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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 preparation 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 (cLeu), 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 CAPIL LARY COLUMN

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

Amino acid	Abbreviation	3°/min		2°/min		RMR of standard solutic	
		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 o isoleucine and *allo*-isoleucine (approximately 1:1).

**The observed retention time and temperature of histidine corresponded to its specific chroma tographic 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-

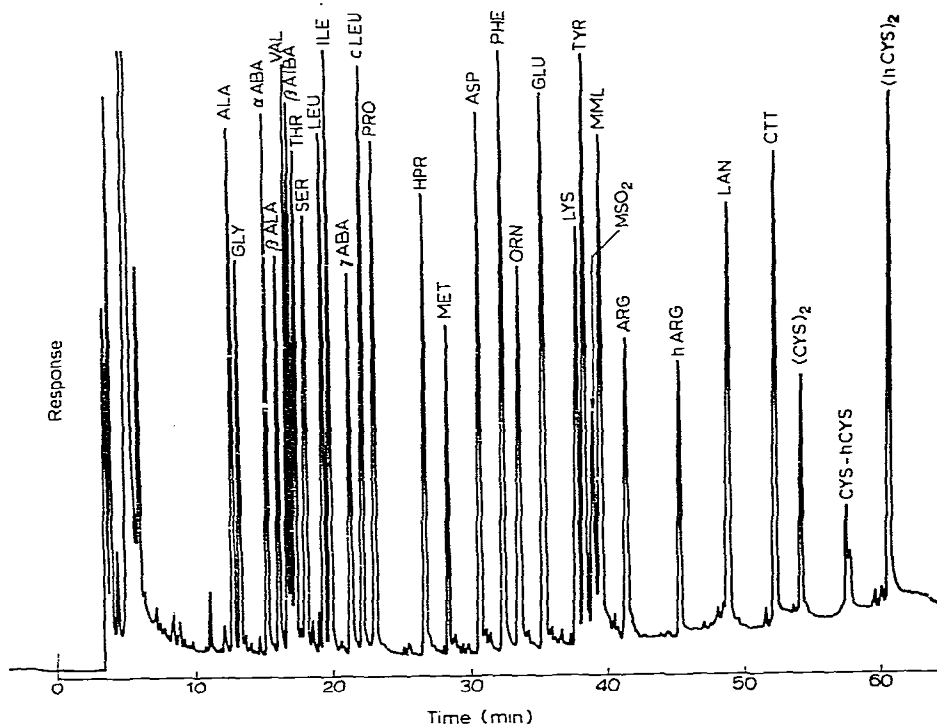


Fig. 1. Gas chromatogram of the isobutyl ester, N(O)-heptafluorobutyrate derivatives of twenty-nine amino acids on a 25 m × 0.23 mm I.D. OV-101-coated glass capillary column. The temperature programme was 3°/min from 90° to 275°. Each amino acid peak represents approximately 50 pmole.

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.

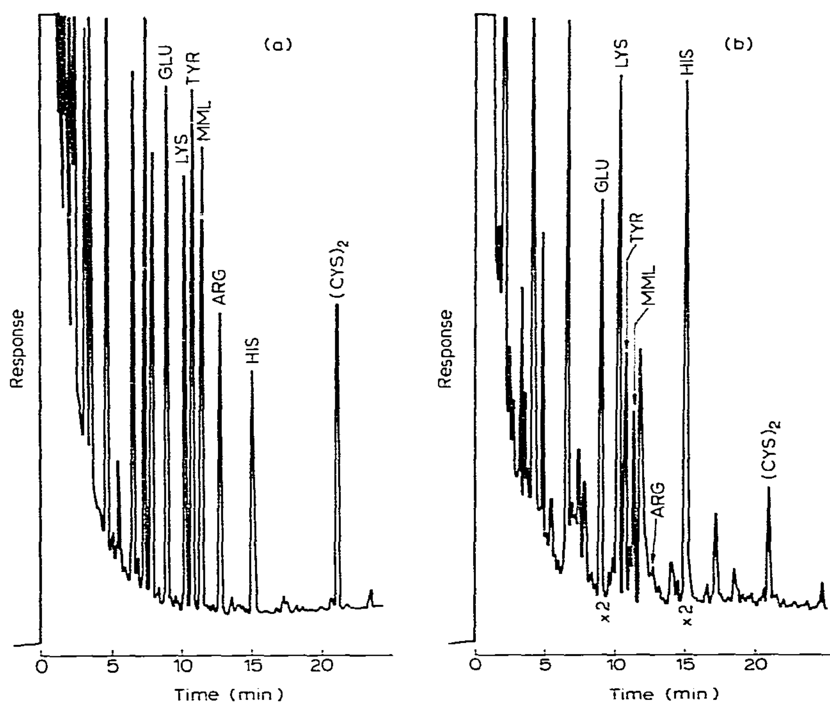


Fig.2. Gas chromatograms of the isobutyl ester, N^{α}-heptafluorobutyrate, N^{τ}-ethoxyformate derivative of histidine: (a) in standard mixture; (b) in urine of a normal patient. The temperature programme was 4°/min from 150° to 240°.

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).

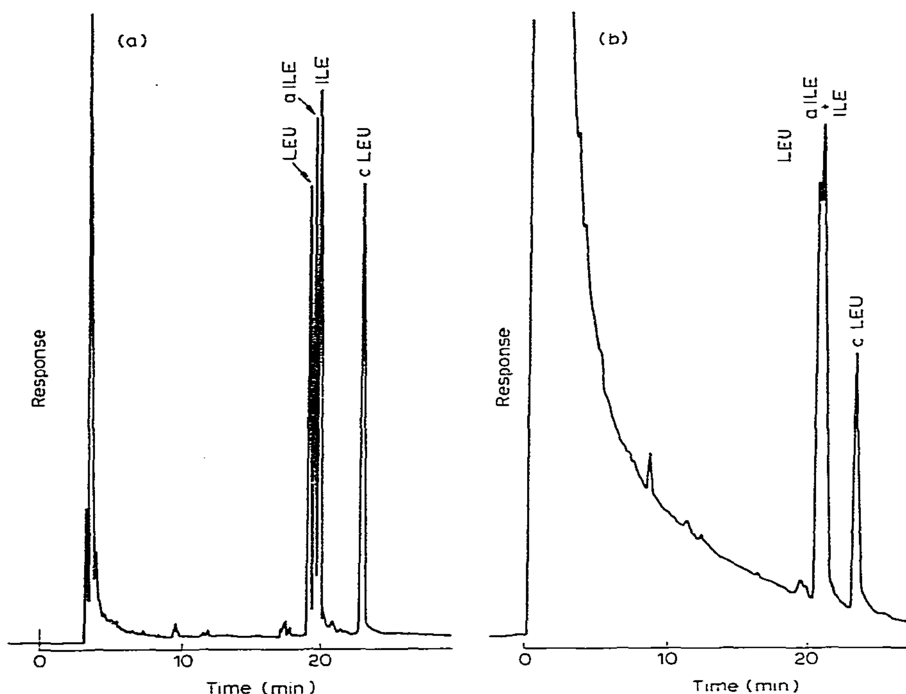


Fig. 3. Gas chromatograms of a mixture of leucine, isoleucine, *allo*-isoleucine and *cyclo*-leucine as IBU, N(O)-HFB derivatives: (a) on an OV-101-coated glass capillary column; (b) on an OV-1-packed column.

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 cLeu 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.

RMR to *c*Leu	Crude sample	Cleaned-up sample		
	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.

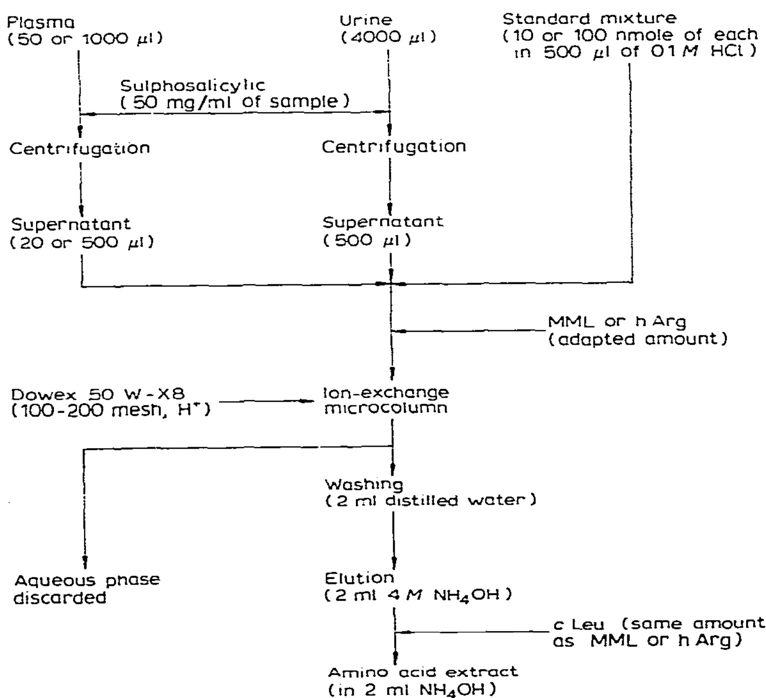
Application to physiological fluids in clinical biochemistry

Identification of chromatographic peaks from biological samples

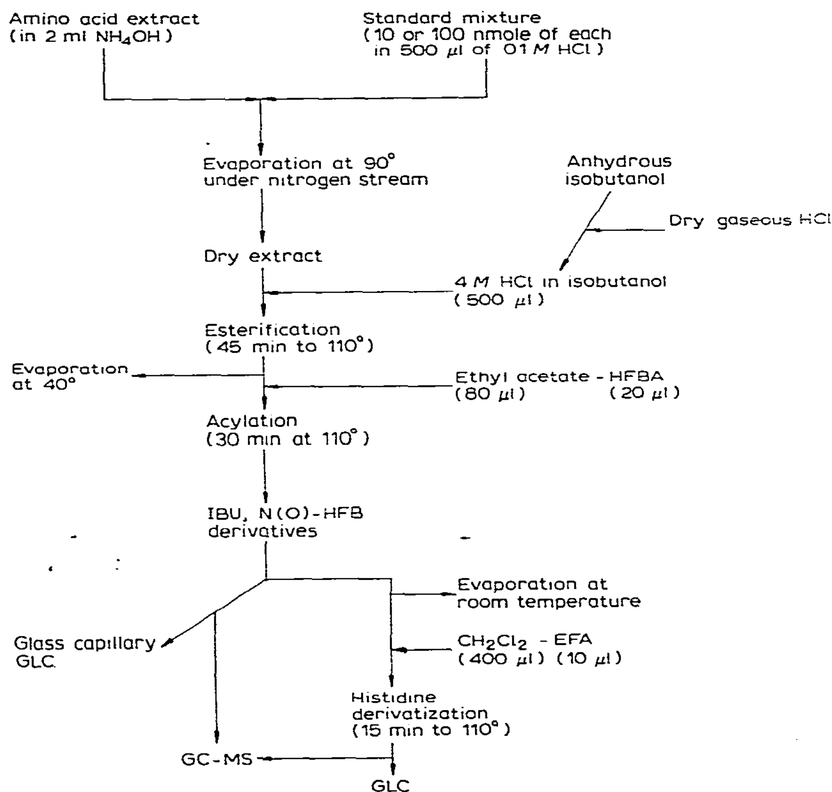
Chromatographic peaks of biological samples were identified by comparison of their retention times with those of a standard amino acid solution. Table I shows the data for two different analytical conditions. The nature of the presumed amino acid was checked by individual spiking off. Finally, exact 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

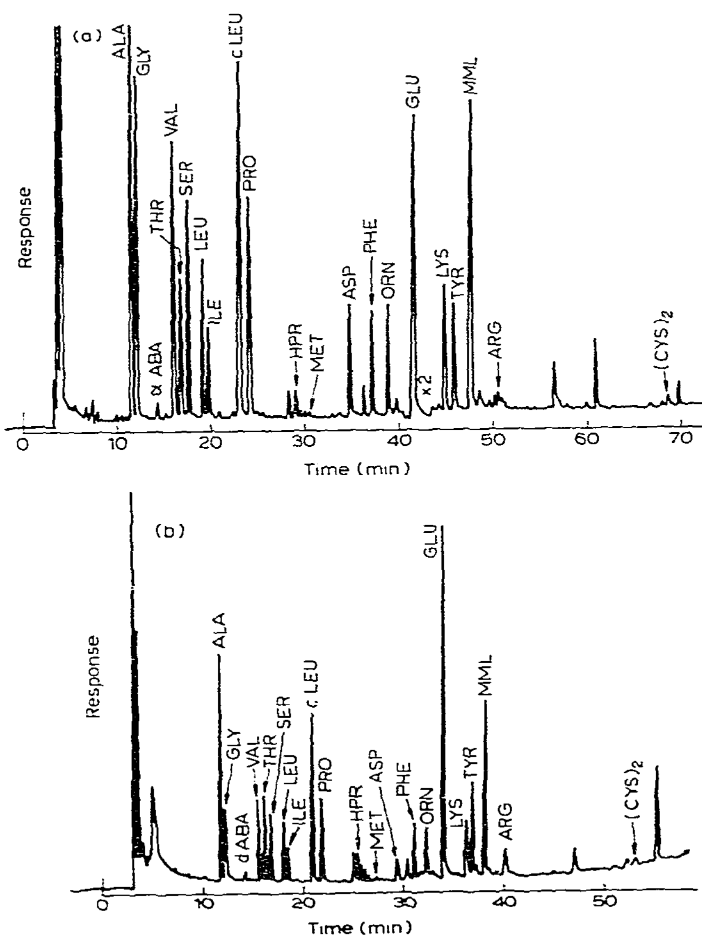


Fig. 4. Gas chromatograms on OV-101-coated glass capillary column of two normal plasma samples subjected to the complete procedure. The temperature programme was 2°/min (a) and 3°/min (b) from 90° to 270°.

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 = $[S—(CH_2)_2—CH(NH—CO—C_3F_7)(COOC_4H_9)]^+$		—	386(2)	386(2)	—	—
b = $[S—CH_2CH(NH—CO—C_3F_7)(COO—C_4H_9)]^+$		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)			—
or				298(82)	298(36)	
b – 74	C_4H_9OH	298(4)	—			—
b – (32 + 56)	$S + C_4H_8$	284(41)	—		284(14)	284(36)
or				284(39)		
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)		—	—
or				270(18)		
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)	$S + COOC_4H_9 + H$	—				
or			252(35)	252(26)	252(29)	—
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)

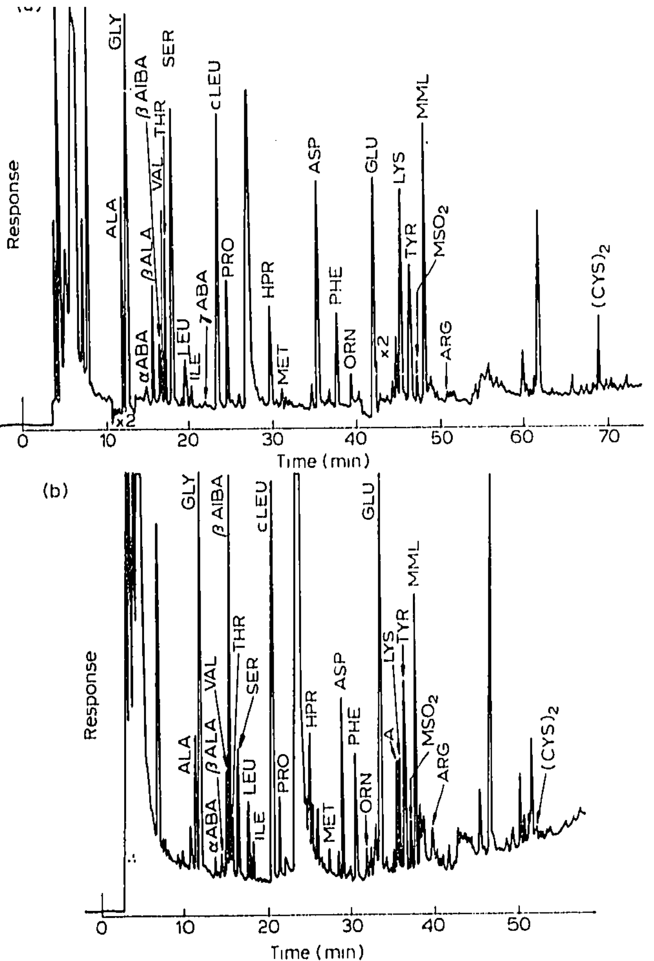


Fig. 5. Gas chromatograms on OV-101-coated glass capillary column of two normal urine samples subjected to the complete procedure. The temperature programme was 2°/min (a) and 3°/min (b) from 90° to 270°.

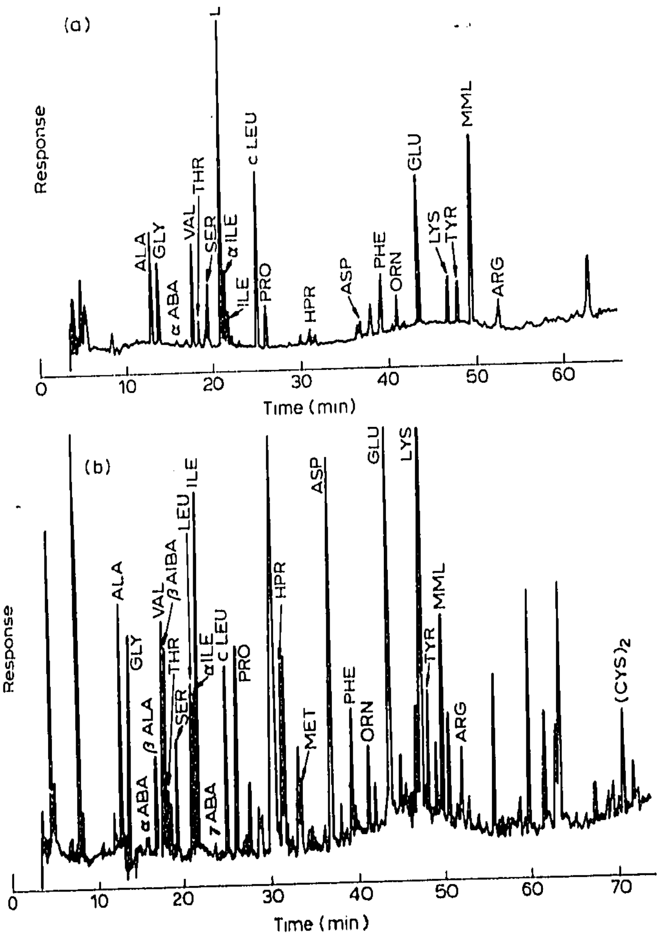


Fig. 6. Gas chromatograms on OV-101-coated glass capillary column of two samples subjected to the complete procedure and obtained from plasma (a) and urine (b) of a newborn infant with maple syrup disease. The temperature programme was 2°/min from 90° to 270°.

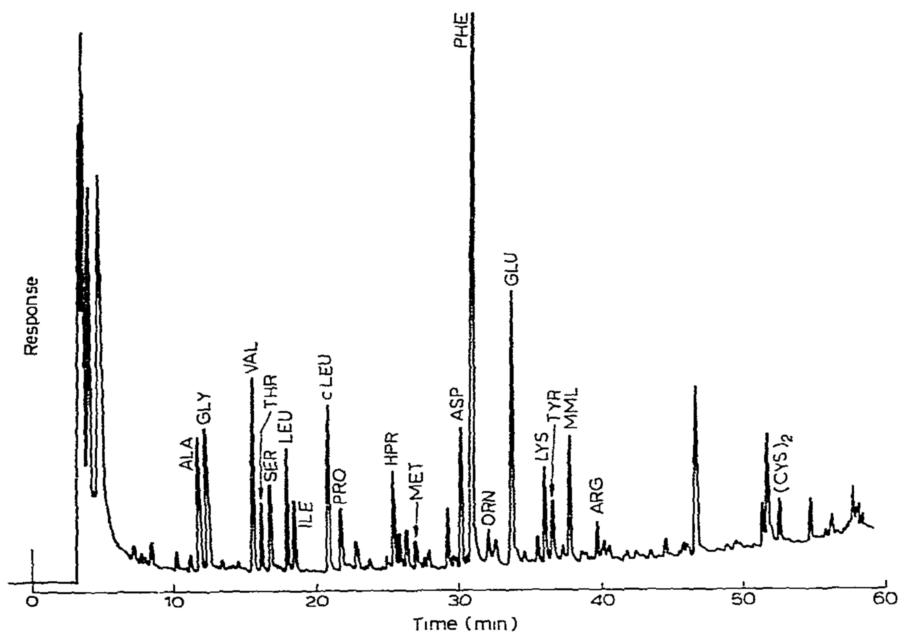


Fig. 7. Gas chromatogram on OV-101-coated glass capillary column of a sample obtained from plasma of a phenylketonuria. The temperature programme was 3°/min from 90° to 270°.

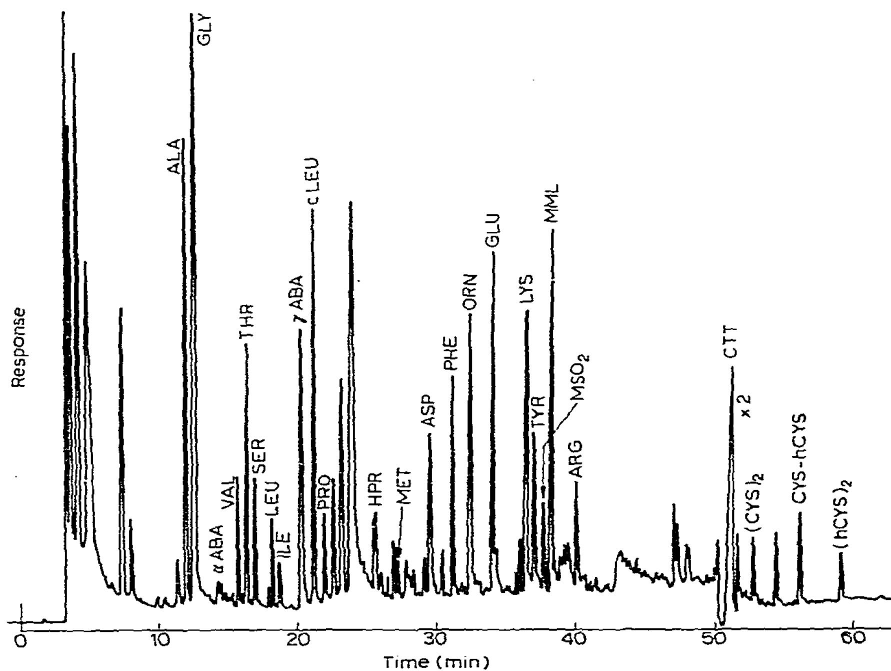


Fig. 8. Gas chromatogram on OV-101-coated glass capillary column of urine amino acids from a patient with hypercystathioninuria. Homocystine is also identified in the sample. The temperature programme was 3°/min from 90° to 270°.

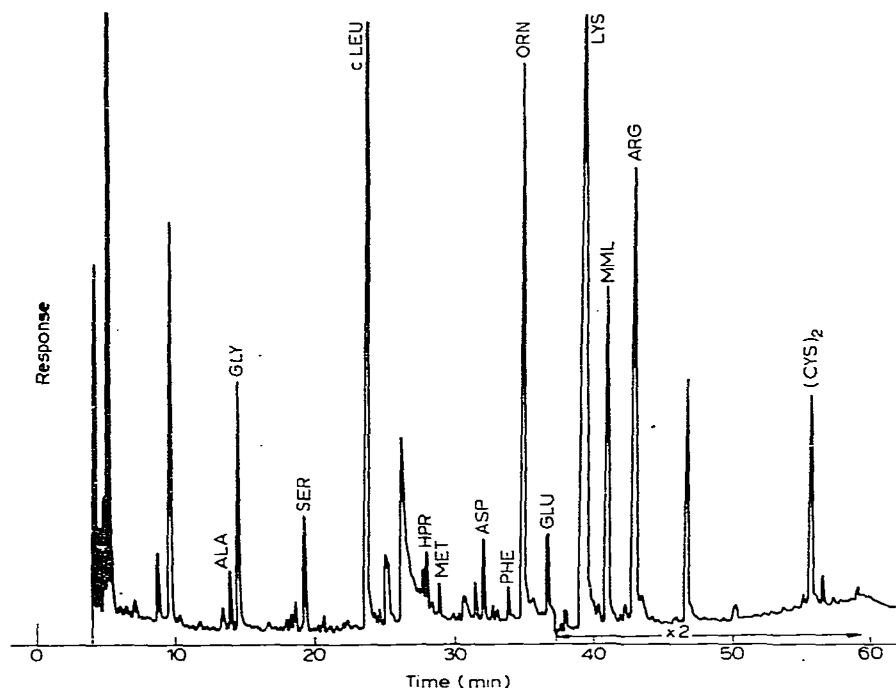


Fig. 9. Gas chromatogram on OV-101-coated glass capillary column of urine amino acids from a patient suffering from ornithine, lysine, arginie, cystinuria. The temperature programme was 3°/min from 90° to 270°.

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 perfcrmed 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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