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6th Int. Symp., Biomedical Applications of Chromatography, Hluboká, May 21–24, 1978

JOURNAL OF CHROMATOGRAPHY BIOMEDICAL APPLICATIONS

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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.

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Radiochromatography

The Chromatography and Electrophoresis of Radiolabelled Compounds

T. R. ROBERTS, *Shell Biosciences Laboratory, Sittingbourne Research Centre, U.K.*

JOURNAL OF CHROMATOGRAPHY LIBRARY - Volume 14

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

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

CONTENTS: Chapters: 1. Introduction. 2. Radioactivity detectors used in chromatography. 3. Radio-paper chromatography. 4. Radio-thin-layer chromatography. 5.Radio-electrophoresis. 6. Radio-column chromatography. 7. Radio-gas-liquid chromatography. 8. Miscellaneous applications related to radiochromatography. Appendix. Subject index.

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Affinity Chromatography

JAROSLAVA TURKOVA, *Czechoslovak Academy of Sciences, Prague.*

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

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

CONTENTS: Chapters: 1. Introduction. 2. The principle, history and use of affinity chromatography. 3. Theory of affinity chromatography. 4. Application of affinity chromatography to the quantitative evaluation of specific complexes. 5. General considerations on affinant—sorbent bonding. 6. Choice of affinity ligands for attachment. 7. Hydrophobic chromatography, covalent affinity chromatography, affinity elution and related methods. 8. Solid matrix supports and the most used methods of binding. 9. Characterization of supports and immobilized affinity ligands. 10. General considerations on sorption, elution and non-specific binding. 11. Examples of the use of affinity chromatography. 12. Immobilized enzymes. Subject Index.

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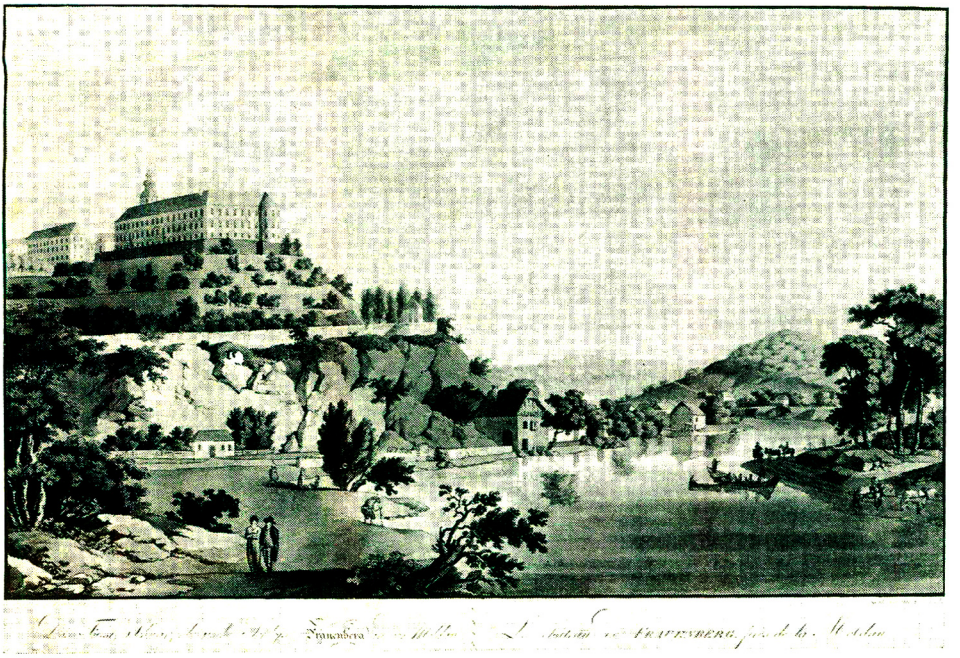

6TH INTERNATIONAL SYMPOSIUM

BIOMEDICAL APPLICATIONS OF CHROMATOGRAPHY

Hluboká (Czechoslovakia), May 21–24, 1978

Edited by:

K. MACEK
(Prague)

I. M. HAIS
(Hradec Králové)

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SIXTH INTERNATIONAL SYMPOSIUM: BIOMEDICAL APPLICATIONS OF CHROMATOGRAPHY, HLUBOKÁ (CZECHOSLOVAKIA), MAY 21–24, 1978

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PREFACE

The Sixth International Symposium "Biomedical Applications of Chromatography" was held in Hluboká castle from May 21st to 24th, 1978. The Symposium was organized by the Chromatography Group of the Czechoslovak Chemical Society and sponsored by The Institue of Physiology, Czechoslovak Academy of Sciences and The Chromatography Group of the Society for Clinical Chemistry and Laboratory Diagnostics of the German Democratic Republic. The symposium was attended by 90 active participants from 14 countries.

The Chromatography Section of the Czechoslovak Chemical Society organized the first symposium already 17 years ago, in 1961, in the castle of Liblice near Prague. This meeting dealt with general problems of paper chromatography [1]. The second [2] and third [3] meetings were also organized in Liblice and for the fourth symposium, Frascati, near Rome was selected [4]. The fifth symposium took place in Carlsbad [5]. It has become a tradition that these symposia were always devoted to advances in a narrow area of a certain chromatographic technique. Since 1966, in cooperation with The Chromatography Group of the Society for Clinical Chemistry and Laboratory Diagnostics of G.D.R., meetings on chromatography in clinical chemistry were regularly held in Leipzig. By coincidence there have also been five of these symposia.

Respecting the fact that currently the application of chromatographic techniques to biomedical research is increasing, it was decided to deal with this topic at the sixth symposium and most probably at the forthcoming symposium also. With the current flood of information it would seem that the most useful form of such a meeting would be a microsymposium with a limited number of participants who are actively working with these methods.

The present symposium was divided into two parts: plenary lectures, and original contributions, with appropriate discussion in sections. The following plenary lectures (not published in this special issue) were presented during the meeting:

(1) E. Jellum: Metabolic profiles

(2) E. Tomlinson: The relation between chromatographic migration and biological activity

(3) K. Macek and J. Wagner: Chromatography in clinico-chemical laboratory

(4) C. Horváth: Biomedical applications of reversed phases

In special sections the following topics were discussed:
(1) Metabolic profiles and screening for metabolic errors
(2) Chromatography in routine laboratory
(3) Relation between chromatographic migration and biological activity
(4) Specialized techniques for biomedical research
(5) Drug monitoring and pharmacokinetic studies

Only a few of the total 43 contributions presented during the symposium are published in extenso in this issue.

KAREL MACEK

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GAS—LIQUID CHROMATOGRAPHY OF ISOBUTYL ESTER, N(O)-HEPTAFLUOROBUTYRATE DERIVATIVES OF AMINO ACIDS ON A GLASS CAPILLARY COLUMN FOR QUANTITATIVE SEPARATION IN CLINICAL BIOLOGY

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SUMMARY

A gas chromatographic method adapted to routine analysis has been developed for quantitative separation on glass capillary columns for free proteic and other known amino acids normally or abnormally found in physiological fluids. The procedure involves ion-exchange chromatography and isobutyl ester, N(O)-heptafluorobutyrate derivatization of free plasma and urine amino acid samples. Derivatized components were ascertained by combined gas chromatography—mass spectrometry. The use of glass for the capillary column is mandatory to achieve qualitative and quantitative analysis of the known occurring amino acids in urine and small plasma samples. Quantitative analysis of several types of human amino acid disorders are presented.

INTRODUCTION

For the last twenty years much work has been undertaken on the problem of gas—liquid chromatography (GLC) of amino acids. Methods of derivatization [1] since the first attempts [2, 3] as well as searches for separative liquid stationary phases [4—6] have been the subjects of many investigations. The relative homogeneity of the family of amino acids due to the clustering of the amino and carboxylic functions on the α-carbon and the diversification of the carbon backbone structure should at first sight bring favourable conditions for easy separation. In addition, the presence of a series of functional groups such as hydroxyl, thiol, and other amino and carboxyl functions should bring a choice of modifications, especially during derivatization, to induce good volatility without thermal decomposition at the high temperatures sustained by polysiloxane liquid stationary phases [7, 8]. In addition, the coupling of the

chromatographic column to the mass spectrometer constitutes an invaluable advantage for structure determination.

Despite the many developments by several workers since 1961 when Johnson et al. [9] succeeded in separating thirty-three amino acids as their *n*-amyl esters, N-acetate, and a year later when Zomzely et al. [10] used *n*-butyl esters, N-trifluoroacetate to separate twenty-one amino acids most of the methods were proposed separately either for protein amino acids or for non-protein biological amino acids [11]. Only Siezen and Mague [8] did separate amino acids belonging to both classes as isobutyl esters, N-heptafluorobutyrate. Apart from some attempts with trimethylsilyl derivatives [12] most of the considerable work of Gehrke's group on the quantitative assay of amino acids has been done with *n*-butyl ester, N-trifluoroacetyl derivatives [13—17]. Other investigations were done on *n*-propyl ester, N-acetyl amino acids [18—20], but alkyl ester, N-heptafluorobutyryl derivatization nowadays appears to be more often used in the form of *n*-propyl [1, 21—24], isoamyl [25, 26] and isobutyl [7, 8, 27, 28] esters.

Therefore the development of a standard method for the routine assay of amino acids in human clinical chemistry necessitated re-investigation of a family of thirty amino acids which comprised the amino acids of protein hydrolysates (eighteen amino acids including hydroxyproline but without tryptophan since it is always partially destroyed by HCl in its esterification phase during derivatization), two plasma metabolites (ornithine and α-aminobutyric acid), and, as described below, seven common urinary amino acids, two alternative primary standards and one secondary standard. This article describes the GLC separation of thirty-two amino acids including *allo*-isoleucine and 5-hydroxylysine, and the development of a quantitative method for physiological fluid analysis of patients, adapted to the routine practice of a clinical biochemistry laboratory.

Results relevant to the research development of the methodology have been or will be published elsewhere, such as experimentation on various liquid phases [29], the specific assay of histidine [30] according to the method of Moodie [31], gas chromatographic—mass spectrometric study of derivative formation and elucidation of fragmentation patterns under various ionization modes [32—36].

MATERIAL AND METHODS

Amino acid standards

A 10^{-3} *M* standard solution of amino acids was prepared in 0.1 *N* HCl from standard solutions or from pure compounds obtained from Technicon (Tarrytown, N.Y., U.S.A.), Sigma (St. Louis, Mo., U.S.A.), K & K Labs. (Plainview, N.Y., U.S.A.) and Calbiochem (Los Angeles, Calif., U.S.A.).

Table I lists the amino acids tested.

Reagents and solvents

Isobutanol, ethyl acetate, methylene dichloride and diethoxyformic anhydride (EFA) were of analysis grade from Merck (Darmstadt, G.F.R.). Heptafluorobutyric anhydride (HFBA) was purchased from Pierce (Rockford, Ill.,

U.S.A.). The ion-exchange resin was Dowex 50W-X8, 100–200 mesh, from Bio-Rad Labs. (Richmond, Calif., U.S.A.). The capillary column from LKB (Bromma, Sweden), purchased from Spiral (Dijon, France), was 25 m × 0.23 mm I.D. and coated with the OV-101 liquid stationary phase.

Gas chromatography and mass spectrometry

The gas chromatograph was a Research Gas Chromatograph, Becker 419 (Becker, Delft, The Netherlands) equipped with a flame ionization detector and the capillary column mentioned above with an all-glass solid injector as described by Ros [37]. Carrier gas was nitrogen with a flow-rate of 2 ml/min. The mass-spectrometric assays were done on an LKB 9000 (Bromma, Sweden), interfaced with a 1% SE-30 packed column, and a Finnigan 3300—Computer 6100 (Sunnyvale, Calif., U.S.A.) or Ribermag GC—MS R10-10 (Rueil-Malmaison, France) both with the capillary column mentioned above.

EXPERIMENTAL

Sample preparqtion and purification

Biological samples were cleaned up by deproteinization with sulphosalicylic acid at a concentration of 50 mg per ml of plasma or urine. For purification by ion exchange a glass column (40 × 3 mm I.D.), fitted on top with a glass reservoir and at the bottom with a glass-wool plug and a PTFE stopcock, was filled up to a height of 1 cm with the Dowex resin (H^+) in water. A volume of 20–500 µl of plasma or 100–1000 µl of urine brought to pH<2.5 by 6 *M* HCl was layered on top of the resin. A calculated amount of primary standard such as N^{ϵ}-monomethyllysine (MML) or homoarginine (hArg) was added: 4–100 nmole in the case of plasma or 20–200 nmole in the case of urine. After adsorption of the liquid layer on the resin, the column was washed with 2 ml of distilled water. Then the amino acids were eluted with 2 ml of 4 *M* NH_4OH, at a flow-rate of 1 drop every 5–10 sec. To the amino acid fraction, collected in a screw-cap tube, *cyclo*-leucine (*c*Leu), the secondary standard, was added in the same amount as the MML or hArg standard. Ammonia was evaporated to dryness by heating at 90° in a sand-bath under a nitrogen stream. Care should be taken to dry the tube thoroughly and the cap avoiding any trace of moisture. For each analytical series, an equimolar standard solution of amino acids underwent the same sample preparation with the same primary and secondary standards in order to calculate the relative molar response (RMR).

Sample derivatization into isobutyl ester, N(O)-heptafluorobutyrate and into isobutyl ester, N(O)-HFB, N^{τ}-ethoxyformate for histidine

After centrifugation the dried sample or amino acid standard remaining in the tube was taken up with 500 µl of anhydrous isobutanol, in which dry gaseous HCl had been dissolved to an approximate concentration of 4 ± 0.5 *M*, and then heated for 45 min at 110° in the sand-bath. After cooling, isobutanol—HCl was evaporated to dryness under a dry nitrogen stream at 40°. Then 80 µl of ethyl acetate and 20 µl of heptafluorobutyric anhydride were added. The acylation reaction was conducted in the sand-bath at 110° for 20 min. The

TABLE I

GLC PARAMETERS OF STANDARD AMINO ACIDS ON OV-101-COATED GLASS CAPILLARY COLUMN

Retention time and temperature are given for two temperature programmes: 3°/min and 2°/min from 90° to 275°. The relative molar response (RMR) to *cyclo*-leucine (*c*Leu) in crude and cleaned-up sample for each amino acid corresponds to the mean ± S.D. from twelve samples routinely made over a six-month period. I.S. = Internal standard.

Amino acid	Abbreviation	3°/min		2°/min		RMR of standard solution	
		Time (min)	Temperature (°C)	Time (min)	Temperature (°C)	Crude sample	Cleaned-up sample
α-Alanine	α-Ala	12.7	128	12.8	115.6	0.99 ± 0.06	1.00 ± 0.05
Glycine	Gly	13.2	129.6	13.3	116.6	0.88 ± 0.04	0.83 ± 0.05
α-Aminobutyric acid	α-ABA	15.2	136.6	15.7	121.4	0.99 ± 0.04	0.96 ± 0.04
β-Alanine	βAla	16.1	138.3	16.7	123.4	0.83 ± 0.04	0.84 ± 0.04
Valine	Val	16.7	140.1	17.5	125	1.14 ± 0.07	1.08 ± 0.07
β-Aminoisobutyric acid	β-AIBA	17	141	17.8	125.6	1.05 ± 0.06	1.03 ± 0.05
Threonine	Thr	17.4	142.2	18.4	126.8	0.91 ± 0.08	0.89 ± 0.08
Serine	Ser	18.1	144.3	19.2	128.4	0.84 ± 0.07	0.83 ± 0.07
Leucine	Leu	19.3	147.9	20.8	131.6	0.91 ± 0.04	0.90 ± 0.04
allo-Isoleucine*	*a*Ile	19.6	148.8	-	-	1.02 -	1.02 -
Isoleucine	Ile	19.8	149.4	21.5	133	1.09 ± 0.08	1.06 ± 0.07
γ-Aminobutyric acid	γ-ABA	21.3	153.9	23.4	136.8	0.70 ± 0.05	0.70 ± 0.06
*cyclo*Leucine	*c*Leu	22.2	156.6	24.6	139.2	1.00 I.S.	1.00 I.S.
Proline	Pro	23.1	159.3	25.8	141.6	0.90 ± 0.06	0.88 ± 0.06
4-Hydroxyproline	Hpr	26.8	170.4	31.1	152.2	0.85 ± 0.04	0.84 ± 0.05
Methionine	Met	28.5	175.5	33.4	156.8	0.59 ± 0.05	0.56 ± 0.05
Aspartic acid	Asp	30.7	182.1	36.7	163.4	0.90 ± 0.06	0.91 ± 0.06
Phenylalanine	Phe	32.4	187.2	39.1	168.2	1.10 ± 0.08	1.10 ± 0.08
Ornithine	Orn	33.6	190.8	41.1	172.2	0.72 ± 0.04	0.68 ± 0.04
Glutamic acid	Glu	35.3	195.9	43.4	176.8	0.98 ± 0.06	0.96 ± 0.05
Lysine	Lys	37.8	203.4	47.1	184.2	0.74 ± 0.05	0.73 ± 0.06
Tyrosine	Tyr	38.4	205.2	47.9	185.8	1.00 ± 0.07	0.96 ± 0.04
Methionine sulphone	MSO_2	39.0	207	48.7	187.4	0.45 ± 0.05	0.44 ± 0.05
N^{ϵ}-monomethyllysine	MML	39.6	208.8	49.7	189.4	0.86 ± 0.04	0.87 ± 0.04
Arginine	Arg	41.4	214.2	52.7	195.4	0.66 ± 0.05	0.62 ± 0.06
Histidine**	His	15.2	210.8	15.2	210.8	0.52 ± 0.05	0.47 ± 0.06
Homo arginine	hArg	45.3	225.9	58.4	206.8	0.66 ± 0.05	0.61 ± 0.06
Lanthionine	Lan	48.8	236.4	63.5	217.0	0.73 ± 0.05	0.70 ± 0.05
Cystathionine	CTT	52.3	246.9	68.5	227.0	0.81 ± 0.06	0.80 ± 0.07
Cystine	$(Cys)_2$	54.2	252.6	71.3	232.6	0.50 ± 0.06	0.48 ± 0.06
Cys-S—S-homoCys	CyshCys	57.5	262.5	76.1	242.2	***	***
Homocystine	$(hCys)_2$	60.6	271.8	80.8	251.6	0.88 ± 0.07	0.84 ± 0.07

*Pure *allo*-isoleucine was not available. Its RMR to *c*Leu was obtained from a molar mixture of isoleucine and *allo*-isoleucine (approximately 1:1).

**The observed retention time and temperature of histidine corresponded to its specific chromatographic conditions: 4°/min from 150° to 260°. Its RMR was related to N^{ϵ}-monomethyllysine.

***The cysteinyl—homocysteinyl disulphide not being available, no RMR could be calculated. Its formation seems (see text) to be a derivatization artifact.

solution, ready for GLC, can be diluted at will with ethyl acetate. All amino acids were completely derivatized as isobutyl ester, N(O)-heptafluorobutyrate (IBU, N(O)-HFB), except histidine. For this compound, the acylating mixture was completely evaporated under a dry nitrogen stream at room temperature. To the dry tube 400 μl of methylene dichloride and 10 μl of diethoxyformic anhydride were added. The reaction to derivatize the imidazole amino group was carried out by heating at 110° for 15 min [30, 31] to give a solution of isobutyl ester, N(O)-heptafluorobutyrate, N-ethoxyformate, (IBU, N(O)-HFB N-EF) histidine directly usable for GLC.

GLC analytical conditions

The most favourable chromatographic conditions were: temperature, 250° for injection port, 270° for detector; gas flow-rate, 250 ml/min for air and 25 ml/min for hydrogen; temperature programming, 2°/min or 3°/min, starting from 90°, for urine and plasma, and 4°/min starting at 150° for isobutyl, N(O)-heptafluorobutyryl, N-ethoxyformyl histidinate.

Quantitation

This important step was performed using the method of internal standardization with *c*Leu. For each amino acid the relative molar response (RMR) [38] to the amino acid standard was established since the absolute molar response cannot be accurately calculated [39] from the electricity produced by the detector (molar response in Coulomb/mole) [40], therefore

$$\text{RMR} = \frac{\text{peak height of amino acid}}{\text{peak height of } c\text{Leu}}$$

Then the amount of each amino acid (in nmole) was expressed as

$$Q = \left(\frac{1}{\text{RMR}_{\text{std}}}\right) \times (\text{RMR}_{\text{sample}}) \times (q_{\text{I.S.}})$$

where RMR_{std} = relative molar response for the standard amino acid solution $\text{RMR}_{\text{sample}}$ = relative molar response for sample amino acid and $q_{\text{I.S.}}$ = nanomoles of *c*Leu added to the biological sample.

To prevent loss of peak height recording due to the electromechanical inertia of the pen recorder, it is advisable to use a reporting integrator such as the HP 3380 or 3385 (Hewlett-Packard France, Orsay, France).

RESULTS

Studies of amino acid standards

Owing to the high complexity of the amino acid composition of physiological fluids, especially in metabolic diseases, the method has been developed for the complete separation of a standard solution of thirty-two amino acids (see Table I).

Derivatization of compounds

From our experience, the isobutyl esters, N(O)-heptafluorobutyrate derivatives have been chosen since they are completely resolved on common station-

ary phases such as SE-30, OV-1 and OV-101. They are very stable and reproducible in packed or capillary columns and, in addition, these phases are widely used in GC—MS. The most critical factors during derivative formation are: reagents should be devoid of any trace of moisture; the preceding reagent should be completely eliminated by evaporation before undertaking the next derivatization step (i.e. removal of isobutanol before acylation, especially from the stopper, and removal of acylating mixture before EFA treatment in the histidine assay). Side-reactions occurring during the over-all procedure are: complete cysteine oxidation into cystine; hydrolysis of glutamine and asparagine into glutamic and aspartic acids; partial (20—25%) hydrolysis of citrulline into ornithine; full conversion of methionine sulphoxide into methionine; partial reaction of cystine and homocystine to produce together cysteinyl, homocysteinyl disulphide.

GLC analysis characteristics

The salient features of the proposed method are: complete resolution of the most common physiological compounds; sensitivity and rapidity; quality control of amino acid quantitation. In addition, a very good correlation of elution pattern to amino acid structure was found.

(a) Chromatographic separation. Fig. 1 demonstrates that a complete reso-

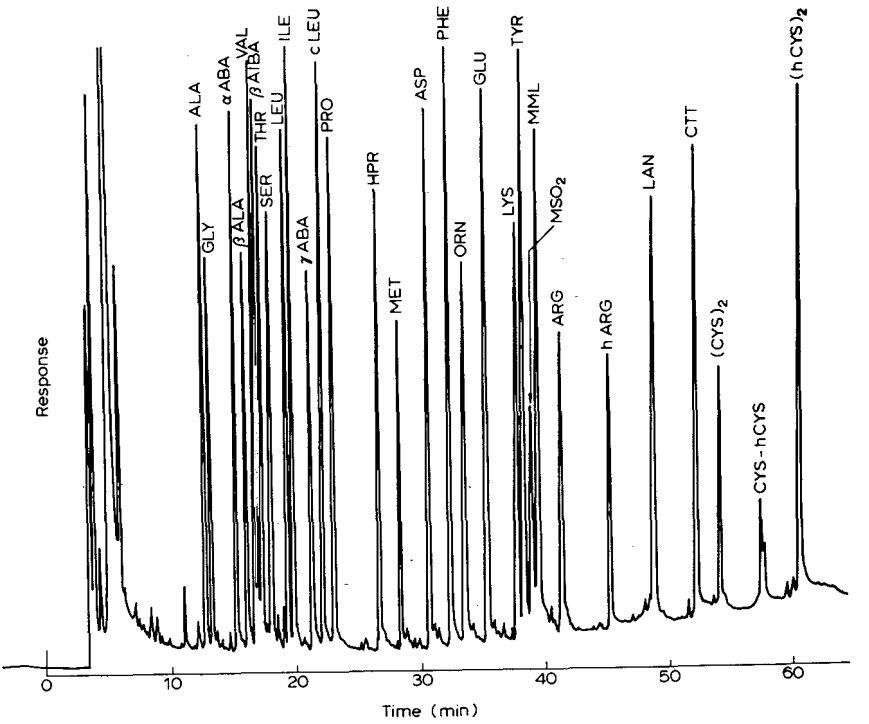


Fig. 1. Gas chromatogram of the isobutyl ester, N(O)-heptafluorobutyrate derivatives of twenty-nine amino acids on a 25 m × 0.23 mm I.D. OV-101-coated glass capillary column. The temperature programme was 3°/min from 90° to 275°. Each amino acid peak represents approximately 50 pmole.

lution of twenty-nine amino acids was achieved on the OV-101 capillary column. They correspond to: (i) the seventeen protein amino acids; (ii) two common plasma metabolites α-aminobutyric acid (α-ABA) and ornithine; (iii) seven urinary compounds of which the occurrence or increased concentration is abnormal: β-alanine (βAla), β-aminoisobutyric acid (βAIBA), γ-aminobutyric acid (γABA), methionine sulphone (MSO_2), lanthionine (Lan), cystathionine (CTT) and homocystine $(hCys)_2$; (iv) *cyclo*-leucine (*c*Leu) used as a secondary standard for quantitative determinations; (v) two alternative primary standards, N^{ϵ}-monomethyllysine (MML) or homoarginine (hArg).

Histidine, which did not give any suitable derivative with this method, should undergo specific derivatization with EFA. Special chromatographic conditions, as specified in the legend to Fig. 2, were devised for this compound.

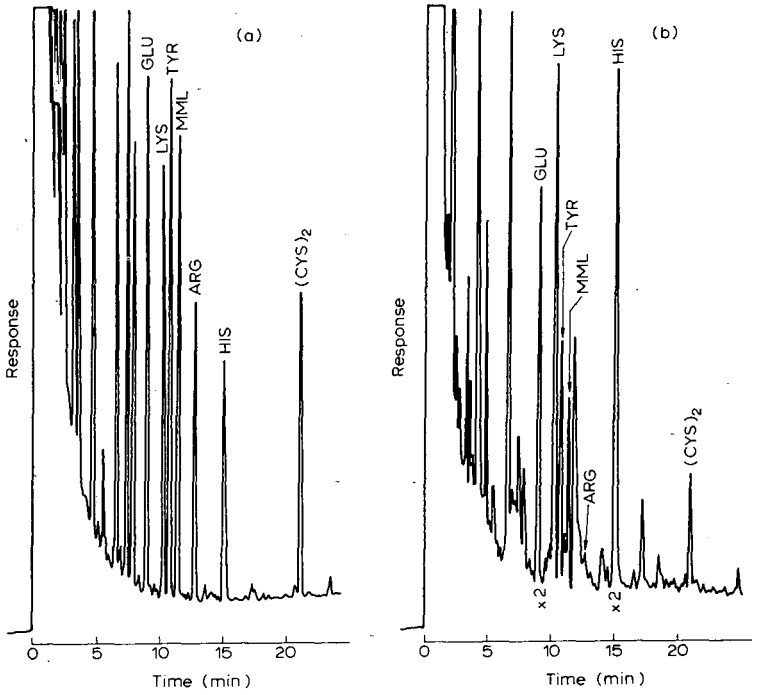


Fig.2. Gas chromatograms of the isobutyl ester, N^{α}-heptafluorobutyrate, N^{τ}-ethoxyformate derivative of histidine: (a) in standard mixture; (b) in urine of a normal patient. The temperature programme was 4°/min from 150° to 240°.

Furthermore, the capillary column allowed the separation of *allo*-isoleucine (*a*Ile) between leucine and isoleucine (Fig. 3a), a situation which was not found up to now using OV-1-coated packed columns (Fig. 3b). Only such a feature brings a clear-cut biochemical diagnosis of maple syrup urine disease.

(b) Practicability: duration and sensitivity of analysis. The time needed to complete the whole assay of the twenty-nine amino acids was between 60 and 75 min depending on the chromatographic conditions (see Experimental).

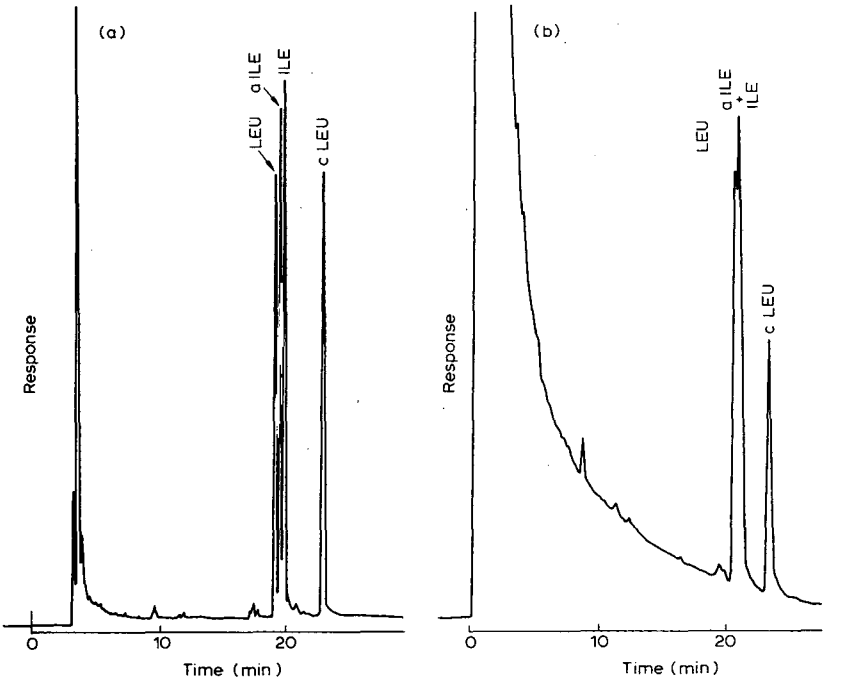


Fig. 3. Gas chromatograms of a mixture of leucine, isoleucine, *allo*-isoleucine and *cyclo*-leucine as IBU, N(O)-HFB derivatives: (a) on an OV-101-coated glass capillary column; (b) on an OV-1-packed column.

Chromatographic conditions resulting in time saving can be adjusted to the specific quantitative assay of small groups of related amino acids: Pro and Hpr; Phe and Tyr; Orn, Lys, Arg and $(Cys)_2$. The practicable sensitivity limits range between 10 and 150 pmole. The lower limit allows use of an initial sample of 20 μl of plasma in order to obtain a peak height five times the noise from the injection of a five hundredth aliquot of the derivatized sample.

(c) Quality control during the different steps of the analysis. (1) Reproducibility. Table I shows the relative molar responses (RMR) to *c*Leu for each of the thirty-one amino acids of the standard solution with ten different preparations of the derivatized standard sample. The coefficient of variation did not exceed 8% with a mean of 5%.

(2) Purification steps. Compounds such as lipids, carbohydrates and pigments should be eliminated by ion-exchange purification prior to derivatization. The most favourable conditions have been established from the standard solution to determine: (i) the loading capacity of the columns used which total 500 nmole of each amino acid together; (ii) no losses after washing with 3 ml distilled water; (iii) the elution by 2 ml of 4 *M* NH_4OH which did not require an excessive time for evaporation; (iv) the flow-rate of elution as already mentioned. Table I shows that under these conditions the RMR of each amino acid of a standard solution cleaned-up or not with Dowex is the same.

(3) Choice of primary standard. MML or hArg was added to the sample before purification, while *c*Leu, the secondary standard, was then added before

derivatization. The RMR of MML to *c*Leu in the amino acid standard solution and biological samples handled together through the same purification steps were compared to the RMR calculated from an amino acid standard solution derivatized without the ion-exchange resin step (see Table II). Altered RMR values would have indicated losses during this crucial stage. In addition MML is used as a secondary standard in the histidine assay since *c*Leu is eluted with the solvent front. During the evaporation of the sample deposited on the Ros injector needle losses of the more volatile derivatives may occur. An increased RMR value of MML to *c*Leu would have been a warning of such a situation.

TABLE II

QUALITY CONTROL ASSAY OF BIOLOGICAL SAMPLES

The samples were subjected to the whole procedure done by comparison of RMR values of MML as the primary internal standard to *c*Leu as the secondary standard in cleaned-up urine and plasma to crude or cleaned-up standard solution. The value of the mean ± S.D. was obtained from twelve preparations.

	Crude sample	Cleaned-up sample		
RMR to *c*Leu	Amino acid standard solution	Amino acid standard solution	Plasma sample	Urine sample
MML	0.86 ± 0.04	0.87 ± 0.04	0.85 ± 0.06	0.93 ± 0.07

(4) Derivative stability. At room temperature most of the derivatives remained stable for three weeks, but Lys and $(Cys)_2$ responses decreased by about 15% in 12 days, and Met and Arg derivatives were rapidly lost. Therefore, derivatized samples should be analysed within three days, a period of time sufficient to check again, but a new standard solution sample should be prepared for each series of analyses.

(d) Relationship between amino acid structure and chromatographic elution. As a general rule the elution order depends on the boiling point and, therefore, on the molecular weight. In the case of isomer families, this order could be related to physicochemical features as follows.

(1) For amino group position isomers the amino group hydrogen exhibits an increase of polarity with increasing distance from the carboxylic group. Therefore, this explains the order of elution of amino butyrate isomers: αABA, βAIBA, γABA and of alanine isomers: Ala, βAla.

(2) The structure of the carbon backbone, such as level of branching or cyclization, modifies the hydrophobic interactions as shown with Leu, *a*Ile, Ile, norleucine (nLeu)*, and *c*Leu or with Val and norvaline (nVal)*.

(3) Hydroxyl substitution by an amino group also increases the retention times: serine and 2,3-diaminopropionic acid*, tyrosine and *p*-aminophenylalanine*.

*Amino acids that are not present on the chromatograms but which have been studied during the search for secondary internal standards.

General procedure

The flow-diagrams shown in Schemes 1 and 2 summarize the various criteria that have been selected after several years of continuous application of the method in clinical biochemistry.

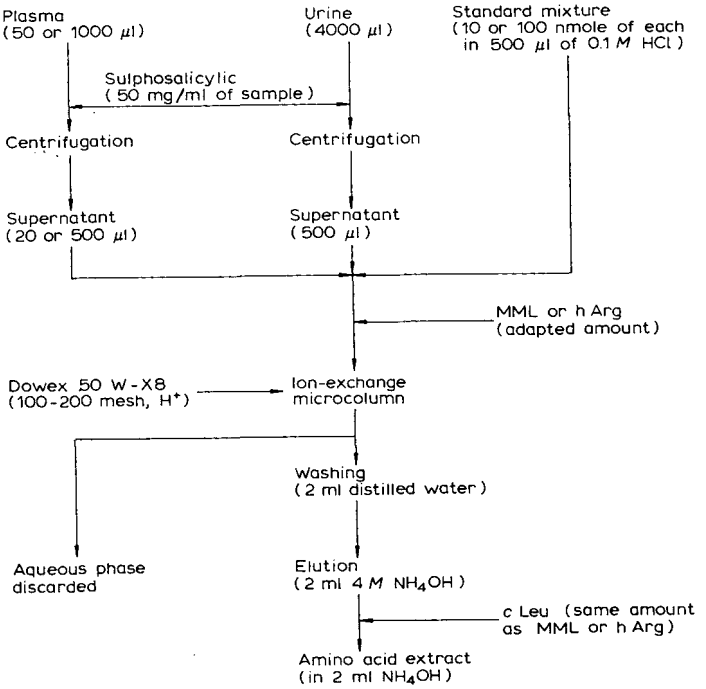
Application to physiological fluids in clinical biochemistry

Identification of chromatographic peaks from biological samples

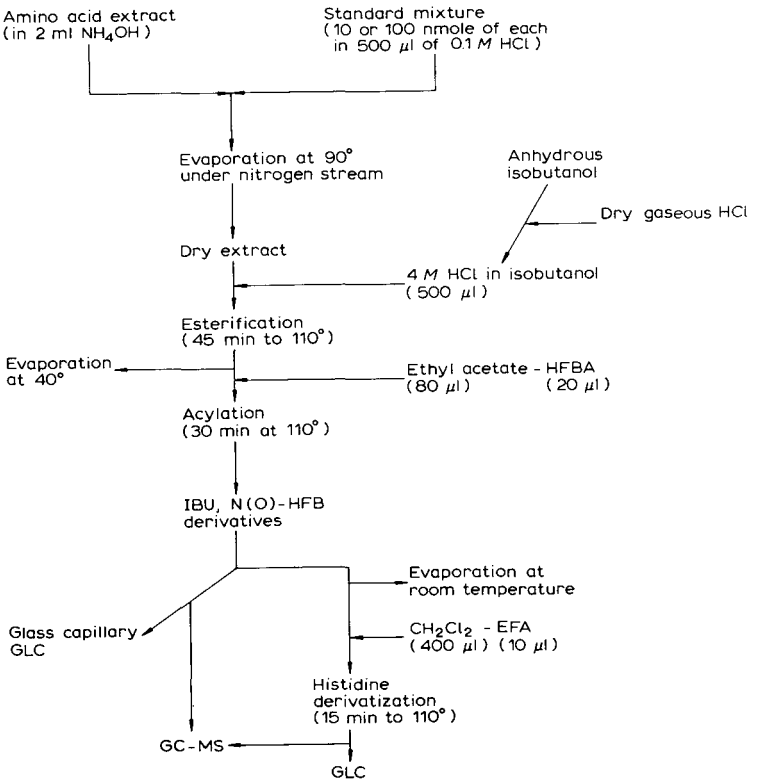
Chromatographic peaks of biological samples were identified by comparison of their retention times with those of a standard amino acid solution. Table I shows the data for two different analytical conditions. The nature of the presumed amino acid was checked by individual spiking off. Finally, exact identifications were obtained by comparison of the mass spectra of the biological compound and of the authentic reference standards obtained by electron impact and chemical ionization (methane, isobutane and ammonia). The detailed results of this study have been partly reported in publications [32, 33] which will be completed by forthcoming articles [29, 30, 34]. However, Tables III and IV give data for sulphur amino acids.

GLC analysis of normal physiological fluid from humans

Figs. 4a and b show the separations of amino acids as isobutyl esters, N(O)-heptafluorobutyrate from two normal plasma samples. The chromatographic



Scheme 1



Scheme 2

settings were different: Fig. 4a was obtained with a temperature program of 2°/min instead of 3°/min as in Fig. 4b. The 15-min increase of the elution time brought a better separation. The advantage of this improvement appears more conspicuous in the case of amino acids of normal urine (Figs. 5a and b) especially when considering the group composed of βAla, Val, βAIBA and Thr, or Hpr and also the group of Lys, Tyr, MSO_2 and MML. Such charts demonstrate clearly the extent of the physiological variations of amino acid concentrations: βAla, βAIBA, Ser and Lys. In Fig. 5b the peak preceding Lys has been identified by CG-MS as 5-hydroxylysine. This amino acid eluted with Lys on packed columns has been found in variable amounts in most urine samples from normal patients assayed so far. Its physiological occurrence and significance remain to be cleared up.

Interest of the capillary column

The chromatographic response of a capillary column compared to a packed one was found to be thirty times more sensitive. This feature is of great interest in the case of plasma amino acids since the plasma sample size can be scaled down to 20—50 µl in the case of premature or new-born infants.

TABLE III

IDENTIFICATION OF SULPHUR AMINO ACIDS BY FRAGMENTATION IN GC–MS UNDER ELECTRON IMPACT IONIZATION: FRAGMENTS PRODUCED BY METHIONINE (Met) AND METHIONINE SULPHONE (MSO_2)

Mass spectrometer settings: accelerating voltage 3.5 kV; trap current 60 μA.

	Fragment	Met	MSO_2
Molecular ion		401(5)	433(0)
Base peak		57(100)	252(100)
M - 55	C_4H_7	346(1)	
M - 56	C_4H_8	345(2)	477(1)
M - 74	C_4H_9OH	*327(18)*	359(5)
M - 79	$H_3C{-}SO_2$		354(2)
M - 100	$CO{-}O{-}C_4H_8$		*333(9)*
M - 101	$CO{-}O{-}C_4H_9$	300(3)	*332(10)*
M - (56 + 61)	$C_4H_8 + H_3C{-}S{-}CH_2$	284(7)	
M - (55 + 75)	$C_4H_7 + H_3C{-}S{-}CH_2{-}CH_2$	*271(32)*	
M - (74 + 61)	$C_4H_9OH + H_3C{-}S{-}CH_2$	*266(11)*	
M - (74 + 74)	$C_4H_9OH + H_3C{-}S{-}CH_2{-}CH$		
or		*253(32)*	
M - (101 + 47)	$COOC_4H_9 + H_3C{-}S$		
M - (101 + 61 + 1)	$COOC_4H_9 + H_3C{-}S{-}CH_2 + H$	238(4)	
$[H_3C{-}S{-}CH_2{-}CH{-}CH{-}COO]^+$		131(7)	
$[H_3C{-}S{-}CH_2{-}CH_2]^+$		*75(47)*	
$[H_3C{-}S{-}CH_2]^+$		*61(65)*	
M - (56 + 79)	$C_4H_8 + H_3C{-}SO_2$		298(4)
M - (74 + 79 + 1)	$C_4H_9OH + H_3C{-}SO_2 + H$		279(3)
M - (101 + 79 + 1)	$COOC_4H_9 + H_3C{-}SO_2 + H$		*252(100)*
M - (100 + 93)	$COOC_4H_8 + H_3C{-}SO_2{-}CH_2$		*240(8)*
M - (100 + 107)	$COOC_4H_8 + H_3C{-}SO_2{-}CH_2{-}CH_2$		226(2)

Pathological plasma and urine

Figs. 6–9 show four examples of amino acid separations and assays in inborn disorders of amino acid metabolism.

In maple syrup disease, plasma (Fig. 6a) and urine (Fig. 6b) amino acid levels and excretions of Val, Leu and Ile were increased while *allo*-isoleucine (*a*Ile) was well separated on the capillary column and was identified by GC–MS.

Fig. 7 from the plasma of an infant affected with an inborn phenylketonuria shows the high increase of Phe.

A congenital defect of cystathionase is illustrated by Fig. 8 with an excretion peak of CTT. Cystinuria (Fig. 9) which is known to result from the alteration of specific re-absorption receptors in kidney tubules leads to increased levels of $(Cys)_2$ and also of Orn, Lys and Arg.

DISCUSSION

In clinical chemistry, gas–liquid chromatography has acquired a widespread application in the field of steroid hormones for many years (see references in

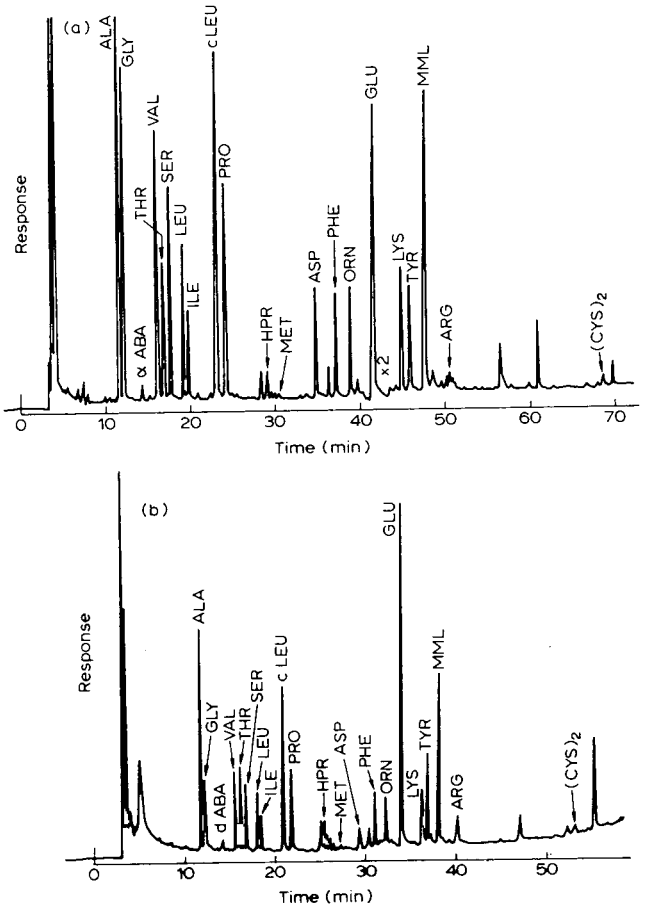


Fig. 4. Gas chromatograms on OV-101-coated glass capillary column of two normal plasma samples subjected to the complete procedure. The temperature programme was 2°/min (a) and 3°/min (b) from 90° to 270°.

ref. 41) since the pioneering work of Luukkainen et al. [42]. In consideration of the prominent work of Gehrke and his associates [12—17, 38], the outcome was not the same for amino acid assay and not such an appreciation of this chromatographic technique can be found in clinical chemistry. Since the beginning of our interest in this field [43—47], during re-investigations of already published methods and in the course of development of specific methods, considerable benefits accrued from the coupling of the gas chromatograph to the mass spectrometer [34, 48] for the determination of eluted amino acids and the elucidation of new compounds belonging to this family [32, 49—52]. The use of GLC for analysis of amino acids of protein and human plasma is well documented [1, 7, 17, 25, 38, 49] but many of the authors, if not all, except Adams [19] had not reached a level of achievement that would open a competition with the well-established ion-exchange liquid chromatography

TABLE IV

IDENTIFICATION OF SULPHUR AMINO ACIDS BY FRAGMENTATION IN GC—MS UNDER ELECTRON IMPACT IONIZATION: FRAGMENTS PRODUCED BY THREE DISULPHIDE AMINO ACIDS AND BY CYSTATHIONINE (CTT) AND LANTHIONINE (Lan)

Mass spectrometer settings: accelerating voltage 3.5 kV; trap current 60 μA. $(Cys)_2$ = cystine; $(hCys)_2$ = homocystine; Cys—hCys = cysteinyl—homocysteinyl disulfide.

	Fragment	$(Cys)_2$	$(hCys)_2$	Cys—hCys	CTT	Lan
Molecular ion $(M)^+$		744(2)	772(1)	758(0)	726(0)	712(0)
Base peak		57	298	57	57	57
M - (101)	$COOC_4H_9$	643(1)	671(1)	—	625(1)	611(1)
M - (101 + 56)	$COOC_4H_9 + C_4H_8$	587(1)	—	601(1)	569(2)	555(3)
M - 213	$H_2N—CO—C_3F_7$	531(2)	—	—	513(6)	499(11)
M - (213 + 74)	$H_2N—CO—C_3F_7 + C_4H_9OH$	—	—	—	439(8)	425(5)
M - (213 + 101 + 1)	$H_2N—CO—C_3F_7 + COOC_4H_9 + H$	—	—	—	411(5)	—
M -(169 + 101 + 56)	$C_3F_7 + COOC_4H_9 + C_4H_8$	—	—	—	400(4)	—
M - (213 + 74 + 56)	$H_2N—CO—C_3F_7 + C_4H_9OH + C_4H_8$	—	—	—	383(4)	369(3)
M - (213 + 74 × 2)	$H_2N—CO—C_3F_7 + (C_4H_9OH)_2$	—	—	—	365(2)	351(4)
M -(213 + 101 + 56)	$H_2N—CO—C_3F_7 + COOC_4H_9 + C_4H_8$	—	—	—	—	342(4)
M -(213 + 101 + 74)	$H_2N—CO—C_3F_7 + COOC_4H_9 + C_4H_9OH$	—	—	—	—	324(5)
M - (213 + 169)	$H_2N—CO—C_3F_7 + C_3F_7$	—	—	—	344(19)	330(11)
M - (213 × 2)	$(H_2N—CO—C_3F_7)_2$	—	—	—	300(21)	286(46)
M - (213 × 2 + 56)	$(H_2N—CO—C_3F_7)_2 + C_4H_8$	—	—	—	244(7)	230(20)
M -(213 × 2 + 74)	$H_2N—CO—C_3F_7 \times 2 + C_4H_9OH$	—	—	—	226(14)	—
a = $\left[S—(CH_2)_2—CH\begin{matrix}NH—CO—C_3F_7\\ \diagdown COOC_4H_9\end{matrix}\right]^+$		—	386(2)	386(2)	—	—
b = $\left[S—CH_2CH\begin{matrix}NH—CO—C_3F_7\\ \diagdown COO—C_4H_9\end{matrix}\right]^+$		372(2)	—	372(3)	—	372(2)

a – 32	S	—	354(60)	354(52)	354(5)	—
b – 32	S	340(32)	—	340(16)	—	—
b – 56	C_4H_8	316(5)	—	316(4)	—	316(3)
a – (32 + 56)	$S + C_4H_8$	—	298(100)	298(82)	298(36)	—
or						
b – 74	C_4H_9OH	298(4)	—			—
b – (32 + 56)	$S + C_4H_8$	284(41)	—	284(39)	284(14)	284(36)
or						
a – (32 + 56 + 14)	$S + C_4H_8 + CH_2$	—	284(11)		—	—
a – (32 + 56 + 14 × 2)	$S + C_4H_8 + CH_2 + CH_2$	—	270(18)	270(18)	—	—
or						
b – (32 + 56 + 14)	$S + C_4H_8 + CH_2$	270(4)			—	—
b – (32 + 74 + 1)	$S + C_4H_9OH + H$	265(21)	—	265(4)	265(9)	265(35)
a – (32 + 101 + 1)	$S + COOC_4H_9 + H$	—	252(35)	252(26)	252(29)	—
or						
a – (32 + 74 + 28)	$S + C_4H_9OH + CH_2 - CH_2$	—				
a – (32 + 100 + 14)	$S + COOC_4H_8 + CH_2$	—	240(10)	240(10)	240(4)	—
b – (32 + 101 + 1)	$S + COOC_4H_9 + H$	238(14)	—	238(8)	238(5)	238(12)

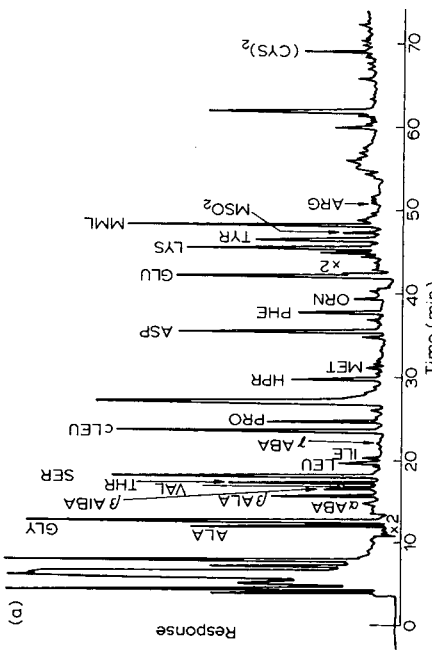


Fig. 5. Gas chromatograms on OV-101-coated glass capillary column of two normal urine samples subjected to the complete procedure. The temperature programme was 2°/min (a) and 3°/min (b) from 90° to 270°.

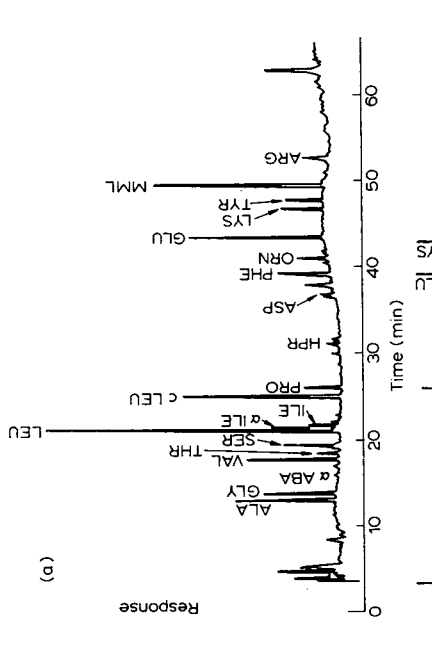


Fig. 6. Gas chromatograms on OV-101-coated glass capillary column of two samples subjected to the complete procedure and obtained from plasma (a) and urine (b) of a newborn infant with maple syrup disease. The temperature programme was 2°/min from 90° to 270°.

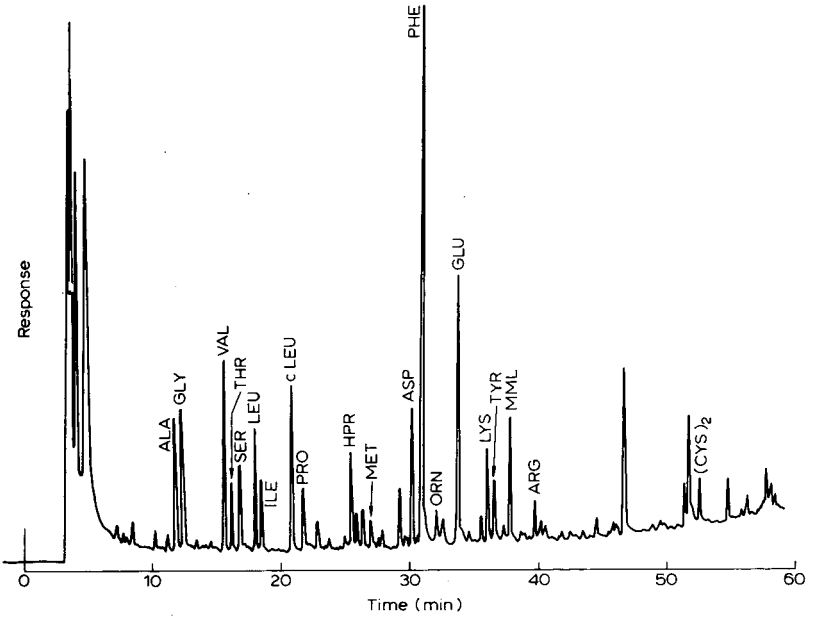


Fig. 7. Gas chromatogram on OV-101-coated glass capillary column of a sample obtained from plasma of a phenylketonuria. The temperature programme was 3°/min from 90° to 270°.

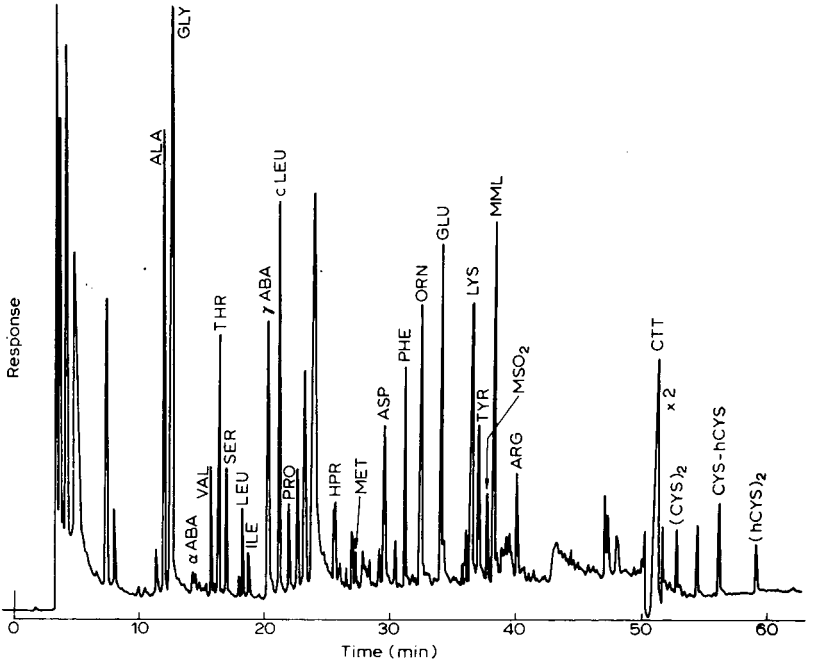


Fig. 8. Gas chromatogram on OV-101-coated glass capillary column of urine amino acids from a patient with hypercystathioninuria. Homocystine is also identified in the sample. The temperature programme was 3°/min from 90° to 270°.

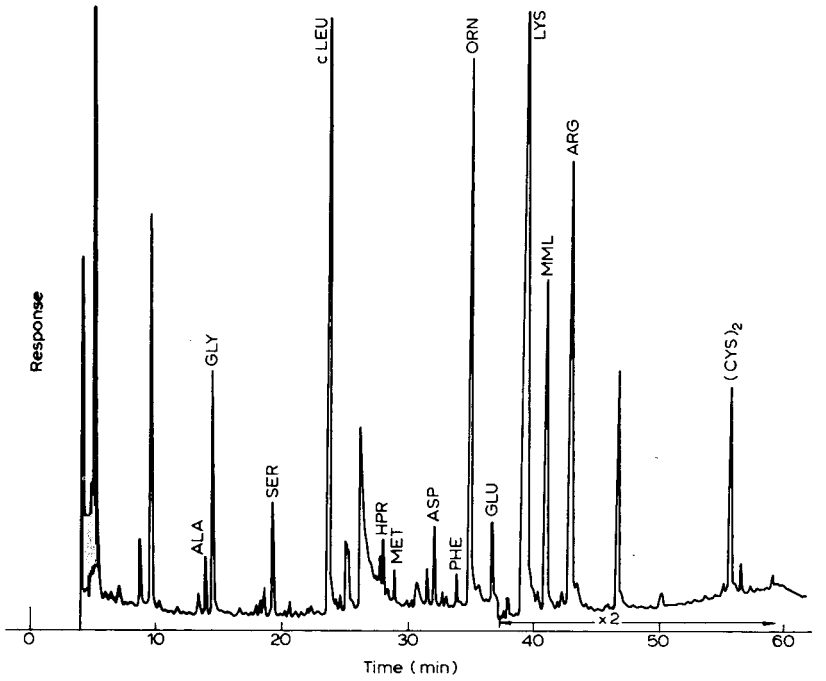


Fig. 9. Gas chromatogram on OV-101-coated glass capillary column of urine amino acids from a patient suffering from ornithine, lysine, arginie, cystinuria. The temperature programme was 3°/min from 90° to 270°.

used in reliable automatic analysers.

Benefitting from the improved separation due to sample purification and use of a capillary column, up to thirty-two amino acids could be easily analysed in human plasma samples on a routine basis in clinical chemistry although histidine still has to be analysed by the method of Moodie [30, 31]. However, the development of a similar method for the analysis of all the urinary amino acids revealed more compelling difficulties to overcome as regards satisfactory routine use for patients. Difficulties in urine amino acid assay arise from the fact that the number of amino acids is greater than in plasma and from the presence of many metabolites having similar GC properties to amino acids and which are detected by flame ionization. Purification of the urine sample by ion-exchange resin and the use of a glass OV-101-coated capillary column were the two improvements that allowed us to separate and quantitate these amino acids in a standard mixture as well as in biological samples.

A complete analysis can be performed in 60 or 75 min. Even with as long a duration as 75 min, required when more than the seventeen protein amino acids have to be separated and quantitated, ion-exchange liquid chromatography cannot compete with GLC in the case of urine samples. Of course one must take into account the time spent for sample purification and derivatization. But six samples can be handled at once. The sensitivity can reach 10 pmole for a signal to noise ratio of five. Such sensitivity allowed the analysis of

very small samples down to 20 μl plasma, such as in the case of infants who cannot sustain withdrawal of large volumes of blood. Cerebrospinal fluid and dialysing fluids from extra-renal clearance of toxic metabolites in cases of inborn errors of amino acid metabolism can be easily analysed, as well as food to control the diet in which toxic amino acids must be restricted. Finally, the possibility of identifying peaks by mass spectrometry by coupling the same column is of great help and is, in many respects obligatory in the field of metabolic disorders. Since the OV-101- or SE-30-coated glass capillary columns used in this method are very reproducible they can be easily interfaced directly on low-resolution mass spectrometers without any separator.

CONCLUSION

The glass OV-101-coated capillary column achieved complete resolution of the protein amino acids by GLC in such a manner that up to twenty-eight amino acids commonly found in urine could be separated in about the same time and then quantitated. The time for a chromatographic run can be adjusted between 60 and 75 min, or less for the analysis of a small group of amino acids. The sensitivity of the glass capillary column with the solid injector allowed us to work with plasma specimens as small as 20 μl. The use of the same column and liquid phase that are commonly found in GC—MS procured ideally a safe control of the method and the possibility of trying to identify any new peak, such as shown by the clear-cut identification of *allo*-isoleucine and by the repeated finding of 5-hydroxylysine in a normal urine sample. These analytical features should stimulate further research in the field of amino acid metabolism and chemical pathology and therefore have far-reaching application.

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SURFACTANT ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF TRYPTOPHAN AND SOME OF ITS METABOLITES IN BIOLOGICAL FLUIDS

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SUMMARY

A rapid, sensitive assay for tryptophan and some of its metabolites in urine, plasma and saliva has been developed using sodium dodecylsulphate as a pairing ion in a surfactant ion-pair high-performance liquid chromatography technique. The method is highly selective for tryptophan which is separated from its main indoleamine metabolites, 5-hydroxytryptophan, 5-hydroxytryptamine and 5-hydroxyindoleacetic acid, and from kynurenine. The usefulness of the assay has been demonstrated in plasma level and urinary excretion studies of orally administered tryptophan.

INTRODUCTION

Tryptophan is metabolised by two primary routes, either through the tryptophan—kynurenine—nicotinic acid pathway [1] or through a series of indoleamines [2]. Abnormal plasma and urine levels of metabolites formed by either pathway have been implicated in diseased states [3—9].

Tryptophan blood levels have been considered [10] as being indicative of mood changes in man and there are several reports of the benefits of tryptophan in the treatment of depression [11, 12]. Tryptophan is found in significant amounts in saliva and it has been suggested [13] that there is an equilibrium between free tryptophan in plasma and in saliva. We are currently investigating whether salivary levels of tryptophan can be used as an indicator of depressive states.

A variety of methods have been employed for the determination of tryptophan in biological fluids, including thin-layer chromatography [14, 15], amino-acid analysis [16—18], gas—liquid chromatography [19, 20], UV spectrometry

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[21], and fluorimetry [22—23]. These methods have proved to be insensitive, time consuming and/or fairly non-selective. Recently, reversed-phase high-performance liquid chromatography (HPLC) [24, 25] and ion-pair HPLC [26] methods have been presented for the analysis of tryptophan and proved to be highly selective. Due to the ability of tryptophan to ionise in acidic solutions, a reversed-phase surfactant ion-pair HPLC system has been used in this study. Such techniques have been shown to have advantages over conventional reversed-phase techniques in terms of flexibility of retention control, peak shape, speed of analysis and to be appropriate for the assay of solutes in biological fluids [27—31].

EXPERIMENTAL

Apparatus

This consisted of a Haskell constant-pressure pneumatic amplifier liquid pump (Olin Energy 24030). Dectection was by UV spectroscopy by either a Jobling fixed-wavelength (280 nm) UV detector (LD 1205) or a Cecil variable-wavelength UV detector (CE 212). The detector output was recorded on a potentiometric detector (Bryans 28000). Injections were made on column with a Specac 8 port-injection valve fitted with a 5 μl internal loop and a 100 μl internal external loop. The column and injection valve temperature were controlled by enclosure in a modified gas-chromatograph oven (Perkin Elmer F11).

Materials

The stationary support was ODS-silica (5 μm Spherisorb ODS) packed into a 100 × 5 mm (I.D.) stainless-steel tube by a dilute slurry technique [32]. The pure samples of tryptophan and its metabolites were from Sigma (Poole, Great Britain). The mobile phase consisted of 50% methanol (HPLC grade, Fisons, Loughborough, Great Britain) in 0.04 mole dm^{-3} di-sodium hydrogen citrate buffer containing decyl, dodecyl or tetradecyl sodium sulphate (Cambrian Chemicals, Croydon, Great Britain). The pH of the mobile phase was adjusted to 2.25 by the addition of 2 *N* hydrochloric acid using a pH meter. Potassium nitrate was used as an unretained compound for the determination of capacity ratios.

Procedures

Samples of urine, blood and saliva were taken from healthy male volunteers. The urine was injected untreated, but the saliva and blood were pretreated to remove cellular matter and protein. The cellular components of blood were removed by centrifugation at 600 *g* for 10 min and the plasma drawn off with a Pasteur pipette. The high molecular weight components of saliva and plasma were removed by centrifugation filtration through a CF 50A Centiflo membrane at 2000 *g* for 20 min, as described by Bauman et al. [33]. This method removed all molecules of molecular weight above 50,000.

Estimations of free levels of tryptophan only were made. It was found that storage of biological samples containing tryptophan (even if frozen at —11°) was not possible, due to the rapid degradation of tryptophan. This is consistent with the findings of Grushka et al. [25], thus all biological samples were chromatographed as soon as possible after collection and preparation.

RESULTS AND DISCUSSION

This work was designed to develop an assay for tryptophan in biological fluids and forms part of a series of investigations into the potential and mechanisms of surfactant ion-pair HPLC [34, 35]. It has previously been demonstrated [34, 35] that chromatographic conditions in surfactant ion-pair HPLC can be optimised by investigating the effect of changing the mobile phase concentration and alkyl chain length of the pairing ion.

Fig. 1 shows the sigmoidal relationship between the alkylsulphate mobile phase concentration and the capacity ratio of tryptophan. This relationship has been attributed [31] to the mixed retention mechanism in surfactant ion-pair HPLC, being a combination of ion-pair distribution and in situ ion exchange [28, 36]. Fig. 2 shows the linear relationship between the logarithm of the

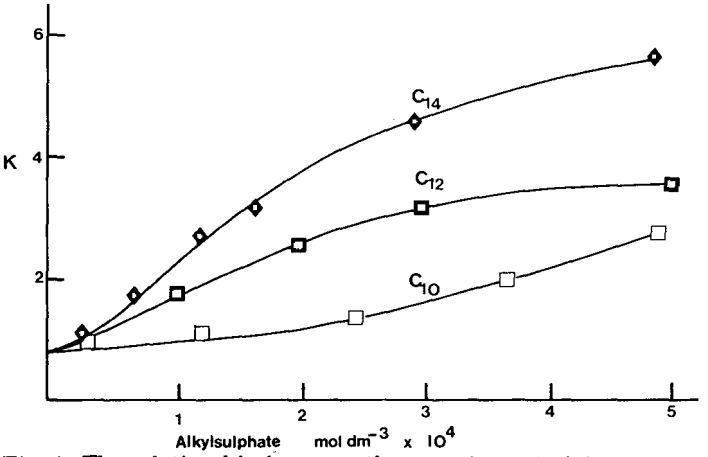

Fig. 1. The relationship between the capacity ratio (κ) of tryptophan and the concentration of decyl (C_{10}), dodecyl (C_{12}) and tetradecyl (C_{14}) sodium sulphate pairing ions in the mobile phase. Conditions: temperature, 30°; flow-rate, 2.0 ml min^{-1}; stationary support, 100 × 5 mm (I.D.) ODS-silica (5 μm); mobile phase, 50% methanol in citrate buffer at pH 2.25 containing alkylsulphate pairing ions.

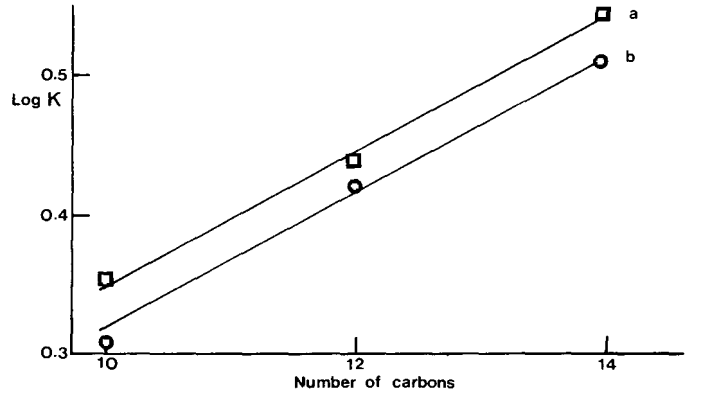

Fig. 2. The relationship between the logarithm of the capacity ratio of tryptophan and the number of carbons in the alkylsulphate pairing ions at mobile phase concentration of: (a) $5.0 \cdot 10^{-4}$ mole dm^{-3}; (b) $4.5 \cdot 10^{-4}$ mole dm^{-3}. Conditions as Fig. 1.

capacity ratio of tryptophan and the number of carbons in the alkylsulphate pairing ion. From Figs. 1 and 2 it is demonstrated that the required capacity ratio for a solute molecule can be achieved by choosing the appropriate pairing ion concentration and chain length, without any further alteration to the mobile phase composition. The assay development was at 30° and the actual determinations in biological fluids were at ambient temperature. The reduction in temperature resulted in a rise in capacity ratio for trypthophan from 3.52 to 4.00 for mobile phase concentration of $4 \cdot 10^{-4}$ mole dm^{-3} dodecyl sodium sulphate.

Fig. 3 shows how column efficiency in surfactant ion-pair HPLC systems is related to the homologue concentration, with high values of reduced plate height (h) being obtained at low pairing ion concentration. Maximum column efficiency is obtained above a $3 \cdot 10^{-4}$ mole dm^{-3} pairing ion concentration. These results are produced below the critical micelle concentration of the pairing ion and it is likely that at higher concentrations micellisation of the pairing ion in the mobile phase will alter column efficiency and capacity ratios.

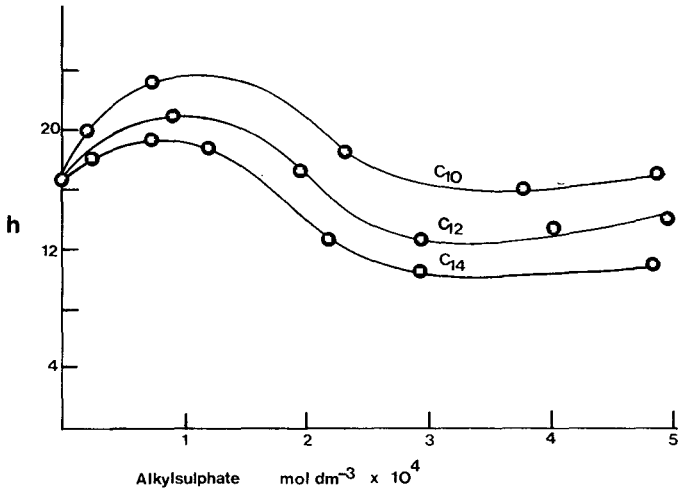

Fig. 3. The effect of alkylsulphate pairing ion concentration and chain length on the reduced plate height (h). Conditions as Fig. 1.

For the analysis of tryptophan in biological fluids, a mobile phase concentration of $4 \cdot 10^{-4}$ mole dm^{-3} sodium dodecylsulphate was used since this resulted in an ideal capacity ratio value for tryptophan and acceptable column efficiency.

All estimations of tryptophan were made on the basis of peak height determinations and by comparison with known standard solutions. The linearity of this response was checked by calibration curves in aqueous solutions and in spiked samples of plasma and saliva (see Table I). The calibration curve for tryptophan in plasma did not pass through the origin due to the presence of endogenous tryptophan in the plasma. The amount of tryptophan in the plasma controls was calculated by extrapolation of the line and was found to be $2.2 \cdot 10^{-3}$ mole dm^{-3} (see Fig. 4). Tryptophan-free saliva controls were obtained by rinsing the mouth with a citric acid solution.

TABLE I

SUMMARY OF THE CALIBRATION PLOTS OF TRYPTOPHAN AGAINST PEAK HEIGHT OR PEAK HEIGHT RATIO IN VARIOUS ENVIRONMENTS

Tryptophan concn. range (mole dm^{-3})	Internal standard concn. (mole dm^{-3})	Environment	Number of points	Correlation coefficient (r)
10^{-5}—10^{-4}	4-Aminophenol (10^{-4})	Aqueous buffer	5	0.996
10^{-6}—10^{-5}	—	Aqueous buffer	5	0.996
10^{-5}—10^{-4}	4-Chloroanaline (3×10^{-4})	Plasma	4	0.997
10^{-6}—10^{-5}	—	Saliva	5	0.987

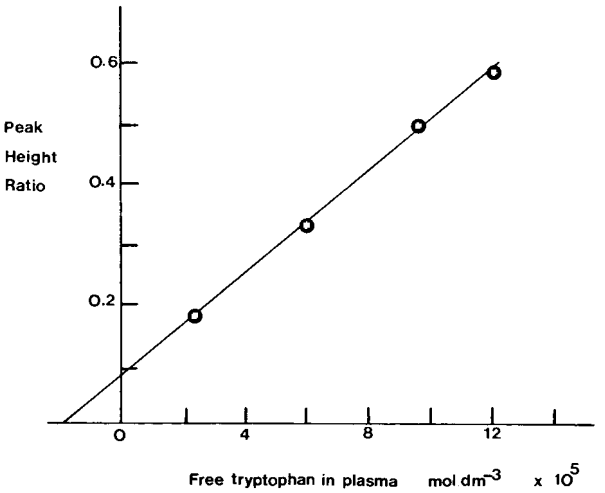

Fig. 4. The relationship between the peak height ratio of tryptophan to internal standard, and the concentration of tryptophan in plasma. Conditions: Temperature, ambient; flow-rate, 2.0 ml min^{-1}; mobile phase, 50% methanol in citrate buffer at pH 2.25 with $4 \cdot 10^{-4}$ mole dm^{-3} sodium dodecylsulphate.

The coefficient of variation of the peak heights produced by ten consecutive 10-μl injections of a 10^{-5} mole dm^{-3} tryptophan solution was 1.68% indicating that four point assays were possible without internal standards, provided that a loop valve injector was used. For aqueous solutions, 4-aminophenol (which eluted before trypthophan) was used as internal standard. For biological fluids, 4-chloroanaline (which eluted after tryptophan) was used as internal standard. For the assays the same internal standard was used in the reference solution as in the biological fluids (see Table I for concentrations of internal standards used). The limit of detection was 10^{-10} moles and for determinations of tryptophan below 10^{-5} mole dm^{-3} injections of 100 μl were required. This resulted in band broadening and incomplete resolution of tryptophan and 4-chloroanaline, so that the internal standard was omitted from these determinations.

The selectivity of the method was demonstrated by injecting controls of plasma, urine and saliva (Fig. 5) and by separation of tryptophan from its metabolites 5-hydroxytryptophan (5-HTP), 5-hydroxytryptamine (5-HT), 5-hydroxy-3-indoleacetic acid (5-HIAA) and kynurenine (Fig. 6). The tryptophan-free saliva contained no peak corresponding to tryptophan. The peaks corresponding to tryptophan in urine and plasma were identified by the addition of authenticated samples of tryptophan.

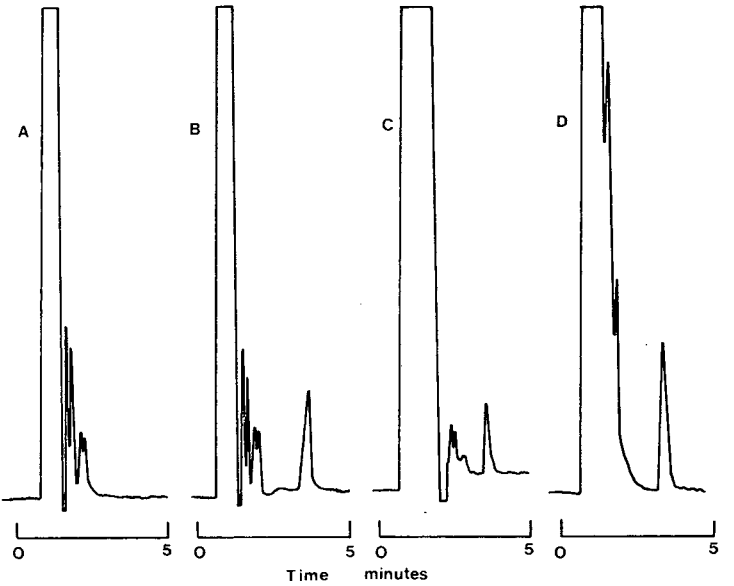

Fig. 5. Chromatographic traces of biological fluids. A = Tryptophan free saliva; B = saliva spiked with $4 \cdot 10^{-6}$ mole dm^{-3} tryptophan; C = plasma; D = urine. Conditions as Fig. 4.

In general, metabolites of biological substances are more polar than the parent compound since this facilitates urinary excretion by the kidney. Fig. 7 shows the important metabolitic pathways of tryptophan. The gain of a hydrophilic substitutent —OH causes a dramatic fall in capacity ratio from 4.0 to 1.5. The loss of an uncharged —COOH to give 5-HT has a lesser effect reducing the capacity ratio from 1.5 for 5-HTP to 1.2 for 5-HT. The principle of retention in surfactant ion-pair HPLC involves both hydrophobic interactions and the interaction between a charged group in the solute molecule (in this case $\equiv\overset{+}{N}-$) and an oppositely charged group ($-SO_4^-$) in the pairing ion. Loss of the charged amino group to give 5-HIAA has the greatest effect on retention such that 5-HIAA is almost unretained having a capacity ratio of 0.5. Splitting of the hydrophobic indole ring to give kynurenine is partly compensated for by the fact that kynurenine has two free charged amino groups available for interaction and so has a capacity ratio of 2.2. This group contribution approach is useful not only in rationalising results but also in the prediction of retention of compounds, particularly if authentic samples are unavailable, as is often the case when drug metabolites or degradation products are under investigation.

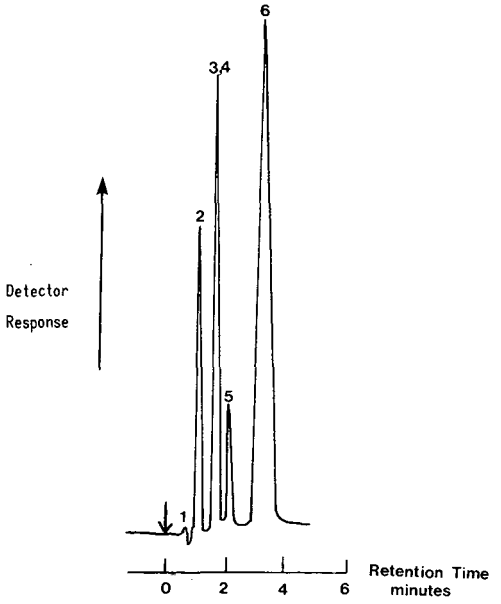

Fig. 6. Chromatographic trace to show the separation of tryptophan from its main metabolites: 1 = solvent (κ = 0); 2 = 5-HIAA (0.5); 3 = 5-HT (1.2); 4 = 5-HTP (1.5); 5 = kynurenine (2.2); 6 = tryptophan (4.0). Conditions as Fig. 4.

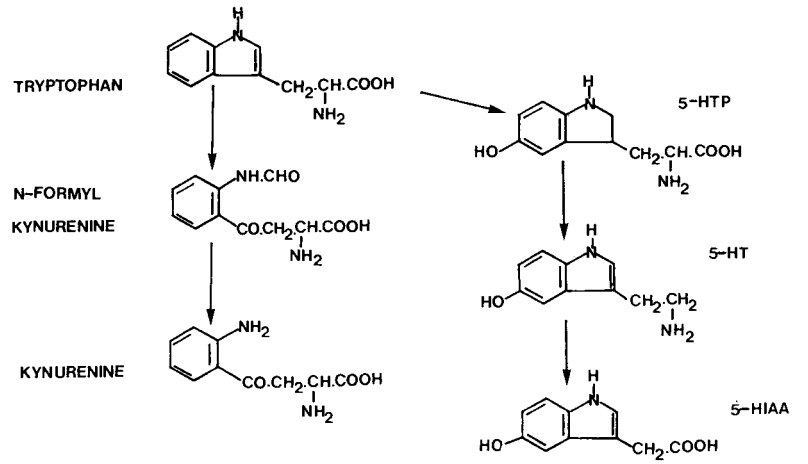

Fig. 7. Summary of the main metabolic pathways of tryptophan.

The urinary excretion profile and bioavailability of tryptophan after a single oral loading dose were determined in fasting male subjects using the developed assay. For the urinary excretion study 1 g (i.e. 2 × 500-mg Optimax tablets) was administered orally and urine samples taken at intervals over a period of 6 h. For the bioavailability study, 2 g (i.e. 4 × 500-mg Optimax tablets) was administered orally and 10 ml blood samples and 10 ml saliva samples taken at

intervals over a period of 6 h. Saliva was collected over a period of 10 min with no attempt made to stimulate salivation artificially. Table II shows the concentration of tryptophan found in the urine and the total amounts of tryptophan excreted with time. Table III shows the concentration of free tryptophan found in plasma and saliva with time. These results confirm those obtained by Ashley et al. [37] in a similar study using fluorimetry as an assay technique, although higher levels of sensitivity have been achieved in this present work.

Salivary levels of tryptophan correlate well with the levels found in plasma, which reinforces the hypothesis that the tryptophan in saliva is in equilibrium with the free tryptophan in plasma. The pharmacological significance of these findings will be reported elsewhere.

TABLE II

THE CONCENTRATION OF TRYPTOPHAN AND THE TOTAL AMOUNT OF TRYPTOPHAN EXCRETED IN THE URINE WITH TIME AFTER A 1 gm ORAL DOSE

Time (h)	Tryptophan concentration in urine		Volume of urine taken	Cumulative amounts of tryptophan excreted	
	(mole $cm^{-3} \cdot 10^4$)	($\mu g\ ml^{-1}$)	(ml)	(moles $\cdot 10^5$)	(mg)
0	1.38	27.9	90	1.24	2.51
0.62	1.22	24.7	80	2.22	4.49
1.55	3.64	73.6	54	4.18	8.45
2.87	1.74	35.2	195	7.58	15.33
5.42	2.09	42.3	71	9.67	19.55

TABLE III

CONCENTRATION OF FREE TRYPTOPHAN IN PLASMA AND SALIVA WITH TIME AFTER A 2 gm ORAL DOSE

Time (h)	Plasma concentration		Time (h)	Saliva concentration	
	(mole $dm^{-3} \cdot 10^5$)	($\mu g\ ml^{-1}$)		(mole $dm^{-3} \cdot 10^5$)	($\mu g\ ml^{-1}$)
0	0.78	1.57	0	0.14	0.28
0.58	1.49	3.02	0.92	0.19	0.38
1.08	2.23	4.51	1.72	0.28	0.57
1.70	2.82	5.71	3.42	0.22	0.44
4.58	1.06	2.14	4.78	0.13	0.27
5.58	0.59	1.19			

CONCLUSIONS:

A surfactant ion-pair HPLC assay which uses low concentrations of pairing ion has been shown to be an effective one for the assay of tryptophan in biological fluids, and should permit the possible salivary/blood level correlations for tryptophan to be well investigated. The flexibility of the approach also permits the various metabolites of similar structure to be well resolved. The study has also provided us with group contribution data for ion-pair systems for possible use in predicting solute retention behaviour.

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WAYS OF COLLAGEN SEPARATION IN PATHOLOGICALLY ALTERED TISSUE

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SUMMARY

A system of chromatographic methods using two successive DEAE-cellulose chromatographic steps and two successive separations on Bio-Gel A-1.5 m has been worked out for the separation of individual collagen types. The success of the procedure is based on the preliminary removal of proteoglycans during the first DEAE-cellulose run. Alternatively it is possible to replace chromatographic steps, following the removal of proteoglycans, with fraction precipitation.

INTRODUCTION

Collagen is an example of a polymorphic protein; at least five different collagen types* have been described in human tissues. Different tissues contain different collagen types and frequently various collagen types occur side by side. In healthy subjects the proportions of individual collagen types are subject only to slight variations during ontogeny. On the other hand, gross changes have been observed under pathological circumstances. For example, in inflamed tissues usually the amount of collagen type III increases [1], in osteoarthrotic cartilages, besides the usual type II, types I and III are also present [2]. Altered proportions of individual collagen types also occur in inborn errors of collagen metabolism.

Despite the common skeleton of the collagen molecule individual collagen types differ in details in the amino acid composition, and in mechanical, deposition and transport properties. Currently five different collagen types are recognized. Of these only collagen type I is partially soluble; all the other collagen types can be brought into solution only after a brief proteolytic

*Since the nomenclature of collagen types and polypeptide chains that consitute the tropocollagen triple helix is rather complex, the reader not familiar with this is referred to ref. [3].

cleavage. This fact determines both some properties of the tissue and the methods of analysis. Immunofluorescence techniques that are capable of differentiating individual collagen types have the advantage of high sensitivity and allow the topographic mapping of the tissue, but they do not allow quantitative evaluation. Fraction precipitation is a simple method, which, however, allows the separation of only some collagen types. Therefore, chromatographic methods alone or in combination with other separation procedures are the methods of choice in the evaluation of healthy and diseased connnective tissue. At present there is not a single chromatographic procedure that offers complete separation of all collagen types. The main aim of this communication was to make a rational selection of chromatographic methods in order to obtain information about the content of individual collagen types in a particular tissue sample.

A system of chromatographic methods has been applied to kidney collagen as the demonstrative example because this tissue contains all collagen types, with the exception of collagen type II, in a reasonable amount.

EXPERIMENTAL

Collagen preparation

Since, apart from a small amount of collagen type I, all collagen types in adult tissues are insoluble, for the isolation of individual collagen types pepsin digestion has to be carried out on the tissue homogenate pre-extracted with citrate buffer of pH 3.7 (collagen:pepsin = 20:1, w/w; 24 h; 25°; pH 2.0 adjusted with hydrochloric acid, repeated once with a double amount of pepsin) [4]. To the pepsin digest sodium chloride was added to produce a final concentration of 0.9 *M* to precipitate collagen. The crude precipitate was then purified by extraction with 0.1 *M* NaCl in 0.05 *M* Tris—HCl buffer (pH 7.5). The collagen solution was then dialysed against 1.0% acetic acid to remove NaCl, and lyophilized.

Removal of proteoglycans by DEAE-cellulose chromatography [5]

In order to make the chromatographic system work, we have found this step essential for further collagen fractionation as proteoglycans, due to their interaction with collagen, interfere with the separation of individual collagen types. Lyophilized collagen was dissolved in 0.2 *M* NaCl in 0.05 *M* Tris—HCl (pH 7.5) and applied to a DEAE-cellulose column (Whatman DE-52, 30 × 5 cm; amount loaded 200 mg), which was equilibrated with the same buffer and cooled to preserve the collagen triple helix. Optimum flow-rate was 100 ml/h. The effluent was monitored by UV absorbance at 230 nm. When no further UV-absorbing material was detected in the effluent, the eluting solvent was changed abruptly to 1.0 *M* NaCl in 0.05 *M* Tris—HCl (pH 7.5) and elution was continued with this buffer until an additional peak was eluted from the column. The first fraction containing purified collagen was freed from non-volatile solutes by dialysis against 1.0% acetic acid and lyophilized [3]. The other (proteoglycan) fraction was discarded.

Collagen prepared in this way is ready for isolation and quantitation of individual collagen types.

Separation of collagen type V from types I and III by DEAE-cellulose chromatography [6]

The sample of purified collagen was dissolved in 0.02 *M* NaCl in 0.05 *M* Tris—HCl, pH 7.5 (2 *M* with respect to urea) and applied to the 2.5 × 20 cm DEAE-cellulose column (Whatman DE-52), refrigerated, and equilibrated with the same buffer. When no further UV-absorbing material (220 nm) was detected, the eluting solvent was changed to a linear gradient of 0.02 *M* NaCl in 0.05 *M* Tris—HCl, pH 7.5 (2 *M* with respect to urea) to 0.3 *M* NaCl in 0.05 *M* Tris—HCl, pH 7.5 (2 *M* with respect to urea), using 500 ml of both buffers. The amount loaded was 140 mg and the flow-rate was 90 ml/h.

The first peak in this separation is formed by collagen types I and III; the peak eluted after the gradient has been introduced is mainly collagen type V.

Identification of collagen type III by agarose (Bio-Gel A-1.5 m) chromatography [7]

The identification of collagen type III is based on the presence of the disulphide bonds in this collagen type; these are absent in collagen type I. A Bio-Gel A-1.5 m column (4.5 × 150 cm) was used with 1 *M* $CaCl_2$ in 0.05 *M* Tris—HCl pH 7.5 as eluent. The UV-absorbance profile exhibits three maxima corresponding to 300,000, 200,000 and 100,000 relative molecular weights. Contrary to the previous separation procedures this one is carried out with denatured collagen. Samples were heated to 43° to ensure denaturation before application to the column. Due to the disulphide bonds, collagen type III is present in the fastest peak, where chain polymers of collagen type I are also present. Peaks with higher retention volumes contain depolymerized collagen type I.

The final proof of the presence of collagen type III was achieved by reduction and alkylation of the fast-running fraction. This was done by dissolving 20 mg of the isolate from the first agarose separation in 10 ml of 8.0 *M* urea (pH 8.0), 0.1 *M* with respect to Tris attained by the addition of Tris-free base. The reaction mixture was flushed with nitrogen, and 2-mercaptoethanol (1.42 ml/l) was added. It was left for 4 h at 37° and then the solution was made 0.02 *M* with respect to iodoacetate. The reaction mixture was left to stand an additional 45 min at room temperature in darkness [4]. After desalting by dialysis and subsequent lyophilization, the whole sample was dissolved in the eluting buffer and subjected to a second Bio-Gel A-1.5 m run. In this case a 3.0 × 150 cm column was used, with the same operating conditions as before [7].

Fraction precipitation. This was carried out by adding NaCl to 1.5 *M* concentration. The type III collagen was collected by centrifugation, dissolved in 1.0% acetic acid, the NaCl removed by dialysis and the collagen type III was lyophilized [4].

CM-cellulose (Whatman CM-52) chromatography [8]. This method was used to characterize the precipitate. The column (1.5 × 8.5 cm) maintained at 40° was equilibrated with 0.01 *M* potassium acetate and 1 *M* urea buffer (pH 4.8). The column was eluted with a linear gradient of 0—0.14 *M* LiCl in 500 ml of buffer.

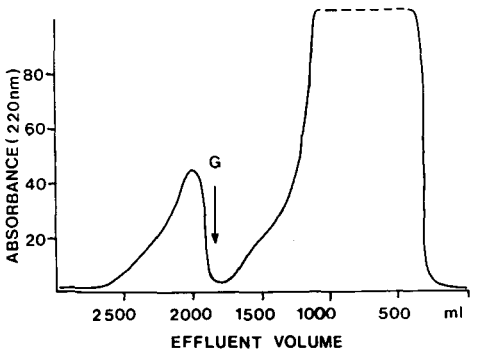
Analysis of the collagenous stroma of pyelonephritic kidney

Human pyelonephritic kidney was the tissue of choice, to demonstrate the versatility of the system of chromatographic separations described above.

RESULTS AND DISCUSSION

As demonstrated in Fig. 1, in the first step proteoglycans were removed by the DEAE-cellulose chromatography. The fraction eluted with 0.2 *M* NaCl + 0.05 *M* Tris—HCl represents proteoglycan-free collagen, which is used for further fractionation. It should be stressed that this step is essential for subsequent isolation of collagen type III by both the appropriate chromatographic procedure and the salting-out method. In both cases, especially however in the salting out method, the presence of proteoglycans causes substantial interference and their contamination of collagen type III is always unacceptable.

The next step is the second DEAE-cellulose chromatography in which a combined peak of collagen types I and III is separated from the bulk of other collagen types (Fig. 2). It is not advisable to attempt the separation of collagen type I and III from other collagen types and proteoglycans in one chromatographic run as no clear separations of individual collagen types can be obtained under such circumstances.



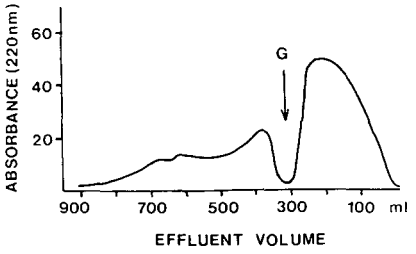


Fig. 1. DEAE-cellulose chromatography of the peptic digest used for the removal of proteoglycans. The arrow (G) indicates the change in the eluting buffer from 0.2 *M* NaCl to 1.0 *M* NaCl (0.05 *M* Tris—HCl, pH 7.5). For further details see Experimental. The peak with low retention volume represents collagen; the other represents proteoglycans.

Fig. 2. DEAE-cellulose chromatography of the purified collagen obtained by the separation shown in Fig. 1. Eluting buffer: 0.02 *M* NaCl (0.05 *M* Tris—HCl, pH 7.5, 2 *M* with respect to urea). G indicates the beginning of the linear gradient 0.02—0.3 *M* NaCl in 0.05 *M* Tris—HCl, 2 *M* with respect to urea (twice 500 ml, 2.5 × 20 cm column).

The separation of collagen types I and III is achieved by molecular sieving. The isolate from the second DEAE-cellulose separation gives on Bio-Gel A-1.5 m a dominant peak which is composed of γ-chains and higher polymers of both type I and type III collagen (Fig. 3). Minor peaks, if present at all, originate essentially from collagen type I. The material from the dominant peak is subjected to reduction and alkylation. Then the second Bio-Gel A chromatography

is carried out. The peaks with smaller molecular weight result from the depolymerized collagen type III (breakage of S—S bonds) and represent the α- and β-fractions of collagen type III. The peak that represents collagen chain polymers is formed by collagen type I and the rest of collagen type III bonded through the non-reducible cross-links (Fig. 3).

Instead of the two BioGel A chromatographic steps it is also possible to separate the collagen types I and III from the isolate of the second DEAE-cellulose run by the salting-out method. By this method collagen type III including the portion bonded with non-reducible cross-links can be isolated in reasonably pure form as shown in Fig. 4.

Collagen type I and type III were identified in all cases by amino-acid analysis and by BrCN-peptides (not shown).

The chemical and also the physical properties of individual collagen types are different and changes in their proportion are reflected in the tissue function. It is useful to obtain information about the proportion of individual collagen

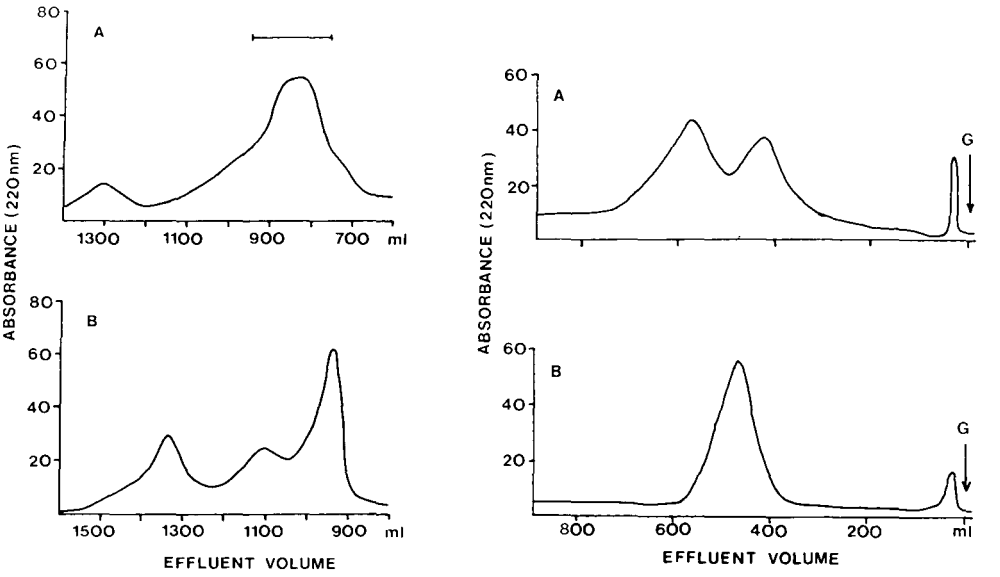


Fig. 3. Bio-Gel A-1.5 m chromatography. (A) A mixture of collagen types I and III (isolate from the second DEAE-cellulose chromatography — Fig. 2) Eluting buffer: 1 *M* $CaCl_2$ in 0.05 *M* Tris—HCl (pH 7.5) and 2 *M* urea. The dominant peak is chain polymers of collagen type I + III; the small peak contains single collagen chains (type I). (B) Rechromatography of the dominant peak from the run in (A) as indicated by the horizontal bar, after reduction and alkylation. For details see Experimental. Elution buffer identical with that of (A). Peaks from right to left: γ-fraction (chain polymers), a mixture of types I and III cross-linked with non-reducible cross-links; β-fraction (chain dimers)—collagen type III; α-chains, single polypeptide chains of collagen type III.

Fig. 4. CM-cellulose chromatography. The column (1.5 × 8.5 cm), maintained at 40°, was equilibrated with 0.02 *M* potassium acetate and 1 *M* urea buffer (pH 4.8). The column was eluted with a linear gradient of 0—0.14 *M* LiCl in 500 ml of buffer. (A) Collagen type I: the supernatant after precipitation with 1.5 *M* NaCl. (B) Collagen type III: the precipitate after precipitation with 1.5 *M* NaCl.

types in diseases affecting the connective tissue in order to understand better the pathological mechanisms involved.

For the system in which separation of individual collagen types is carried out by chromatographic methods only, the amount of collagen needed for a complete separation is of the order of 100 mg. The advantage of this procedure is that very pure collagen fractions are obtained. On the other hand, if fraction precipitation follows the preliminary removal of proteoglycans by DEAE-chromatography the amount of collagen needed can be cut down to about 10 mg. Quantitation can be done by hydroxyproline assay or by optical rotation measurement.

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LONGITUDINAL URINARY TRACE AMINE EXCRETION IN A HUMAN MALE

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SUMMARY

The urinary excretion of a β-phenylethylamine (PE), *m*-tyramine (*m*TA), *p*-tyramine (*p*TA) and tryptamine (TR) in their unconjugated (free) and conjugated (except tryptamine) forms, was examined in a male human subject over a total period of 28 days. The average excretion values were (in μg per 24 h, mean ± standard error): PE, free 3.19 ± 0.21, conjugated 6.85 ± 0.52; *m*TA, free 98.0 ± 2.2, conjugated 106.1 ± 18.6; *p*TA, free 427 ± 12, conjugated 571 ± 142; and TR, free 79.4 ± 2.8.

This data, when considered along with other published information, permits the suggestion that probably *m*TA and *p*TA in the unconjugated form are exclusively formed endogenously; whether or not this also pertains to PE and TR is less clear. In all cases, the conjugated amines derive from both exogenous and endogenous sources.

INTRODUCTION

The normal urinary excretion of the trace amines, β-phenylethylamine (PE), *m*-tyramine (*m*TA), *p*-tyramine (*p*TA), and tryptamine (TR) in the human is well documented (see recent review by Boulton [1]). In the unconjugated form, the values (as μg per 24 h, mean ± standard error; n = 16—21) listed by Slingsby and Boulton [2] are: PE, 4.9 ± 1.0; *m*TA 83 ± 7; *p*TA, 489 ± 40; and TR, 100 ± 13, respectively. The variation in the excretion of the amine conjugates is much larger in all cases. Abnormalities in the excretion of both the free and conjugated forms of the trace amines have been claimed to occur in Parkinsonism, migraine, depression, schizophrenia, phenylketonuria, mania and hypertension [1—3].

In a recent investigation [2, 4], a significant proportion of mentally disordered individuals admitted to a general hospital psychiatric ward exhibited increases in their urinary excretion of unconjugated *p*TA, PE and TR. As a consequence of this, we have decided to investigate, in a longitudinal manner, the

excretion of PE, *m*TA, *p*TA and TR in their free and conjugated forms (except TR), in normal subjects and in selected patients suffering from certain mental disorders. This particular paper describes the excretion of the above amines in a human male, who ingested a "normal" diet and certain likely precursors labelled with deuterium, over a period of 28 days.

MATERIALS AND METHODS

All of the solvents, chemicals and materials used were commercially available with the exception of the deuterated internal standards [1,1,2,2-tetradeutero-2-(4-hydroxyphenyl)ethylamine, (*p*-tyramine-d_4); 1,1,2,2-tetradeutero-2(3-hydroxyphenyl)-ethylamine, (*m*-tyramine-d_4); 1,1,2,2-tetradeutero-2-phenylethylamine, (β-phenylethylamine-d_4), and 1,1-dideutero-2-indolylethylamine (tryptamine-d_2)], which were prepared as previously described [5, 6]. With the exception of tryptamine, all the amine internal standards were tetradeuterated. The use of d_4 internal standards possesses two distinct advantages when compared with the dideutero standards that were incorporated when the integrated ion current (IIC) method was first introduced [2, 6]. First, the correction factor (incorporated into the formula used to calculate the amounts present) is much smaller and secondly, and more importantly, the use of d_4 standards permits not only the quantitation of the endogenous (protonated) amine but also any amines synthesised from precursors labelled with a different number of deuteriums (see ref. 28 for further details).

The amino acid and amine precursors listed in Fig. 2—5, suitably labelled with deuterium, were synthesised in gram quantities for this study by Dr. Bruce Davis of the Psychiatric Research Division in Saskatoon.

Twenty-four hour urine samples starting with the first voiding each day, were collected onto 10 ml conc. HCl in polyethylene bottles; after recording the total volume, a 250-ml aliquot was removed and stored at −16° until analysed. The collection period was over 16 days in May, 1976, followed by a 12 day period in May, 1977 (28 days in total), during which time various deuterated likely metabolic precursors (see below) of the amines were ingested. The content of the diet which was "normal" for North America, was recorded. Precursors labelled with deuterium were ingested, in equally divided doses (at 8 a.m. and 6 p.m.), according to the following regimen: DL-phenylalanine-d_2 (3 gm) on day 2; DL-*p*-tyrosine-d_2 (3 gm) on day 6; DL-tryptophan-d_5 (3 gm) on day 10; β-phenylethylamine-d_2 (200 mg) on days 13 and 24.

At the time of analysis (the general procedure is summarized in Fig. 1) samples were completely thawed and mixed before aliquots were removed (10 ml in the case of PE, 1 ml for *m*TA, *p*TA and TR). Each aliquot was diluted to 15 ml, to which was added each internal standard (*p*TA-d_4, 1000 ng; *m*TA-d_4, 200 ng; PE-d_4, 200 ng; and TR-d_2, 500 ng). The aliquot was then adjusted to pH 7.0 with 2 *N* NaOH and percolated through a Bio-Rad AG 50W-X2 ion-exchange chromatography column (6 × 1 cm) prepared as described by Kakimoto and Armstrong [7]. The initial eluate and first water wash were retained for the determination of conjugated amines. The eluted amine fractions (ethanol—ammonium hydroxide—water, 65 : 25 : 10, in the case of *m*TA, *p*TA and TR, and methanol—hydrochloric acid, 86 : 14 in the case of PE) were

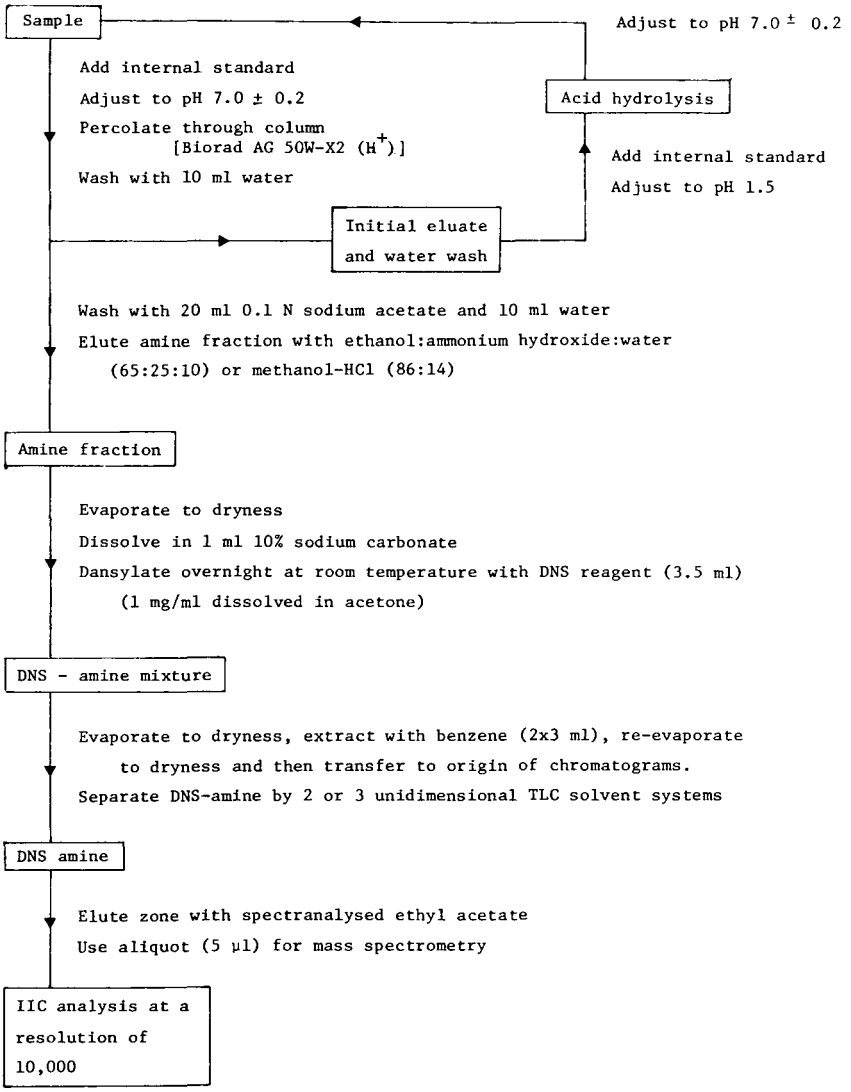

Fig. 1. Isolation, hydrolysis, derivatisation, chromatographic separation and mass spectral analysis of urinary trace amines.

dried under reduced pressure at 45°, dissolved in sodium carbonate (1 ml), dansylated, and after dansylation transferred to the origin of a silica-gel thin-layer chromatographic plate. PE was isolated by three successive unidimensional separations in solvent systems 1, 2 and 3 (see Table I), TR in systems 1 and 4 and the tyramines in systems 1 and 5. Complete details concerning the transfer and isolation of the zones, their comparison with standards run in parallel and their mass spectrometric analysis will be found in ref. 2 and the reviews by Philips [8] and Durden [9].

Conjugated amines were analysed in an identical manner in the same sample following hydrolysis of the column effluent and wash (30 min at 100° at pH 1.5).

TABLE I

CHROMATOGRAPHIC PROPERTIES OF THE DNS DERIVATIVES OF SOME TRACE AMINES

All separations on commercially prepared silica gel plates (Brinkman Instruments, Rexdale, Ontario, Canada).

Solvent system	R_F values for DNS derivatives of:			
	PE	*m*TA	*p*TA	TR
(1) Choroform—*n*-butyl acetate, 5 : 2	0.79	0.55	0.55	0.49
(2) Benzene—triethylamine, 8 : 1	0.60	—	—	—
(3) Carbon tetrachloride—triethylamine, 4 : 1	0.25	—	—	—
(4) Benzene—triethylamine, 4 : 1	—	—	—	0.11
(5) Benzene—triethylamine, 12 : 1	—	0.46	0.42	—

RESULTS

The urinary excretion profiles for free and conjugated PE, *m*TA, *p*TA and TR (free only) are shown in Figs. 2—5 and Table II. It can be seen that the daily variation in the excretion of the unconjugated tyramines is extremely small (*m*TA, 98 ± 2.2; *p*TA, 427 ± 12; in μg per day, respectively). A somewhat greater variation, although still relatively small, was seen in the case of PE

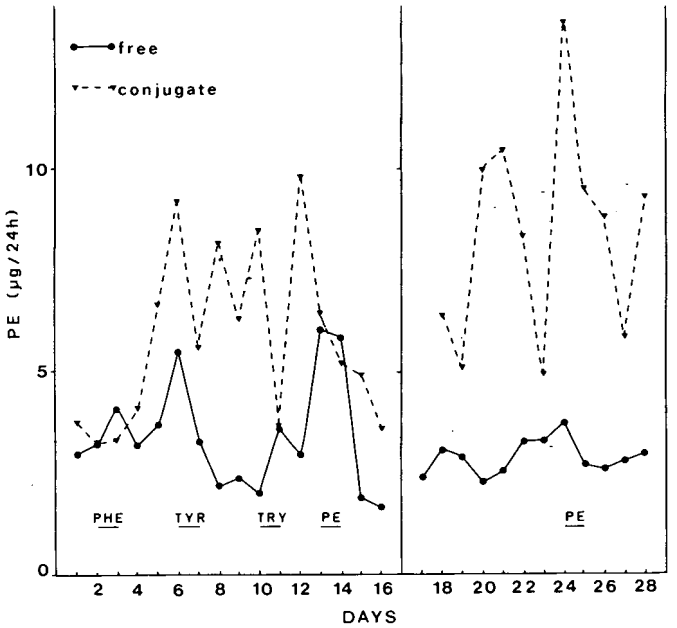

Fig. 2. Urinary excretion of conjugated and unconjugated β-phenylethylamine in a human male. The deuterated amino acids, phenylalanine, (PHE), tyrosine (TYR) and tryptophan (TRY) and deuterated phenylethylamine (PE) were ingested on days 2, 6, 10, 13 and 24 as described in the text.

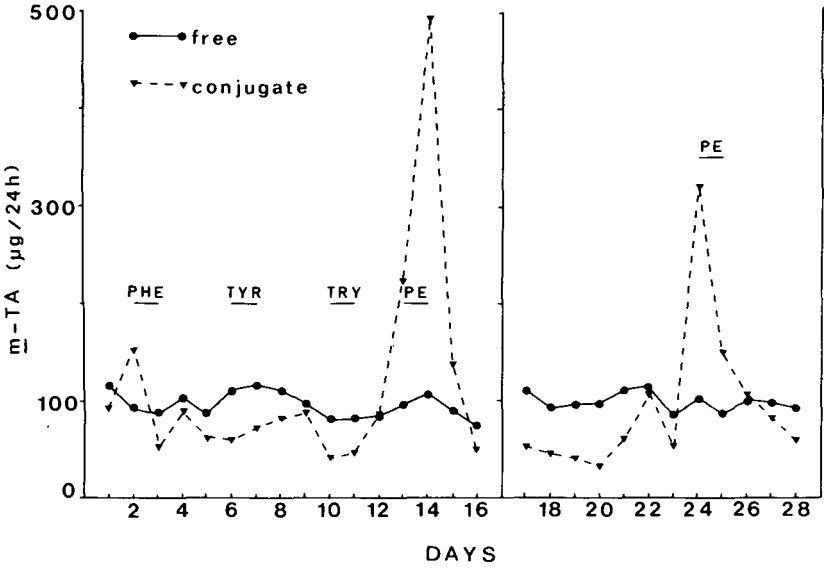

Fig. 3. Urinary excretion of conjugated and unconjugated *meta*-tyramine in a human male (see Fig. 2 and text for further details).

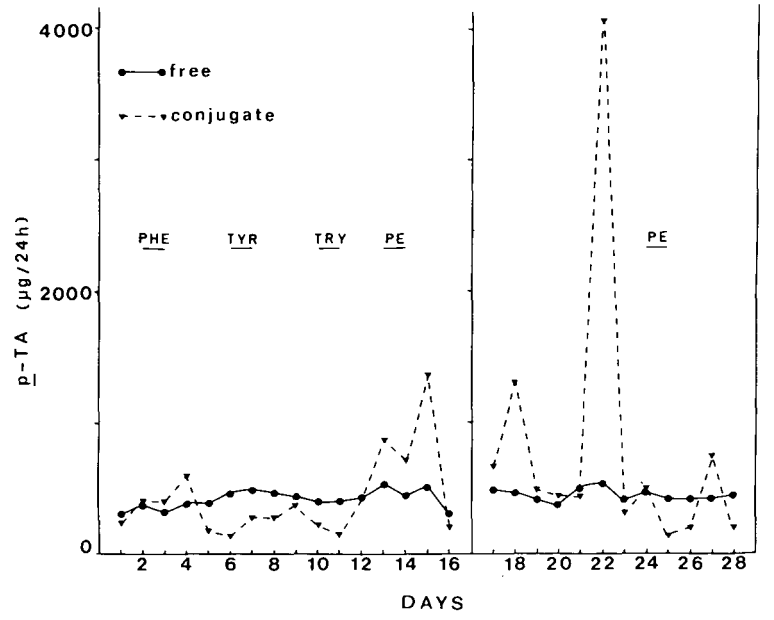

Fig. 4. Urinary excretion of conjugated and unconjugated *para*-tyramine in a human male (see Fig. 2 and the text for further details).

(3.2 ± 0.2) and TR (79.4 ± 2.8) (in µg per day).

The ingestion of the precursor substances exhibited little, if any, effect on the excretion of the unconjugated tyramines.

The excretion of all the amines in their conjugated forms was much more

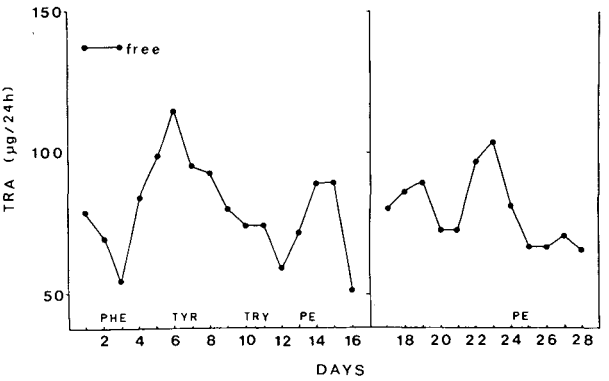

Fig. 5. Urinary excretion of unconjugated tryptamine in a human male (see Fig. 2 and the text for further details).

TABLE II

URINARY EXCRETION OF SOME TRACE AMINES IN A HUMAN MALE

All values as Mean ± S.E. are in µg per 24 h.

Amine	Free		Conjugated	
	Level	Range	Level	Range
β-Phenylethylamine	3.19 ± 0.21	1.67— 6.06	6.85 ± 0.52	3.27— 13.62
m-Tyramine	98.0 ± 2.2	74.7 —117.39	106.1 ± 18.6	34.4 — 494.0
p-Tyramine	427 ± 12	298 —549	571 ± 142	134 —4065
Tryptamine	79.4 ± 2.8	50.8 —115.3	—	—

variable, with large increases sometimes following ingestion of some of the precursors (see Figs. 2—5).

The measurement of conjugated TR after either alkaline or enzymatic hydrolysis was not possible.

DISCUSSION

The question of the origin of urinary trace amines, particularly the tyramines, has been debated for some considerable time; the principal suggestions have been that they arise from: (1) exogenous sources (diet and gut bacteria); (2) endogenous sources; or (3) a combination of endogenous and exogenous sources [1—3, 7, 10]. We believe the available evidence now permits a more definitive conclusion to be advanced concerning the origins of *m*TA and *p*TA; namely, that unconjugated tyramines (*meta* and *para*; *ortho* is not considered here) arise from endogenous sources while the conjugated tyramines are made up of a relatively constant endogenous component along with a variable exogenous component. The argument that the unconjugated tyramines may arise from the gut flora (aerobic or anaerobic) with any variations being a consequence of change in the intestinal flora composition [10], could equally well be interpreted as the availability of, and changes in, the absorption of the

precursor substances which in vivo are then converted to the tyramines. As can be seen from Figs. 3 and 4, Table II and ref. 5, however, the excretion of the unconjugated tyramines occurs with remarkable consistency and little, if any, variation.

For any substance found in urine to be considered to be of endogenous origin it would have to be excreted in a stable, consistent and reproducible manner over time in any particular individual, be excreted with relatively little variation among individuals in any particular species, and be normally present in tissues with enzymes for its synthesis and degradation also being present in those tissues. It is now clear that unconjugated *m*TA and *p*TA meet these criteria.

The average daily excretion of unconjugated *m*TA and *p*TA in a control human population is about 83 and 489 μg (83 ± 7 and 489 ± 40 respectively, mean ± standard error of the mean, $n = 21$) [2]. Such figures agree well with those published by Boulton and co-workers [5, 6, 11, 12, 14], and others [4, 7—10, 13] at other times and in different laboratories; as can be seen, any variation is surprisingly small. In a longitudinal study in a single individual as reported in this publication, even when there was an interruption of a year in the middle of the urine collection period, and when various potential precursors were ingested, the excretion of unconjugated *m*TA and *p*TA remained remarkably stable and reproducible (98 ± 2.2 and 427 ± 12, μg per day respectively). Further data supporting an essentially exclusive endogenous origin for the unconjugated tyramines are that virtually no free *p*TA was excreted following ingestion of *p*TA (up to 100 mg as free base) although between 2 and 6% was excreted in the free form when *p*TA was injected intravenously [11, 15] and that no *p*TA was excreted in the free form following the ingestion of foods known to be rich in *p*TA. In this latter experiment [12] there was an almost stoichiometric relationship between the *p*TA ingested and the conjugated *p*TA and free and conjugated *p*-hydroxyphenylacetic acid excreted. The unambiguous identification and quantitation of *m*TA and *p*TA in peripheral tissues, in the brain and in subcellular fractions prepared from brain, have recently been established [16—19]. In addition, it has recently been shown [20] (see also 1, 21) that decarboxylation of tyrosine in mammals is unlikely to be the primary synthetic origin of *p*TA. *m*TA arising from *meta*-tyrosine by simple decarboxylation is unlikely to occur since *meta*-tyrosine is not a normal constituent of foods or proteins. Instead, it is now known that *p*TA and *m*TA arise by hydroxylation of PE and dehydroxylation (perhaps associated in some situations with a subsequent decarboxylation) of catecholic precursors; hydroxylation probably represents the major route of synthesis for *p*TA and dehydroxylation the major route for *m*TA [18, 21—28]. The above data, we believe, make it very likely that unconjugated *m*TA and *p*TA are predominantly, if not exclusively, of endogenous origin.

It is clear from Figs. 2—5 and Table II, however, that the conjugated forms of these amines are excreted with considerable daily variation and are affected in some cases by the ingestion of possible precursors. We conclude that they arise from both exogenous and endogenous sources.

The situation with respect to PE and TR is not as clear. These amines are formed predominantly by decarboxylation in mammalian tissues as well as in

bacteria and even in the unconjugated form, are excreted with relatively large day-to-day fluctuations. At this time it is not possible to be sure of their origin, although it must be noted that they are normal constituents of peripheral and cerebral tissues [1, 16—19, 21, 29—33] and they are oxidised and lost after injection at remarkably fast rates [34, 35].

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CHROMBIO. 250

GAS CHROMATOGRAPHIC EVALUATION OF PLASMA TRIGLYCERIDE COMPOSITION IN HYPERLIPIDEMIA

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SUMMARY

The molecular distribution of human plasma triglycerides was studied by gas—liquid chromatography. Triglycerides of the total plasma lipids are separated into fractions according to molecular weight. Individual fractions consist of molecules containing constant numbers of carbon atoms in the acyl chains. The carbon number serves to designate the fraction. In normal plasma it is possible to separate plasma triglycerides into four main fractions: C_{48}, C_{50}, C_{52}, and C_{54}.

The reference group consists of 199 apparently healthy subjects (93 men, 106 women) and is stratified according to age. Triglyceride values were adjusted to age and sex. Parameters of triglyceride fractions C_{48}—C_{56} were calculated after arcsin transformation of percentage values.

The group of hypertriglyceridemic subjects totals 113 (56 men, 57 women). Their adjusted triglyceride values exceed the 95 percentile of the reference group values. Analysis of the triglyceride fractions gives the following results:

(1) In more than a quarter of the hypertriglyceridemic patients a different molecular distribution was demonstrated. Mostly it was fraction C_{56} that was increased in proportion.

(2) There seems not to be an anomaly of triglyceride molecular distribution associated with any type of hyperlipoproteinemia. Nevertheless, in a family with type III hyperlipoproteinemia we observed a constant increase of C_{56} and higher fractions.

Fraction C_{56} and higher molecular weight fractions contain one or more twenty-carbon or longer fatty acids chains. We conclude that hypertriglyceridemia in about one quarter of cases is associated with a disorder of the metabolism of triglyceride molecules containing C_{20} or longer fatty acid chains.

INTRODUCTION

Hypertriglyceridemia is known as an important risk factor of coronary heart disease even if the causal relationship has not yet been proved [1,2]. In all

previous studies on hyperlipidemia only total plasma triglyceride levels have been assessed. This is the consequence of methodological difficulties in analysis and quantitation of triglyceride molecular species in large-scale clinical studies.

In 1967 Kuksis and collaborators proposed gas chromatographic (GC) analysis of the plasma lipid profile [3]. This was a good step forward because the method affords a better view of plasma triglyceride composition with a relatively simple technique. In principle Kuksis' method separates triglycerides according to molecular weight. Individual triglyceride fractions are identified by their "carbon number". The carbon number is the sum of all the carbon atoms of the fatty acyl chains attached to glycerol in the individual molecule.

In our laboratory we have increased possibilities for the quantitation of triglyceride fractions [4]. At present we have several thousands of analyses for evaluating the technique for clinical purposes. This present paper reports on the comparison of hypertriglyceridemia and the reference group.

METHODS

Reference group

The reference group is composed of 199 persons (93 men, 106 women) selected according to the following criteria. Exclusion of all subjects with (1) weight index exceeding the value of 1.1; (2) arterial hypertension; (3) hyperglycemia; (4) hyperuricemia; (5) functional disorder of the thyroid; (6) xantomatus manifestations on the skin; (7) corneal arcus senilis.

We excluded all subjects with a history of manifest signs of illness, all subjects with a history of (a) myocardial infarction; (b) hysterectomy; (c) cholecystectomy; (d) thyroid surgery; (e) familial hyperlipoproteinemia; (f) viral hepatitis within the last five years.

Further criteria for exclusion were (1) hormonal contraception in women; (2) regular alcohol consumption; (3) heavy smoking habits (more than 20 cigarettes a day); (4) special dietary habits.

Group of hyperlipidemic patients

The group of hyperlipidemic patients consisted of 113 persons (56 men, 57 women) with a plasma triglyceride value, adjusted for age and sex, of more than 144 mg/dl. This critical value is significant on a 5% level. No difference was made for lipoprotein type hyperlipidemia according to Fredrickson's classification, but the majority of patients were classified as combined hyperlipidemia. No exclusion was made for patients with secondary hyperlipidemia associated with obesity, alcoholism and chemical diabetes, but in the majority of patients the underlying primary disease factor of hyperlipidemia was not evident.

Chemicals

Acetone, methanol and chloroform, all of analytical grade, were obtained from Lachema (Brno, Czechoslovakia). Isooctane analytical grade was provided by International Enzymes (Windsor, Great Britain). Cholesteryl butyrate was obtained from Applied Science Labs. (State College, Pa., U.S.A.). Florisil 60—100 mesh was supplied by Fluka (Buchs, Switzerland). The purity of all solvents was checked by GC.

Chemical analysis

Blood samples were collected from fasting subjects into test-tubes containing 1 mg EDTA per ml of blood and centrifuged within 1 h at 4° (10 min at 1000 *g*). Extraction and isolation of neutral lipids from plasma was made in a one-step procedure. The principle of this method, which will be described in another paper, is the sorption of polar lipids on Florisil simultaneously with extraction. Aliquots of the extracts were taken into 10-ml flasks, dried under nitrogen and stored at -20°. Samples were stable for 3 months. Just before GC analysis the samples were dissolved in the internal standard solution of cholesteryl butyrate (200 ng/μl) in the mixture isooctane—chloroform (80:20, v/v). Analyses were performed on a Perkin-Elmer F 30 gas chromatograph equipped with autosampler PS 4950 or on a Perkin-Elmer F 17 gas chromatograph with manual injection of the samples. Both gas chromatographs were equipped with a dual column system with FID detection. Glass columns (0.5 m × 2.0 mm I.D.) were packed with 1% OV-1 on Gas-Chrom Q 100—120 mesh. The injector was thermostatted at 300°; the oven temperature was programmed as follows: initial temperature 180°, programme rate 5°/min, final temperature 350°. Helium flow-rate was 100 ml/min. The gas chromatographs were combined with a Perkin-Elmer M-2 Calculating Integrator or a Perkin-Elmer Sigma 10 Laboratory Data system. Data were processed by a Computer MDS-2400 using a special programme [4].

Precision and accuracy control

Precision of the results in time was controlled by means of quality control (K card). Coefficients of variation for individual fractions of the lipid profile were as follows: 1.21 for free cholesterol; 1.13 for cholesterol esters; 5.39, 1.23, 1.14 and 2.14 for fractions of cholesteryl esters of carbon number 41, 43, 45 and 47, respectively; 1.11 for total cholesterol; 1.99 for triglycerides; 4.26, 1.96, 2.13, 3.23 and 4.41 for fractions of triglycerides of carbon number 48, 50, 52, 54 and 56, respectively.

For the quality control we used a model mixture of pure compounds, the composition of which was similar to that of plasma. Results of the total cholesterol and triglyceride determination in plasma were compared with those measured enzymatically using commercial sets of chemicals from Boehringer (Mannheim, G.F.R.) Cat. Nos. 15732 and 138355. The two methods were in good agreement: the correlation coefficients of 30 duplicate measurements were 0.986 and 0.998 for total cholesterol and triglycerides, respectively. Detailed results of the quality control will be published separately.

Processing and analysis of data

Regression analysis was used to calculate adjusted log triglyceride confidence limits. For classification of triglyceridemia the antilog critical value was calculated as 144 mg/dl, which represents the 95 percentile of the reference group.

Percentage values of triglyceride fractions were transformed by arcsin function before computing means and standard deviations. The distribution of transformed values was approximately normal.

Data processing was performed on a programmable calculator HP 97 (Hewlett-Packard) using commercial and our own programmes.

RESULTS

The values for total plasma triglyceride in the reference group study are summarized in Fig. 1. The curves represent the relationship of triglyceride to age in men and women. Regression functions are the exponentials and show the change of triglyceride level with increasing age as well as the difference between the sexes.

Contrary results are evident concerning the molecular composition of plasma triglyceride. The percentage molecular composition of triglycerides in the same group of subjects is shown in Table I. The uniformity of the molecular distribution is evident for all age groups in men as well as in women and no difference between the sexes could be observed.

The fraction C_{56}, which contains two eighteen-carbon chains and one twenty-carbon fatty acyl chain has a content of about 1% on average. The value of 6.28% is considered as elevated at the 5% level of significance. Table II summarizes the comparison of the molecular composition of plasma triglycerides in the hypertriglyceridemia and reference groups. The increase of triglyceride fraction C_{56} was found in 28 of the total number of 111 patients. In the 199 subjects of the reference group the same fraction was found in 9 individuals only. The fourfold table test is significant for the difference. The increased frequency of the augmented cholesteryl fraction with carbon number 47 can also be seen.

We were interested to see if there was some relationship between the anomaly and lipoprotein type according to Fredrickson's classification [5]. Table III shows lipoprotein type in 28 patients with heterogeneous hypertriglyceridemia; all lipoprotein types of hyperlipidemia are involved. The difference in frequencies reflects more the incidence of individual types in the population than the preference of a particular lipoprotein type for heterogeneous hyperglyceridemia.

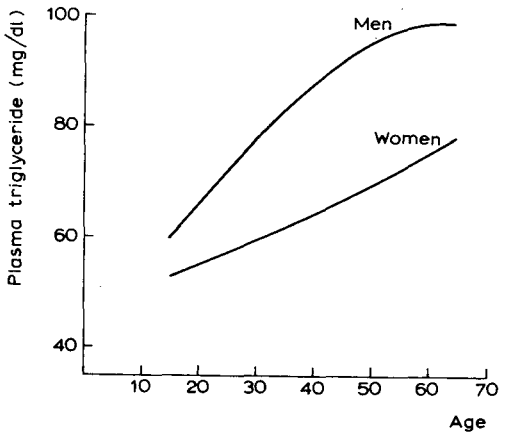

Fig. 1. Fitted regression lines for plasma total glyceride with age in men and women. Regression equations: for men $Y = 40.6062 \exp(0.027882x - 0.000219x^2)$; for women $Y = 47.4108 \exp(0.007576x)$. Coefficients are calculated from log values and re-transformed to arithmetic values.

TABLE I

PLASMA TRIGLYCERIDE COMPOSITION IN RELATION TO AGE IN MEN AND WOMEN

Age (years)	*n*	Triglyceride composition (weight percent)					
		C_{48}	C_{50}	C_{52}	C_{54}	C_{56}	C_{58}
Men							
15—19	17	4.8*	17.6	48.2	28.3	1.1	0
20—29	16	6.3	20.3	48.5	24.7	0.2	0
30—39	16	5.4	19.1	49.6	24.6	1.2	0
40—49	17	5.5	19.5	48.8	25.1	1.1	0
50—59	15	5.9	19.5	48.6	25.8	0.2	0
60 and over	12	5.9	19.1	49.2	23.8	1.9	0
Women							
15—19	20	5.3	18.2	47.0	29.5	0	0
20—29	20	6.6	20.4	49.6	23.4	0	0
30—39	19	5.9	19.6	47.3	24.3	2.9	0
40—49	18	5.1	17.8	49.1	26.8	1.2	0
50—59	19	6.5	19.3	50.6	23.3	0.4	0
60 and over	10	6.4	17.7	48.3	24.9	1.9	0.8

*The values are the mean for each age group.

TABLE II

FREQUENCY OF PLASMA TRIGLYCERIDE FRACTION C_{56} AND CHOLESTERYL ESTER FRACTION C_{47} IN HYPERLIPIDEMIA AND IN REFERENCE GROUP

Group	*n*	Frequency of augmented fraction	
		Triglyceride C_{56}	Cholesteryl ester C_{47}
Hyperlipidemia	113	28	23
Reference	199	9	5
Fourfold table test (*P*)		<0.001	<0.001

TABLE III

LIPOPROTEIN TYPE IN HETEROGENEOUS HYPERTRIGLYCERIDEMIA

Group	*n*	Lipoprotein type		
		IIB	III	IV
Heterogeneous hypertriglyceridemia	28	4	3	21

In Table IV the incidence of the molecular composition anomaly in a family in which genetic factors for hyperlipidemia predominate is demonstrated. The index patient, aged 48, female, reveals a classical picture of type III hyperlipoproteinemia with all clinical and laboratory signs present. The total plasma triglyceride is 1068 mg/dl and its molecular composition is quite abnormal. The same is true for her mother, who is 78 years and has a plasma triglyceride level of 650 mg/dl. In this patient the fractions with two twenty-carbon acyl chains (carbon number 58) and with three twenty-carbon acyl chains (carbon number 60) are augmented. This finding was constant in both patients over long-term observation with repeated triglyceride analyses. One sister of the index patient is normal; another sister as well as the children show a mild form of type IV hypertriglyceridemia with normal molecular composition.

TABLE IV

PLASMA TRIGLYCERIDE COMPOSITION IN A FAMILY RUNNING TYPE III HYPERLIPOPROTEINEMIA

Patient	Position in family	Age (years)	Triglyceride (mg/dl)	Lipoprotein type	Weight percent		
					C_{56}	C_{58}	C_{60}
J.H. 609	Father	74	163	N	0	0	0
M.H. 765	Mother	78	650	III	5.7	3.7	1.4
M.P. 658	Index patient	48	1068	III	9.8	7.3	0
J.P. 766	Husband	47	228	IV	3.5	0	0
V.P. 607	Sister	38	172	IV	3.5	0	0
J.B. 606	Sister	50	140	N	5.0	0	0
J.S. 611	Daughter	24	179	IV	0	0	0
M.P. 604	Daughter	22	160	IV	0	0	0
J.P. 605	Son	25	287	IV	0	0	0

Interesting is the effect of treatment of the index patient which is shown in Table V. Before the treatment started the patient was highly hypertriglyceridemic with abnormal molecular composition of triglyceride. The treatment was introduced with diet and Clofibrate. After 5 months' therapy a dramatic fall of total plasma triglyceride was registered. The content of triglyceride fraction C_{56} was on the border of significance.

TABLE V

PLASMA TRIGLYCERIDE COMPOSITION IN TYPE III HYPERLIPOPROTEINEMIA: EFFECT OF TREATMENT

Conditions	Triglyceride (mg/dl)	Weight percent	
		C_{56}	C_{58}
Before treatment	1079	9.8	7.3
After treatment (diet, Clofibrate) for 5 months	92	5.4	0

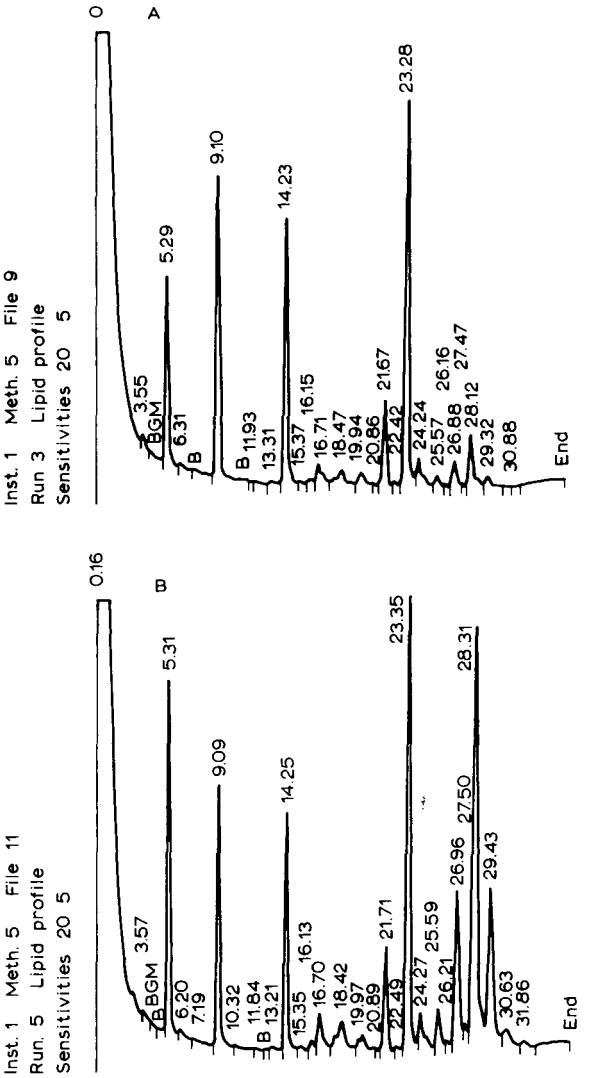

Fig. 2. Gas chromatograms of normal (A) and hyperlipidemic (B) plasma neutral lipid. Individual compounds are identified according to their elution times (min): (A): 5.29 = cholesterol; 9.10 = cholesteryl butyrate (internal standard); 14.23 = cholesteryl benzoate (standard for laboratory control); 15.37, 16.15, 16.71, 18.47 = diglycerides and decomposition products of phospholipids; 19.94, 20.86, 21.67, 22.42, 23.28, 24.24 = cholesteryl esters with carbon numbers 41, 42, 43, 44, 45, 47, respectively; 25.57, 26.16, 26.88, 27.47, 28.12, 29.32 = triglycerides with carbon numbers 48, 49, 50, 51, 52, 54 respectively. (B): 5.31 = cholesterol; 9.09 = cholesteryl butyrate (internal standard); 14.25 = cholesteryl benzoate (standard for laboratory control); 15.35, 16.13, 16.70, 18.42 = diglycerides and decomposition products of phospholipids; 19.97, 20.89, 21.71, 22.49, 23.35, 24.27 = cholesteryl esters with carbon numbers 41, 42, 43, 44, 45, 47, respectively; 25.59, 26.21, 26.96, 27.50, 28.31, 29.43, 30.63, 31.86 = triglycerides with carbon numbers 48, 49, 50, 51, 52, 54, 56, 58, respectively. Gas chromatograms were recorded by means of a Sigma 10 Data System. Vertical lines under the baseline are the marks of start and end of the integration. Sample volume 2 μl; chart speed, 5 mm/min; other analytical conditions are given in the text.

DISCUSSION

Our results demonstrate that in hypertriglyceridemia there is an anomaly of plasma triglyceride composition in about 25% of cases. This heterogeneous hypertriglyceridemia can be proved by GC analysis of intact plasma lipids (Fig. 2).

The increased triglyceride fraction consists of molecules with carbon number 56. In some cases an augmented content of fractions C_{58} and C_{60} was found. It is evident that in such cases there must be some metabolic disturbance of fatty acids with twenty-carbon and twenty-two-carbon chains. Not only triglyceride but also the cholesteryl ester fraction loaded with twenty-carbon fatty acyl chains is augmented.

It is not clear if the anomaly in triglyceride composition is the result of hypertriglyceridemia or if it is its background cause. Further studies that will try to answer this question and to help towards a better understanding of the underlying pathogenetic factors of hyperlipidemia are in progress.

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CHROMBIO. 251

SPECTRUM OF TOTAL FATTY ACIDS IN CEREBROSPINAL FLUID DETERMINED BY GAS CHROMATOGRAPHY

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SUMMARY

A simple gas—liquid chromatographic method for the determination of the spectrum of fatty acids in a small volume of cerebrospinal fluid (CSF) is presented. In a group of 49 neurological patients it has been found that in the CSF of the controls (n = 12) there are the following main fatty acids: oleic (27.28%), palmitic (23.23%), stearic (12.21%), linoleic (7.66%), myristic (5.02%), and palmitoleic (4.51%). Altogether 28 fatty acid methyl esters (FAMEs) from 12:0 to 22:2 have been tentatively identified. The majority of them appeared irregularly, sometimes in less than in 10% of cases. The composition of FAMEs in the CSF of patients with lumbar discopathy and with acute ischaemic cerebrovascular accidents does not differ from the control group. A significant difference ($P<0.01$) has been found in the group of hypophyseal adenomas in which the amounts of practically all saturated FAMEs with an odd carbon number are elevated. The same applies to the 18:0 and 20:0 compounds. In the group of atrophic and degenerative CNS processes the palmitic and stearic acids predominated to the detriment of oleic and linoleic acids.

INTRODUCTION

Lipids of the cerebrospinal fluid (CSF) and their analysis have been a relatively less well-known area in liquorology. The lipid concentration in the CSF is several thousand times lower than in the central nervous tissue and several

hundred times lower than in the serum [1]. Apart from the striking quantitative differences the CSF lipids differ from nerve tissue and from the serum in the percentage representation of lipid classes and their subfractions [2]. Free fatty acids (FAs) and phosphatidylethanolamine occur in relatively higher concentrations in the CSF than in the serum [3]; lysophosphatidylcholine is nearly absent from the CSF, and the molar ratio of esterified to free cholesterol is 1:1 [4]. A significant difference has been found between the serum and CSF cholesterol ester pattern [3]. Whereas in the serum cholesterol ester pattern cholesterol linoleate predominates, in cholesterol esters of the CSF saturated and monounsaturated FAs prevail [2,3].

The aim of this study was to ascertain the CSF total FA profile as a preliminary stage in the determination of the FA spectrum in single samples of CSF of classes of lipids other than cholesterol esters.

MATERIALS

CSF obtained by lumbar puncture has been examined in 49 neurological patients (Table I). The control group was formed by 12 patients (4 women, average age 34.5 years, and 8 men of average age 40.8 years) without any sign of organic damage to the CNS, and in whom the CSF analysis corresponds to the criteria for normal composition. In the majority there were vertebrogenic syndromes and different functional disorders of the CNS. The group of vascular cerebral ischaemic accidents was formed by 16 patients with the anamnesis of an acute cerebral accident (11 men with a mean age of 67.5 years, and 5 women of mean age 62.5 years), whose CSF was not sanguinolent and spectrophotometrically correspond to encephalomalacia [5]. Into the group of discopathies, formed by 7 patients (average age 52 years) we classified such patients whose perimyelographic examination showed a prolapse or protrusion of a disc and whose CSF gave a picture of a protein—cytological dissociation with the protein level above 41 mg/100 ml. In the group of degenerative diseases were one patient with an amyotrophic lateral sclerosis, one patient with morbus Aran-Duchene, a case of perinatal encephalopathy with an evolutional anomaly, one case of myelodysplasia, one case of cerebral atrophy and six cases of olivopontocerebellar atrophy. The adenomas of the hypophysis

TABLE I

BASIC CLINICAL DISORDERS ON GROUPS STUDIED

Group	*n*	Age (years)	Protein (mg/100 ml)
Control	12	36	34.83
Discopathies	7	52	51.29
Vascular disorders	16	70	61.94
Degenerative disorders	11	44	33.66
Hypophyseal adenoma	3	48	47.83

(n=3) were diagnosed by X-ray, and verified through operation. There were two cases of endocrinologically silent adenomas and one case of a prolactin-producing adenoma.

METHODS

A 3-ml volume of centrifuged CSF was extracted with chloroform—methanol (1:1) [6]. The total lipid extract was evaporated to dryness in a stream of nitrogen, dissolved in 0.5 ml of benzene—methylacetate (1:4, v/v) and boiled for 20 min in a water-bath in sealed test-tubes with 4.5 ml of 10—12% boron trifluoride in methanol (w/v). The methylesters (FAMEs) obtained after shaking four times with 5 ml of light petroleum (b.p.<40°) were isolated on a micro-column of silicic acid (1 g of Kieselgel (Merck) 80—120 mesh) and eluted with 5 ml of 2% ethyl ether in light petroleum. After evaporation of the solvent in a stream of nitrogen the residue was dissolved in 20 μl of chloroform and 1—2 μl were injected into the glass column (1.8 m × 4 mm I.D.) of a Perkin Elmer F-30 gas chromatograph. The stationary phase was 13% DEGS on Chromaton N AW DMCS, 0.125—0.160 mm (Lachema, Brno, Czechoslovakia). The carrier gas was nitrogen at a flow-rate of 75 ml/min. The analysis was at 175°; the temperature of the injector block and of the detector was 250°. The recorder was set at 5 mV, the chart speed was 0.5 cm/min. The chromatograms were evaluated by means of an integrator SIP 1. Tentative identification of the individual FAMEs was made from the graphs on the basis of the logarithms of the various retention times compared with those of commercial standards (Serva, Heidelberg, G.F.R.). For the structural identification of FAMEs that were not always present or present only in small quantities, suitable techniques such as mass spectrometry were not available. Calculation of the FAME mass percentage was done without any internal standards or corrections.

RESULTS

Table II gives the reproducibility of the quantitative analysis and the standard deviation (S.D.) of fourteen determinations of a mixture of five standard saturated FAMEs. The chromatogram of the qualitative mixture of the commercial standards is shown in Fig. 1, whereas Fig. 2 shows the chromatogram

TABLE II

THE REPRODUCIBILITY OF THE STANDARD FAME MIXTURE ANALYSIS

FAME	Given	Found	S.D. (n=14)	S.D. (%)
15:0	15.5	15.57	0.601	3.86
16:0	17.2	17.20	0.563	3.27
17:0	19.7	19.56	0.465	3.38
18:0	22.8	22.83	0.568	2.49
19:0	24.8	24.83	0.488	1.97

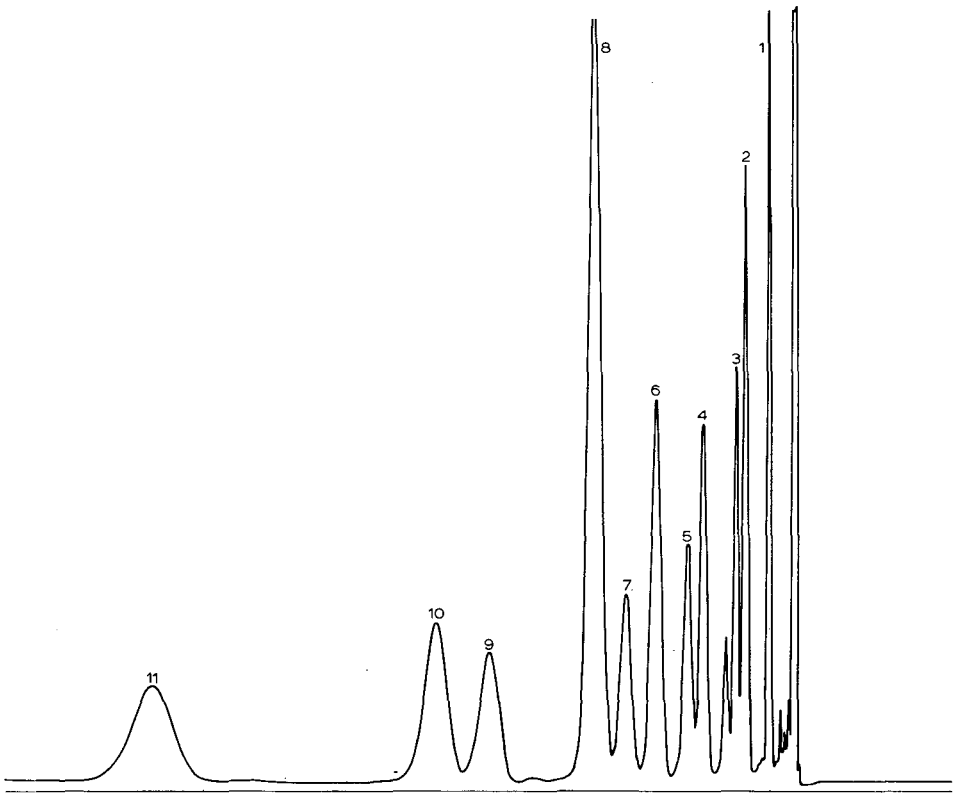

Fig. 1. Chromatogram of the qualitative mixture of commercial standards (Serva, Heidelberg, G.F.R.). 1=14:0, 2=16:0, 3=16:1, 4=18:0, 5=18:1, 6=18:2, 7=20:0, 8=18:3, 9=22:0, 10=22:1, 11=24:1.

of the quantitative commercial mixture of 15:0, 16:0, 17:0, 18:0 and 19:0 standards. Fig. 3 shows the trace of GC analysis of FAMEs in normal CSF. Fig. 4 shows the trace of the GC analysis of FAMEs in CSF from a patient with hypophysical adenoma. A graphic representation of the FAMEs in all examined groups (excluding the discopathies which are identical with the controls) is shown in Fig. 5. The spectrum of FAMEs in the groups under study is given in Table III.

Owing to the large variability and small number of cases it was not possible to use the Student's *t*-test for statistical evaluation. We therefore used the non-parametric sequential test as described by Wilcoxon [7]. The significant values on the 0.05 level are marked ↑ or ↓, those on the 0.01 level by ⇑ or ⇓. We have identified a total of 28 FAMEs. Although the group of ischaemic vascular cerebral accidents does not differ in the distribution of the constantly occurring FAMEs, we have found certain differences in the distribution of short FAMEs; this will be presented elsewhere [8].

The discopathy group does not differ essentially from the controls. The degenerative diseases show a statistically highly significant reduction of 18:1 and 18:2, and an increase in 16:0 and 18:0 and in some odd-numbered FAMEs

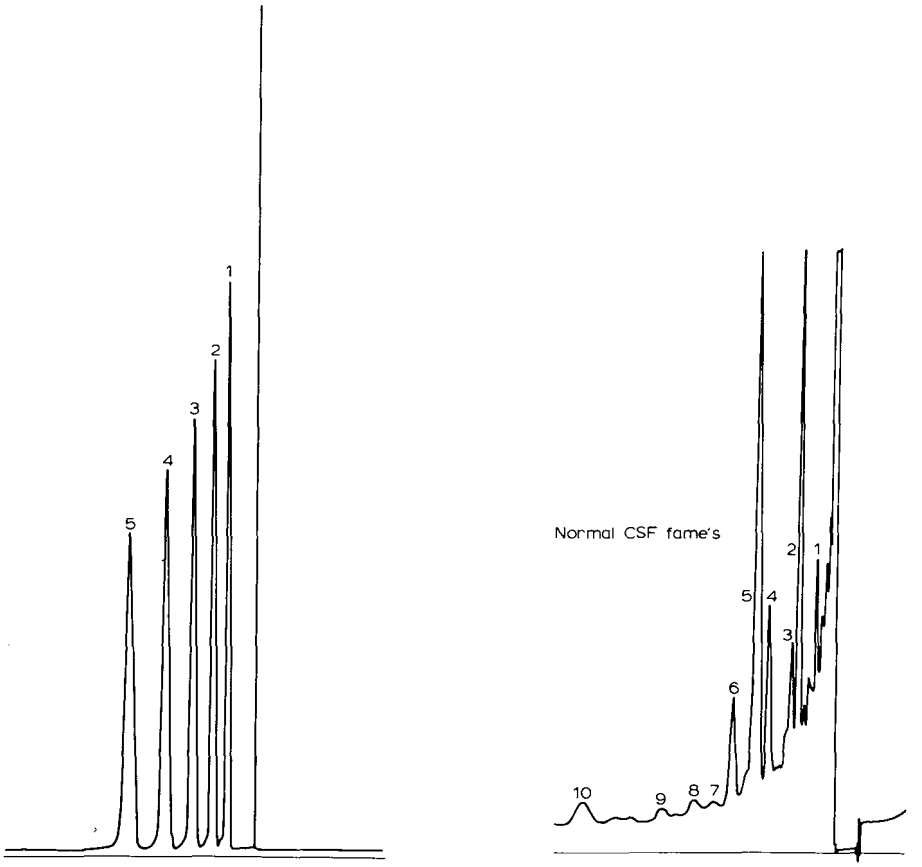

Fig. 2. Chromatogram of 2.5 μg of a quantitative mixture of commercial standards (Applied Science Labs.). 1=15:0, 2=16:0, 3=17:0, 4=18:0 and 5=19:0.

Fig. 3. Chromatogram of a normal CSF. 1=14:0, 2=16:0, 3=16:1, 4=18:0, 6=18:2, 7=20:0, 8=18:3, 9=20:1, 10=22:1.

15:0, 17:0). Still more significant were the changes in FAMEs in the small group of hypophyseal adenomas. There were increases in 15:0, 17:0, 19:0, 20:0 and 21:0 and a marked decrease in 18:1 and 18:2.

The average values presented (Table III) do not reflect, however, the true profile of FAME occurrence, because many FAs do not appear constantly. Fig. 6 shows the frequency of occurrence of the individual FAMEs in the set studied. We can see that the regular occurrence of FAME is proved for the following acids: myristic, palmitic, plamitoleic, stearic, oleic and linoleic acids, which appear in disease groups such as those of the controls, vascular accidents and degenerative diseases. Some FAMEs appear in less than 10% of examined cases.

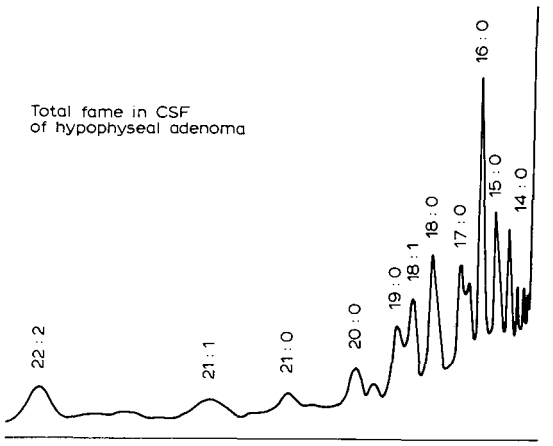

Fig. 4. Chromatogram of CSF total fatty acids from a patient with hypophyseal adenoma.

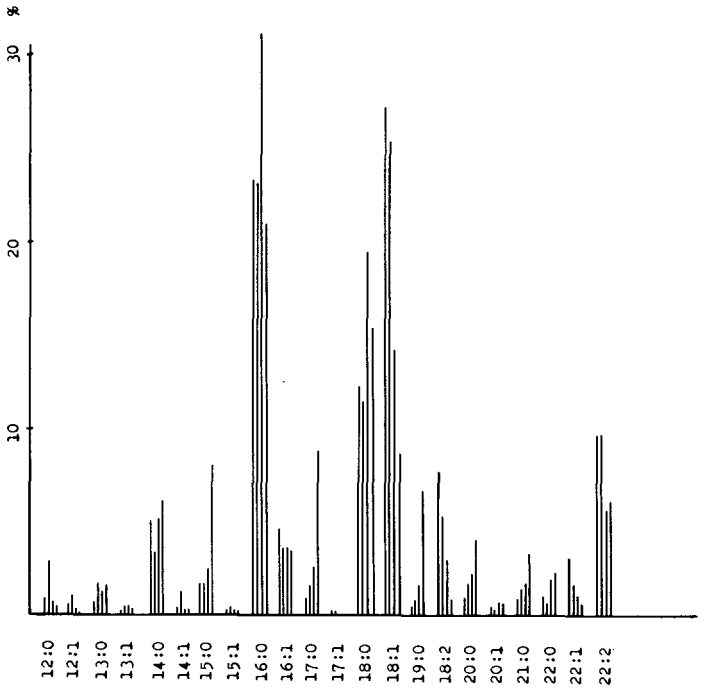

Fig. 5. Relative distribution of FAMEs in four of the groups studied: (from left to right) controls, vascular diseases, degenerative diseases and hypophyseal adenoma.

DISCUSSION

FAMEs for determination by gas—liquid chromatography (GLC) can be prepared by acid- or base-catalysed esterification or transesterification. The methanolic solutions of boron trifluoride and hydrochloric and sulphuric acid form

TABLE III

RELATIVE DISTRIBUTION OF FAMEs IN THE CSF IN CONTROLS AND PATIENTS WITH NEUROLOGICAL DISORDERS

FAME	Controls (n=12)	Discopathies (n=7)	Vascular disorders (n=16)	Degenerative diseases (n=11)	Hypophyseal adenoma (n=3)
12:0	0.87.	1.61	2.89	0.65	0.42
12:1	0.37	0.83	1.66	0.28	0.00
13:0	0.63	0.68	1.61	1.16	1.51
13:1	0.08	0.18	0.31	0.39	0.00
14:0	5.02	4.83	3.38	5.10	6.07
14:1	0.26	0.87	1.19	0.11	0.00
15:0	1.58	1.21	1.53	2.35 ↑	7.87 ⇑
15:1	0.07	0.40	0.28	0.03	0.00
16:0	23.24	19.64	23.51	31.16 ⇑	21.12
16:1	4.51	4.55	3.43	3.59	3.40
17:0	0.83	0.63	1.50	2.59 ⇑	8.67 ⇑
17:1	0.05	0.00	0.08	0.00	0.00
18:0	12.21	9.48	11.44	19.45 ⇑	15.33
18:1	27.28	29.48	25.53	14.33 ⇓	8.56 ⇓
19:0	0.39	0.55	0.72	1.60	6.69 ⇑
19:1	0.00	0.71	0.00	0.00	0.00
18:2	7.66	6.63	5.22	2.89 ⇓	0.65 ⇑
20:0	0.85	0.93	1.55	2.19	4.03 ⇓
18:3	0.03	0.00	0.08	0.00	0.00
20:1	0.29	0.43	0.19	0.50	0.47
20:2	0.00	0.05	0.00	0.00	0.00
21:0	0.58	1.24	1.22	1.56	3.28 ⇑
21:1	0.00	0.09	0.00	0.00	1.39
22:0	0.08	1.08	0.56	1.79	2.17
22:1	2.87	2.33	1.48	0.98	0.70
21:2	0.06	0.67	0.00	0.00	0.00
23:0	0.04	0.00	0.00	0.00	1.56
22:2	9.44	11.02	9.70	5.59	6.06

↑ = $P<0.05$
⇑ = $P<0.01$

on heating FAMEs from free FAs and their esters. Under these conditions the different fractions of lipids react differently. MEs are formed most quickly from free FAs and from triglycerides, and most slowly from cholesterol esters and sphingomyelins. The mixtures of lipid fractions react according to the proportional representation of the individual components. A higher efficiency can be achieved only by prolonging the heating period, for instance in a sealed ampoule on a boiling water-bath. The longer the heating the higher the probability of forming artefactual compounds: methoxymethylesters of unsaturated FAs and methoxymethylcholesterol. Their quantity is also proportional to the concentration of mineral acid in the methanol [9—12].

The base-catalysed transesterification of FA esters by sodium methoxide proceeds at normal temperatures about 1000 times more quickly than the

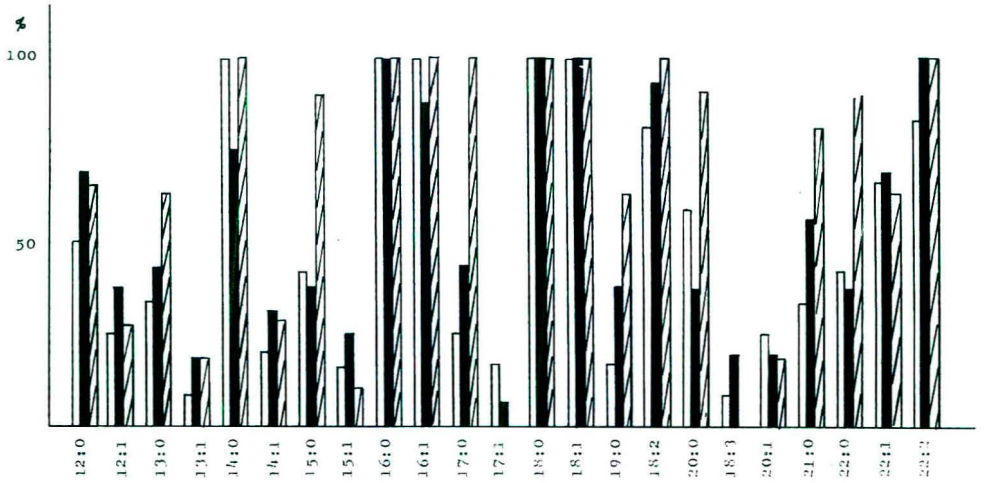

Fig. 6. Frequency of the individual FAMEs of CSF in the groups of controls (□) and cerebrovascular (■) and degenerative (▨) disorders.

saponification of these esters. Saponification of the FA esters can be accelerated by raising the temperature in the case of cholesterol esters and sphingomyelins. At temperatures higher than 90° and in alkaline conditions cumulation of isolated double bonds of unsaturated FAs takes place [13].

The data on transmethylation or on the preparation of FAMEs for GLC analysis are very numerous and often contradictory. We can state that if the more gentle transmethylation process is chosen, the more incomplete is the transmethylation, especially of sphingomyelins and cholesterol esters, as well as of FFAs and triglycerides (Fig. 5). If more drastic processes are chosen, especially with a high temperature, artefacts are easily formed (Fig. 7). Boron trifluoride was chosen for the preparation of MEs for its universal character and recommended efficiency especially with FFAs. The transmethylation process has been modified so that all lipids in an environment rich in methyl groups are brought into solution. This has enabled us to use methylacetate with a 20% admixture of benzene as the solvent for lipid extraction prior to transmethylation. The completeness of transmethylation with boron trifluoride depends on the age of the reagent and on the quality of the methanol. The reaction does not run satisfactorily if the environment is not completely water-free.

Comparison of the results of different laboratories that have used different methods of transmethylation is always problematic, particularly for the different preparatory procedure of FAMEs. Blomstrand et al. [14] were the first to study the FA spectrum in liquor lipids by menas of GLC. They ascertained that, contrary to serum, the CSF contains predominantly palmitic and oleic acids. We have ascertained, first by paper chromatography [15] and later by thin-layer chromatography [2,3] that in the CSF cholesterol esters predominate over saturated and monounsaturated fatty acids, contrary to the serum, in which cholesterol linoleate is the main ester. Tuna et al. [16] compared the composition of FAs in serum and liquor. Fourteen FAMEs were determined by

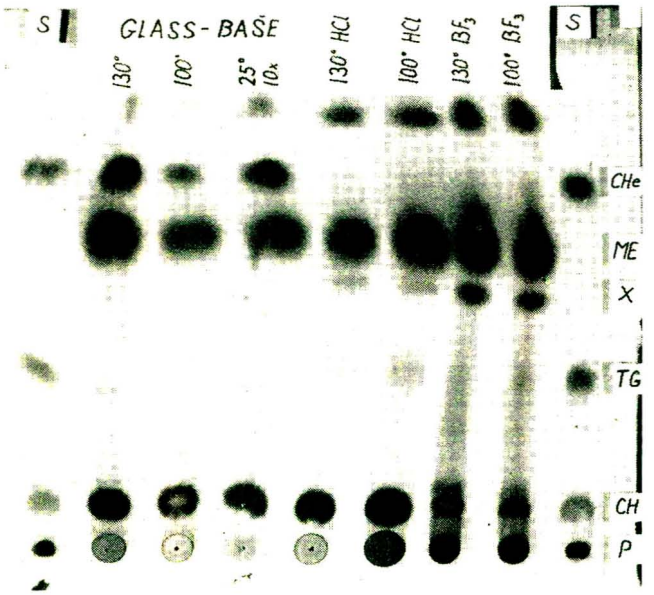

Fig. 7. Thin-layer chromatography of different procedures for preparing methyl esters from serum total lipids. Silica gel G; solvent, *n*-heptane—ethyl ether—acetic acid (85:15:1, v/v); detection by charring after $(NH_4)_2SO_4$ spray. CHe= esterified cholesterol, ME= methylesters, TG= triglycerides, CH= free cholesterol, P= phospholipids, X= artefacts (methoxymethylesters?).

means of GLC. The acids of highest concentration in the liquor were verified as palmitic, oleic, palmitoleic and stearic acids. Polyenoic FAs showed very low concentrations: linoleic acid approximately 4%, and arachidonic acid approximately 1.5%. The same authors found no substantial differences when comparing the FA spectrum in demyelinating diseases with that in controls. In the degenerative diseases they found a similar spectrum to that of the controls (Tuna et al. [17]). The composition of FAs in liquor has been treated in various papers by Farstad and Skaug [18]. They found that the highest concentration is of 18:1 (22.8—43.8%), and a constant further occurrence of 14:0 (2.6—7.7%), 16:0 (9.7—18.7%) and 18:0 (4.2—9.0%). Of the remaining FAs the following showed a measurable concentration: 14:1, 15:0, 15:1, 17:0, 18:2 and 18:3. The identification of single FAs, especially of the critical pairs 16:0—16:1 and 18:0—18:1, is not convincing enough, however. Moreover, at the end of the chromatogram a high fraction occurs, characterized as 22:6, which the author [19] identified later as an artefactual one. In our chromatograms as well, obviously owing to the drastic transmethylation ratio by 10—12% boron trifluoride, artefactual MEs with cumulated double bonds appeared.

Farstad's paper [19] established, by identification of 15 different FAs, that the physiological oscillation of individual FAs in the CSF is enormous. The importance of their determination in different neurological and psychiatric diseases has also been evaluated by Farstad in a subsequent paper [20]. He evaluated the aliphatic FAMEs up to 22:0 in 84 patients but he did not identify the branched ones. Each diagnostic group has shown a significant variation

of values for individual FAMEs. It was not possible to ascertain a significant change of individual fatty acids in any of the examined groups of psychoneuroses, headaches, muscle diseases, psychoses, intra cerebral tumours, epilepsy and encephalopathy. Oleic and linoleic acids were lower in the liquor than in the serum. However, myristic acid was five times more frequent in the CSF, comprising 6—9%. Seidel and Lindlar [21] found a striking and surprisingly high percentage of myristic acid in all examined groups of liquor lipids: in cholesterol esters over 21%, in triglycerides over 17%, and in phosphatides over 25%. However, oleic acid was surprisingly depressed; in all examined lipids it formed approximately only 10%. We have examined the spectrum of total FAs in the CSF of 100 neurological patients. In a number of cases the FAME spectrum differed significantly; a detailed report is being presented elsewhere (Skorkovská et al. [22]).

Sastry and Stancer [23] who studied the FAME pattern in isolated fractions of phospholipids (lecithins, phosphatidylethanolamines, sphingomyelins) in children, concluded that the main acid in lecithins is palmitic acid (44.3%); stearic acid amounted to 16.5% and oleic acid to 14.1%. Myristic acid only accounted for 2.7%. In the phosphatidylethanolamines there was 37.5% of palmitic acid and 25.1% of stearic acid. In sphingomyelins palmitic acid contributed 30.6% and stearic acid 35.1%. The oleic acid in phosphatidylethanolamines formed 6.2% and in sphingomyelins 2.2%.

In 1970 we compared the FA profiles of some main classes of lipids in the CSF of healthy persons and of chronic alcoholics. We studied the spectrum of cholesterol esters, triglycerides, phosphatidylethanolamines and phosphatidyl cholines by means of GLC after their prior separation on thin layers of silica gel G. Owing to a small concentration of lipids it was necessary to work with pooled liquor samples from six healthy and nine alcoholic subjects. Each class of lipids studied showed its own specific spectrum. The distribution of FAMEs in triglycerides and in cholesterol esters is shown in Table IV.

In the spectrum of phosphatidylcholines palmitic and oleic acids predomi-

TABLE IV

FATTY ACID COMPOSITION OF CHOLESTERYL ESTERS AND TRIGLYCERIDES IN CSF AND SERUM (S) OF CONTROLS

Fatty acid	Cholesteryl esters		Triglycerides	
	CSF	S	CSF	S
16:0	23.2	11.0	41.9	24.8
16:1	11.2	3.9	5.2	5.2
18:0	0.6	1.0	14.1	5.1
18:1	37.0	20.9	27.9	36.9
18:2(*n*-6)	21.4	53.4	2.8	14.3
18:3(*n*-6)	0.2	0.5	0.5	0.3
18:3(*n*-3) + 20:1	0.4	1.0	0.7	3.2
20:3(*n*-6)	0.3	0.5	0.2	0.6
20:4(*n*-6)	4.7	5.2	4.6	3.6
22:6(*n*-3)	0.4	0.7	1.7	2.0

nated, together comprising more than 70% of all the FAMEs. In the FAME spectrum of the phosphatidylethanolamines a high percentage of 20:6 and 22:6 has been found (Tichý et al. [24]). Arnetoli et al. [25] determined the FAs in CSF by GLC. In agreement with other authors they found the following highest concentrations for the acids: 16:0 (36.21%), 18:1 (23.74%), 16:1 (6.6%) and 18:2 (4.52%). Amaducci et al. [26] shared their experience in examining liquors of more than 300 neurological patients. They found a considerable variability, especially in the percentage representation of 18:2. Its increase was related to the reduction in 14:0. Owing to the fact that both FAMEs have quite different concentrations in CSF and serum, the authors came to the conclusion that the change in the amounts of these acids in liquor may well demonstrate damage to blood—brain barrier (in this case the linoleic acid content increases). A comprehensive survey of the problems of CSF lipids and FAs has been made by Pilz [1] in his monograph.

The group which we studied is a small one and not very homogeneous. In the control group particularly, minority fractions show considerable variation, resulting in a lack of statistical significance of some deviations found. The FAME spectrum in discopathies with increased proteinosis and in the acute ischaemic cerebrovascular accidents do not differ either in the main FAs or in those acids which appear in the CSF only irregularly. The most striking finding appears to be the statistically significant increase in the odd-numbered FAMEs, 15:0, 17:0, 19:0 and 21:0, in three cases of hypophyseal adenoma, where the increase is compensated by a statistically significant decrease in 18:1 and 18:2. The insufficiently homogeneous group of degenerative processes showed an increase in 16:0 and 18:0 to the debit of 18:1 and 18:2. All charges were statistically highly significant.

It would be premature to try to explain these findings. It remains a fact that in the CSF there are some changes in the pattern of total FAs in some neurological disorders.

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REVERSED-PHASE THIN-LAYER CHROMATOGRAPHY IN THE PARAMETRIZATION OF LIPOPHILICITY OF SOME SERIES OF ARYLALIPHATIC ACIDS

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SUMMARY

In the series of arylacetic acid and β-aryl-*n*-butyric acids, chromatography was carried out on a thin layer of silica gel impregnated with silicone oil, with 50% acetone as mobile phase. A separation mechanism in this system was evaluated using relationships between R_M values and the concentration of the lipophilic solvent in the silica-gel layer. It was found, that both partition and adsorption mechanisms participate, and that the adsorption effect increases with decreasing lipophilicity of the acids. The dichotomy of the mechanism manifests itself in the non-linear course of the relationships between R_M values and π parameters, or fragmental constants f derived from the partition system *n*-octanol—water. Such relationships can be expressed by a single quadratic dependence between lipophilic parameters and R_M values, or by two separate linear expressions with different slopes for different regions of substituent lipophilicities. The linear dependence between R_M and π at the lower range of lipophilicity is most probably made possible by significant linear dependence between π parameters and molecular surface areas of the substituents.

INTRODUCTION

Partition chromatography is one of the most important methods for the experimental evaluation of lipophilicity in quantitative structure—activity relationships. As evident from studies published by Martin and co-workers [1, 2], the relationship between R_M values and the logarithm of the partition coefficient can be expressed by eqn. 1. The quantity R_M is defined by Bate-Smith and Westall [3], P_s is the partition coefficient determined in a system identical with the chromatographic system, V_S and V_M are volumes of stationary and mobile phases, respectively. Provided the Collander [4] linear relationship (eqn. 2) is valid for the chromatographic system and for the reference system [5, 6] *n*-octanol—water, eqn. 1 can be rewritten as eqn. 3. An analogous linear relationship holds also when using π parameters instead of log P, or fragmental constants f [7]. These equations are valid on the a priori assumption that the partition mechanism prevails and that the adsorption effects are negligible.

$$R_M = \log P_s + \log (V_S/V_M) \quad (1)$$

$$\log P_s = a \cdot \log P + b \quad (2)$$

$$R_M = a \cdot \log P + c \quad (3)$$

The partition chromatography may be carried out by various methods differing in the character of the stationary phase. In paper chromatography [8, 9] a paper impregnated with a polar solvent is usually used. When reversed-phase thin-layer chromatography is performed an absorbent as support for lipoid stationary phase is used. Amongst others, silicone oil [10—13], *n*-octanol [14, 15] and liquid paraffin [15, 16] have been preferentially used as suitable solvents.

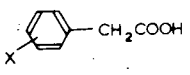
In many recent papers concerning chromatographic determination of lipophilicity, the partition mechanism has been assumed but not, however, always proved. Only recently Hulshoff and Perrin [17, 18] have described the possibility of verifying the partition mechanism in reversed-phase thin-layer chromatography. Deriving a condition for the validity of the partition mechanism, they started from eqn. 1 assuming that a ratio of volumes of both phases is linearly dependent on a volume concentration of lipophilic solvent in the stationary phase according to eqn. 4. Substituting into eqn. 1, eqn. 5 was obtained which holds for dissociable compounds, provided the dissociation is prevented by proper selection of the pH of the chromatographic system. Eqn. 5, on substituting for R_M and taking antilogarithm, yields eqn. 6, expressing the relationship between R_F values and C_{oil}. It is evident from eqn. 5 that a linear relationship with slope equal to 1 holds between R_M values and the log of the concentration of a lipophilic solvent in the stationary phase.

$$V_S/V_M = k \cdot C_{oil} \quad (4)$$

$$R_M = \log P_s + \log k + \log C_{oil} = \log C_{oil} + \text{constant} \quad (5)$$

$$1/R_F = P_s \cdot k \cdot C_{oil} + 1 \quad (6)$$

Hulshoff and Perrin [17] have found that in a system with Kieselguhr G impregnated with oleyl alcohol as stationary phase, the chromatography of phenothiazines was directed by a partition mechanism. However, verification of this mechanism for silica gel impregnated with a suitable lipoid solvent has not so far been made. We have been dealing with this problem in the series of arylacetic (I) and β-aryl-*n*-butyric (II) acids which were chromatographed on a silica-gel thin layer impregnated with silicone oil with 50% acetone as mobile phase.



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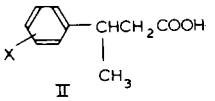

II

EXPERIMENTAL

Chromatography

For the preparation of the stationary phase, 25 g of silica gel G F_{254} were shaken for 90 sec with a mixture of x% (v/v) of silicone oil, 6 ml of acetone and diluted with dioxane to 50 ml. The glass plates (10 cm × 20 cm) were covered with a 0.25-mm layer of a slurry of the support using standard equipment. The volatile components of the impregnating solution were evaporated off within 16 h at 20°.

Solutions of 1% of acids I and II in methanol were prepared, and 5-μl samples were applied to the plate 3 cm from the lower edge. After evaporating off the methanol at 20°, ascending one-dimensional chromatography was carried out using 50% acetone containing a buffer (pH 3.4) as mobile phase. The chromatographic chamber had been equilibrated for 16 h with the mobile phase. The temperature was kept at 20°. When 15 cm migration was attained, the plates were removed and, after evaporating off the remaining mobile phase, acids I and II were visualized in UV light (λ = 254 nm). For evaluation of R_M—log C_{oil} relationships, each chromatogram containing five compounds was repeated three times; the mean R_F values were taken for calculation of R_M. For evaluation of π—R_M dependencies, each chromatogram contained six compounds; two acids serving as reference samples were repeated on each chromatogram. In the chromatograms evaluated, the R_F values of the standards did not differ by more than 0.02.

Sample preparation

Arylacetic acids (I) were prepared from the corresponding substituted benzyl chlorides and sodium cyanide in dimethylsulphoxide with subsequent hydrolysis [19]. Benzyloxyphenylacetic acid (Io) and its *m*-chloro- (Ip) and *m*-methoxy- (In) derivatives were prepared [20] by the reaction of benzyl chloride with esters of *p*-hydroxyphenylacetic acid, or its *m*-chloro- and *m*-methoxy-analogues in the presence of sodium methoxide with subsequent hydrolysis.

β-Aryl-*n*-butyric acids (II) were prepared by the method of Asano et al. [21], which has been described in detail elsewhere [22, 23].

Calculations

In the regression analysis, the π parameters derived [24] for arylacetic acids were used. The π parameters for alkoxy groups and for higher alkyls were calculated from the value for the methoxy group, or the methyl, and from the following increments [25]: $\Delta\pi$ = 0.5 for CH_2, $\Delta\pi$ = —0.2 for branching. For calculation of $\Sigma\pi$ for disubstituted derivatives, a difference between the lipophilicity [7] of the remaining aromatic parts —C_6H_4— and —C_6H_3= was taken into consideration, so that the value 0.23 corresponding [26] to 0.5 log P of hydrogen was substracted from the sum of both substituents [9]. The values of the fragmental constants f were taken from ref. 7.

For calculation of the molecular surface areas of substituents, the spheric areas of single atoms were used, according to Bondi [27], because of the absence of generally accepted pear surface areas [28]. The values of Van der

TABLE I

VAN DER WAALS' RADII AND SURFACE AREAS OF ATOMS

Atom	Radius* (A)	Surface area (10^2A^2)
C	1.7	0.363
H	1.1	0.152
O	1.4	0.246
Cl (aromatic)	1.8	0.407
Br (aromatic)	1.9	0.453

*Values are taken from ref. 28.

TABLE II

CORRECTION VALUES OF VAN DER WAALS' SURFACE AREA FOR SPHERE OVERLAPPING DUE TO COVALENT BONDING

Bond	Bond length (*A*)	Correction value ($10^2 A^2$)
C—C	1.5	0.203
C⋯C (aromatic)	1.4	0.214
C—H	1.1	0.132
C—O	1.4	0.162
C—benzene*	1.5	0.101
H—benzene*	1.1	0.091
O—benzene*	1.4	0.091
Cl—benzene*	1.8	0.091
Br—benzene*	1.9	0.091

*Correction value includes only overlapping of the atom bound to the aromatic nucleus.

Waals' radii and calculated surface areas of atoms are listed in Table I together with the bond lengths and correction values for sphere overlapping due to covalent bonding (Table II). For the atoms directly bound to the aromatic nucleus, the correction corresponds only to overlapping of the bound atom. For 3,4-disubstituted derivatives, the area of one hydrogen atom was subtracted from the sum of the surface areas.

The coefficients in the regression equations were calculated from experimental results by multiple regression analysis using the least-squares method on a Hewlett-Packard 9820 computer. The statistical significances of the regression equations were tested by the standard deviation s, the coefficient of multiple correlation r, and the Fischer-Snedecor criterion F. Individual parameters were statistically evaluated by the Student's t-test at the minimal significance level $\alpha = 0.005$; the exceptions in eqns. 12 and 22 are noted in the text.

RESULTS AND DISCUSSION

To determine the relationships between R_M values and concentration of silicone oil in the silica gel, the support was impregnated with 2.5, 3.5, 5.0, 6.2 and 7.5% of silicone oil. The influence of dissociation on chromatographic behavior of acids I and II was suppressed by suitably arranging the pH of the mobile phase. At pH 3.4, β-(*m*-bromophenyl)-*n*-butyric acid, the most acidic compound in both series (pK = 6.85 in 50% acetone), exists almost exclusively in unionized form.

The experimental results for five arylacetic and five β-aryl-*n*-butyric acids are summarized in Table III and Fig. 1. The linear dependences were evaluated by regression analysis; the results are summarized in Table IV. The variability of the slopes from 0.45 to 0.82 clearly demonstrates that the mechanism of chromatographic separation of the acids is not uniform. Provided the value of the slope can be taken as a measure of the contribution of the partition mechanism to the chromatographic process, the results show that the share of this mechanism increases with increasing lipophilicity of the substituents in both series of acids. The ratio of partition and adsorption mechanisms is also apparently influenced by other physico-chemical characteristics of these acids. The slopes for arylacetic acids (I) are generally higher than those for β-aryl-*n*-butyric acids (II), although the total lipophilicity, expressed as log P, is lower for arylacetic acids.

In both series of acids, we have studied an effect of a non-uniform mechanism of chromatographic separation upon relationships between R_M and other lipophilic quantities. The lipophilicity of the substituents was expressed either by π parameters, or by fragmental constants f. The chromatogra-

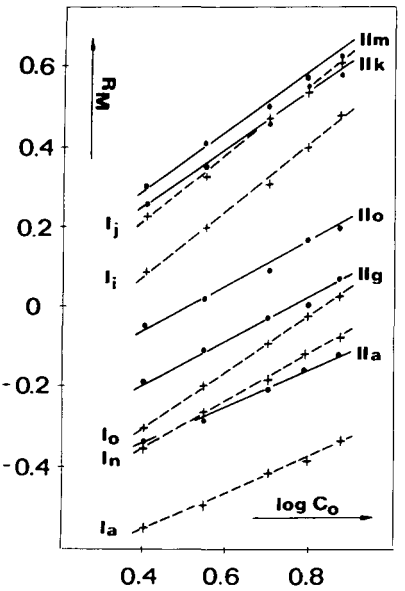

Fig. 1. Effect of the concentration of silicone oil (C_o) in the impregnating mixture on R_M values of arylacetic acids (I, - - -) and β-aryl-*n*-butyric acids (II, - - -).

TABLE III

R_M VALUES OF ACIDS I AND II FOR 2.5—7.5% SILICONE OIL IN THE IMPREGNATING MIXTURE

Compound		Silicone oil (%, v/v)									
No.	X	2.5		3.5		5.0		6.2		7.5	
		R_F	R_M	R_F	R_M	R_F	R_M	R_F	R_M	R_F	R_M
Arylacetic acids											
Ia	4-CH_3O	0.78	−0.55	0.755	−0.49	0.72	−0.41	0.705	−0.38	0.68	−0.33
In	3-CH_3O,4-$C_6H_5CH_2O$	0.685	−0.34	0.645	−0.26	0.60	−0.18	0.565	−0.11	0.54	−0.07
Io	4-$C_6H_5CH_2O$	0.665	−0.30	0.61	−0.19	0.547	−0.09	0.51	−0.02	0.48	0.03
Ii	4-*n*-$C_6H_{13}O$	0.447	0.09	0.385	0.20	0.327	0.31	0.295	0.40	0.247	0.48
Ij	3-Cl,4-*n*-$C_6H_{13}O$	0.37	0.23	0.32	0.33	0.25	0.47	0.225	0.54	0.193	0.62
β-Aryl-*n*-butyric acids											
IIa	H	0.685	−0.34	0.65	−0.27	0.62	−0.21	0.59	−0.16	0.57	−0.12
IIg	3-Br	0.61	−0.19	0.565	−0.11	0.52	−0.03	0.50	0	0.46	0.07
IIo	4-$C_6H_5CH_2O$	0.53	−0.05	0.49	0.02	0.45	0.09	0.405	0.17	0.38	0.21
IIk	4-*i*-C_5H_{11}	0.355	0.26	0.31	0.35	0.257	0.46	0.22	0.55	0.215	0.56
IIm	4-*n*-$C_6H_{13}O$	0.335	0.30	0.28	0.41	0.24	0.50	0.213	0.57	0.193	0.62

TABLE IV

CALCULATED VALUES OF SLOPES AND INTERCEPTS OF THE GENERAL EQUATION $R_M = a \cdot \log C_{oil} + b$

Experimental values from Table III were employed; $n = 5$.

Compound	π_X	Log P	a	b	r	s	F	Eqn. No.
Arylacetic acids								
Ia	0.01	1.46**	0.457	-0.734	0.998	0.006	732	(7)
In	1.09*	2.54	0.572	-0.570	0.998	0.007	954	(8)
Io	1.33*	2.78	0.692	-0.572	0.999	0.005	2986	(9)
Ii	2.51	3.96	0.808	-0.238	0.998	0.012	642	(10)
Ij	2.96	4.41	0.821	-0.105	0.999	0.010	1081	(11)
β-Aryl-n-butyric acids								
IIa	0	2.15**	0.455	-0.521	0.999	0.005	1301	(12)
IIg	0.91	3.06	0.522	-0.397	0.995	0.011	310	(13)
IIo	1.31***	3.46	0.551	-0.277	0.994	0.013	253	(14)
IIk	2.25	4.40	0.699	-0.023	0.996	0.014	389	(15)
IIm	2.51	4.66	0.666	0.040	0.999	0.006	1535	(16)

*Values are calculated from eqn. 20.
**Log P values of phenylacetic and β-phenyl-n-butyric acids are taken from ref. 25.
***Value is calculated from eqn. 30.

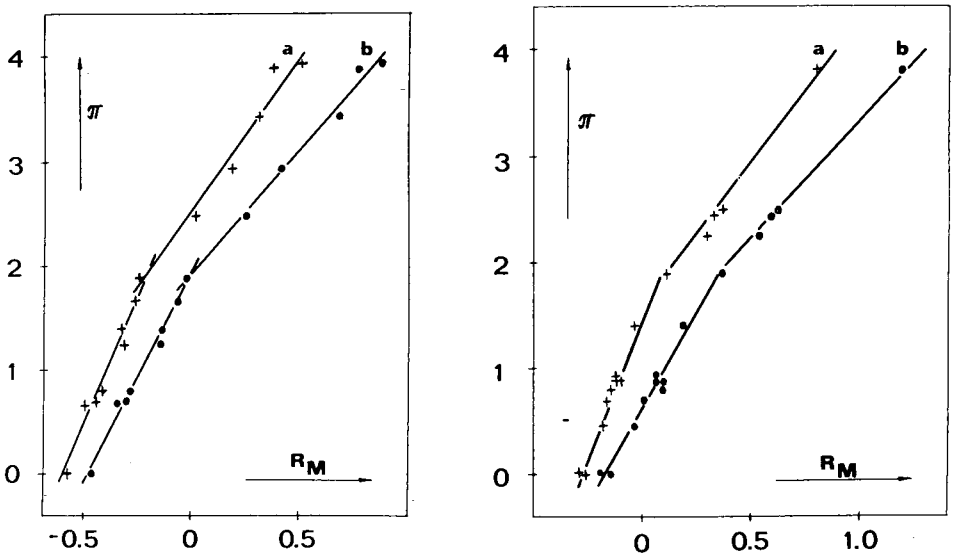

Fig. 2. Arylacetic acids: relationships between π and R_M values. (a) 2.1% Concentration of silicone oil. (b) 4.2% Concentration of silicone oil.

Fig. 3. β-Aryl-n-butyric acids: relationships between π and R_M values. (a) 3.5% Concentration of silicone oil. (b) 7.5% Concentration of silicone oil.

TABLE V

CHROMATOGRAPHIC PROPERTIES OF ARYLACETIC ACIDS

No.	X	π		f		A_W	2.1% Silicone oil		4.2% Silicone oil	
		Tab.	Calc.*	Tab.	Calc.**	($10^2 A^2$)	R_F	R_M	R_F	R_M
Ia	4-CH_3O	0.01	0.11	1.97	2.08	0.416	0.793	-0.585	0.747	-0.47
Ib	3-Cl	0.68	0.58	2.65	2.61	0.316	0.76	-0.50	0.693	-0.35
Ic	4-Cl	0.70	0.73	2.65	2.79	0.316	0.74	-0.45	0.673	-0.315
Id	4-*i*-C_3H_7O	0.81	0.83	2.96	2.90	0.816	0.726	-0.42	0.66	-0.29
Ie	3-Cl,4-*i*-C_3H_7O	1.26	1.34	3.65	3.47	1.071	0.66	-0.29	0.585	-0.15
If	4-*i*-C_3H_7	1.40	1.38	3.42	3.51	0.722	0.68	-0.33	0.58	-0.14
Ig	4-*t*-C_4H_9	1.68	1.61	4.01	3.77	0.922	0.653	-0.275	0.543	-0.08
Ih	4-*i*-C_4H_9	1.90	1.72	3.95	3.90	0.922	0.643	-0.255	0.525	-0.04
Ii	4-*n*-$C_6H_{13}O$	2.51	2.61	4.62	4.87	1.417	0.496	0.01	0.36	0.25
Ij	3-Cl,4-*n*-$C_6H_{13}O$	2.96	3.02	5.31	5.31	1.672	0.40	0.18	0.28	0.41
Ik	3-Cl,4-*n*-$C_7H_{15}O$	3.46	3.61	5.84	5.93	1.872	0.33	0.31	0.173	0.68
Il	4-2'-Ethylhexyl	3.90	3.76	6.07	6.08	1.722	0.30	0.37	0.147	0.76
Im	3-Cl,4-*n*-$C_8H_{17}O$	3.96	3.94	6.37	6.26	2.072	0.24	0.50	0.12	0.87
In	3-CH_3O,4-$C_6H_5CH_2O$***	—	1.09	—	3.19	1.545	0.705	-0.38	0.625	-0.22
Io	4-$C_6H_5CH_2O$***	—	1.33	—	3.45	1.190	0.693	-0.36	0.585	-0.15
Ip	3-Cl,4-$C_6H_5CH_2O$***	—	1.81	—	3.99	1.445	0.64	-0.25	0.505	-0.01

*Values are calculated from eqn. 20.
**Values are calculated from eqn. 22.
***Not included in the regression analysis.

TABLE VI

CHROMATOGRAPHIC PROPERTIES OF β-ARYL-*n*-BUTYRIC ACIDS

No.	X	π		A_W	3.5% Silicone oil		7.5% Silicone oil	
		Tab.	Calc.*	(10^2A^2)	R_F	R_M	R_F	R_M
IIa	H	0	0.12	0.061	0.65	−0.27	0.58	−0.14
IIb	4-CH_3O	0.01	−0.06	0.416	0.657	−0.28	0.61	−0.19
IIc	4-CH_3	0.45	0.48	0.321	0.60	−0.18	0.525	−0.04
IId	4-Cl	0.70	0.66	0.316	0.59	−0.16	0.495	0.01
IIe	4-*i*-C_3H_7O	0.81	0.93	0.816	0.58	−0.14	0.45	0.09
IIf	4-Br	0.90	0.86	0.363	0.57	−0.12	0.46	0.07
IIg	3-Br	0.91	0.86	0.363	0.565	−0.11	0.46	0.07
IIh	4-C_2H_5	0.90	0.93	0.522	0.56	−0.10	0.45	0.09
IIi	4-*i*-C_3H_7	1.40	1.26	0.722	0.52	−0.03	0.39	0.19
IIj	4-*i*-C_4H_9	1.90	1.82	0.922	0.435	0.11	0.30	0.37
IIk	4-*i*-C_5H_{11}	2.25	2.32	1.122	0.335	0.30	0.225	0.54
IIl	4-*n*-C_5H_{11}	2.45	2.48	1.122	0.32	0.33	0.20	0.60
IIm	4-*n*-$C_6H_{13}O$	2.51	2.54	1.417	0.30	0.37	0.193	0.62
IIn	4-2'-Ethylhexyl	3.90	3.88	1.722	0.136	0.80	0.06	1.19
IIo	4-$C_6H_5CH_2O$**	—	1.31	1.190	0.505	−0.01	0.38	0.21

*Values are calculated from eqn. 30.
**Not included in the regression analysis.

phy in both series of acids was performed with two concentrations of silicone oil in the silica gel. The experimental results are summarized in Table V (for the acids I) and in Table VI (for the acids II) and on the corresponding graphs (Figs. 2 and 3). A non-linearity of the dependence between R_M and π is clearly visible from the plots.

Eqn. 17 gives the π—R_M linear relationship for the 2.1% concentration of silicone oil, and eqn. 19 for the 4.2% concentration of silicone oil in the series of arylacetic acids. Equations 18 and 20 show the parabolic dependences between π and R_M. The quadratic term $R_M{}^2$ becomes significant on the significance level $\alpha = 0.005$ for the higher concentration of the silicone oil; for the lower concentration, the significance of this term declines to $\alpha = 0.025$ (the confidence intervals are introduced in brackets). Application of the fragmental constants f yielded similar results. Eqns. 21 and 22, corresponding to eqns. 19 and 20 are given for the sake of comparison. Since the introduction of the fragmental constants instead of π did not improve the correlation, only the π values were used in further analysis.

The relationships between lipophilic parameters and R_M values can be substituted in the given range of lipophilicity, by two separate linear dependences for lower and higher lipophilicity. For the first group of acids (Ia—Ih), eqns. 23 and 25 were derived, and for the second group (Ig—Im), eqns. 24 and 26. A comparison of these equations reveals significant differences in the slopes in the sense of decreasing slopes with increasing lipophilicity. This experimental finding shows that a change of Gibbs energy accompanying the separation in the chromatographic system used tends to be lower in a region of higher lipophilicity.

	n	r	s	F	
$\pi = 3.543\ (\pm 0.570)\ R_M + 2.422(\pm 0.215)$	13	0.988	0.206	472	(17)
$\pi = 3.426(\pm 0.487)\ R_M - 1.394(\pm 1.361)\ R_M{}^2 + 2.603(\pm 0.293)$	13	0.994	0.163	381	(18)
(4.2% of silicone oil)					
$\pi = 2.836(\pm 0.410)\ R_M + 1.693(\pm 0.184)$	13	0.991	0.186	582	(19)
$\pi = 3.251(\pm 0.412)\ R_M - 0.985(\pm 0.771)\ R_M{}^2 + 1.855(\pm 0.169)$	13	0.997	0.111	830	(20)
$f = 3.088(\pm 0.520)\ R_M + 3.843(\pm 0.233)$	13	0.988	0.236	430	(21)
$f = 3.600(\pm 0.552)\ R_M - 1.212(\pm 1.034)\ R_M{}^2 + 4.042(\pm 0.226)$	13	0.996	0.149	549	(22)
(2.1% of silicone oil)					
$\pi = 5.232(\pm 1.778)\ R_M + 3.105(\pm 0.723)$	8	0.982	0.127	161	(23)
$\pi = 2.994(\pm 0.921)\ R_M + 2.551(\pm 0.281)$	7	0.990	0.143	241	(24)
(4.2% of silicone oil)					
$\pi = 4.080(\pm 0.915)\ R_M + 1.991(\pm 0.246)$	8	0.992	0.084	371	(25)
$\pi = 2.405(\pm 0.496)\ R_M + 1.929(\pm 0.267)$	7	0.995	0.097	538	(26)

The experimental results of chromatography of β-aryl-*n*-butyric acids using silica gel containing 3.5 and 7.5% of silicone oil (Table VI) were processed in a similar manner. The regression equations (eqns. 27—34) are summarized in Table VII. Also in this case the relationships between π and R_M were expressed both by the linear (eqns. 27 and 29) and quadratic dependences (eqns. 28 and 30). Replacement of the parabolic expression by two linear relationships for different regions of the lipophilicity, yielded eqns. 31 and 32 for a 3.5% concentration of the silicone oil, and eqns. 33 and 34 for the 7.5% concentration. The equations show that analogous rules hold as in the series of arylacetic acids.

The relationships between R_M values and the concentration of silicone oil demonstrate the presence of adsorption effects in the chromatographic separation of both series of acids. Notwithstanding, the linear dependences between R_M and π values were observed even in the groups of acids I and II with lower lipophilicity. It is probable that an adsorption of the molecules on a solid support is affected by the surface areas of the molecules. Therefore, we have undertaken the calculation of molar surface areas (A_W) of the substituents with the aim of searching for the relationship between A_W and π. Such relationships are given by eqns. 35 and 36 for the substituents of acids Ia—Im and IIa—IIn, respectively.

Taking these linear correlations into account, it is understandable that the

TABLE VII

RELATIONSHIPS BETWEEN π AND R_M VALUES OF β-ARYL-*n*-BUTYRIC ACIDS

General equation: $\pi = a \cdot R_M - b \cdot R_M^2 + c$.

Eqn. No.	Silicone oil (%)	Series	a	b	c	n	r	s	F
27	3.5	IIa—IIn	3.542(±0.531)	1.138(±1.129)*	1.232(±0.159)	14	0.989	0.172	525
28	3.5	IIa—IIn	4.030(±0.678)		1.316(±0.155)	14	0.994	0.131	460
29	7.5	IIa—IIn	2.914(±0.333)		0.641(±0.147)	14	0.993	0.132	898
30	7.5	IIa—IIn	3.481(±0.491)	0.695(±0.489)	0.622(±0.094)	14	0.998	0.082	1168
31	3.5	IIa—IIj	5.073(±0.900)		1.447(±0.151)	10	0.992	0.080	465
32	3.5	IIj—IIn	2.989(±1.449)		1.461(±0.412)	5	0.994	0.099	236
33	7.5	IIa—IIj	3.583(±0.661)		0.612(±0.176)	10	0.995	0.083	430
34	7.5	IIj—IIn	2.462(±0.378)		0.967(±0.272)	5	0.999	0.031	2358

*Confidence interval of the quadratic term on the significance level $\alpha = 0.01$.

linear relationships between π and R_M in both regions of lipophilicity are not affected by adsorption effects.

	n	r	s	F	
$\pi = 2.036\, A_W - 0.280$	13	0.959	0.401	138	(35)
$\pi = 2.180\, A_W - 0.225$	14	0.947	0.361	105	(36)

CONCLUSIONS

Summarizing the results obtained it is possible to observe that a non-uniform mechanism of the chromatographic separation of the acids being investigated manifests itself in significant departures from linearity in the relationships between π and R_M values. The role of a purely partition mechanism in this chromatographic system is influenced by the lipophilicity of the compounds, and most probably by certain other physicochemical characteristics. A non-linear relationship between lipophilic π parameters and R_M values can be replaced either by a quadratic dependence or by two lines with different slopes in different regions of the lipophilicity. In the broader region of lipophilicity the R_M values thus obtained do not represent the conventionally used measure of lipophilicity, that is lipophilic parameters derived from the octanol—water system. Therefore, the determination of the separation mechanism plays an important role in the chromatographic evaluation of lipophilicity. The use of the above-mentioned R_M values in correlating some biological activities of acids I and II is in progress in our laboratory.

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CHROMBIO. 253

WATER AND OLEYL ALCOHOL AS STATIONARY LIQUID PHASES IN GAS—LIQUID CHROMATOGRAPHY

DETERMINATION OF PARTITION COEFFICIENTS OF VOLATILE SUBSTANCES FOR ANALYSIS OF QUANTITATIVE STRUCTURE—ACTIVITY RELATIONSHIPS

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SUMMARY

Indirect determination of partition coefficients of volatile substances in the system oleyl alcohol—water for analysis of quantitative structure—activity relationships is discussed. Water and oleyl alcohol were used as stationary liquid phases in gas—liquid chromatography. In order to correct the results for adsorption effects, two new procedures using a standard substance were suggested. The procedures were compared with a procedure used by Conder et al. Comparison of molar hydration enthalpies and entropies derived from experimental results of a series of aliphatic alcohols with calorimetric data proved that all procedures under consideration yielded true partition coefficients.

Regression analysis of quantitative structure—activity relationships (QSAR) of congeneric compounds is accomplished using the so-called hydrophobic parameters (for example partition coefficients in the *n*-octanol—water or oleyl alcohol—water systems). The determination of partition coefficients of volatile substances is, however, often very difficult and time-consuming. This was the reason for the development of gas—liquid chromatographic (GLC) methods using water and oleyl alcohol as stationary liquid phases (SLP).

The partition coefficient of a compound in the system oleyl alcohol—water is to a fair approximation equal to the ratio of partition coefficients in the systems oleyl alcohol (saturated with water)—N_2 and water—N_2 which may be determined by GLC.

It was Karger and coworkers [1—7] who suggested water as a GLC phase

about ten years ago. However, it was found that interface adsorption of solutes in the column system has a decisive influence upon the retention volumes. Conder and coworkers [8,9] suggested a procedure making it possible to eliminate the influence of adsorption and thus to determine true partition coefficients. From theoretical considerations we proposed two procedures using a structurally related compound as a standard and giving equally reliable results in a shorter time.

We started from the following considerations. Rachinskii [10], Conder et al. [8] and Berezkin and Fateeva [11] formulated several equations describing the dependence of the retention volume V_R on various parameters. For infinite dilution we may summarize their results in eqn. 1.

$$V_R = K_R\, d^{-1}\, \omega + K_R^H\, A^H + K_R^L\, A^L + K_R^{HL}\, A^{HL} \tag{1}$$

where K_R^H, K_R^L and K_R^{HL} represent adsorption constants of the compound R at the corresponding interfaces of the column (H, support and column material—gas interface, surface saturated with water from the gas phase: L, liquid—gas interface; HL, solid—liquid interface). A^H, A^L, A^{HL} represent the corresponding surface areas; K_R is the partition coefficient in the system bulk liquid phase—gas phase; ω is the amount of SLP in the column, and d the density of SLP. For further considerations parameters α ("covering ratio"), β and $V_{R,O}$ may be introduced by means of eqns. 2, 3 and 4.

$$\alpha = \frac{A^{HL}}{A^{Ho}} \tag{2}$$

$$\beta = \frac{A^L}{A^{HL}} \tag{3}$$

$$V_{R,O} = K_R^H\, A^{Ho} \tag{4}$$

where A^{Ho} stands for the total surface of the support and column materials (saturated with water from the gas phase).

By means of assumptions 5 and 6

$$K_R^L = k_1\, K_R^H \tag{5}$$

$$K_R^{HL} = k_2 K_R^H \tag{6}$$

$$\bar{K}_R = \frac{V_R}{\omega}\, d \tag{7}$$

where k_1 and k_2 represent substance-independent constants (at least for structurally related substances), eqn. 1 may be rewritten as

$$V_R = K_R\, d^{-1}\, \omega + V_{R,O}\, \epsilon_{\alpha,\beta} \tag{8}$$

$$\epsilon_{\alpha,\beta} = 1 - \alpha\,(1 - k_1\beta - k_2) \tag{9}$$

$\epsilon_{\alpha,\beta}$ represents a substance-independent constant which is linearly dependent on α and β (eqn. 9). This constant characterizes, in fact, the quality of column preparation. The retention volume $V_{R,O}$ is a measurable quantity.

Eqn. 10, describing in another form the extrapolation procedure according to Conder et al., follows from eqn. 8

$$\overline{K}_R = K_R + V_{R,O}\ \epsilon_{\alpha,\beta} \cdot \frac{d}{\omega} \tag{10}$$

The apparent partition coefficient $\overline{K}_R$ is given by eqn. 7.

Extrapolation in the coordinate system $\overline{K}_R$; $1/\omega$ yields values of true partition coefficients (eqn. 11).

$$\lim_{1/\omega \to 0} \overline{K}_R = K_R \tag{11}$$

Eqn. 8 is valid also for the standard substance S. Hence

$$\overline{K}_R = K_R + \gamma_{R/S}\,(\overline{K}_S - K_S) \tag{12}$$

where

$$\gamma_{R/S} = \frac{V_{R,O}}{V_{S,O}} \tag{13}$$

As $\gamma_{R/S}$ represents a measurable quantity (the ratio of retention volumes of substances R and S in the column, used without a condensed water phase, with water molecules adsorbed on the surface from the gas phase only), it is possible to use eqn. 12 for the determination of K_R values, knowing the K_S value. A comparison of the values found by means of this method with values obtained by the method according to Conder et al. is shown in Table I.

As K_R, K_S and $\gamma_{R/S}$ are constants independent of ω and $\epsilon_{\alpha,\beta}$, eqn. 12 describes a linear dependence of the corresponding apparent partition coefficients of the substances R and S measured with various columns, i.e. eqn. 14.

$$\overline{K}_R = a\,\overline{K}_S + b \tag{14}$$

As far as the true partition coefficient of the standard substance (K_S) is known, and there is a sufficient number of measurements for determining the constants a and b, the K_R value may be calculated by means of eqn. 15

$$K_R = a\,K_S + b \tag{15}$$

The validity of the described relationships has been verified with a series of about 50 compounds [12]. Examples of the results obtained are shown in Figs. 1 and 2.

The validity of the assumptions represented by eqns. 5 and 6 was tested by the following experiments. The dependences of the retention volumes of compounds under study on the covering ratio, α, were investigated in a glass col-

TABLE I

MOLAR ENTHALPIES AND ENTROPIES OF HYDRATION AND SOLVATION (OLEYL ALCOHOL) OF ALIPHATIC ALCOHOLS

Gas phase standard state: ideal gas at 1 atm. Solution standard state: mole fraction $x = 1$, $T = 298.15°K$. The thermodynamic characteristics were calculated assuming a parabolic temperature dependency of Gibbs energies of hydration or solvation.

Compound	$-\Delta H_h^o$ (kJ mol^{-1})	$-\Delta H_h^{o*}$ (kJ mol^{-1})	$-\Delta H_s^{o**}$ (kJ mol^{-1})	$-\Delta S_h^o$ (J mol^{-1} deg^{-1})	$-\Delta S_s^{o**}$ (J mol^{-1} deg^{-1})
Methanol	45.71	45.22	39.4	143	118
Ethanol	53.18	52.75	47.3	169	138
n-Propanol	57.79	57.74	46.8	186	128
Isopropanol	59.31	58.59	46.2	193	131
n-Butanol	61.53	61.37	52.2	200	136
tert.-Butanol	64.43	64.10	47.8	213	134
n-Amyl alcohol	65.72**		57.3	217**	145
2-Methyl butanol-2	68.77		50.9	229	136
Cyclopentanol	68.53		56.6	214	141
n-Hexanol	69.60***			232***	
Cyclohexanol	71.33			222	
n-Heptanol	73.70**			248**	
n-Octanol	77.96**			265**	

*Calorimetric data according to Hill [13] and Krishnan and Friedman [14].

**The partition coefficients were measured using the method suggested by Conder and coworkers [8,9] (see eqn. 10).

***The partition coefficients were measured according to the method described by Conder and coworkers [8,9] and one of the suggested procedures (eqn. 12) using *n*-amyl alcohol as the standard substance. Both methods gave the same results. All other values were measured by means of the suggested procedure (eqn. 12) using *n*-amyl alcohol as the standard substance.

umn packed with glass spheres. Corresponding amounts of water were applied to different lengths of the column in such a way that the mean film thickness of water remained constant in the wetted part of the column. According to our experience the covering ratio α in this part of the column could be assumed as equal to 1. In the uncovered part of the column surface (saturated with vapour from the gas phase only) α equals zero. The average covering ratio was therefore considered to be equal to the ratio of the wetted length of the column to its total length. In those experiments the covering ratio changed proportionally to the amount of water ω. The results obtained with di-(*n*-butyl)-ketone and *n*-hexyl acetate are shown on the left-hand side of Fig. 1.

In further experiments, the total surface of the column was wetted with various amounts of water in a range of ω values in which the value of α amounts to 1. In those cases the average thickness of the liquid phase changed especially with the amount of water. The changes of the β values (eqn. 3) were relatively small in the selected range of the ω values. The results are shown on the righthand side of Fig. 1. The dependence degenerates to a single strait line

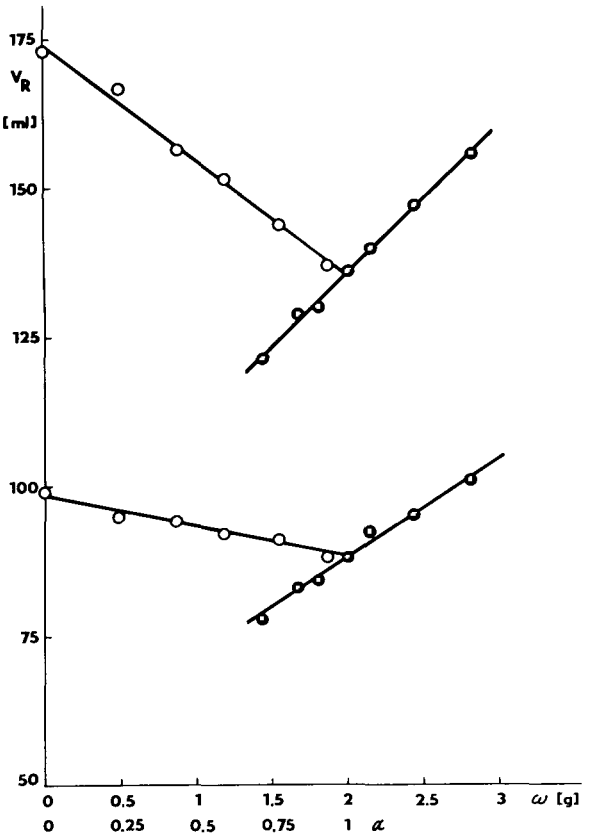

Fig. 1. Dependences of the retention volume V_R on the amount ω of the SLP (water) and the covering ratio α (○), and on the amount ω of the SLP with $\alpha = 1$ (●). $T = 310.2°K$. Di-(*n*-butyl)ketone (above), *n*-hexyl acetate (below).

with compounds whose interface adsorption in the column was small in comparison with their bulk liquid—gas partitioning. The results obtained with all compounds under study were analogous and the data fulfilled eqn. 8 within the limits of the measuring errors. The linear dependences obtained (according to eqn. 14 and presented in Fig. 2) demonstrate the validity of the assumptions for the system under study (column, support material—glass; liquid phase—water). The results obtained permit the conclusion that eqns. 12 and 14 may be used for determination of true partition coefficients.

Direct proof that results obtained by the suggested methods represent true partition coefficients is not as simple as published data on partition coefficients show considerable discrepancies. We were therefore obliged to find a round-about way. Using the method described by Conder and co-workers [8, 9] and one of the methods suggested, we determined temperature dependences of the partition coefficients and calculated from these data the molar enthalpies and entropies of hydration. As there are exact calorimetric data available for a series of aliphatic alcohols [13,14], a comparison of the results could be achieved (see Table I).

Results of analogous experiments with oleyl alcohol as SLP are shown in

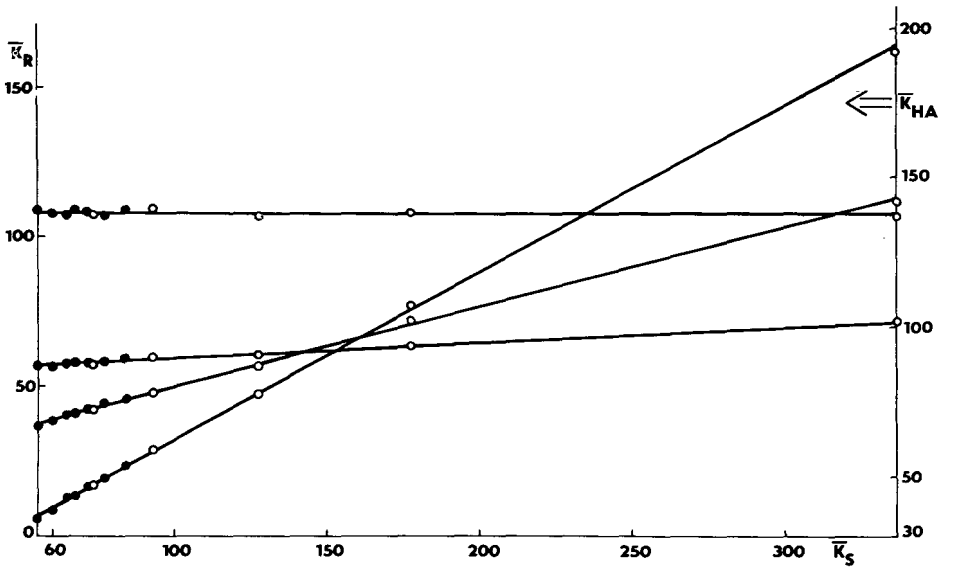

Fig. 2. Linear dependences of apparent partition coefficients $\bar{K}_R$ of the compounds (from the top) methyl acetate, *n*-propyl acetate, *n*-amyl acetate and *n*-hexyl acetate (HA), on the corresponding apparent partition coefficient $\bar{K}_S$ of the standard substance (di-(*n*-butyl)ketone). T = 310.2°K. For the meaning of symbols (•, ○) see Fig. 1.

Table I (molar enthalpies and entropies of solvation).

The comparison shows that the methods under consideration render it possible to measure true partition coefficients in the systems water—N_2 and oleyl alcohol—N_2. The methods were developed for capillary and packed columns so that partition coefficients in the system water—N_2 in the range 1—50,000, and in the system oleyl alcohol—N_2 in the range 100—50,000, are reproducible and may be measured with errors of about 1—2%.

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A SPECIFIC AND SENSITIVE METHOD FOR THE DETERMINATION OF THE ANTICOAGULANT PHENPROCOUMON IN PLASMA

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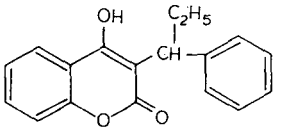
SUMMARY

A specific thin-layer chromatographic assay for phenprocoumon has been developed with a sensitivity of 5 ng/ml of plasma, using only 0.2 ml. This sensitivity is more than 20 times higher than that of the published methods. The drug is extracted from acidified plasma, an aliquot of the extract is applied to a silica-gel thin-layer plate and separated from interfering substances. The quantity of phenprocoumon is determined by fluorescence densitometry in situ. The standard deviation of the whole procedure is less than ± 3%. The new procedure permits pharmacokinetic studies with low doses of phenprocoumon to be performed on volunteers. Furthermore, due to the high sensitivity of the method, it is possible to determine the free drug fraction of this highly protein-bound substance in the plasma of patients. It was shown that, in the therapeutic concentration range, phenprocoumon is bound by about 99.5% to the plasma proteins. Since the assay is simple and quick to perform, a large series of plasma samples can be analysed without any problems.

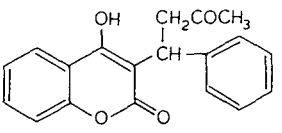
INTRODUCTION

The coumarin derivative phenprocoumon (3-[1'-phenylpropyl]-4-hydroxycoumarin, active principle of Marcoumar®) is an orally administered anticoagulant, which is structurally related to warfarin. Several analytical procedures have been described in the last few years for the determination of this drug, such as fluorimetry [1], gas chromatography [2—5] and thin-layer chromatography (TLC) [6, 7]. All these assays have a sensitivity of 100—200 ng/ml of plasma. This sensitivity is sufficient for the determination of therapeutic plasma levels of phenprocoumon. However, for extensive pharmacokinetic studies with volunteers, rather high doses of the drug have to be administered to obtain measurable plasma concentrations over a sufficient period of time [1, 2, 8, 9]. Due to the high activity of phenprocoumon, such quantities involve risks for the volunteers. Therefore, an assay for the drug in plasma

Phenprocoumon



Warfarin



with a considerably higher sensitivity was desirable to reduce this danger.

Phenprocoumon is strongly bound to the plasma proteins [1, 8, 10, 11]. For the determination of the free drug in plasma of patients, the sensitivity of the published methods is not sufficient and erroneous results could occur, i.e. due to the large variation of the sample blank.

To facilitate study of the pharmacokinetics after single oral and i.v. administration of phenprocoumon and to determine the non-protein-bound drug in plasma of patients, a sensitive and specific TLC procedure was developed for the measurement of the intact substance.

MATERIALS AND METHODS

Reagents

All chemicals must be of analytical grade and were purchased either from Merck (Darmstadt, G.F.R.) or from Fluka (Buchs, Switzerland).

Buffer solutions

Phosphate buffer (pH 7.4): add 22 ml of 1 *N* HCl to 24 g of Na_2HPO_4, dilute to 1000 ml with distilled water and adjust the pH to 7.4 with 0.1 *N* HCl or 0.1 *N* NaOH.

Acetate buffer (pH 4.0): dissolve 33 g of $CH_3COONa \cdot 12H_2O$ in 900 ml distilled water, adjust the pH to 4.0 with acetic acid and dilute to 1000 ml.

Thin-layer chromatography

The TLC was performed on silica gel 60 F_{254} 0.25 mm precoated thin-layer plates from Merck, measuring 20 × 20 cm. The thin-layer plates had to be cleaned with the solvent methanol—triethylamine (80:20) by developing for 15 cm. The plates were then dried for 5 min at 140° and cooled to room temperature. The lower edges of the plates were impregnated to 3 cm with the mixture pentane—triethylamine (100:10). This impregnation of the application zone is necessary to stablize phenprocoumon on the silica gel. After evaporation of pentane at room temperature, the samples were applied with 10-μl pipettes (Desaga, Model 130083). The developing fluid consisted of chloroform—methanol—triethylamine (95:15:5).

Fluorescence measurement

The fluorescence of phenprocoumon on thin-layer plates can be used directly for quantitative analysis. The measurements were performed with a Zeiss chromatogram spectrophotometer PMQ II.

Standard solutions

Standard solutions in organic solvents: 20 mg of phenprocoumon were dissolved in 10 ml of acetone (stock solution I). One ml of this solution was diluted to 100 ml with acetone (stock solution II). Further dilutions for checking the extraction yield were prepared by dilution of this stock solution II with ether.

Since phenprocoumon shows some instability in organic solvents, the standards in organic solvents cannot be stored longer than 1—2 weeks.

Standard solutions in plasma: 10.0 mg of phenprocoumon were dissolved in 0.5 ml 2.5% sodium hydroxide and diluted with phosphate buffer (pH 7.4) to 20 ml. Four ml of this stock solution were diluted with distilled water to 10 ml (= 200 μg/ml). One ml of this solution was mixed with blank plasma and brought to 20 ml (plasma standard containing 10 μg/ml of phenprocoumon). Further plasma standards containing 5 μg down to 5 ng/ml plasma were prepared by diluting aliquots of this plasma standard with blank plasma. The plasma standards were stored in portions of 1—2 ml at — 20°. No decomposition of phenprocoumon was observed in plasma stored at — 20°.

PROCEDURE

Extraction

The following procedure was used for concentrations of 0.1—1 μg/ml plasma. Pipette 0.2 ml of plasma to be analysed, 0.05 ml of acetate buffer (pH 4) and 0.5 ml of isopropylchloride into 5-ml stoppered centrifuge tubes. Shake for 5 min on a reciprocating shaker, and centrifuge for 5 min at 700 *g*. Along with eight plasma samples, process three standard plasmas (i.e. 1, 0.5 and 0.1 μg/ml) according to the expected plasma levels.

Apply 20 μl (two times 10 μl) of the extracts to a thin-layer plate prepared as described above. The distance from the bottom and side-edges of the plate should be 1.5 cm, between the spots 1.4 cm. For localization of phenprocoumon after separation, place 5 μl of the stock solution II to a boundary point.

The quantities of plasma and solvents used for extraction depend on the expected plasma levels. The relevant data are given in Table I.

TABLE I

AMOUNTS OF PLASMA AND ISOPROPYLCHLORIDE TO BE USED FOR THE DETERMINATION OF PHENPROCOUMON

Expected concentration range of phenprocoumon in plasma (μg/ml)	Amount of plasma to be extracted (ml)	Amount of isopropylchloride (ml)	Volume to be applied to the thin-layer plate (μl)	Plasma standards to be used (μg/ml)
1—10	0.1	1	10	1, 5, 10
0.1—1	0.2	0.5	20	0.1, 0.5, 1.0
0.005—0.1	0.2	0.5	40	0.01, 0.05, 0.1

Chromatography

Develop the thin-layer plate in a jar lined with filter paper to achieve vapour saturation. Equilibrate with the mobile phase (chloroform—methanol—triethylamine, 95:15:5) for about 10 min before use. Developing distance: 8 cm from application points. Dry the plate for 3 min at room temperature and then for 1 min in a drying oven at 30—35°. Mark the position of the phenprocoumon zone under shortwave UV light (254 nm).

Fluorescence densitometry

Scan the chromatogram with a Zeiss chromatogram spectrophotometer. We used a model PMQ II with the following instrument settings: mercury lamp, excitation wavelength 312 nm; entrance diaphragm 6 mm, slit-width 1 mm; secondary filter M365 nm; ordinate 2 or 4 times extended, damping 1, scanning speed 10 cm/min, paper speed 10 cm/min.

Calculation

In the concentration range 0.1—10 ng per spot the peak heights of the fluorescence signals are directly proportional to the quantity applied. The linear regression curve of the plasma standards used is determined with the aid of a suitable calculator. With this regression curve the concentrations of the plasma samples to be determined are calculated according to the fluorescence peak-heights measured.

RESULTS

Analytical variables

Extraction. The recovery of phenprocoumon added to blank plasma was 95% and higher in the concentration range 0.005—10 μg/ml using the extraction conditions mentioned above. Therefore, the addition of an internal standard for compensation of the extraction variation is not necessary.

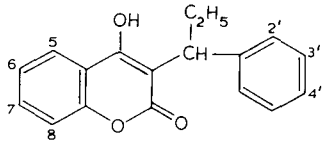
Stability of phenprocoumon on silica-gel thin-layer plates. It is a well-known fact that phenprocoumon decomposes very quickly on silica-gel thin-layer plates [7]. We found that the degradation rate of phenprocoumon on silica gel changed from one plate to another of the same batch. For quantitive densitometry it was therefore absolutely necessary to stabilize the drug on the silica-gel plates. This problem was solved by impregnating the starting zone with triethylamine. With this procedure the degradation of phenprocoumon could be almost completely abolished, but the samples still have to be applied to the silica-gel thin-layer plates within 10—15 min. The reason for the need for this stabilization is not known.

Chromatography. With the developing solvent chloroform—methanol—triethylamine (95:15:5) phenprocoumon has an R_F of 0.45. The R_F of some possible metabolites of phenprocoumon are given in Table II. The addition of triethylamine to the mobile phase considerably enhances and stabilizes the fluorescence of phenprocoumon. The chromatograms can still be scanned after some hours of storage at room temperature without a noticeable loss of sensitivity. Fig. 1 shows a scan of a thin-layer plate with a series of plasma standards, measured under the conditions described above.

TABLE II

THIN-LAYER CHROMATOGRAPHY OF PHENPROCOUMON DERIVATIVES

Solvent system: chloroform—methanol—triethylamine (95:15:5).



Compound	R_F
Phenprocoumon	0.45
5-Hydroxyphenprocoumon	0.39
6-Hydroxyphenprocoumon	0.23
7-Hydroxyphenprocoumon	0.14
8-Hydroxyphenprocoumon	0.28
2'-Hydroxyphenprocoumon	0.33
4'-Hydroxyphenprocoumon	0.21

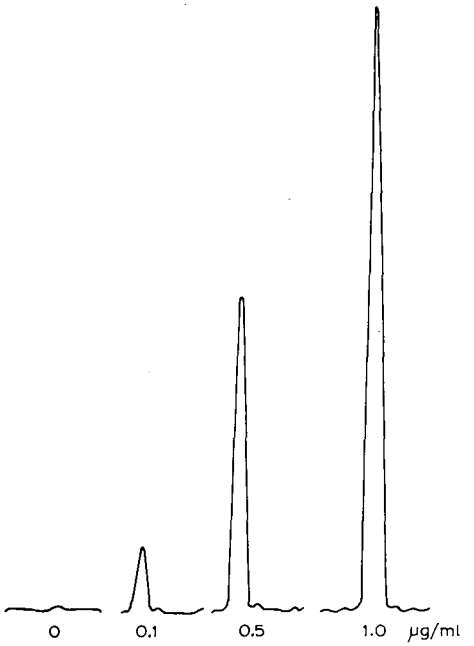


Fig. 1. Fluorescence densitometric scan of phenprocoumon plasma standards after extraction and thin-layer chromatography. Quantities: 0.2 ml plasma, 0.05 ml buffer pH 4, 0.5 ml isopropylchloride. 20 µl applied to the plate. Ordinate = arbitrary units.

Specificity. The extraction and chromatographic separation of the plasma extracts guarantee a high degree of specificity.

Sensitivity. The lower limit of detection of the procedure is about 5 ng/ml plasma. With less effort it should be possible to increase the sensitivity to about 1 ng/ml (i.e. the concentration of the plasma extract).

Reproducibility of the method. The same plasma samples were analysed at least twice on different days. With these results a relative standard deviation of the whole procedure could be calculated and was found to be less than ± 3% for concentrations down to 20 ng/ml plasma. For concentrations around 5 ng/ml this value rose to ± 10%.

Efficiency. The procedure for determination of phenprocoumon is very simple to perform. Since no time-consuming steps, such as back-extraction or evaporation, are needed, thirty or more analyses of unknown samples can be done in one day.

Analysis of samples

Phenprocoumon plasma levels after i.v. administration of the drug. With the method described above, plasma levels of phenprocoumon can be determined for a long period after a single i.v. or oral dose. The plasma concentrations of a volunteer who received a single dose of 3 mg phenprocoumon i.v. are given in Table III. These data demonstrate that, with this method, which has a sensitivity more than 20 times higher than the published ones, it is possible to study the pharmacokinetics of phenprocoumon with single doses of 3 mg. This low dose can be given to volunteers without taking any special precautions.

Free phenprocoumon in plasma. To illustrate the usefulness and the possibilities of the new procedure, the protein binding of phenprocoumon was determined by equilibrium dialysis. Spiked plasma and plasma of a patient

TABLE III

PLASMA CONCENTRATION OF PHENPROCOUMON AFTER A SINGLE i.v. DOSE OF 3 mg

Volunteer J.W.

Time after injection	Plasma concentration of phenprocoumon * (μg/ml)
Zero-plasma	0.0
5 min	0.800
10 min	0.434
20 min	0.319
30 min	0.265
45 min	0.204
1 h	0.180
2 h	0.160
4 h	0.144
8 h	0.140
1 day	0.122
2 days	0.110
4 days	0.091
6 days	0.072
8 days	0.060
16 days	0.038
24 days	0.031
32 days	0.024
64 days	0.012

*Mean of at least three determinations.

TABLE IV

PROTEIN BINDING OF PHENPROCOUMON IN BLANK PLASMA SPIKED WITH THE DRUG

Plasma concentration (μg/ml)	Free phenprocoumon (%)	Protein-bound phenprocoumon (%)
50	0.5	99.5
10	0.4	99.6
5	0.4	99.6
1	0.4	99.6
0.5	0.5	99.5

TABLE V

PROTEIN BINDING OF PHENPROCOUMON IN PLASMA OF A PATIENT RECEIVING 1.6 mg/day OF THE DRUG

Days after first medication	Plasma concentration of phenprocoumon (μg/ml)	Free phenprocoumon (%)	Protein-bound phenprocoumon (%)
12	1.75	0.5	99.5
16	1.80	0.4	99.6
23	1.90	0.4	99.6

treated with phenprocoumon were dialysed against phosphate buffer (pH 7.4). The data from this experiment are summarized in Tables IV and V.

DISCUSSION

A TLC procedure has been developed for the determination of phenprocoumon in plasma. The method has several important advantages compared with the published assays. These include high specificity and sensitivity, good reproducibility, the need for only small quantities of plasma for the analysis, and a low time requirement.

Until now for single-dose pharmacokinetics 20—50 mg of phenprocoumon have been administered to volunteers to give interpretable plasma levels [3, 8, 9]. These high doses are critical for volunteers since the drug is very active and haemorrhages could occur. Therefore, in some cases the antidote vitamin K was administered simultaneously [9], but it has not been proved that this co-medication has no influence on the pharmacokinetic behaviour of phenprocoumon. The new procedure allows reliable pharmacokinetic studies with single doses of 3 mg which are harmless to volunteers. The high accuracy of the method makes it possible to measure even very low concentrations accurately, and to observe the plasma levels over a long period of time.

Free phenprocoumon could only be determined previously in spiked plasma samples in a concentration range that was much higher than the therapeutic one. Two important aspects could not be tested. Firstly it was not proved

whether or not the protein binding of phenprocoumon is higher in the therapeutic plasma levels. Also, it was not known if the protein binding is the same in spiked plasma as in plasma of patients medicated with the drug.

Our method gave us the possibility to answer both questions. In the range of 0.5—50 μg/ml plasma the protein binding of phenprocoumon is constant and amounts to 99.5% or more. Furthermore, there is no significant difference in the protein binding of phenprocoumon added to blank plasma and the protein binding of phenprocoumon in plasma of a patient medicated with this drug.

Since the described assay is simple, normal analytical-grade solvents can be used, and since it is not time-consuming, extensive pharmacokinetic studies with phenprocoumon are practicable. Eventually, future work can also answer the question of whether there is a correlation between the free phenprocoumon in plasma and the biological activity.

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CAFFEINE DETERMINATION IN RAT PLASMA

A COMPARATIVE STUDY OF MICROMETHODS

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SUMMARY

Confronted with the need for a sensitive (1 mg/l), specific and reproducible (<5%) method for the determination of caffeine in small plasma samples (10—50 μl) of small laboratory animals, two existing methods, radioactive labelling and gas chromatography, were adapted. The first step, common to both methods, is chloroform extraction, followed by either gas-chromatographic analysis or radioactivity measurement. These methods were compared by using a common internal standard, labelled caffeine, measured before and after the extraction step. The initial requirements were fulfilled and the correlation of the results proved to be excellent.

These methods can be extended to animals other than the rat, and to organs or biological fluids other than plasma. The very small amount of plasma needed for the determination allows pharmacokinetic studies to be conducted in small laboratory animals. Further, by combining the two methods it is possible to perform rather complex investigations, such as evaluating the extent of caffeine metabolism and, at the same time, determining levels in the case of chronic administration.

INTRODUCTION

Several methods have been proposed for the determination of caffeine in human blood: after extraction by means of an organic solvent (generally benzene or chloroform), caffeine is measured by UV spectrophotometry [1—4] or by gas chromatography (GC) [5,6]. Sensitivity and specificity are two major problems in this type of determination. Sensitivity has been gained by using large plasma samples (>2 ml) whose extracts are concentrated. Plasma levels of 1—2 mg/l could be determined in this manner. Specificity has been obtained by column separation in the case of GC and tentatively by subtracting blank values when using UV absorption. Apart from the high variability of the blank values observed, such a procedure does not differentiate caffeine from various

endogenous or exogenous absorbing compounds. Routh [7] proposed a differential method to compensate for the absorption due to the presence of theophylline and theobromine, two important metabolites of caffeine. Study of the metabolic pathway of caffeine [8—12], however, has revealed the presence of a great variety of metabolites which are being investigated in the case of chronic caffeine administration [13]. More recently, radioimmunoassay [14] high-pressure liquid chromatography [14,15], and thin-layer chromatography (TLC)—densitometry [16] have been proposed for the measurement of caffeine in the plasma.

A potential major draw-back of these methods is that none of them utilizes a real internal standard to control the whole procedure of extraction, quantitation and reproducibility.

For small laboratory animals (such as rats and mice), the plasma volume required is too large for the above methods to be applicable. Isotopic labelling has been used mainly for metabolic studies [9, 10, 12, 17] or for investigations of transfer processes [18, 19]. In the present investigation it was our intention to adapt two existing methods, isotopic labelling and GC, for small samples (10—50 μl) and to correlate the results by using a common internal standard, [1-CH_3-^{14}C]caffeine, which would in addition allow us to evaluate the recovery over the whole procedure.

EXPERIMENTAL

Biological samples

Adult Sprague-Dawley male and female rats, weighing 200—300 g, were used. All the animals were fasted for one night before caffeine administration.

Pure aqueous or NaCl isotonic solutions of caffeine benzoate were labelled with (1-CH_3-^{14}C)caffeine (New England Nuclear, Boston, Mass., U.S.A.). Caffeine solutions were administered either orally to unanaesthetized animals or intravenously to rats under ether narcosis. Blood was collected in heparinized tubes under ether anaesthesia by aortic puncture. Plasma samples were frozen until analysis, for a time period which never exceeded four weeks. Urine samples were taken from some animals several hours after caffeine administration. The dose of caffeine varied from 0.10 to 50 mg/kg body wt. The rats were generally given 5 μCi, and exceptionally up to 50 μCi. The time interval between administration and blood collection was either 2 min or between 0.5 and 8 h. We assumed that, in plasma, 2 min after i.v. administration, the concentration of the caffeine metabolites is negligible and that the activity measured is essentially due to caffeine alone.

Radioactivity measurements

Radioactivity was measured directly in 10-μl plasma samples and in chloroform extracts of these samples. For simple counting, 10 μl plasma were extracted in 500 μl chloroform; for TLC analysis, 250 μl plasma were extracted in 800 μl chloroform. All the extractions took place in the presence of a buffer (pH 10 glycine buffer according to Sörensen) and chloroform was always evaporated. All the samples were dissolved in Soluene-100 (Packard), except for TLC studies where the residues after chloroform evaporation were dissolved in 0.05 *N* HCl.

TLC was performed on silica-gel plates (Merck No. 5737) with chloroform—ethanol (9:1) or acetone—*n*-butanol—chloroform—ammonia 25% (30:40:30:10) as solvent mixtures for one- or two-dimensional migration, respectively [20]. After separation, a radiographic film (Kodak Kodirex) was placed against the plate for 1—4 weeks. The silica gel spots located on the film were scraped from the plate and their activities were counted. The spots were identified by means of labelled standards applied in parallel.

The samples were counted in a spectrometer (TriCarb, Packard), after addition of a standard PPO—POPOP—toluol—ethanol scintillator. Quenching was controlled by channel ratio and by external standardisation.

Gas chromatography

Most of the samples used in determinations of isotopic labelling were also analysed by GC using a slightly different extraction method. To 50-μl plasma samples in 0.3-ml conical vials, 5 μl citrate buffer (pH 6) and 50 μl chloroform were added. The contents of each vial were mixed for 30 sec and then centrifuged at 200 *g* for 10 min. Two μl were drawn from the chloroform layer under the lipoprotein interface and injected into a gas chromatograph (Perkin-Elmer 3920) equipped with a heated injection port, a glass column (1.8 m × 2 mm I.D.) packed with 3% SE-30 on Chromosorb W AW DMCS (80—100 mesh), and a flame ionization detector. The temperature conditions were 250° for the injector and the detector and 190° for the column. The carrier gas was N_2 plus HCOOH vapour at a flow-rate of 30 ml/min. No internal standard was used. Peaks were quantified (Hewlett-Packard 3380A integrator) by comparison with standard solutions covering a range corresponding to that of a given experiment, before and after each series.

RESULTS AND DISCUSSION

Benzene, toluene, and diethyl ether—chloroform and diethyl ether—acetone mixtures have also been used to extract caffeine from plasma. In our hands chloroform gave the best results in preliminary trials. By changing the pH value from 5 to 12, the recovery of caffeine remained unchanged. However, the pH value had a strong influence on the extraction of caffeine metabolites, either basic or acidic, as shown by TLC of extracts of urine samples. In order to study the demethylated metabolites of caffeine, the extractions for radioactive measurements were conducted at pH 10. On the other hand, in order to avoid the introduction of more polar molecules into the GC column, citrate buffer (pH 6) was used for the GC determinations.

When measuring caffeine by GC the polarity of the molecule provokes a loss of sensitivity and an asymmetrical form of the peak at low concentrations. Since the volatility of the caffeine is low, the column temperature needs to be high and the deactivation by silyl groups, generally used for this purpose, is not sufficient. We used a method proposed by Welton [21] for the analysis of barbiturates, which consists of adding formic acid vapour to the carrier gas. By applying this method, we could obtain symmetrical peaks, with no lengthening of the retention time, down to 0.5 ng caffeine in 1 μl of injected chloroform.

Another problem encountered in this type of determination is the use of an internal standard. A molecule chemically different from caffeine can not be considered as far as the extraction step is concerned, which is indeed the most critical one. Having already an isotopic internal standard for the over-all purpose of this study, we preferred external calibration for the GC determinations.

Statistics

All the experimental errors are expressed as the variation coefficient ($s/\bar{x}$) or as the ratio of the slope error over the slope (s_b/b).

Specificity

The specificity of the determinations was assessed in two different ways. Chloroform extracts, analysed by TLC, showed that if a single spot was to be attributed to caffeine 2 min after i.v. administration, two other spots could be detected after 1 h or more. The identification of caffeine was performed by two-dimensional TLC. Further, the product corresponding to the peak attributed to caffeine in the gas chromatograms has been identified as pure caffeine by mass spectrometry (MS) using a GC—MS method (LKB 2091; GC conditions identical to those used above, except for the use of pure helium as carrier gas).

Linearity and reproducibility

The linearity of both extraction and detection methods was verified by adding known amounts of caffeine, ranging from 1 to 25 mg/l, to rat plasma. Linear relationships were obtained by both methods ($r > 0.99$).

The reproducibility of each method was determined at different concentration levels (1—20 mg/l) by repeat extractions and determinations under identical conditions. In all cases, the reproducibility was found to be better than 5%.

Recovery

The assumption that labelled caffeine could be used as a common internal standard for both methods was verified by counting the total activity of a plasma sample, the total activity of its chloroform extract and the activity of the caffeine spot after TLC separation and scraping. The recovery of the activity of a caffeine standard spot compared with that of the solution before migration was found to be 90 ± 3%.

Therefore chloroform extracts from plasma samples collected 2 min after i.v. administration were applied to the TLC plates. The plates were divided into 10 strips (1-cm wide) which were scraped for measurement of their activity. The strips corresponding to the location of the caffeine standard spot contained 94 ± 6% ($n = 9$) of the total activity. When the time before collection of the samples was longer than 2 min, the activity of the extracts decreased to 67% after 1 h, 19% after 4 h and 1.8% after 8 h. Linear relationships were obtained by plotting the concentration of caffeine measured by radioactivity counting or by GC against the total activity of plasma collected 2 min after i.v. administration of different concentrations. Values for the slopes of the regression lines were 1.02 ± 0.02 ($r = 0.999$) for ^{14}C-labelling and 0.93 ± 0.02 ($r = 0.999$) for GC. Thereafter, values obtained by GC were corrected for by this recovery co-

efficient. The difference observed between the two methods was due to the fact that the solvent volume ratios were not the same, being 10:1 and 1:1, respectively.

Correlation between the methods

Both methods were correlated by measuring the caffeine content of 12 plasma samples collected more than 30 min after oral administration of solutions ranging from 1 to 50 mg/kg body wt. A value of 0.993 was obtained for the correlation coefficient (slope = 1.04 ± 0.04) (Fig. 1). Table I shows the main characteristics of the methods.

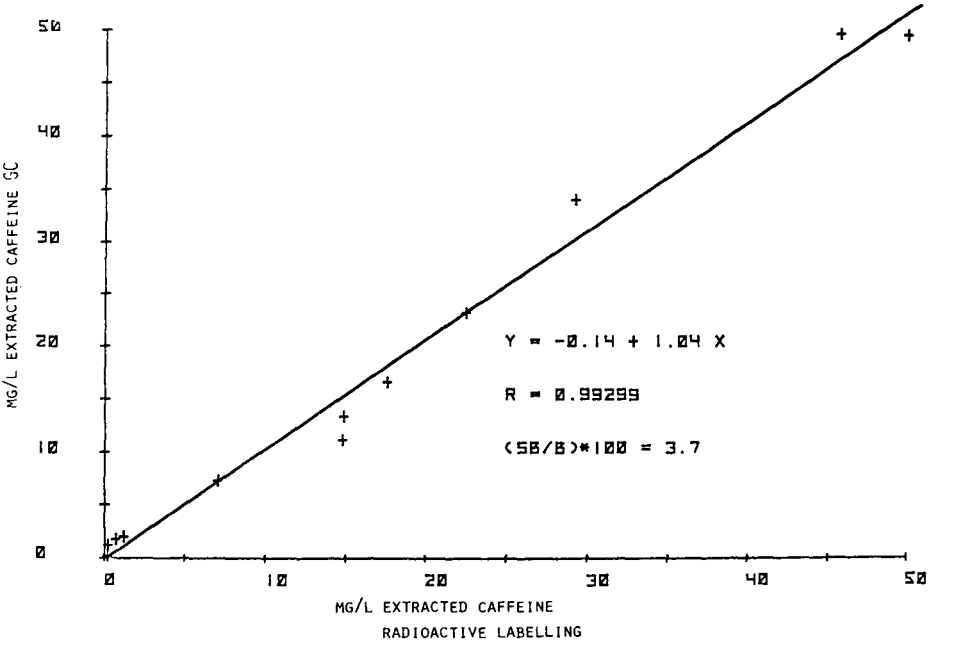

Fig. 1. Correlation between the methods.

CONCLUSIONS

We have adapted two existing methods, radioactive labelling and GC, for the determination of caffeine in very small plasma samples (10—50 μl). These methods were compared by using a common internal standard, labelled caffeine, measured before and after the extraction step. The correlation of the results proved to be excellent.

These methods can be extended to animals other than the rat, and to organs or biological fluids other than plasma. The very small amount of plasma needed for the determination allows one to conduct pharmacokinetic studies in small laboratory animals. Further, by combining the two methods, it is possible to perform complex investigations, such as obtaining an evaluation of the extent of caffeine metabolism, and, at the same time, determining levels in the case of chronic administration.

TABLE I

METHODS OF CAFFEINE DETERMINATION USED IN THIS WORK

	GC	^{14}C	^{14}C + TLC
Plasma (ml)	0.05	0.01	0.05
Reproducibility	5%	5%	5%
Sensitivity (mg/l)	0.1	According to specific activity	According to specific activity
Recovery	93%	100%	94%
Specificity:			
Substance	Caffeine	Caffeine + radioactive metabolites	Caffeine
Control	Mass spectrometry	TLC autoradiography	TLC autoradiography

ACKNOWLEDGEMENTS

We gratefully acknowledge Dr. H.P. Würzner for valuable discussions and Ms J. Markovic for skillful technical assistance.

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Note

The detection of phospholipids with acid fuchsin—uranyl nitrate reagent and its application for the estimation of phosphatidylcholine–sphingomyelin ratio (L : S) in amniotic fluid by thin-layer chromatography

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The methods recently developed for predicting, before birth, infants potentially at risk from neonatal respiratory distress syndrome (RDS) undoubtedly represent a major advance in obstetric care. When positive results are obtained these methods enable the obstetrician to terminate a complicated pregnancy well before term, safe in the knowledge that the infant will not develop RDS; negative results warn that the fetal lungs are not mature and that delivery should be delayed even if the pregnancy may appear to be at term [1—3].

Amniotic fluid lecithin assay

The main component of surfactant is phosphatidylcholine (lecithin) and a method for measuring the amniotic fluid lecithin concentration by thin-layer chromatography (TLC) was developed [4]. Using this method, it was shown that an amniotic fluid lecithin level of about 3.5 mg/100 ml was critical: if it was above that level, RDS would not occur, but if it was below, RDS was almost inevitable.

The "shake" test

This is a quick and simple test for amniotic fluid surfactant based on the stability of bubbles formed by shaking amniotic fluid treated with ethanol. The test depends on the ability of the pulmonary surfactant in amniotic fluid to maintain a stable foam after shaking [5].

Amniotic fluid lecithin—sphingomyelin ratio (L : S index)

In 1971 Gluck et al. [6] developed a semiquantitative method for measuring the L : S ratio by TLC. The value of the L : S ratio depends on the fact that the concentration of lecithin in amniotic fluid is less than sphingomyelin until about 35—36 weeks gestation, at which time a surge in the lecithin concentration increases the L : S ratio, indicating fetal pulmonary maturation. The authors postulated that an L : S ratio of 2.0 or more indicates maturation of the fetal lungs with no risk of RDS, whereas infants with an L : S ratio of less than 2.0 have some clinical RDS, the lower the L : S ratio, the more severe the RDS.

Many modifications of the original method have been published in recent years [3, 7—13], and here we describe our present technique.

EXPERIMENTAL

A 2-ml sample of the centrifuged amniotic fluid was added dropwise to 2 ml of methanol, shaken and then 2 volumes of chloroform were added. After 2 min shaking and centrifugation the lower phase was removed and evaporated in a water bath at 60° under a stream of nitrogen. The dry residue was dissolved in chloroform (about 100 μl). A 20—30-μl volume of the chloroform solution was applied as a 1-cm line to the Silufol silica-gel sheets (4 × 10 cm; Sklárny Kavalier, Votice, Czechoslovakia). The foil was developed in chloroform—methanol—conc.NH_4OH (70:30:5) or chloroform—methanol—water (65:35:5). After a short drying at laboratory temperature the chromatograms were detected with acid fuchsin—uranyl nitrate reagent [14, 15]. Two-dimensional TLC was carried out with chloroform—methanol—conc.NH_4OH (70:30:5; 1st. dimension) and chloroform—acetone—methanol—acetic acid—water (5:2:1:1:0.5; 2nd. dimension). In some cases Vaskovsky's reagent [16] for phosphorus determination was used. After the detection washing of the chromatograms with ethanol is necessary. Two-dimensional chromatography was performed mainly for the verification of the results obtained by the one-dimensional technique. Densitometric scanning was performed with ERI 10 apparatus using filter No. 3 (VEB Zeiss, Jena, G.D.R.).

RESULTS AND DISCUSSION

Our present method was tested on 90 different amniotic fluids obtained by transabdominal amniocentesis (Figs. 1 and 2). The preparation of a calibration curve (phosphatidylcholine and sphingomyelin) is very useful for obtaining the more representative values of L : S ratio (Fig. 3). The simultaneous TLC of standard solutions of phosphatidylcholine and sphingomyelin is convenient.

Sometimes a double zone of sphingomyelin was observed (Fig. 1). In such cases the values of both fractions were summated and expressed as a total percentage of sphingomyelin.

The valuation of our modification was made by TLC of 14 samples of the same amniotic fluid. The mean value of the L : S ratio was 1.66 and the standard deviation ± 0.30. In the correlation with various clinical parameters it was observed that an L : S ratio of less than 2.0 indicates a risk of RDS.

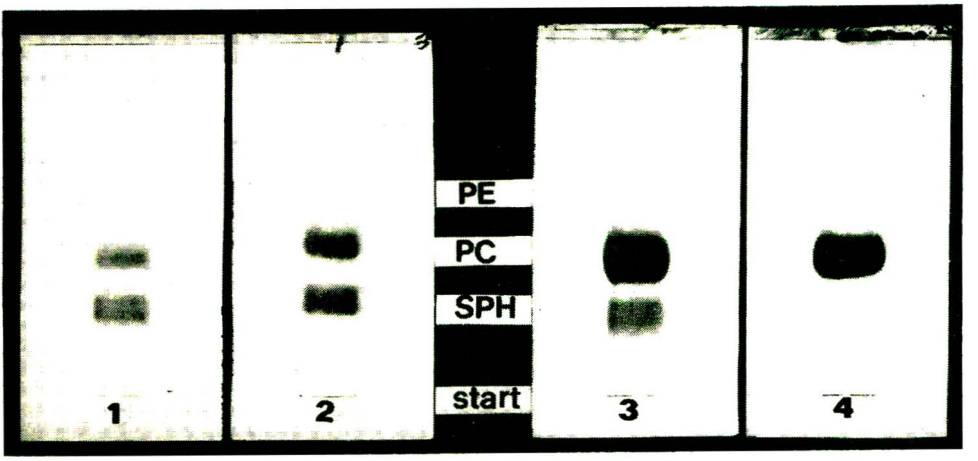

Fig. 1. One-dimensional chromatography of L:S ratio in amniotic fluid. Sorbent:Silufol sheets (4 × 10 cm). Solvent system:chloroform—methanol—conc. NH_4 OH (70:30:5). Detection:acid fuchsin—uranyl nitrate. SPH = sphingomyelin; PC = phosphatidylcholine; PE = phosphatidylethanolamine. L : S ratio: 1 = 0.9 (32 weeks of gestation); 2 = 1.3 (33 weeks); 3 = 2.7 (36 weeks); 4 = 8.5 (35 weeks).

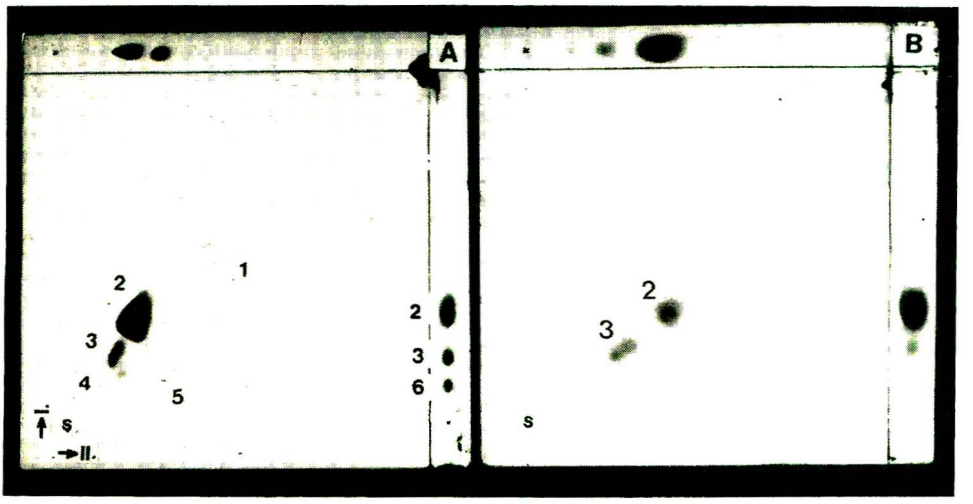

Fig. 2. Two-dimensional chromatography of phospholipids in amniotic fluid. Sorbent: Silufol sheets (10 × 10 cm). Solvent systems: 1st. dimension (I): chloroform—methanol—conc.NH_4OH (70:30:5); 2nd. dimension (II): chloroform—acetone—methanol—acetic acid—water (5:2:1:1:0.5). Detection: acid fuchsin—uranyl nitrate. A and B = two different samples of amniotic fluid. 1 = Phosphatidylethanolamine; 2 = phosphatidylcholine; 3 = sphingomyelin; 4 = phosphatidylinositol; 5 = phosphatidylserine; 6 = lysophosphatidyl—choline.

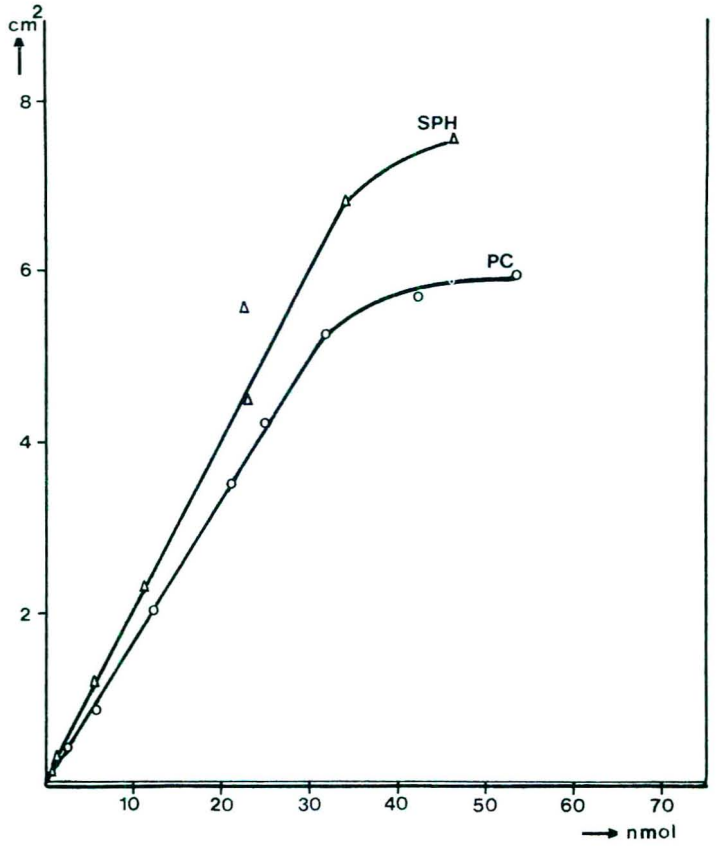

Fig. 3. Calibration curves of phosphatidylcholine and sphingomyelin after TLC on Silufol sheets and the detection with acid fuchsin—uranyl nitrate reagent. Densitometry: ERI 10 apparatus. PC = phosphatidylcholine; SPH = sphingomyelin.

The detection of phospholipids with acid fuchsin—uranyl nitrate reagent is very suitable for the semiquantitative evaluation of these substances in amniotic fluid. The application of the above-mentioned staining procedure is especially advantageous for detection of phospholipids carrying one negative charge (phosphate group) and one positive charge (choline or ethanolamine group) in their molecule [17].

In comparison with other TLC methods our modification has some advantages, especially in the use of a new staining procedure. According to our experiences we consider the present method as a simple and rapid determination of L : S ratio in routine laboratory practice.

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Note

A simple thin-layer chromatographic method for the estimation of hippuric acid: comparison with a photometric and a gas chromatographic method

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Hippuric acid is the main metabolite of toluene which is often used instead of benzene. Approximately 60% of inhaled toluene is expired. The toluene remaining in the body is oxidized to benzoic acid and after being conjugated, mainly to glycine, it is excreted in the urine as hippuric acid [1]. Off-set printers, for example, are affected by exposure to toluene. Furthermore, the estimation of hippuric acid is important in diagnosis for liver function [2] and other clinical [3,4] or pharmacological [5] problems. For the estimation of hippuric acid photometric [2,6], isotachophoretic [7] and chromatographic [8—10] methods have already been described. Photometric methods are unspecific, and isotachophoretic and gas chromatographic (GC) methods are expensive in both cost and time. Paper and thin-layer chromatographic (TLC) methods [9,10] render it possible to make a specific estimation with relatively small expense.

We have developed a routine TLC method, which allows simple, quick and specific, quantitative analysis of hippuric acid as well as application in the field of screening. Quantitation can be made either densitometrically in situ, or photometrically after elution, or by both methods.

The specificity and the importance in the field of occupational health of this TLC method has been compared with the modified GC method of Sedivec [11], and with photometric estimation after reaction with benzene sulphochloride [6].

METHODS

Thin-layer chromatography

Depending on the exposure, 1—5 μl urine (for semi-quantitative analyses, a millionth of the 24-h urine volume) are applied to silica gel (Silufol®; Kavalier, Sklárny, Czechoslovakia) and developed twice by the two-step procedure

up to 10 cm in chloroform—acetone—glacial acetic acid (40:10:5). After drying for 10 min at 150° the spots are detected with the help of 5% *p*-dimethylaminobenzaldehyde in acetic anhydride and heated again for 10 min at 150° (Fig. 1). When formic acid is used in the solvent instead of acetic acid, the colour intensity is reduced by about 20%. Contrary to alcohols ethyl acetate does not dissolve unreacted reagents. Estimation is done either semi-quantitatively by comparison of the colour intensity of standards and samples, or by quantitative densitometry across the chromatographic run with a variation coefficient of 6.50% for standards and 15.6% for urine samples, or by photometry at 468 nm after elution with 3 ml ethyl acetate with a coefficient of variation for standards and urine below 5%.

We used the electrophoresis scanner ERI 65 m® (Carl Zeiss, Jena, G.D.R.) in remission. No linear calibration curves have been obtained by means of this scanner.

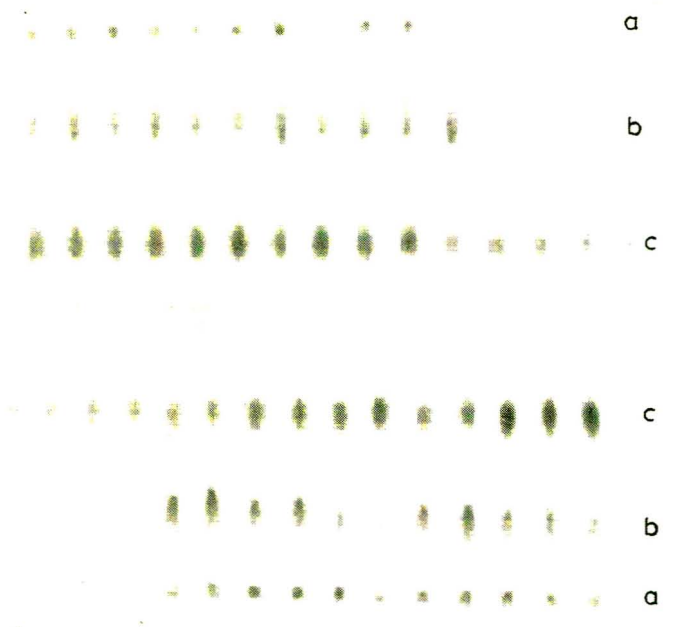

Fig. 1. Chromatogram of hippuric acid in urine on Silufol. Both sides of the plate were used for analyses. Spots: (a) unknown (violet); (b) unknown (green, not stable); (c) hippuric acid (red). Methylhippuric acid runs further than hippuric acid.

Gas chromatography

Five ml of urine were acidified with dilute sulphuric acid and saturated with ammonium sulphate. The extraction is made with ethyl acetate, containing phenacetin as internal standard, and derivatization is with diazomethane. After the addition of glacial acetic acid aliquots of this solution are chromatographed.

Technical data: Chromatograph Model GCHF 18.3® (VEB Chromatron, G.D.R.); steel column 1 m × 4 mm I.D. with 4.1% neopentylglycol succinate

on Chromosorb W AW, 80—100 mesh; carrier gas N_2 at a flow-rate of 40 ml/min; column temperature 197°; range 0—1000 μg hippuric acid. For the elimination of tailing by alteration of the column, it is better to make a hippuric acid standard daily. The photometric estimation was similar to that of Tomokuni and Ogata [6].

RESULTS

The regression equation of $y = 0.436 + 0.447x$ ($r = 0.971$, $n = 51$), which describes the connection between the values obtained densitometrically and photometrically after elution, as well as mean values of both estimations ($\bar{x}_{elution} = 1.62 \pm 1.15$, $\bar{y}_{densitometry} = 2.64 \pm 2.50$) show that at a good correlation the densitometrically estimated values are systematically higher. The linearity of the calibration curve of the estimation of hippuric acid is given at a sensitivity of about 5×10^{-8} g per spot up to 60 μg. In the measured concentration range of 0—17.6 μg hippuric acid per μl urine a very good correlation has been found between the GC and TLC estimations ($y_{TLC} = 0.996x_{GC} - 0.23$; $r = 0.998$, $n = 120$), but the correlation between the TLC method and the method of Tomokuni and Ogata was not satisfactory. It was found that the values above 10 μg hippuric acid when measured photometrically are systematically lower than when measured by TLC, and vice versa below 6 μg hippuric acid.

These results show that the TLC method represents a sufficiently exact and rational procedure for the estimation of hippuric acid. The number of samples per laboratory assistant and per day is approximately 200 analyses in the semiquantitative analysis and approximately 80 analyses in the quantitative analysis after elution. The reference range for the hippuric acid contents evaluated by means of the TLC method with a control group is 456 ± 213 mg/24 h. A strictly linear correlation has been found between exposure to toluene and the excretion of hippuric acid by means of this method.

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Note

High-performance liquid chromatographic determination of aminosalicylate, sulfapyridine and their metabolites

Its application for pharmacokinetic studies with salicylazosulfapyridine in man

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Salicylazosulfapyridine (SASP; trade name Azulfidine from Pharmacia, Uppsala, Sweden) is the drug of first choice for the treatment of Crohn's disease and ulcerative colitis [1, 2]. It is split in the colon by gut bacteria to its two biologically active moieties aminosalicylate (AS) and sulfapyridine (SP). AS is eliminated mainly in acetylated form (AcAS). SP is metabolised to its N-acetyl-(AcSP) and 5-hydroxyl-(SPOH) derivate, and these two major metabolites are conjugated to their corresponding glucuronides. The acetylation rate is genetically controlled [3] and plasma concentrations of 20—50 μg total SP per ml are regarded as therapeutic (for review see ref. 4).

While this metabolism has been thoroughly investigated [5, 6], the pharmacokinetic properties of the drugs are almost unknown. Therefore we have developed specific and sensitive high-performance liquid chromatographic (HPLC) assays for the determination of AS, SP and their corresponding metabolites and studied the pharmacokinetics of these drugs in 7 healthy volunteers and 4 patients with Morbus Crohn or ulcerative colitis. In addition, since the reported side effects were associated with high plasma levels and mainly directed to slow acetylators, we also monitored these concentrations and phenotypes in 65 patients.

EXPERIMENTAL

The determination of SP and its metabolites has recently been described [7]. In Fig. 1 the extraction procedure for the determination of AS and AcAS is

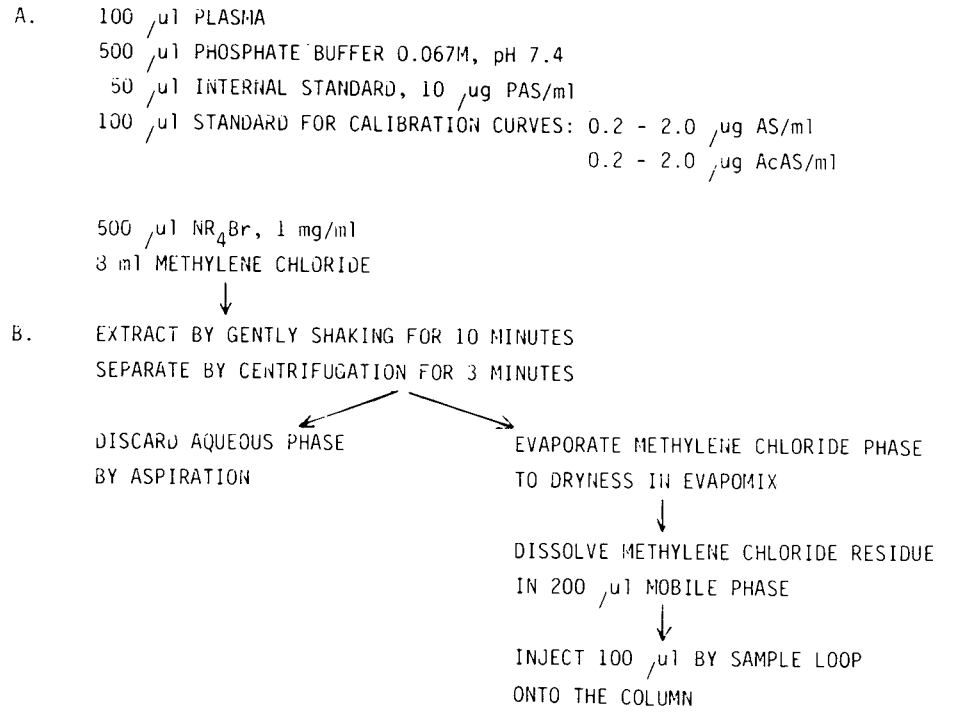

Fig. 1. Scheme of the extraction procedure for the determination of AS, its acetylated metabolite, AcAS, and the added internal standard, PAS.

outlined. Plasma (100 µl), the added internal standard *p*-aminosalicylate (PAS) and trimethylcetylammoniumbromide (NR_4Br) were extracted by methylene chloride. The evaporated residue of the organic phase was redissolved in 200 µl of the mobile phase and injected on to a reversed-phase column (250 × 3 mm; Nucleosil RP-18; particle size 10 µm). The mobile phase consisted of 50% methanol containing 1 g/l N,N,N,-trimethylcetylammoniumbromide. To achieve the sensitivity necessary to measure the low plasma levels of the salicylates a fluorescence-monitor had to be used (excitation 310 nm, emission 430 nm).

The separation of the three compounds was performed within 45 min by a flow of 1.3 ml/min. In the blank plasma, even if amplified ten-fold, no interfering peaks were observed (Fig. 2). If adding known amounts, AS was eluted first, close to the internal standard (PAS). The best sensitivity (factor 10) demonstrated AcAS (third peak). Since the unchanged AS had the most disadvantageous fluorometric spectrum, its peak was relatively small in the patient samples.

The pharmacokinetics of SASP was evaluated from single dose cross-over experiments (4 g SASP and 1.25 g SP one week apart) in 7 healthy volunteers and from steady state studies in 4 patients with Morbus Crohn or ulcerative colitis. Multiple blood samples (10 ml) were drawn for 65 and 36 h, respectively, following the single oral dose. During the steady state 8 specimens were collected following the last dose (3 g SASP/die). In 65 patients one blood sample was drawn routinely prior to the next morning dose.

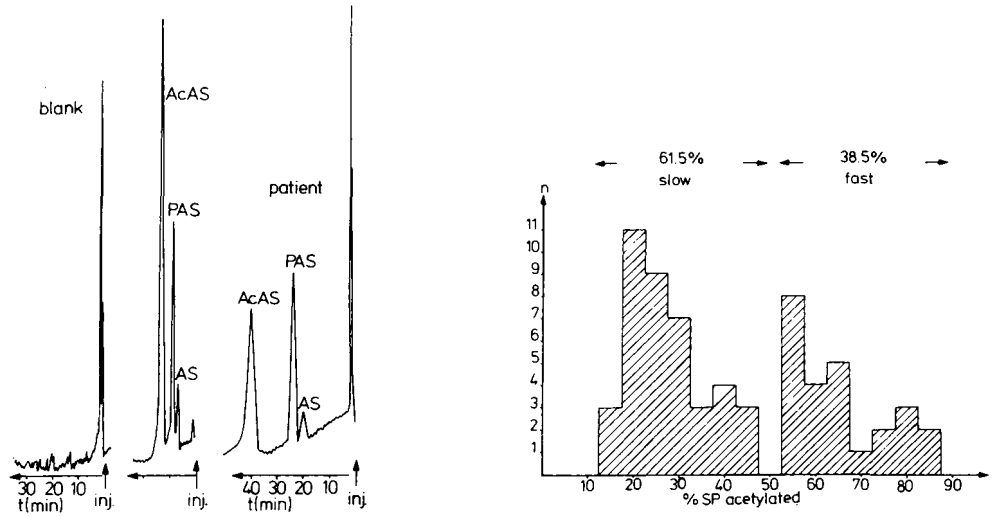

Fig. 2. Typical chromatograms of AS, its acetylated metabolite, AcAS, and the internal standard, PAS. For comparison an extract of a blank plasma (left) and of a patient's sample (right) was injected on to the column. Column: RP-18, 10 μm, 250 × 3 mm. Mobile phase: 50% methanol, 1 g NR_4Br/l. Flow-rate 1.3 ml/min. Excitation: 310 nm. Emission: 430 nm.

Fig. 3. Histogram of the genetically controlled acetylation phenotype of SP in 65 patients with Morbus Crohn or ulcerative colitis.

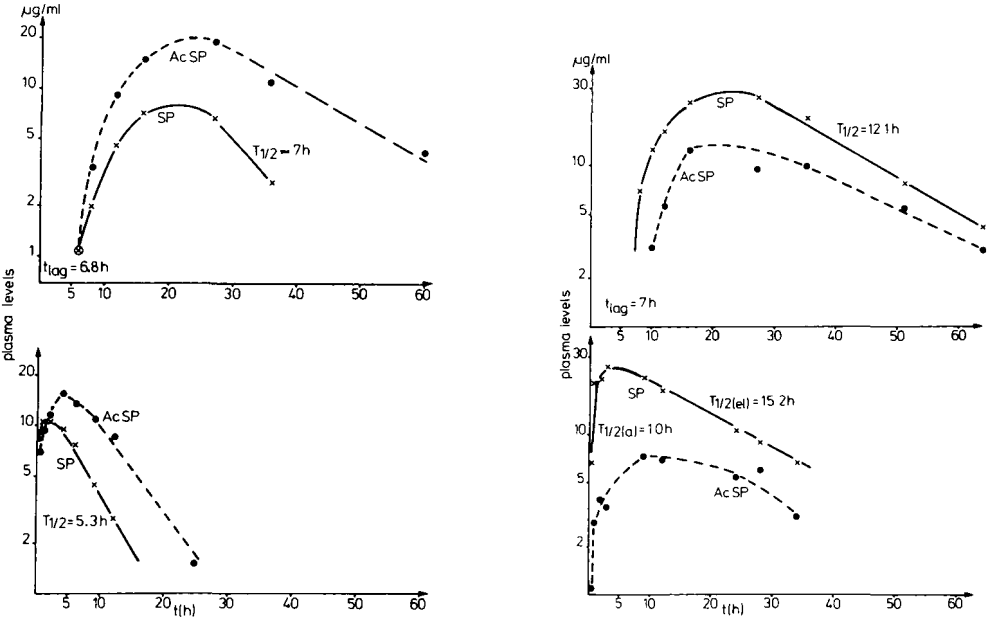

Fig. 4. Plasma level—time profiles: x, SP; •, AcSP. Both in the same fast acetylator following a single oral dose of 4 g SASP (top) and the direct oral application of 1.25 g SP (bottom).

Fig. 5. Plasma level—time profiles: x, SP; •, AsSP. Both in the same slow acetylator following a single oral dose of 4 g SASP (top) and the direct oral application of 1.25 g SP (bottom).

RESULTS

The magnitude of the plasma concentrations of SP and its metabolites are dependent on the genetically determined phenotype. The results of these estimations are given in a histogram, Fig. 3. From the 65 patients tested 40 (61.5%) can be regarded as slow acetylators with an acetylation rate of 12 to 7%.

In Fig. 4 typical plasma level time curves of SP and AcSP following the single oral doses of 4 g SASP (top) and 1.25 g SP in suspension (bottom) in one individual are shown. Following the administration of SASP, SP could be detected with a lag time of 6.8 h. After this time also, the metabolite was visible. Maximal concentrations were reached within 24 h followed by a monoexponential decline with an elimination half-life ($t_{1/2}$) of about 7 h. The time profile of the major metabolite AcSP was similar, but its concentrations were much higher than those of SP in this so-called fast acetylator.

After direct application of SP the drug could be measured already in the first plasma sample (0.5 h). Absorption was rapid with a $t_{1/2}$ between 0.3 and 2 h. Following the peak level the decline of the concentrations could be characterised by a $t_{1/2}$ of 5.3 h. Again, the concentrations of the metabolite exceeded those of the parent compound.

In Fig. 5 the plasma level:time profiles of a slow acetylator were monitored. Again, after oral administration of SASP a lag time and after direct application of SP, rapid absorption was found. While the shape of the curves is similar, significant quantitative differences exist during the elimination phase and in the ratio of the concentrations: $t_{1/2}$ is prolonged to 12—15 h and plasma levels of the metabolite are considerably lower than the corresponding SP values.

In Fig. 6 plasma levels of AS and AcAS are shown in a fast acetylator (top)

TABLE I

MEAN VALUES OF IMPORTANT PHARMACOKINETIC PARAMETERS OF SP IN 7 SLOW ACETYLATORS AND 4 FAST ACETYLATORS.

Parameter	Slow acetylators	Fast acetylators
A. Following the SASP-dose		
Lag time (h)	5.5	
t_{max} (h)	19.8	
$t_{1/2}$ (A') (h)	4.6	
C_{max} (μg/ml)	30.5	7.2
$t_{1/2}$ (el) (h)	15.2	6.6
B. Following the SP-dose		
t_{max} (h)	2.8	
$t_{1/2}$ (A) (h)	1.1	
C_{max} (μg/ml)	21.6	16.7
$t_{1/2}$ (el) (h)	13.0	4.6
Cl (ml/min)	48	155
Vd_{β} (l/kg)	0.8	1.2
Plasma protein binding		
SP = 49.6%	AcSP = 73.5%	

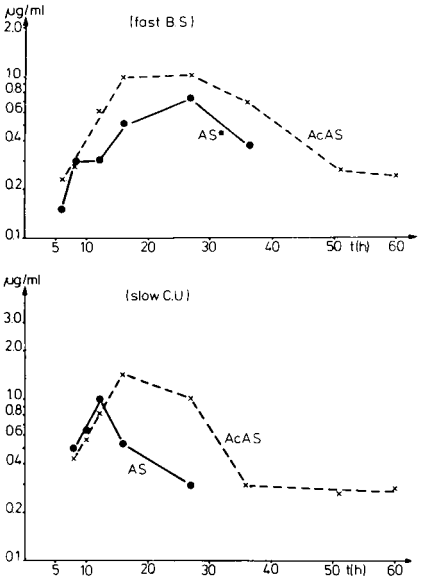

Fig. 6. Plasma level—time profiles: SA and AcAS in a fast acetylator (top) and a slow acetylator (bottom) following the oral administration of 4 g SASP (measured fluorometrically).

and a slow acetylator (bottom) following the single oral dose of 4 g SASP. The low concentrations demonstrate some fluctuations and in all cases the metabolite exhibits higher concentrations than the original compound.

From the decline of the SP concentrations and the area under these curves the most important pharmacokinetic parameters can be calculated. They are summarized in Table I. In the upper part of the table data were obtained after administration of SASP. For more correct calculations SP itself should be administrered as was done in the lower part of this table. According to the concentration ratios the indiviudals were divided into slow and fast acetylators.

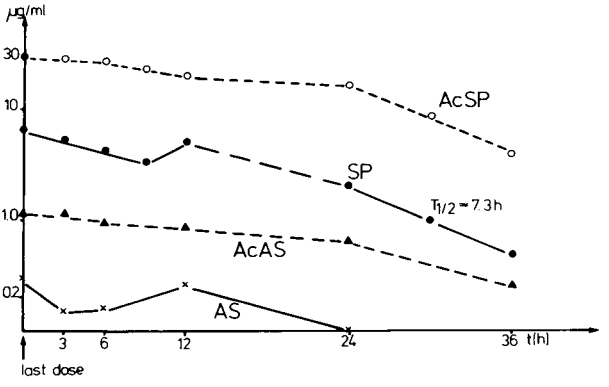

Fig. 7. Plasma level—time profiles: •, SP; x, AS; ○, AcSP; ▲, AcAS. Patient with Morbus Crohn treated chronically with 3 g SASP/die. Treatment stopped for 36 h.

Rate of absorption, either expressed as $t_{1/2}$ (a) or t_{max}, was almost identical in both groups. However, the maximal concentrations of SP were higher in the slow acetylators. A significant difference ($p = 0.0053$) was observed in the elimination $t_{1/2}$. The prolonged $t_{1/2}$ (el) seen in the slow acetylators was due to a much lower total body clearance, $\overline{Cl}$ ($p = 0.0016$). SP and AcSP are bound to plasma constituents to 49.6 and 73.5%, respectively, which corresponds to literature data on the saliva:plasma ratio of 0.5 and 0.25, respectively [8]. The apparent distribution volume, Vd_β, of approximately 1 l/kg was similar in both groups.

In Fig. 7, plasma levels of SP, AcSP, AS and AcAS were monitored for 36 h in a patient treated chronically with 3 g SASP/die. Following the last dose the initial plasma level decline of SP is interrupted by the late absorption peak. Thereafter the concentrations declined with a $t_{1/2}$ of 7.3 h. In this fast acetylator the metabolite exceeds SP. Concentrations of the salicylates are much lower than those of the sulfonamides.

DISCUSSION

The rapid and simultaneous measurements of SP and its major metabolite AcSP in a single plasma sample allows determination of the acetylator phenotype and the plasma levels. Since therapeutic and toxic effects of SP seem to be related to the plasma concentrations, the latter being much more frequent in slow acetylators, these monitorings might improve the safety and effectivity of the therapy with SASP. In addition, knowledge of the pharmacokinetic properties of the administered drugs is an elementary basis for their rational use. It is very important to consider that in the slow acetylators steady state plasma levels of SP will be much higher, since their $\overline{Cl}$-rate is significantly reduced. In the past, SP was regarded as the biologically active moiety of the SASP molecule. However, from two papers published recently [9, 10] it became evident that AS also has a therapeutic effect. So, plasma level measurements of this drug and its major metabolite (AcAS) might be of some help in increasing the therapeutic efficacy of SASP and in evaluating its mechanism of action which might involve the prostaglandins [11].

The high polarity of the salicylates render necessary their extraction into an organic solvent. This problem can be solved by the addition of NR_4Br. The basic NR_4^- ion can form an ion pair with the acidic counter ion (salicylates). AS, AcAS and the internal standard are extracted at pH 7.4 into the organic phase (methylene chloride). The mobile phase (50% methanol) for the elution from reversed-phase HPLC has also to contain NR_4Br, so the compounds are eluted as ion-pairs. The low fluorescence sensitivity of AS does not permit detection of less than a 0.3 μg/ml in a 1-ml plasma sample. However, the HPLC procedure represents a more specific method than the direct fluorescence determination described by Hannson [12].

In applying our methods, 62% of the patients could be regarded as slow acetylators with a $t_{1/2}$ of 13.0 h and 38% as fast acetylators with a $t_{1/2}$ of 4.6 h. This bimodal distribution was caused by significant differences in $\overline{Cl}$.

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Biomedical Applications

CHROMBIO.267

CLOSING REMARKS

On behalf of participants, guest speakers and discussants we heartily thank the organizers and the sponsors as well as the Czechoslovak Government Authorities who permitted this scientific event to take place.

First, the name of this meeting itself: Biomedical Applications of Chromatography is an encouraging sign that the technical application to medical sciences and medical practice of an important analytical technique such as chromatography can nowadays be the whole theme of an international congress.

The introduction of separation methods with zone electrophoresis and paper chromatography appeared thirty years ago as revolutionary methods which readily imposed themselves due to the simplicity of the technology and the relatively low cost of the equipment. Nevertheless, the need for quantitative separation methods orientated the efforts of the analysts to develop, for medical use, methods based on liquid column or gas column chromatography. From a pulse feed of known value and deposition on top of the column, peaks of separated compounds can be eluted quantitatively provided that a detector can function to the physiological levels of the compounds and that the assessed compounds are devoid of interfering substances.

Such practical considerations constitute the major difference between an existing fundamental research method and its application as a routine working-laboratory technique in clinical chemistry. Practicability does not only involve accuracy and selectivity but also repeatability at costs appropriate to the human health care services.

In most congresses on analytical methods there is a section devoted to biomedical applications. The biomedical label is often a propitiatory invocation summoning the protection of the Gods on the congress and obtaining the support of those who believe, even unconsciously, in the aura of the Medicine Man which remains in the Physician. Therefore, particularly earnest thanks should be addressed to the scientific committee and the organizers who have dared to choose "Biomedical Application" as the unique topic of this meeting. That is to say the organization of a meeting centered on the demonstration and discussion of techniques which might solve the every day difficulties that a clinical chemist encounters when wishing to use routine chromatographic methods of separation and quantitation to satisfy the clinician who requires more and more informative analyses based on the selection of specific substrates and compounds related to the anatomo-clinical description of diseases.

Plenary lectures brought to the audience the most outstanding information on theoretical and practical trends in chromatography and constituted an excellent introduction to the goal of this symposium. They comprised a demonstration by Dr. Jellum that the complexity of physiological fluids can be overcome, at least partly, by judicious coupling of gas—liquid chromatography to mass spectrometry, pertinent remarks and advice from Dr. Macek on the wise use of chromatographic methods in the clinical laboratory, a brilliant essay by Dr. Tomlinson correlating chromatographic behaviour and biological activity of substrates, and an enthralling demonstration by Dr. Horváth of the potency of reversed-phase chromatography in biomedical chemistry, a technology that many of us use unwittingly.

Opportunity was then given to speakers and discussants to cover a number of subjects through five communication and poster sessions as specified in the Preface.

In the field of chromatographic applications of clinical interest, seven communications covered application to lipid analysis of lipid profiles and lipid class assays in atherosclerosis and dyslipidemia. Evaluation of lipid amounts in each class and especially among the different classes of lipoproteins is a critical factor in atherosclerosis screening. This disease was responsible for 1,037,460 deaths out of a total of 1,977,000 in the U.S.A. during the year 1973.

Similarly, specific analytical separations are more and more necessary for increasing the recognition of inborn errors of metabolism, both to facilitate early therapy and also to improve understanding of these genetic defects and the altered metabolic pathways involved. Even if it is thought uneconomic to detect the rarest types of these diseases, much profit in terms of scientific and medical knowledge can be expected from the use of selective and specific analytical separation of metabolites.

Due to the limitations of liquid ion-exchange chromatography in the identification of new compounds, especially at low concentrations, gas—liquid chromatography of amino acids and metabolites became one of the focal points of this meeting. Five communications showed the need for gas-phase separation of amino acids and metabolites together with the possibility of using this method coupled with mass spectrometry on a routine scale with the utmost advantage in the identification of compounds and in establishing abnormal pathways.

New methods for the chromatographic assay of either small molecules like amines, organic acids, nucleotides, steroids and cyclic compounds or of biopolymers such a collagens and ^{125}I-labelled hormones testify to the extension of chromatographic separation to many classes of biological compound in the clinical laboratory.

The development of separation methods in clinical biology and advanced functional exploration constitutes a definite trend in the medical sciences towards obtaining more specific biological information related to fundamental knowledge of chemical mechanisms within living cells.

The development of quantitative separation method is not only necessary for the assay of endogenous compounds and their metabolites but becomes increasingly indispensable in assessing exogenous compounds such as medicinal drugs and xenobiotic products brought into human contact.

The position held in medicine by X-rays and biological investigations has such importance that any precise diagnosis cannot be achieved without these paraclinical techniques. While it becomes fundamental to draw the interest of advised physicians towards clinical chemistry, it is still important for the clinical chemist to keep control of this medical science. Because of the complexity and cost of assays involving chromatographic methods, the clinical chemist is obliged to decide the validity and the necessity for obtaining information. It is essential to avoid development of costly but unuseful assessments, otherwise, the role and prestige of clinical chemistry, which has made such an effort to adapt itself to modern technology, will be endangered. Conversely, the clinical chemist is committed to bringing to the patient a very accurate and precise technology. Assay must be not only precise, i.e. the repetition of the technique for a given specimen must provide not only consistent results ensuring a low variation coefficient, but also accurate ones, i.e. the result should be as close as possible to the theoretical or the expected value. There are patients rather than diseases and in healthy people physiological values may vary widely. Methodology must be founded on quality control which will give confidence in the produced results. Assays cannot be repeated simply to increase accuracy and precision, not only because the specimen size may be too small but principally to avoid, for the patient's sake, delays in obtaining a result, and for the sake of the national economy, the increasing cost of health care and welfare. Routine laboratory methods must be controlled by reference methods, themselves controlled by definitive methods. For example, in the field of free steroid hormone assays in the blood, the quality of radioimmunoassays (RIA) should be controlled by gas-chromatographic assays which have to be themselves controlled by isotope dilution—mass fragmentography which appears at present to be the definitive method. The production of results by RIA with large coefficients of variation, between 20% and 50% for some steroid hormones, implies that the method is not reliable in routine use and that organisations bearing the expenses of health care may have the right to demand better methods or to refuse payment of the bill for poor results. This example shows the necessity for developing worldwide cooperation in biochemical standardization by chromatographic and chromatographic—mass spectrometric methods. It implies the need to produce stable standards labelled with heavy isotopes. The labelling of the carbon chain or nucleus by at least two ^{13}C nowadays seems a reasonable goal which is sufficiently inexpensive to ensure isotopic dilution—mass spectrometric assessments on an M+2 isotopic peak less contaminated by natural isotopes than M+1, and close to M, the molecular ion, to avoid isotopic effects during the chromatographic separation.

In conclusion, the present evaluation of the utmost importance and opportuneness of this meeting should be clearly understood in the light of the increasing role that chromatography will play among the existing methods of clinical chemistry.

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75 Years of Chromatography

A Historical Dialogue

L. S. ETTRE *and* A. ZLATKIS (Editors).

Journal of Chromatography Library - Volume 17

On the occasion of the 75th anniversary of the invention of chromatography, this book compiles the personal stories of 59 pioneers of the various chromatographic techniques (including five Nobel Prize laureates). In their contributions to this volume, these pioneers review the events which influenced them to enter the field; explain the background of their inventions; summarize their activities and results during their professional lives; and discuss their interactions with other scientists and other disciplines.

This book is more than a nostalgic recollection of the past for those who have been in chromatography for some time. It also provides, for the younger generation of chromatographers, a unique record of how present-day knowledge was accumulated.
The final chapter is devoted to "Those who are no longer with us".

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