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GAS CHROMATOGRAPHY OF AMINO ACIDS IN URINE AND HAEMOFILTRATE

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SUMMARY

Capillary gas chromatography of amino acid derivatives obtained from biological material shows a large number of previously unknown constituents. The profiles of amino acids obtained from urine of healthy individuals and haemofiltrate of uraemic patients indicate that haemofiltration removes some amino acids to a considerably higher extent from uraemic patients than the kidney does from healthy persons. For instance, if haemofiltration is required three times a week, an approximately ten-fold amount of the essential amino acid methionine and a forty-fold amount of the essential amino acid leucine is lost compared to their excretion in urine by a healthy individual over the same period.

INTRODUCTION

Haemofiltration is employed to remove toxic compounds in renal failure. The efficiency of this procedure may be estimated by comparison of profiles obtained from urine of healthy individuals and haemofiltrate of uraemic patients. The isolation of the amino acid fraction from biological liquids is achieved by ion-exchange chromatography [1—4]. For further separation, the amino acids are usually converted into the corresponding phenylthiohydantoins

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[5] or dansyl derivatives [6—8]. These derivatives show a characteristic UV absorption [9, 10], allowing their analysis by liquid chromatography even if large amounts of other compounds are still present. Unfortunately, however, those amino acids that do not react with phenylisothiocyanate or 1-dimethylaminonaphthalene-5-sulphonylchloride escape detection, e.g. those compounds with a substituent at the amino group. Gas chromatography (GC), combined with mass spectrometry, is a powerful method for the analysis of mixtures of organic compounds, enabling not only quantitation of known compounds but also structure elucidation of compounds from complex mixtures. This technique requires derivatization of amino and carboxyl groups to obtain sufficient volatility.

Based on the earlier work of Zomzely et al. [11], Gehrke and co-workers [12, 13] developed a method in which the amino acids are converted to the corresponding trifluoroacetylated butyl esters (TAB derivatives). Later, the preparation of a large number of other esters and acyl derivatives was described. Nevertheless, TAB derivatives and also N-heptafluorobutyryl-*n*-propyl esters of amino acids were used only occasionally to analyse amino acids obtained from biological liquids [14, 15]. The application of this method, and especially the use of ion-exchange chromatography, was recently critically reviewed by Labadarios et al. [16]. Previously, separation was done with packed columns. The resolution of packed columns is poor, especially if mixtures of compounds of natural origin are analysed. Often, two and more compounds are hidden under one peak. The application of glass capillary columns, offering a much higher separation power compared to packed columns, was introduced by Desgres et al. [17]. He used successfully isobutyl esters of N(O)-heptafluorobutyrate derivatives to analyse amino acids in blood and urine samples. We report here on the separation of butyl esters of (N(O)-heptafluorobutyrates (HBB derivatives) of amino acids occurring in urine and haemofiltrate using capillary columns.

EXPERIMENTAL

Amino acids

The twenty proteinogenic amino acids, 1-methylhistidine, 3-methylhistidine, 3-hydroxyproline and N-α-methyllysine were purchased from Sigma (Munich, F.R.G.). The ion-exchange resin Amberlite IR-120 (20—80 mesh) was bought from Serva (Heidelberg, F.R.G.). Cycloleucine, norleucine and *trans*-4-(aminomethylcyclohexane)carboxylic acid (Aca) were bought from EGA (Steinheim, F.R.G.). Standard solutions of amino acids (10 mmol/l) were prepared by dissolving the amino acids (purchased from Sigma) in 0.1 *M* hydrochloric acid and several drops of ethylmercaptan.

Reagents and solvents

Methanol, *n*-butanol, ammonia solution (25%), mercaptoethane, heptafluorobutyric anhydride (HFBA), chloroethylformate, acetylchloride and hydrochloric acid (32%) were of analytical-reagent grade from Merck (Darmstadt, F.R.G.). Methylene chloride was distilled and chromatographed on Alumina Woelm® B, activity I (25 g per 100 ml). Trifluoroacetic anhydride (TFA) and trifluoroacetic acid (TFAA) were purchased from EGA.

Gas chromatography—mass spectrometry

For GC the equipment was a Carlo Erba 4160 analytical gas chromatograph equipped with a flame ionization detector, either a home-made WCOT capillary column (I), 30 m × 0.33 mm, coated with OV-101 according to the static method [18] or a fused-silica WCOT capillary column (II), 50 m × 0.22 mm, with chemical bonded phase OV-1701 (permanent desactivation, coating efficiency 80%) purchased from Chrompack (Middelburg, The Netherlands). The carrier gas was hydrogen at a flow-rate of 2 ml/min. Peak areas were determined with the Kontron PSI computer system and the system software Kontron INTEGR (Kontron, Eching, F.R.G.).

Chromatographic parameters

Injector temperature 260°C; detector temperature 280°C; temperature programming 3°C/min starting from 80 to 280°C (column I) or from 130 to 260°C (column II). Mass spectral data were calculated with a Varian MAT 312 mass spectrometer equipped with the data system MAT SS 200 (PDP 11/34 computer). The ionization energy was 70 eV and the total-ion current was registered at 20 eV. The mass spectrometer was connected to a Varian 3700 gas chromatograph equipped with the capillary columns mentioned above.

Sample preparation

Clean-up of urine and haemofiltrate for amino acid analysis [2, 12, 13]. A 5-ml volume of urine or 15 ml of uraemic haemofiltrate were brought to pH 1.8—2.0 by addition of 50% hydrochloric acid. The internal standard, 10 μmol of *trans*-4-(aminomethylcyclohexanecarboxylic acid (norleucine or cycloleucine) dissolved in 100 μl of water—methanol (1:1), was added. The acidified biological liquid was brought onto an ion-exchange column (5 × 1 cm I.D.) of Amberlite IR-120, 20—80 mesh in the hydrogen form. The column was rinsed with 200 ml of water. Then amino acids and peptides were eluted with 100 ml of 1 *M* aqueous ammonia. The solution was concentrated under reduced pressure in a round-bottomed flask to a volume of ca. 2 ml on the rotary evaporator. The concentrate was transferred to a Reacti vial (Pierce, Rockford, IL, U.S.A.) with a Pasteur pipette. The rotary evaporator flask was rinsed with 1 ml of water and the washings were added to the concentrate in the Reacti vial. Finally, the water was removed by freeze-drying.

Conversion of amino acids into the heptafluorobutyric-n-butyl esters (HBB) and trifluoroacetyl-n-butyl esters (TAB). The freeze-dried sample was dissolved in 1 ml of 2 *M* methanolic hydrochloric acid (prepared by mixing equal amounts of methanol and acetyl chloride) [19]. The tightly closed Reacti vial was heated for 20 min to 120°C in a Reacti-Therm® module from Pierce. After cooling to room temperature, the excess methanolic hydrochloric acid was evaporated to dryness under a stream of nitrogen at 60°C. The residue was transesterified with 3 *M* butanolic hydrochloric acid prepared in the same way as methanolic hydrochloric acid, and heated to 120°C for 40 min. The excess reagents were evaporated under a stream of nitrogen, a few drops of methylene chloride were added and the solvent was removed under nitrogen. A 50-μl volume of dichloromethane and 150 μl of HFBA (or TFA) were added. After

ultrasonication for a few minutes, the closed vial was placed in the Reacti-Therm module at 150°C for 12 min (if acylated with HFBA) or for 5 min (if acylated with TFAA). After cooling to room temperature, the mixture was evaporated under a stream of nitrogen, 150 μl of methylene chloride were added and the solvent was removed under nitrogen to remove traces of water. Finally, the residue was dissolved in 20 μl of dichloromethane and 0.5 μl of this solution was analysed by GC.

TABLE I

RELATIVE MOLAR RESPONSE OF THE PROTEIN AMINO ACIDS (HBB DERIVATIVES)

Amino acid	RMR* (mean ± S.D.)
Alanine	0.39 ± 0.05
Valine	0.53 ± 0.07
Glycine	0.37 ± 0.05
Threonine	0.50 ± 0.07
Serine	0.54 ± 0.07
Leucine	0.67 ± 0.07
Isoleucine	0.20 ± 0.03
Proline	0.60 ± 0.07
Cysteine	0.36 ± 0.06
Hydroxyproline	0.72 ± 0.09
Methionine	0.60 ± 0.07
Aspartic acid**	0.76 ± 0.08
Phenylalanine	0.98 ± 0.09
Glutamic acid**	0.88 ± 0.07
Tyrosine	0.90 ± 0.05
Lysine	0.87 ± 0.06
Arginine	0.60 ± 0.14
Tryptophan	0.55 ± 0.10
Histidine (EOC derivative)	0.76 ± 0.08

*Mean of ten analyses. Relative to the internal standard, Aca.
**Includes asparagine and glutamine.

Quantitation

For quantitation, an equimolar amount of the internal standard and amino acid standard mixture were used to determine the relative molar response (RMR) of each amino acid with respect to the internal standard (Table I). The RMR was used to determine the amino acid concentration in urine and in haemofiltrate samples (Tables II and III):

$$\text{RMR} = \frac{\text{peak area for AA}}{\text{peak area for I.S.}}$$

where AA denotes amino acid and I.S. denotes internal standard.

The amount of each amino acid was determined by:

$$\text{Concentration} = \frac{\text{peak area for AA}}{\text{RMR} \times \text{peak area for I.S.}} \times \text{concentration I.S.}$$

TABLE II

AMOUNTS OF AMINO ACIDS IN URINE (mg per 24 h)

Amino acid	GC analysis according to Adams [20]	GC analysis (HBB derivatives) (amount urine 1550 ml per 24 h)		Ion-exchange chromatography according to Stein and Moore [21]
		mg per 24 h	mmol per 24 h	
Alanine	14— 65	62	0.69	20— 70
Glycine	54—160	190	2.53	70—200
Valine	2— 8	8	0.07	—
Isoleucine	12— 35	20	0.15	10— 30
Leucine	20— 72	11	0.08	10— 25
Proline	1— 7	4	0.03	—
Threonine	18— 55	45	0.38	15— 50
Serine	30— 70	23	0.22	25— 75
Aspartic acid*	14— 52	30	0.91	—
Methionine	2— 6	2	0.01	—
Cysteine	6— 15	11	0.09	10— 20
Hydroxyproline	2— 12	14	0.11	—
Glutamic acid**	20— 90	82	0.56	—
Histidine	80—225	223	1.44	110—320
Lysine	8— 36	30	0.21	10— 50
Arginine	1— 6	6	0.03	—
Tryptophan	0— 4	0	0.00	—
Tyrosine	10— 42	14	0.08	15— 50

*Includes asparagine.
**Includes glutamine.

TABLE III

AMOUNTS OF AMINO ACIDS IN HAEMOFILTRATE (HBB DERIVATIVES)

Amino acid	mg per 20 l and 48 h	mmol per 10 l and 24 h	Haemofiltrate/urine ratio
Alanine	1160	6.51	9.43
Glycine	749	4.99	1.97
Valine	586	2.50	35.71
Threonine	195	0.82	2.16
Serine	212	1.00	4.54
Leucine	342	1.30	16.25
Isoleucine	94	0.36	2.40
Proline	850	3.69	125.00
Cysteine	217	0.89	9.89
Hydroxyproline	37	0.14	1.27
Methionine	49	0.16	16.00
Aspartic acid*	132	0.50	0.55
Phenylalanine	367	0.90	—
Glutamic acid**	1100	3.74	6.68
Tyrosine	117	0.32	4.00
Lysine	399	2.73	13.00
Arginine	312	0.90	30.00
Tryptophan	18	0.04	—
Histidine	163	0.52	0.36

*Includes asparagine.
**Includes glutamine.

The recovery of amino acids after ion exchange was determined as follows: (1) An aliquot of an amino acid standard solution (10 μmol/ml) was brought onto the ion-exchange column and eluted with 1 *M* ammonia solution (100 ml). (2) After addition of internal standard (10 μmol/ml) to the eluate, the sample was lyophilized and derivatized as mentioned above. (3) The sample was dissolved in methylene chloride and 0.5 μl of this solution was chromatographed. (4) The peak areas were determined and correlated with peak areas obtained after derivatization without the ion-exchange step.

The recovery for protein amino acids was 97—99%.

RESULTS

Reference mixtures of some synthetic α-amino acids were analysed according to the method of Kaiser et al. [2] in the form of their trifluoroacetylated and heptafluorobutyrylated butyrates: it proved necessary to remove any traces of the acylating agent before GC separation, since even traces of the reagent destroyed the phase OV-101 of the capillary column. Removal of the acylating reagent and the acid formed thereof is even more difficult if synthetic mixtures are replaced by complex biological samples; therefore evaporation must be repeated several times after the addition of solvent. If the evaporation was supported by a strong nitrogen stream, considerable losses of amino acids were observed, especially for the lower homologues and hydroxy-containing amino acids. Only a gentle stream of nitrogen should therefore be applied for this purpose. Loss of histidine also is observed, mainly due to the low solubility of the reaction product in butanolic hydrochloric acid [22]. The amino acids were, therefore, first converted into methylates and these were then transesterified into butyrates. Furthermore, analysis of histidine on packed columns requires the on-column conversion of the monoacyl into the diacyl derivative by co-injection of acetic anhydride [23]. Although this is possible if packed columns are used, capillary columns coated with OV-101 lost their selectivity very quickly. Since the monoacylated derivative is quite polar it shows a broad peak, excluding quantitation; therefore we converted the monoacylated histidine butyrate into the ethyloxycarbonyl derivative (EOC) by treatment with chloroethyl formate according to the method of Moodie [24] (Fig. 1). Consequently, if a mixture containing histidine is analysed with capillary columns, one run is required to analyse the EOC histidine derivative and another to determine the other amino acids.

If the butyrate of tryptophan was treated with TFAA, even under mild conditions we observed four peaks in the glass capillary chromatograms. The main peak (retention index 2089) corresponded to the expected di-TFA-*n*-butyl

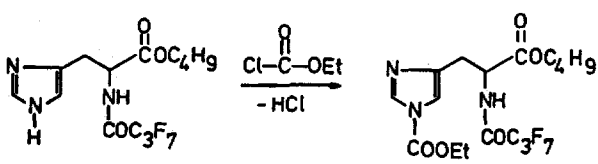


Fig. 1. Conversion of the monoacylated histidine butyrate into the ethyloxycarbonyl derivative (EOC) by treatment with chloroethyl formate.

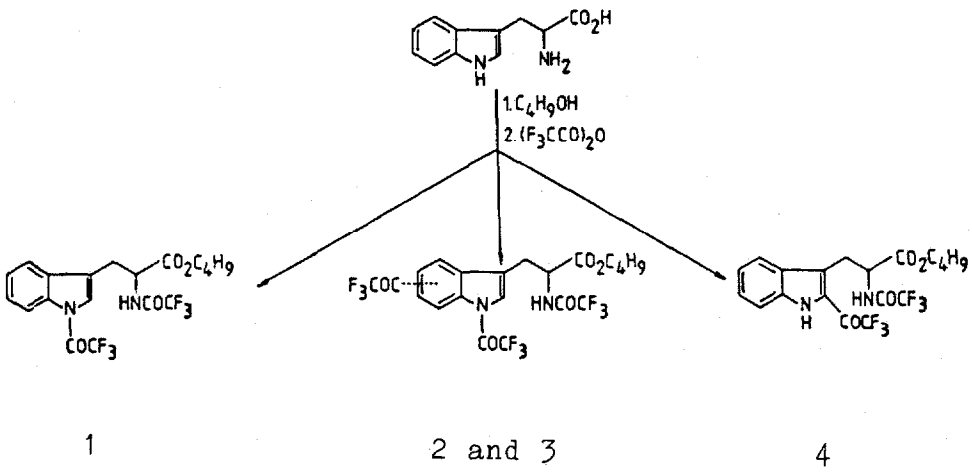
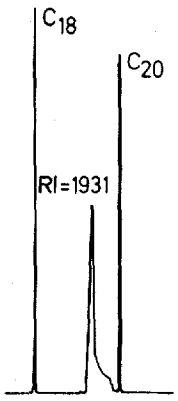


Fig. 2. Derivatization of tryptophan with *n*-butanol—hydrochloric acid and trifluoroacetic acid anhydride.

ester of tryptophan (1 in Fig. 2). Two additional small peaks (retention indices 1981 and 2083) (2 and 3 in Fig. 2), according to their mass spectra, corresponded to products containing an additional $COCF_3$ function in the benzene ring, while the fourth peak (retention index 2191) obviously was due to a di-TFA-*n*-butyl ester of tryptophan (4 in Fig. 2) (an isomer of the main peak). We assume that these peaks would escape detection if packed columns were used. If HFBA was used as the acylating reagent, only one peak corresponding to the diheptafluorobutyric-*n*-butyl ester of tryptophan was produced.



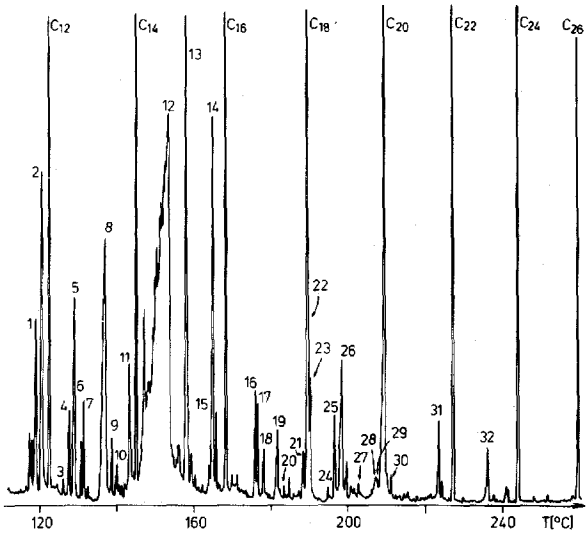


Fig. 3. Peak of arginine TAB (retention index 1931) on a capillary column coated with OV-101.

Fig. 4. Urine profile of the TAB amino acid derivatives (OV-101) of a 29-year-old healthy male individual (24 h urine). For peak identification, see Table IV.

Oxidation of cysteine to cystine caused losses of the former. To avoid these losses, mercaptoethane was added to the urine samples [25]. Furthermore, we assume that this reducing substance prevents oxidation of tryptophan.

Arginine analysed in the form of its TAB derivative on a capillary column coated with OV-101 shows a broad peak (Fig. 3). We assume that this behaviour is a result of partial decomposition of arginine TAB on the capillary column.

To avoid these problems, we checked the possibility of separating HBB derivatives on a fused-silica capillary column with chemical bonded phase OV-1701. These derivatives are less volatile than the isobutyl esters described by Desgres et al. [17]; therefore losses during work-up procedure due to too high a volatility are reduced. HFBA (an acylating reagent of similar strength to TFAA) produces derivatives that are more stable to hydrolysis [26] and show

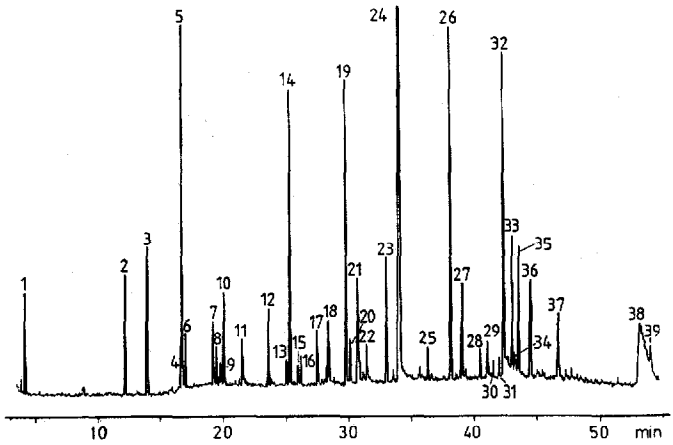


Fig. 5. Urine profile of the HBB derivatives (OV-1701) of a 26-year-old healthy woman (24-h urine). For peak identification, see Table V.

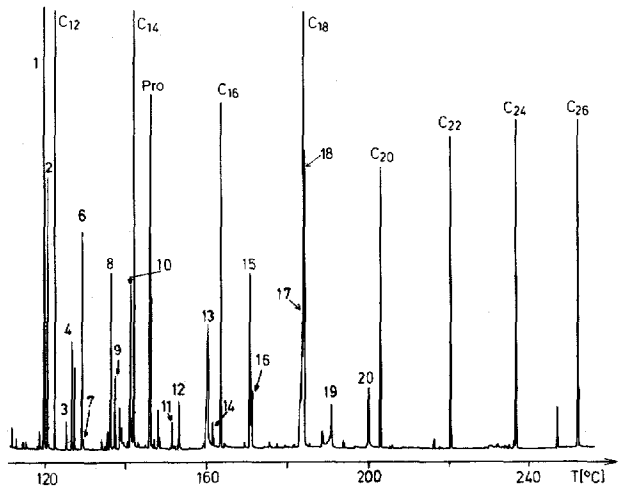


Fig. 6. Amino acid profile (TAB derivatives, OV-101) obtained from haemofiltrate of a 52-year-old uraemic patient. For peak identification, see Table VI.

significantly lower retention times than TAB derivatives, enabling us to reduce the time for a GC analysis.

Typical urinary amino acid profiles of healthy male adults are reproduced in Figs. 4 and 5. The corresponding compounds and retention indices or retention times are listed in Tables IV and V.

The GC separation of amino acids in the form of their HBB derivatives on column II (fused-silica WCOT capillary column, OV-1701) compared to separation on column I (WCOT capillary column, OV-101) offers the advantage of reduced losses of cysteine, arginine, histidine, 1-methylhistidine and 3-methylhistidine and improves the separation of glutamic acid from lysine and tyrosine. The determination of proline is not possible on column I if TAB derivatives are

TABLE IV

RETENTION INDICES OF TAB AMINO ACID DERIVATIVES IN URINE

Peak No.*	Compound	Retention index (OV 101)
1	Alanine	1159
2	Glycine	1173
3	*α-Aminoisobutyric acid*	1235
4	Threonine	1246
5	Serine	1257
6	Valine	1280
7	β-Aminoisobutyric acid	1282
8	N-Methylproline	1328
9	Leucine	1349
10	Isoleucine	1358
11	Nicotinic acid	1384
12	Creatinine	1440
13	Unknown	1512
14	Unknown	1570
15	Unknown	1580
16	Aspartic acid**	1676
17	Phenylalanine	1678
18	Ornithine	1690
19	Unknown	1724
20	Unknown	1742
21	N(O)-TFA-tyrosine	1769
22	Glutamic acid***	1807
23	Lysine	1811
24	N-α-Methyllysine	1851
25	2-Aminoadipic acid	1868
26	1-Methylhistidine plus 3-methylhistidine	1888
27	Glycylproline	1931
28	N-TFA-tyrosine	1976
29	Histidine (monoacyl)	1983
30	N-α-Acetyllysine	2019
31	Prolylhydroxyproline	2058
32	Unknown	2308

*See Fig. 4.
**Includes asparagine.
***Includes glutamine.

TABLE V

RETENTION TIMES OF HBB AMINO ACID DERIVATIVES IN URINE AND HAEMOFILTRATE

Peak No.*	Compound	Retention time (min)	Peak	Compound	Retention time (min)
1	Unknown	4.26	22	Methionine	31.13
2	Unknown	12.18	23	Aspartic acid**	32.99
3	Alanine	14.01	24	Creatinine +	
4	Valine	16.57		phenylalanine	34.00
5	Glycine	16.76	25	Unknown	35.63
6	Threonine	17.06	26	Glutamic acid***	38.10
7	Serine	19.27	27	Tyrosine	39.03
8	β-Aminoisobutyric		28	Unknown	40.43
	acid (AIBA)	19.50	29	Unknown	41.05
9	Leucine	19.85	30	Ornithine	41.51
10	Isoleucine	20.11	31	Unknown	41.79
11	Unknown	21.50	32	Unknown	42.32
12	Cycloleucine	23.56	33	Aca (I.S.)	43.03
13	Proline	24.99	34	Arginine	43.21
14	Unknown	25.29	35	Lysine	43.52
15	Cysteine	25.95	36	1-Methylhistidine	44.49
16	Unkown	26.15	37	3-Methylhistidine	46.66
17	Unknown	27.48	38	Histidine	53.20
18	Hydroxyproline	28.19	39	Unknown	53.93
19	Unknown	29.76			
20	Unknown	30.11			
21	Unknown	30.69			

*See Figs. 5 and 7.
**Includes asparagine.
***Includes glutamine.

used, since the peak for this amino acid is hidden under the overloading peak for creatinine as shown in Fig. 4. Otherwise, if HBB derivatives are separated on column II, the peaks of creatinine and phenylalanine HBB are not resolved.

Profiles of TAB and HBB amino acids obtained from uraemic haemofiltrate are shown in Figs. 6 and 7. Data are listed in Tables V and VI. The profiles of urine and haemofiltrate are clearly distinguished. Proline is present in urine in only small amounts (excretion 1—7 mg/day) [20] but is a major compound in uraemic haemofiltrate. The amount of proline removed from the body in one haemofiltration treatment was calculated to be of the order of ca. 850 mg per 20 l. Since some patients need a haemofiltration treatment every second day, the total loss of proline can be calculated to be at least 425 mg/day. Similarly, the essential amino acids methionine and leucine are present in haemofiltrate in concentrations exceeding their amount by several fold in urine of healthy adults (see histogram, Fig. 8). These results confirm the observation of Mann [27] who found that haemofiltrates of uraemic dogs contain a ten-fold amount of methionine, compared to plasma of healthy dogs. The removal of the essential amino acids must be considered in the future to improve the treatment.

In addition to urine of healthy individuals, profiles of amino acids from

uraemic haemofiltrate were characterized by a change in the ratios Ala/Gly and Val/β-aminoisobutyric acid (β-AIBA). The Ala/Gly ratio in urine is about 1:3 while the Gly/Ala ratio in haemofiltrate is about 2:3. The Val/β-AIBA ratio in urine is about 1:8 while the Val/β-AIBA ratio in haemofiltrate is 20:1—8:1. The relative proportion of the essential amino acid leucine in haemofiltrate

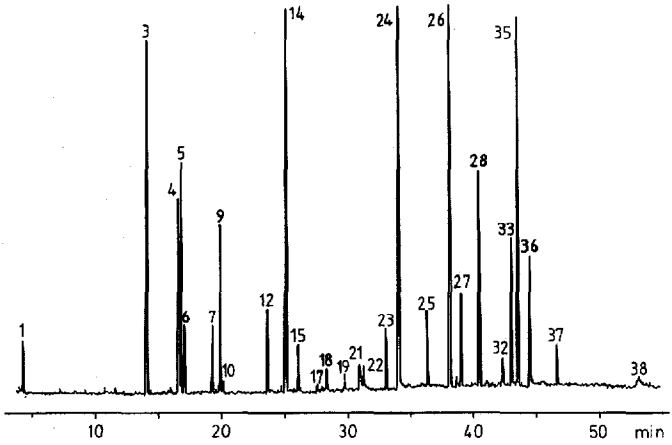


Fig. 7. Amino acid profile (HBB derivatives, OV-1701) from haemofiltrate of a 52-year-old uraemic patient. For peak identification, see Table V.

TABLE VI

RETENTION INDICES OF TAB DERIVATIVES IN HAEMOFILTRATE

Peak No.*	Compound	Retention index (OV 101)
1	Alanine	1159
2	Glycine	1173
3	α-Aminoisobutyric acid	1235
4	Threonine	1246
5	Serine	1257
6	Valine	1280
7	β-Aminoisobutyric acid	1282
8	Leucine	1349
9	Isoleucine	1358
10	Norleucine (I.S.)	1389
11	Hydroxyproline	1489
12	Pyroglutamic acid	1505
13	Methionine	1568
14	Phenylglycine	1584
15	Aspartic acid**	1676
16	Phenylalanine	1678
17	Glutamic acid***	1807
18	Lysine	1811
19	1-Methylhistidine plus 3-methylhistidine	1888
20	Histidine (monoacyl)	1983

*See Fig. 6.
**Includes asparagine.
***Includes glutamine.

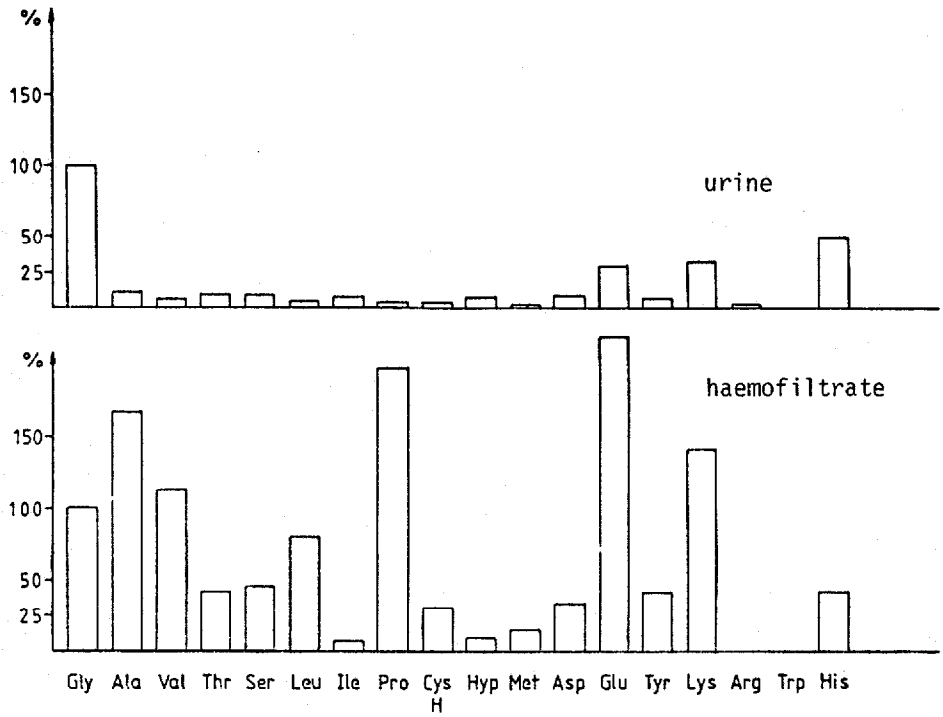


Fig. 8. Histogram of amino acids (HBB derivatives) obtained from urine and haemofiltrate (Gly = 100%).

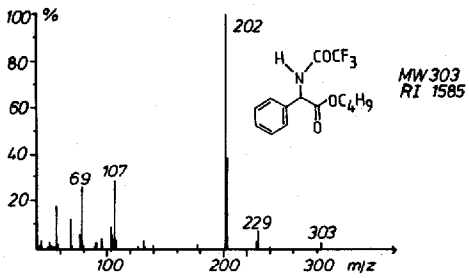


Fig. 9. Mass spectrum of the TAB derivative of phenylglycine.

(ca. 79% of the amount of glycine) is much higher than in urine of healthy individuals (ca. 6% of the amount of glycine). In one out of three samples of haemofiltrate, we found an additional amino acid not present in urine or other biological samples: phenylglycine. This compound shows an identical mass spectrum and the same retention index as the TAB derivative of synthetic phenylglycine (Fig. 9). Also, N-methylproline was not previously known to occur in urine. This compound was identified by comparison of its mass spectrum with the mass spectrum of a synthetic sample prepared by the method of Ingram [28].

DISCUSSION

Although GC methods for the determination of naturally occurring amino acids need careful sample preparation, they prove to be very useful for

detecting previously unknown compounds and for obtaining an overview of metabolic states. Since amino acids are involved in many metabolic diseases, the method may be used to attain a better insight into these diseases and to aid diagnosis, as shown in the case of uraemic patients.

The observation that large amounts of proline, lysine, leucine and methionine are lost in the course of haemofiltration was not previously realized. Although it is well known that the kidney function cannot be totally substituted by the filter, which does not have reabsorption qualities, the loss of essential compounds should be recognized and substitution therapy should be taken into account.

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