in relation to the clinical effect to be studied more specifically than by radioimmunoassay.

INTRODUCTION

Digitoxin is prescribed in cases of cardiac failure and has a notably small therapeutic index. Although nowadays digoxin is used more frequently than digitoxin, it is still not certain which of these drugs is the most useful. In discussing this point [1, 2], the differences in the pharmacotherapeutic properties of digitoxin and digoxin become important in three main areas: protein binding [3], biological half-life and interactions with other drugs [4]. Digoxin seems to be preferred in cases of heart insufficiency alone, while digitoxin is suitable in cases of heart insufficiency combined with renal insufficiency [3]. To solve

such problems, it is essential that further research into the biological half-lives, interactions with other drugs and the protein binding of these two important drugs should be carried out.

A method is required that is very sensitive, accurate, specific, rapid, cheap and can be performed in any clinical laboratory. These requirements present the clinical pharmacist with a challenging analytical problem. Various procedures for the determination of digitoxin published during the past 10 years are mostly very time consuming or not specific enough [5—10]. Smith [11] developed a radioimmunoassay (RIA) method that is very sensitive, accurate, simple and requires only 1 h, but there are disadvantages related to digitoxin metabolism [12] and to the fact that patients with maintenance dosages of digoxin give false-positive values corresponding to levels of digitoxin of 1—2 ng/ml in serum. Another disadvantage is that laboratories which lack radiochemical equipment cannot use this procedure. Therefore, in order to eliminate these disadvantages, we decided to develop a new method [13—15], based on a fluoridensitometric approach as already applied to kinidine [16] and to amitriptyline and nortriptyline [17]. The conventional TLC method has been compared with the RIA method and the results are discussed.

EXPERIMENTAL

In establishing this method for the determination of digitoxin we tried to optimize important factors such as the thin-layer material, the sample application procedure, the use of one-dimensional or multi-dimensional development and the purity of the reagents. In this method, the ratio of digitoxin to interfering substances must be relatively high because of the low therapeutic blood level of digitoxin.

The method involves a single extraction of digitoxin from serum, concentration of the extract followed by thin-layer chromatography, a fluorigenic reaction with an acidic vapour and artificial light, quantitative measurement of the fluorescence of digitoxin with a densitometer and calculation of the amount in an unknown sample by interpolation from a calibration graph.

Apparatus and reagents

The TLC plates were Kieselgel 60 DC-Fertigplatten of dimensions 20 × 20 cm (E. Merck, Darmstadt, G.F.R.). A 10- and a 25-μl Hamilton syringe (with a PTFE-coated plunger and a PTFE gasket tip) with a Hamilton repeating dispenser were used. A Desaga chromatography tank, a Vortex-Genie Vibromixer, a GLC-2 centrifuge with a maximum speed of 4955 *g*, a Bolex Lite 2M quartz-halogen lamp (1000 W) and a Vitatron TLD-100 densitometer were used.

Chloroform (spectroscopic quality) and hydrochloric acid (pro analysi; minimum concentration 37%) were obtained from Merck. Methanol (minimum 99.5%), ethanol (99.5%) and acetone (99.5%) were obtained from J.T. Baker Chemicals (Deventer, The Netherlands); digitoxin, Ph. Ned.* Ed. VII or Merck; digitoxigenin bisdigitoxoside, digitoxigenin monodigitoxoside, digitoxigenin and digitoxin, Boehringer (Mannheim, G.F.R.); digoxin, Ph. Ned. Ed. VII or Merck; digoxigenin bisdigitoxoside, digoxigenin monodigitoxoside and digoxigenin, Boehringer. The stock solution of digitoxin was prepared by dissolving 10.000 mg of digitoxin in 100 ml of chloroform, and was stored in a refrigerator. Standard solutions of 1 and 2 ng/μl of digitoxin in chloroform were used.

*Pharmacopoeia Neerlandica.

Extraction

A 2-ml volume of serum is shaken for 2 min with 8 ml of chloroform in a 10 ml glass-stoppered centrifuge tube in a Vibromixer and the mixture is centrifuged for 15—20 min at 1417 *g*. Then 7 ml of the supernatant are transferred into a 10-ml glass-stoppered tube with a tapered base of volume 0.2 ml. The chloroform extract is evaporated to dryness at 40° in the tube by the passage of dried compressed air for 20—30 min to avoid adsorption. The residue is dissolved in 200 μl of chloroform by mixing vigorously again for 2 min to maintain high accuracy for digitoxin at the low concentrations involved.

Chromatography

With a 25-μl Hamilton syringe (right-angled), volumes of $x_1 = 20\,\mu l, x_2 = 40\,\mu l$ and $x_3 = 50\,\mu l$ of the unknown solution (x) and volumes of $b_1 = 20\,\mu l$, $b_2 = 40\,\mu l$ and $b_3 = 50\,\mu l$ of serum blanks are applied on a thin-layer plate, together with 4 ng (S_1), 9 ng (S_2), 7 ng (S_3), 14 ng (S_4), 5 ng (S_5) and 15 ng (S_6) of standard solutions of digitoxin using a 10-μl Hamilton syringe. The sequence of application is $S_1, b_1, x_1, S_2 ; S_3, b_2, x_2, S_4 ; S_5, b_3, x_3, S_6$. This is a large number of standards, which may be decreased by duplicate spotting of a few standards, so that on one plate more unknown samples can be examined because of the linearity and reproducibility of the method (see *Reproducibility of the method).* The centres of the spots are 1.5 cm apart and 1.8 cm from the edge of the plate. Before it was decided which solvent to use, at least 10 solvent systems were tested, because of the need to effect a separation from both metabolites and interfering substances in serum, and also to take account of the study of Zullich et al. [18] on the metabolism of digitoxin in rats. The eluent selected was chloroform—methanol—acetone—water (64:6:28:2), the development being carried out without a saturated tank with an elution time of about 20 min. The elution distance is 10 cm and the plate is dried directly with a warm hair drier to avoid sideways diffusion.

Fluorigenic reaction and detection

The determination of digitoxin and digoxin in biological material is not possible because of the lack of sensitivity of the fluorescent product being formed by a number of reagents. Only Seipel et al. [19] and Van Oostveen [20] have determined digitoxin in serum and digoxin in urine, respectively, with a TLC method using fluoridensitometry. The fluorescence was generated by a chloramine—trichloroacetic acid spray reagent. Other workers [21—26] have performed spectrofluorimetric determinations on pharmaceutical preparations. Some other spray reagents [27] and solvents for TLC mentioned in the literature lack stability and sensitivity, while Hoeke and Exler [28] and Frijns [23, 24] generated fluorescence by treatment with hydrogen chloride vapour under the influence of heat and artificial light, respectively. There was a low sensitivity or detection limit and the fluorescence colours were not reported.

As hydrogen chloride vapour gives a better sensitivity than other reagents and a reagent in the form of a vapour gives a homogeneous partition over the entire plate, we developed a more sensitive and less time-consuming modified assay. An increase in fluorescence intensity can be obtained by covering the chromatogram with a non-volatile fluid flim such as liquid paraffin (Ph. Ned. Ed. VII).

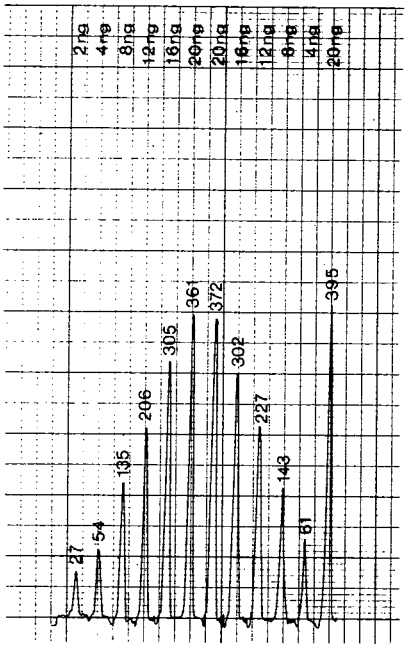
To obtain optimal fluorescence, the TLC plate is placed in a tank saturated with hydrochloric acid for at least 1 h, in the absense of daylight, and is subsequently irradiated with artificial light (quartz-halogen lamp) for 12—13 min. This process converts digitoxin and digoxin and their metabolites into fluorescent compounds. Under UV light, an orange-yellow fluorescence is observed; after removing the acid from the plate by placing it in a drying oven for 1—2 min, the fluorescence appeared to be light yellow. This fluorescence is stable for days, provided that the plate is kept in the dark.

The spots are quantified by measuring the fluorescence directly with a Vitatron TLD-100 densitometer. The operating conditions are as follows: light source, mercury lamp; mode, In II (+); level, f; coarse zero, 7; damping, 2; span, 10; excitation filter, 365 nm; emission filter, 536 nm (optimum for digitoxin and metabolites); diaphragm, 1.0; swing, 2; scanning speed, 1 cm/min; paper speed, 0.5 cm/min; integrator, 8.

RESULTS AND DISCUSSION

Linearity

It appeared that there was a linear relationship between the amount of digitoxin applied to the plate up to 20 ng and the corresponding fluorescence of the digitoxin derivative (integrated peak area) (Figs. 1 and 2). The numbers at the top of the peaks (integrated peak area) in Fig. 1 represent the fluorescence of the spots on the TLC plate. Digitoxin is spotted in duplicate in multiples of 2 ng. The spots are scanned on their R_F values, because a calibration graph is used,



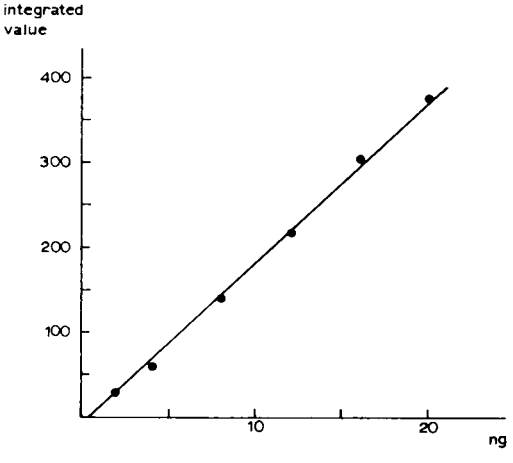


Fig. 1. Densitogram of different amounts of pure digitoxin.

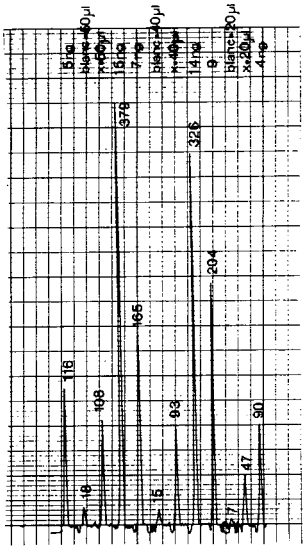
Fig. 2. Calibration graph for digitoxin (2—20 ng).

the shape of the spots (signal to noise ratio) and time saving. The calibration graph (Fig. 2) of digitoxin is constructed from the average value of the duplicate determinations from the densitogram of different amounts of pure digitoxin in Fig. 1.

To confirm these results, a number of duplicate determinations were carried out on solutions containing 2, 4, 8, 12 and 16 ng of the drug. After statistical analysis of the results [29], it appeared that the deviation from linearity was not statistically significant at a threshold value of 5%.

Calculation of digitoxin in an unknown sample

Fig. 3 shows a densitogram for the determination of digitoxin in an unknown sample. The unknown x is spotted in amounts of 20, 40 and 50 μl; blanks of the same amounts are run at the same time to give another impression of the sensitivity, accuracy and precision of the method. The calibration graph from which the digitoxin in the unknown sample is determined by interpolation (Fig. 4) shows again the good linear relationship. From the average value of the number of pulses of the unknown sample of digitoxin (x_1, x_2 and x_3) minus the avarage value (b_1, b_2 and b_3) of the serum blank, the amount in nanograms can be calculated directly from the calibration graph. It may be also sufficient to make the calculation from one or two reference standards (see *Reproducibility of the method*), depending on the purpose.



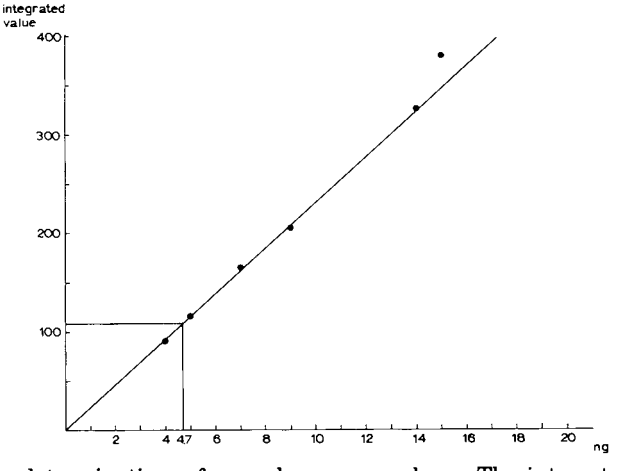


Fig. 3. Densitogram to show the determination of an unknown sample, x. The integrated value for each spot is given at the top of the curve.

Fig. 4. Calibration graph for the determination of the digitoxin in the unknown sample from Fig. 3.

Specificity of the method

The separation of digitoxin and its main metabolites and digoxin was carried out with the above solvent system. The dihydro products, with much lower fluorescence intensities and which rarely occur as metabolites, were not used.

TABLE I

hR_F VALUES OF DIGITOXIN, ITS MAIN METABOLITES AND DIGOXIN

Stationary phase: Kieselgel 60 (Merck, Fertigplatten). Solvent system: chloroform—methanol—acetone—water (64:6:28:2). Running distance, 10 cm; environment, unsaturated; fluorigenic reaction, 75 min under influence of HCl vapour in the dark and 12 min with both HCl vapour and artificial light.

Substance	hR_F value
Digitoxin	35
Digitoxigenin bisdigitoxoside	39
Digitoxigenin monodigitoxoside	44
Digitoxigenin	56
Digitonin	Origin
Digoxin	24

In Table I, it is shown that digitoxin is well separated from its main metabolite digitoxigenin bisdigitoxoside, other metabolites and digoxin.

Reproducibility of the method

A volume of 20—100 μl of digitoxin was added to blank serum the mixture was shaken for 2 min in a Vibromixer. The serum was incubated for at least ½ h. Table II summarizes the results of a series of recovery experiments in the therapeutic range of 10—50 ng/ml of digitoxin in serum and indicates the accuracy of the TLC method. In Fig. 5, the results of a recovery experiment for digitoxin are shown in a densitogram. The unknown x was spotted in a volume of 30 μl from the 200 μl of extract. The blank was also spotted in a volume of 30 μl. After calculation, we obtained a recovery of 114% at the 20 ng/ml level, which is above the average found value. In the experiment shown in Fig. 5, a standard of 8 ng was spotted six times ($n = 6$). These integrated values indicate the precision of the method and how it is influenced by the quantitative application, the chromatography and the fluorigenic reaction. The average value was 313 counts with a standard deviation of 8.3 and a relative standard deviation of 2.6%.

TABLE II

RECOVERY OF DIGITOXIN FROM SERUM ON CONSECUTIVE DAYS

Day	Digitoxin serum concentration (ng/ml)*				
	10	20	30	40	50
1	121	114	100	87	94
2		113	95	91	91
			96		
3	90	105	94	93	84
4	118		98		

*The overall recovery (n = 17) for digitoxin is 99.1% with a standard deviation of 11.2% with a range of 84—121 ng/ml.

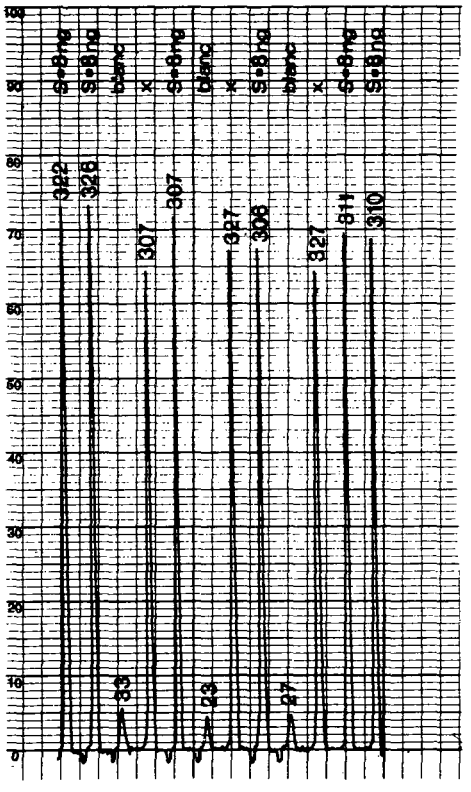


Fig. 5. Densitogram showing the results of a recovery experiment for digitoxin. For details, see text.

It appeared that the quality of the Merck plates was not consistent and both the reproducibility and the detection limit could be influenced to a large extent in different charges. Therefore, control of the charge is necessary. The relative standard deviation for 5 ng of digitoxin on one plate should not be more than 6%, when the detection limit is less than 0.5 ng.

All previous specific methods based upon chromatographic techniques are time consuming. All other methods for use with biological material are based on the affinity of digitoxin to biological receptors. Moreover, the specificity is dependent on the quality of the biological system, which may be uncertain. Our fluoridensitometric TLC method is principally suitable for determining both digitoxin and digoxin in any biological material, e.g., urine, faeces, heart tissue and muscle tissue.

Although the recoveries and the other features outlined above were acceptable, there were differences between the values obtained by fluoridensitometry and by RIA. The results of both methods applied to 11 patients are shown in Table III.

Since Vöhringer and Rietbrock [12] found that digitoxin is the main substance in the chloroform extract and all values obtained by the RIA method are

TABLE III

DETERMINATION OF SERUM LEVELS OF DIGITOXIN IN PATIENTS BY BOTH THE RIA AND TLC METHODS

Standard deviation 0.25 (n = 11); relative standard deviation of the mean ratio, S_{rel} = 53%.

Patient	RIA method [11]	TLC method]13]	Ratio serum levels, TLC/RIA
1	33	11.9	0.36
2	48	14.1	0.29
3	49	16.7	0.34
4	56	20.1	0.34
5	25	21.3	0.85
6	25	2.2	0.09
7	62	24.9	0.40
8	17	5.7	0.34
9	10	9.5	0.95
10	33	18.6	0.56
11	17	10.9	0.64
			Mean: 0.47

higher than those obtained by the TLC method (Table III), there must be a co-determination of other substances in the radioimmunoassay. Further, it can be concluded from the work of Vöhringer and Rietbrock [12] that it is important not to co-determine the main metabolites, because of changing of the ratio of chloroform-soluble and -insoluble metabolites in the first 48 h from 8.4 to 3.3, and because of the different half-lives of digoxin, digitoxin and their metabolites.

In the fluoridensitometric TLC method, there is no co-determination, and therefore this method is specific and is not influenced by structurally related compounds. The difference between the values obtained by the two methods (Table III) might be due to a lack of specificity in RIA or partly to a lack of attainment of equilibrium in the fluorimetric recovery experiments, although our recovery was almost 100% with reasonable accuracy. Hence the efficiency of recovery of the TLC method cannot be the reason for the difference in serum levels or for the difference in the precisions.

Any lack of specificity in the RIA method might be due to the co-determination of interfering substances such as metabolites of digitoxin, structurally related drugs and their metabolites and other structurally related substances. When the values obtained by the RIA and TLC methods do not bear a constant ratio to each other or there is an average ratio with a small standard deviation, while both methods have good precision, it can be concluded that the extent of the lack of specificity of the RIA method will be expressed by the standard deviation of the ratio of the serum levels obtained in the two methods.

CONCLUSION

A TLC method that is not too time consuming, not too expensive, with good accuracy and precision and specific and sensitive for the determination of digi-

toxin in the serum of patients in the therapeutic and toxic range of 10—60 ng/ml has been developed.

Improvements may come from investigating not only the sample preparation and the use of programmed multiple development [14, 15, 30, 31] and of high-performance TLC material [14, 15, 32], but also a better densitometric integration system. The result could be a fluoridensitometric TLC method that has advantages over RIA. As the fluorigenic reaction of digoxin and methyldigoxin surpasses even that of digitoxin, it seems possible that digoxin (lower therapeutic level 1—2 ng/ml) could likewise be assayed by fluoridensitometry.

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Biomedical Applications

CHROMBIO.007

Note

Identification of an interfering compound in the gas—liquid chromatographic determination of N^2,N^2-dimethylguanosine★

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(Received April 26th, 1976)

In the search for biological markers for cancer, the minor methylated nucleosides and pseudouridine, components of transfer RNA, have been suggested as indicators of cancerous growth. Since most of the minor nucleosides are excreted from the body in the urine, a gas chromatography method [1] has been developed to monitor the levels of pseudouridine (ψ), N^2,N^2-dimethylguanosine (m_2^2G) and N^1-methylinosine (m^1I) in urine. Reports by Waalkes et al. [2, 3] have indeed shown elevated levels for these markers in the urine of cancer patients with Burkitt's lymphoma, lung, colon, breast and other cancerous tumours.

In approximately 5—10% of the urine samples from cancer patients, an abnormally high m_2^2G value has been observed. Examination of these samples by gas chromatography—mass spectrometry (GC—MS) has confirmed the presence of a major interfering compound co-eluting with m_2^2G, which could not be readily resolved by the use of other GC columns. This paper reports on the isolation and identification of this interference.

EXPERIMENTAL

Reagents and equipment

A 402 F&M gas chromatograph with a two column oven bath, two hydrogen flame detectors, two differential electrometers, a linear temperature programmer, and a Honeywell Electronik 18 recorder was used for all gas chromatographic analysis. All gas chromatographic separations were done with a column of 3% SE-30 on 100—120 mesh Supelcoport.

★Contribution from the Missouri Agricultural Experiment Station and National Cancer Institute. Journal Series No. 7534. Approved by the Director.

A CEC 21-110 mass spectrometer interfaced by means of a "Llewellyn type" silicone membrane separator with a Varian 1500 gas chromatograph was used for all GC—MS work. The mass marker was provided by a Jeolco JEC-6 spectrum computer. All high-resolution measurements were made either by peak matching or using a Jeolco JMA-1C-0 automatic data analyzer micro densitometer to read exact masses from the photographic plates.

A Waters Assoc. M-6000 high-pressure solvent delivery system was used as the pump for the liquid chromatographic isolation. A 30 cm × 0.9 cm column filled with Bio-Rad Aminex A-5 cation-exchange resin was used for the sample purification. The detector was a Laboratory Data Control Duo Monitor set to monitor at 254 and 280 nm.

A Cary Model 10 ultraviolet—visible spectrophotometer was used for all UV spectra.

A Bruker HX-90 Fourier Transform 90-MHz nuclear magnetic resonance (NMR) spectrometer equipped with a Nicolet 1080 signal average computer was used for ^{13}C and ^{1}H NMR spectra.

All other reagents and supplies were the same as previously reported [1].

Isolation of unknown compound

The unknown compound was isolated from urine by use of a modification of the charcoal adsorption method published by Chang et al. [1] followed by cation-exchange chromatography. The urine sample was filtered through a 3-μm millipore filter and then applied to a charcoal column. The ratio of urine to charcoal was normally 5:2 (v/v). The quantity of urine usually processed was 25—50 ml. After application of the urine sample to the column, it was washed with about 25 ml of water per milliliter of charcoal. The column was also washed with 1% pyridine in water with a volume of 25 ml being used per milliliter of charcoal. The unknown was then eluted with 95% ethanol. The volume of ethanol used was twice the column bed volume. The eluate from the charcoal column was taken to dryness at 60—70° under nitrogen gas and the residue dissolved in 1 *N* acetic acid. Approximately 1 ml of acetic acid was used for each 15 ml of urine. This solution was then filtered through a 1-μm millipore filter.

The elution characteristics of the unknown was then determined in the following manner. The equivalent of about 2 ml of urine in 1 *N* acetic acid was then placed on a cation-exchange resin column and eluted with deionized water using a UV detector to monitor the elution. The elution position of the unknown was determined by collecting all UV-absorbing peaks. Each fraction was evaporated to dryness, then derivatized as the trimethylsilyl derivative by the procedure of Chang et al. [1]. The derivatized fractions were checked by GC retention time and GC—MS. The unknown was eluted after three column dead volumes.

The unknown, in the eluate from the charcoal column, was then isolated on a preparative scale by loading the equivalent of 15—20 ml of urine on the cation-exchange column and collecting the appropriate fraction. The purified material was rechromatographed on the same column with approximately 60% of the peak being collected. These purified fractions were dried at 60—70° under a stream of nitrogen. The homogenity of the fraction was checked by gas—liquid and thin-layer chromatography. This residue was used for these structural investigations:

The trimethylsilyl and d_9-trimethylsilyl derivatives were subjected to both low- and high-resolution mass spectrometry.

The IR spectrum was obtained as a pellet in potassium bromide.

The ^1H and broad band ^1H decoupled ^{13}C NMR spectra of the unknown were obtained using ^2H_2O as the solvent with tetramethylsilane and hexafluorobenzene as the respective reference or lock compounds (capillary insert).

An ultraviolet spectrum of the unknown was obtained from its solution in water at pH's 1, 7, and 10.

RESULTS AND DISCUSSION

The mass spectral fragments from the trimethylsilyl and d_9-trimethylsilyl derivatives of the unknown are presented in Table I. Table II presents the ^{13}C and ^1H NMR spectral data.

TABLE I

MASS SPECTRAL DATA OF UNKNOWN

TMS-derivative *m/e* (intensity)	d_9-TMS-derivative *m/e* (intensity)	No. of silicon atoms
73 (100)	82 (100)	1
147 (84)	162 (78)	2
217 (69)	232 (74)	2
223 (51)	235 (27)	2
295 (96)	313 (92)	2
375 (71)	402 (54)	3
464 (7)	499 (8)	4
672 (2.5)	714 (3.0)	5
687 (0.3)	732 (0.3)	5

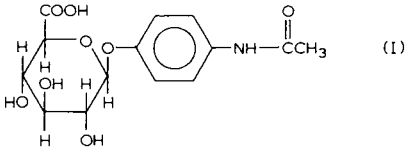
TABLE II

NMR DATA ON UNKNOWN

^{13}C NMR		^1H NMR	
δ	intensity	δ	intensity
23.28	1	2.71	3
70.83	1	4.26	4
71.83	1	7.88	4
73.23	1		
75.82	1		
101.89	1		
118.01	2		
124.81	2		
137.86	1		
154.69	1		
173.79	1		
178.00	1		

The IR spectrum showed a broad band centered at 3400 cm^{-1} and multiple bands between 1650 and 1800 cm^{-1}. These absorption bands are indicative of hydroxyl groups and multiple carbonyls. The ultraviolet spectrum showed a maximum at 241 nm, a shoulder at 280 nm, and typical end absorption and did not vary upon changing the pH. These are typical characteristics of a substituted aromatic ring, lacking ionizable functionality in conjugation with the aromatic system. The absence of phosphorus and sulfur was verified by chromatographing the derivatized unknown on a gas chromatograph equipped with a flame photometric detector in both the P and S modes.

The above data taken in conjunction with the following NMR and MS spectral designations are consistent with the assignment of the following structure of the glucuronide of 4-hydroxyacetanilide (I) to the unknown.



(I)

The acetyl methyl group appears as a singlet at δ 2.71 in the ^{1}H NMR spectrum and at δ 23.78 in the ^{13}C NMR spectrum. The methide protons of the glucuronic acid group appear as a multiplet centered at δ 4.26 in the ^{1}H NMR and the corresponding carbons are located at δ 70.83, 71.83, 73.23, 75.82, and 101.89 (anomeric carbon) in the ^{13}C NMR spectrum. The *para* disubstituted benzene ring is observed as a characteristic AB quartet at δ 7.88 in the ^{1}H NMR spectrum and at δ 118.01, 124.81, 137.86 (N-bound C), and 154.69 (O-bound C) in the ^{13}C NMR spectrum. The exact mass measurement of 687.2999 for the molecular ion is in good agreement with this elemental composition. This compound would be expected to be silylated at five sites as shown by the mass spectrum of the d_9-trimethylsilyl derivative. Additional proof is provided by the fact that the ultraviolet spectrum of 4-hydroxyacetanilide in water is identical with that for this unknown.

The origin of the glucuronide of 4-hydroxyacetanilide from the human body would be through the administration of 4-hydroxyacetanilide (paracetamol), which is a common acetylsalicylic acid substitute.

Final confirmation of the origin for the unknown was obtained by the administration of paracetamol to two people. The unknown was not present in the urine before, but was confirmed to be present by GC—MS after administration of paracetamol. This further substantiates the structural assignment of the unknown to the glucuronide of 4-hydroxyacetanilide.

ACKNOWLEDGEMENT

The authors wish to express their deep appreciation to Mr. Roy Rice for the mass spectra and Dr. Robert W. Zumwalt and Mr. Kenneth Kuo for many useful discussions.

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Journal of Chromatography, 143 (1977) 109—111
Biomedical Applications

CHROMBIO.003

Note

Improved radioimmunoelectrophoretic assay of human serum thyroxine-binding globulin

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(Received February 27th, 1976)

Direct determination of human serum thyroxine-binding globulin (TBG) concentration was first described by Freeman and Pearson [1], using two-dimensional immunoelectrophoresis and autoradiography. The method was time consuming, and costly in its use of antiserum and [^{125}I]thyroxine (T_4). A more rapid assay resulted from the adaptation of the Laurell monorocket technique for use in TBG measurement [2, 3]. Barbital, an inhibitor of T_4—TBG binding, was used as a buffering medium, as originally described by Laurell [4]. Improved results were reported by Drysdale et al. [5], who used a phosphate buffer to increase the binding of [^{125}I]T_4 to TBG and an antiserum-free starter gel to stabilize the [^{125}I]T_4—TBG complex. In this report we describe a radioimmunoelectrophoretic assay of TBG, in which labelling of serum samples with high specific activity [^{125}I]T_4 occurs during electrophoresis, thereby simplifying sample preparation and speeding the assay.

MATERIALS AND METHODS

Materials

High-specific-activity [^{125}I]T_4 (approximately 600 mCi/μmole) was synthesized by the method of Weeke and Örskow [6]. Antiwhole human serum was obtained from Wellcome (Beckenham, Great Britain). Antiserum to a human α-globulin fraction, rich in TBG, was produced in sheep. The fraction for immunization was obtained by electrophoresis of whole human serum in agarose gel. The globulin fraction was cut from the undried gel, emulsified with complete Freund's adjuvant, and injected intramuscularly. RP-XOmat X-ray film was obtained from Kodak UK (London, Great Britain). Agarose and other reagents were obtained from BDH (Poole, Great Britain).

Experimental procedure

Laurell monorocket electrophoresis was carried out using 0.05 *M* phosphate buffer at pH 7.4 according to the method of Drysdale et al. [5]. Electrophoresis continued for 12 h, at a voltage of 2 V/cm across the plate.

In an important modification, high specific activity [^{125}I]T_4 was added to the starter gel (Fig. 1) in a concentration of 500 nCi/ml. Antigen wells, 2 mm diameter, were punched in the starter gel 5 mm from the starter gel—antibody gel interface, and serum samples of 2 μl were added to each well.

The antibody gel consisted of either a 25% dilution of Wellcome antiwhole human serum or a 2% dilution of anti-α-globulin antiserum.

The dried electrophoretic plates were subjected to autoradiography for 12 h followed by rapid processing in an RP-XOmat Processor, Model M 6-N [2].

RESULTS

The results of a typical assay using anti-α-globulin antiserum are shown in Fig. 1. Use of 25 × 8 cm glass plates allowed measurement of twenty samples and six standards in one assay.

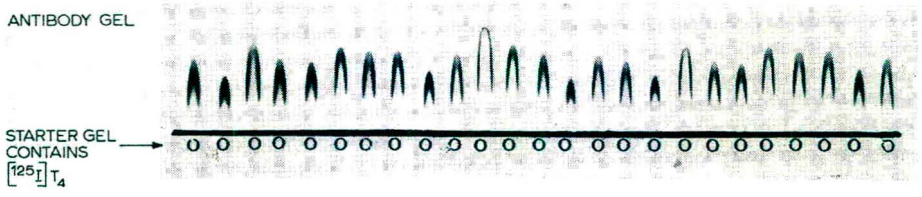

Fig. 1. Autoradiograph showing TBG peaks. Antibody gel contains sheep anti-α-globulin.

TBG results were expressed as a percentage of a working standard serum [5]. In thirteen consecutive assays, measurement of a single standard yielded a mean of 48 (±2.7 S.D.), giving a relative standard deviation of ±5.6%. The sensitivity of the method was 5% of the working standard serum, although smaller amounts could be measured by increasing the sample size.

DISCUSSION

The use of monorocket electrophoresis for TBG measurement has been described using both barbital [2, 3] and phosphate buffers [5]. In both systems, serum samples were individually labelled and incubated with [^{125}I]T_4 before electrophoresis. In the modified method described here, labelling of TBG with [^{125}I]T_4 occurs during electrophoresis, thereby simplifying the assay and reducing the time required for completion. The antigen wells were situated near the starter gel—antibody gel interface, so that [^{125}I]T_4, which has a greater electrophoretic mobility than TBG in the buffer system used, could flow past the serum samples during electrophoresis. The use of a high-specific-activity preparation of [^{125}I]T_4 enables a reduction in the time needed for autoradiography, and reduced the total time for TBG measurement to 24 h.

The assay could be performed using a commercial antiwhole human serum,

but clearer peaks were obtained, using less antiserum, when an antibody to α-globulin was used. The method is sufficiently rapid, precise and inexpensive to make it an acceptable alternative to the indirect and expensive T_3 uptake test.

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Journal of Chromatography, 143 (1977) 112—114
Biomedical Applications

CHROMBIO.009

Note

Thin-layer chromatographic method for the quantitative analysis of paracetamol (N-acetyl-*p*-aminophenol) in blood plasma

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Paracetamol* is a commonly used non-prescription analgesic. It is potentially a hepato-toxic drug, and the determination of the plasma levels of the drug is helpful in the management of patients who have ingested an overdose of paracetamol [1]. Various methods for the estimation of paracetamol in body fluids have been critically reviewed recently [2]. Thin-layer chromatography (TLC) has been used to separate paracetamol from body constituents prior to its quantitation by ultraviolet absorptiometry [3].

This note describes a simple and rapid method for the estimation of "free" paracetamol in plasma. It consists of extraction and concentration of drug from a small volume of plasma. The drug is separated from other plasma constituents by TLC, and is made visible by spraying with Folin—Ciocalteu reagent which is specific for phenols. Quantitation is effected by measuring the intensity of coloured spots directly on the plate by a densitometer.

EXPERIMENTAL

All the chemicals used were of analytical grade, and were used without any further purification. Paracetamol was kindly supplied by McNeil Labs., Don Mills, Canada. Precoated silica gel plates 10 × 20 cm (EM Labs., Elmsford, N.Y., U.S.A.; Cat. No. 5610) were used. Premade Folin—Ciocalteu [4] reagent was purchased from BDH, Toronto, Canada. A Clifford densitometer (Model 445) was used to measure the intensity of coloured spots.

*In North America, paracetamol is known as acetaminophen.

Standard solutions

Paracetamol: 100 mg was weighed and dissolved in 100 ml of absolute methanol. Its concentration was checked by measuring its absorbance (λ_{max} 249 nm, $A^{1\%}_{1cm}$ 897) [5]. The solution was stored at 4°.

Plasma standards: 20 mg/l, 10 mg/l, and 5 mg/l were prepared by adding 2 ml, 1 ml and 0.5 ml of the above stock solution respectively to 100 ml of filtered plasma. These standards were divided into 2-ml aliquots and frozen.

Extraction

Samples of 0.5 ml of plasma (test, 20, 10 and 5 mg/l standards) were pipetted into PTFE-lined screw-capped 12 × 120 mm culture tubes. To each tube, 5 ml of dichloromethane was added and the tube was vortex mixed. About 2 g of anhydrous sodium sulphate were added to each tube, which was again vortex mixed. The tubes were centrifuged. Three ml of dichloromethane extract from each tube were transferred to correspondingly labelled 16 × 100 mm disposable glass tubes, and evaporated to dryness in a water-bath at 50°. The tubes were cooled and the residue was dissolved in 100 µl of methanol by vortex mixing. The tubes were kept tightly stoppered to prevent evaporation of methanol. From each tube, 10 µl of methanolic extract were applied to a thin-layer plate (along the length edge) in this sequence: S_{20}, S_{10}, S_5, x, x, S_5, S_{10}, S_{20}.

The plate was developed in chloroform—acetone (60:40) up to a height of about 8 cm. The plate was dried in the fume hood for 10 min, and was sprayed with 10% sodium carbonate followed by Folin—Ciocalteu reagent, diluted 1:2 with deionized water. The plate was allowed to dry in air for about 10 min and was then scanned in the densitometer for the measurement of colour intensity using 650-nm filter. A standard curve for the standards of 5, 10 and 20 mg/l versus their average peak heights was plotted. The concentration of the unknown was determined from the standard curve and its average peak height.

RESULTS AND DISCUSSION

Paracetamol has been recovered in 50% yield in the present procedure using a single extraction with dichloromethane. Extraction efficiency was somewhat better (70%) in a single extraction with diethyl ether. However, overall precision of the method was not so good when diethyl ether was used in place of dichloromethane to extract paracetamol from plasma. There was no improvement in recovery when the pH was lowered to 4 or 2 [6]. A number of solvents have been described for thin-layer separation of paracetamol on silica gel [5]. Paracetamol moves on silica gel plates without trailing in the developing solvent selected for this procedure (R_F = 0.42). Folin—Ciocalteu reagent proved to be the best visualisation agent for paracetamol among a large number of available reagents for the detection of paracetamol [5]. This reagent has adequate sensitivity to detect paracetamol at therapeutic concentrations. Extracts of drug-free plasma or plasma containing a number of drugs that are commonly ingested in overdose (barbiturates, salicylates, hydantoins, methylprylon, meprobamate) do not produce coloured spots after TLC and spraying with this detection reagent. However,

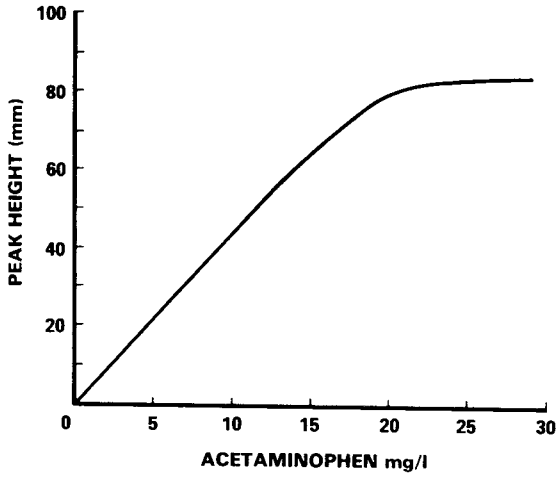

Fig. 1. Standard curve (peak height versus concentration) for the estimation of paracetamol (acetaminophen) in plasma.

the possibility of a phenolic metabolite of any of these drugs producing a spot with similar R_F value and colour to those of paracetamol cannot be ruled out.

This procedure is linear up to 20 mg/l (Fig. 1). In case a test specimen shows paracetamol concentration higher than 20 mg/l, TLC is repeated by spotting 10 μl of extracts of each of the standards, but only 5 μl of the extract of test specimens.

This procedure shows a within batch variation of 2.9% at a concentration of 5 mg/l (n = 7) and a between batch variation of 6.3% (n = 9) at a concentration of 8 mg/l.

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Biomedical Applications

CHROMBIO. 025

Book Review

Fortschritte chromatographischer Methoden und ihre Anwendung in der klinischen Biochemie, (IV. Symposium Chromatographie in der klinischen Biochemie, Leipzig, 24. und 25. Mai 1973; Ergebnisse der experimentellen Medizin, Vol. 20), edited by J. Wagner, VEB Verlag Volk und Gesundheit, Berlin, 1976, XX + 263 pp., price M 30.00.

In 1976 the 5th Symposium on Chromatography in Clinical Biochemistry took place at Leipzig, organized jointly by the Chromatography Sections of the Gesellschaft für klinische Chemie und Laboratoriumsdiagnostik der D.D.R., of the Chemische Gesellschaft der D.D.R. and of the Czechoslovak Chemical Society. The proceedings of the preceding Symposium appeared shortly before. In spite of the delay between the Symposium and the appearance of its account, the information has not become so outdated as to be without interest to those using or about to use chromatographic methods in clinical chemistry and biochemistry. The reason may be that, in clinical laboratory methodology, as in some other branches of science and technology, further development mostly supplements, modifies, extends and rediscovers already known approaches without rejecting them as obsolete and unfit for use. For instance, the experience with the organization of work in the gas chromatography (GC)—mass spectrometry—computer laboratory in Oslo and the way in which new nosological entities were discovered by these techniques there (plenary lecture by Jellum et al.), and the review (or rather concise monograph, 20 pp.) of M. Doss on the chromatography and pathobiochemistry of porphyrins will remain sources of inspiration and enlightenment for a long time.

The proceedings contain some 15 papers from the German Democratic Republic and 26 from 9 other European countries. The style and consistent spelling show that the manuscripts have undergone thorough linguistic revision. Misprints abound; fortunately, they do not interfere with the understanding.

The communications show that the organizers, Drs. Wagner and Macek, succeeded in attracting protagonists in their respective fields and that chromatography has reached a high level of quality and interest in European medical research.

The book is introduced by a historical paper that recalls the introduction of chromatography in 1903 with Tswett's advanced views on the necessity to unite the chemical and (ultra)structural approaches in biology. Jellum's fascinating plenary lecture has already been mentioned. Five sections deal

with certain classes of compounds.

In the amino-acid field, derivatization to 4-substituted 2,2-bis(chlorodifluoromethyl)-1,3-oxazolidinones (Hušek) seems very promising.

For steroids, thin-layer chromatographic and GC methods (the latter especially as used by Gleispach for quantitative purposes) were given attention.

The lipids section includes papers with both technical and diagnostic bias. Thus Mangold and Mukherjee described their quantitative method of evaluation of thin layers inside a test-tube by mineralization and flame ionization or thermal conductivity detecting and presented a review on the biochemistry of alkoxy and alkenoxy lipids.

In the protein field, gel permeation, collagen-gel and other types of chromatography have been used for proteins and GC for carbohydrates in glycoprotein hydrolysates.

Among other classes of compounds, four papers were devoted to catecholamines, their metabolites and related substances. In addition to the review by Doss, two further papers dealt with porphyrins. Endogenous sucrosuria was described by Gorodetskii. An anti-doping laboratory bus used for the supervision of cycling contests was displayed by Chundela and Šlechtová. Surprisingly high levels of halothane were found by GC in the air of an operating theatre (Schöntube and Schädlich). Glass-adherence and plastic-membrane chromatography of whole cells was presented by Přistoupil.

The book culminates with its final section on affinity chromatography introduced by a brief but stimulating review by Fischer. An elegant application was that of Hampl and Stárka: sexual-hormone-binding globulin was purified on a column of 5α-androstane-3β,17β-diol 3-hemisuccinate coupled to Enzacryl AA. "Affinity elution" by ligand solutions from cellulose phosphate columns is described by Schulz et al.

Clinical biochemists and biochemically orientated physicians may find some valuable stimuli and suggestions in perusing the book.

Hradec Králové (Czechoslovakia) I.M. HAIS

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BIOMEDICAL APPLICATIONS

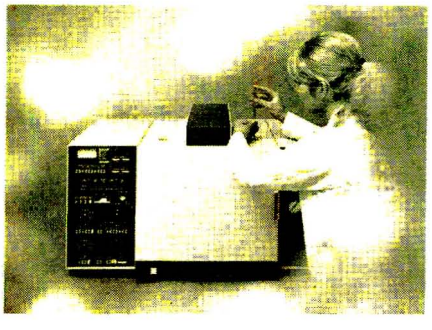
NEWS SECTION

APPARATUS

N-926

DUAL-COLUMN GAS CHROMATOGRAPH

The new Varian GC 3700 is a dual-column gas chromatograph with newly designed injectors, detectors, pneumatics, column oven, digital controls and automation features. The modular design allows interchangeability and upgradeability of all components. The automatic linear temperature programmer provides accurate



control over the range −99° −420°. The instrument is available as a TCD instrument, as a dual flame-ionization, or a dual electron-capture, detector instrument. Also, a combination of the TC, FI and EC detectors is available. Combined with Varian's AutoSampler and CDS-111 Chromatography Data System the instrument can provide complete automation from sample injection to final report.

N-932

DATA SYSTEM FOR AUTOMATED CHROMATOGRAPHY

The new Varian data system (CDS-111) automatically integrates and calculates the data from gas and liquid chromatographs, automatically quantitating most chromatograms on its own. The areas of simple and complex peaks can be measured. Internal standard, external standard, calibration factor, relative response factor, area percent and normalized area percent methods, separately or in combination, are used to calculate the results in forms such as weight percent, mole percent or volume percent. The system interfaces with most gas and liquid chromatographs. In combination with the Varian GC 3700, the instrument can automate the whole chromatographic process from injection to final report. The latter shows the chromatographic results, as well as GC and AutoSampler parameters (e.g. carrier-gas flow, temperature, vial and rack number).

N-933

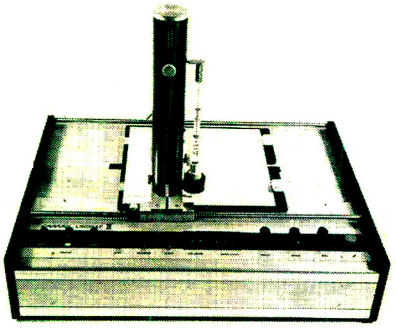
HARD-LAYER TLC PLATES

New England Nuclear is now manufacturing hard-layer TLC plates that feature a silica-gel coating and an inert, organic binder. The hard-layer surface can be written on without chipping. Suitable for a wide range of analyses, the prescored plates are additionally designed for autoradiographic visualization.

N-929

SAMPLE APPLICATOR FOR TLC

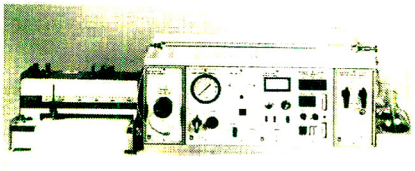
The Camag Linomat III has been designed for the quantitative application of samples in the form of narrow bands onto TLC layers, the sample solution being sprayed off the tip of a syringe using nitrogen as carrier gas. Thus practically any volume can be sprayed onto the layer in the form of a narrow uniform band without disturbing the layer surface. Operational data are pre-selectable and electronically controlled: sample volume, speed of application, and band length (continuously adjustable from 0 mm to 199 mm). The delivered volume is digitally displayed.

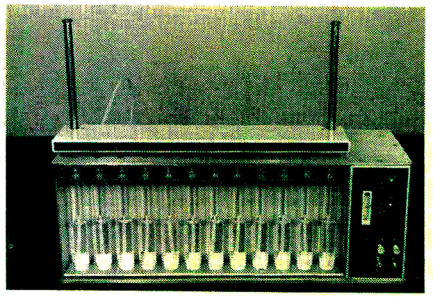


N-925

NEW HIGH-PERFORMANCE LIQUID CHROMATOGRAPH ON U.S. MARKET

A modular high-performance liquid chromatograph has been introduced to the U.S. by the LC Instrument Co. (a subsidiary of Lachat Chemicals). UV, RI or fluorescence detectors are available. The unit is available with or without a gradient pump. The unit is available in two models: one with minimum components, the other with any desired combination of components.





N-928

CAMAG ELUQUICK

Eluquick is a new Camag instrument for the quantitative elution of sample zones from TLC sheets and is particularly useful in serial analysis where sample quantities are not extremely small. Sample areas are cut out, placed in test-tubes and elution solvent added. After a pre-set soaking time a stream of air is passed through the liquid for a time also pre-set, effecting a thorough but gentle mixing so that the adsorbent layer does not flake off the sheet. The clear eluate is removed by decanting, pipetting, aspirating, etc. 12 samples can be simultaneously eluted in the Eluquick.

N-937

MICRO-HYDROGENATOR

A simple apparatus for the hydrogenation of small quantities of material is described in Supelco's Bulletin 759. The apparatus can be used quantitatively, for example, to determine total unsaturation, or qualitatively as in the conversion of unsaturated parent compounds for subsequent GLC analysis. The micro-hydrogenator was designed to work in the micro- and low-milligram range, 100 mg or less.

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

CHEMICALS

N-965

AGARO-GEL™ HYDRATED FILMS

Agaro-Gel™ Hydrated Film (Product No. A 1266) is a water-saturated non-buffered agarose gel. It is intended to be used as a support medium for various electrophoresis techniques such as protein, lipoproteins, hemoglobulin and isoenzymes separations. The gel is well suited for Sigma Procedure No. 705-EP colorimetric (LDH Isoenzymes).

The gel size (2¼ × 4½ in.) is adaptable for use with most commercially available electrophoresis equipment.

N-930

DRUGS OF ABUSE KITS

Kits of analytical standards for drugs of abuse (exempt from DEA regulations) are now available from Analabs. The kits cover the routine problem areas of opiates, morphine substitutes and barbiturates. Each standard is dissolved in high-purity *tert*-butyl alcohol (in some cases with a small amount of methanol added).

N-938

NEW EM LABORATORIES CATALOGUE

EM Laboratories (E.Merck) have recently issued a new catalogue – "Products for Chromatography" – listing their complete range of sorbents, pre-coated TLC and HPLC products, pre-packed columns, spray reagents, reference standards and auxiliary products. Details of Template and a listing of technical bulletins and reprints available are also included.

PROCEDURES

N-936

CARRIER GAS PURITY EFFECT

Available from Precision Gas Products is a technical paper outlining the effects of carrier gas purity in gas chromatography. The paper details results of several experiments using various, purposely contaminated samples and purification techniques.

N-966

COLORIMETRIC LDH ISOENZYME ASSAY

Following electrophoresis, the zones of LDH isoenzyme activity are visualized after incubating samples in a solution containing optimal amounts of NAD, L(+)lactate, tetranitroblue tetrazolium and phenazine methosulfate. The color reagent is more sensitive than existing methods involving use of nitroblue tetrazolium. Sigma Procedure No. 705-A is for use with agarose gels, such as Agaro-Gel™ Hydrated Film, Product No. A 1266, while Sigma Procedure No. 705-C is designed for cellulose acetate electrophoresis.

NEW BOOKS

Adverse effects of environmental chemicals and psychotropic drugs, Vol. 2, Neurophysiological and behavioural tests, edited by M. Horváth, Elsevier, Amsterdam, Oxford, New York, 1976, xv + 334 pp., price Dfl. 103.00, US$ 39.75, ISBN 0-444-41498-3.

Pharmacological and chemical synonyms; A collection of names of drugs, pesticides and other compounds drawn from the medical literature of the world, compiled by E.E.J. Marler, Excerpta Medica, Amsterdam, New York, 6th ed., 1976, ca. 520 pp., price Dfl. 195.00, US$ 74.95, ISBN 90-219-9249-3.

Amino acids, peptides and proteins – An introduction, by H.-D. Jakubke and H. Jeschkeit, Macmillan, London, 1976, 336 pp., price ca. £ 10.50, ISBN 0-333-17886-6.

Art in biosynthesis – The synthetic chemist's challenge, Vol. 1, by D. Ranganathan and S. Ranganathan, Academic Press, New York, London, 1976, xi + 249 pp., price US$ 11.00, £ 6.05, ISBN 0-12-580001-0.

Fortschritte chromatographischer Methoden und ihre Anwendung in der klinischen Biochemie (IV. Symposium Chromatographie in der klinischen Biochemie, Leipzig, 24. und 25. Mai 1973; Ergebnisse der experimentellen Medizin, Vol. 20), edited by J. Wagner, VEB Verlag Volk und Gesundheit, Berlin, 1976, xx + 263 pp., price DM 30.00 (unbound).

CALENDAR OF FORTHCOMING MEETINGS

February 28–March 4, 1977
Cleveland, Ohio, U.S.A.

28th Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy

Contact:
John E. Graham, Program Chairman, Koppers Company, Inc., 440 College Park Drive, Monroeville, Pa. 15146, U.S.A.

April 26–28, 1977
Bratislava, Czechoslovakia

5th International Symposium. Improvements and Application of Chromatography in the Chemical Industry

Contact:
Ing. J. Remeň, Analytická sekcia ČS VTS, pri. n. p. Slovnaft, 82300 Bratislava, Czechoslovakia

May 22–27, 1977
Davos, Switzerland

International Symposium on Microchemical Techniques 1977

Contact:
Dr. W. Merz, BASF A.G. Untersuchungslaboratorium, WHU, D-6700 Ludwigshafen, G.F.R. (Further details published in Vol. 117, No. 1)

July 17–22, 1977
Birmingham, Great Britain

4th SAC Conference

Contact:
Dr. A. Townshend or Dr. A.M.G. Macdonald, Chemistry Department, The University, P.O. Box 363, Birmingham B15 2TT, Great Britain

September 12–16, 1977
Leipzig, G.D.R.

International Symposium on Control Mechanisms in Bio- and Ecosystems

Contact:
Wissenschaftlich-Technische Gesellschaft für Mess- und Automatisierungstechnik in der Kammer der Technik der DDR, PSF 1315, 1086 Berlin, G.D.R.

September 27–30, 1977
Salzburg, Austria

3rd International Symposium on Column Liquid Chromatography

Contact:
Professor Dr. J.F.K. Huber, Analytisches Institut der Universität Wien, Währinger Strasse 38, A-1090 Vienna, Austria

November 7–11, 1977
Amsterdam, The Netherlands

Chromatography '77. 12th International Symposium on Advances in Chromatography

Contact:
Professor A. Zlatkis, Chemistry Department, University of Houston, Houston, Texas 77004, U.S.A.

GENERAL INFORMATION

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

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

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

Title. The title of the paper should be concise and informative. Since titles are widely used in information retrieval systems, care should be taken to include the key words. The title should be followed by the authors' full names, academic or professional affiliations, and the address of the laboratory where the work was carried out. If the present address of an author is different from that mentioned, it should be given in a footnote. Acknowledgements of financial support are *not* to be made in a footnote to the title or name of the author, but should be included in the Acknowledgements at the end of the paper.

Summary. Full-length papers and review articles should have a summary of 50–100 words which clearly and briefly indicates what is new, different and significant. In the case of French or German articles an additional summary in English, headed by an English translation of the title, should also be provided. (Short communications and Notes are published without a summary.)

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

References. References should be numbered in the order in which they are cited in the text, and listed in numerical sequence on a separate sheet at the end of the article. The numbers should appear in the text at the appropriate places in square brackets. In the reference list, periodicals [1], books [2], multi-author books [3] and proceedings [4] should be cited in accordance with the following examples:

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 R. D. Marshall and A. Neuberger, in A. Gottschalk (Editor), *Glycoproteins*, Part A, Elsevier, Amsterdam, 2nd ed., 1972, Ch. 3, p. 251.
4 R. H. Doremus, B. W. Roberts and D. Turnbull (Editors), *Growth and Preparation of Crystals, Proc. Int. Conf. Crystal Growth, Coopertown, N.Y., August 27–29, 1958*, Wiley, New York, 1958.

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

Proofs. One set of proofs will be sent to the author to be carefully checked for printer's errors. Corrections must be restricted to instances in which the proof is at variance with the manuscript. "Extra corrections" will be inserted at the authors' expense.

Reprints. Fifty reprints of Full-length papers, Short communications and Notes will be supplied free of charge. Additional reprints can be ordered by the authors. An order form containing price quotations will be sent to the authors together with the proofs of their article.

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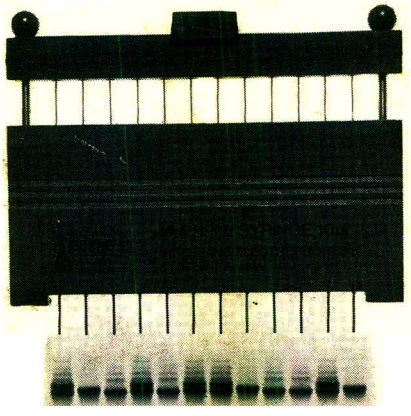

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