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DETERMINATION OF URINARY AMINO ACIDS BY MEANS OF GLASS CAPILLARY GAS—LIQUID CHROMATOGRAPHY WITH ALKALI-FLAME IONISATION DETECTION AND FLAME IONISATION DETECTION

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SUMMARY

The determination of amino acids as their N(O)-heptafluorobutyryl-isobutyl ester derivatives by glass capillary gas—liquid chromatography has been studied. Separations of amino acids obtained from insulin hydrolysate and human urine analysed with a flame ionisation detector, an alkali-flame ionisation detector and an electron-capture detector are shown. The quantitative results of urinary amino acids of one deep-sea diver are presented.

INTRODUCTION

The determination of amino acids is usually performed by well-established ion-exchange chromatography followed by post-column derivatisation and photometric detection [1]. The amino acid analyser is a single purpose instrument which is expensive both to purchase and to run and requires skilled manipulation for successful operation. The advantages of analytical speed are particularly relevant to the analysis of physiological samples which require several hours for analysis by ion-exchange chromatography.

Amino acids, being multifunctional, require derivatisation of reactive groups before they can be analysed by gas—liquid chromatography (GLC). A large number of derivatisation techniques and different derivatives have been employed for the successful quantitation of amino acids (see reviews, refs. 2—

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4). The most extensively studied amino acids derivatives include the N-acetyl *n*-amyl esters [5], the N-trifluoroacetyl (TFA) methyl esters [6—10], the N-TFA *n*-amyl esters [11], the N-TFA *n*-butyl esters [12—20], the N-heptafluorobutyryl (HFB) *n*-propyl esters [21—24], the N-HFB isobutyl esters [25—30] and the N-HFB isoamyl esters, [31, 32]. Separation of amino acids by GLC is usually done with packed columns, but more recently capillary columns have been used [33—35].

Gehrke et al. [36] studied the interaction of the sensitive amino acids arginine, histidine and cystine as their N-TFA *n*-butyl ester derivatives with solid support and two stationary phases. They showed that a dual-column method was essential for the quantitative determination of amino acids [37]. They also showed that non-polar columns were essential for the quantitative determination of these amino acids. The N(O)-HFB isobutyl esters of amino acids were preferred because these derivatives could be separated on the non-polar stationary phases SE-30, OV-101 and OV-1, and their separation was better than that shown with other derivatives on the same stationary phases [24, 32, 38].

The alkali-flame ionisation detector (AFID), sometimes known as the thermionic detector, has appeared in several forms since it was first described [39]. However, its employment is qualified by the wide variation in optimal running conditions [40—44]. With each detector, a knowledge of the operating parameters and of the limitations of the detector are essential to obtain maximum sensitivity and reliability. The Pye AFID differs from other designs in that it has a three-electrode system and employs rubidium chloride [45]. It is shown that this detector may be operated in various modes to improve the simplicity of operation for routine use, to obtain selective response for nitrogen-containing compounds, with minimum response to hydrocarbons [46].

Butler and Darbre [47] used the AFID for the analysis of fourteen N-TFA methyl esters of amino acids but no work was reported with basic amino acids. Adams et al. [33] used a wall-coated open tubular (WCOT) column coated with mixed stationary phase Carbowax 20M and Silar 5CP (1 : 1, w/w) together with an AFID to separate N-acetyl *n*-propyl amino acid esters obtained from human sera, urines and protein hydrolysates, but no data on relative molar response (RMR) values were given.

GLC with glass capillary columns, when used for determination of amino acids, offers higher speeds of analysis, better resolution and improved sensitivity of detection than with packed columns. Due to the complexity of physiological samples (especially urine samples), a single peak may be due to several compounds. It was relatively easy to find different stationary phases that could separate three of four metabolites [48] but it was difficult to find different stationary phases that could separate all the protein amino acids. Most publications indicate the use of the flame ionisation detector (FID). In this investigation other detectors were studied, in particular the AFID because of its specificity for nitrogen-containing compounds, and the electron-capture detector (ECD) because of its very high sensitivity. Three detectors, FID, AFID and ECD were studied in conjunction with support-coated open tubular (SCOT) columns and applied to some physiological and non-physio-

logical samples. Determination of amino acids with ECD has been reported separately [49]. This work was mainly carried out in order to study urine samples of deep-sea divers.

EXPERIMENTAL

Materials

A 2 m*M* standard solution of amino acids was prepared in 0.1 *M* HCl from pure compounds obtained from Sigma (St. Louis, MO, U.S.A.) and Aldrich (Milwaukee, WI, U.S.A.). Heptafluorobutyric anhydride (HFBA) was purchased from Fluka (Buchs, Switzerland). The ion-exchange resin, Dowex 50W-X8, 200—400 mesh, was from Sigma. Reacti-vials (1 ml) (Pierce, Rockford, IL, U.S.A.) were used for derivatisation. Other reagents were prepared according to the descriptions of MacKenzie and Tenaschuk [28, 29].

Gas chromatography

Gas chromatography was carried out with the following gas chromatographs: a Hewlett-Packard Model 7620A fitted with a 2-mCi ^{63}Ni ECD, a Pye Series 104 Model 24, fitted with an FID; a Pye Series 104 Model 24, fitted with an AFID. Integration of the peak areas was carried out with Hewlett-Packard Model 3370A and Vidar Autolab 6300 digital integrators. A glass capillary column (25 m × 0.4 mm I.D.) was coated with 5% Chromosorb R and 15% OV-101 by a single-step coating method [50]. Direct injection on to the column with sample volumes of up to 2.0 μl were made without an inlet heater [51]. Samples were injected at column temperature, 60°C, which was programmed at 30°C/min to 90°C then at 4°C/min to 250°C.

Ion-exchange clean-up procedure

A glass column (5 cm × 3 mm, I.D.), with a glass-wool plug at the bottom, was packed with Dowex 50W resin (H^+) in water up to a height of 1 cm. A urine sample (100—500 μl), to which a calculated amount of internal standards (norleucine and homoarginine) was added, brought to pH 1.0 with 6 *M* HCl, was layered on top of the resin. All the non-cationic impurities were washed through the resin with 2 ml of deionised water. The amino acids were eluted with 2 ml of 4 *M* ammonia at a flow-rate of one drop every 5—10 sec. Excess ammonia was evaporated to dryness at 90°C under a stream of nitrogen with care being taken to dry the sample at the bottom of the reacti-vial.

Insulin hydrolysis

Insulin was hydrolysed with 6 *M* HCl at 110°C for 24 h in a sealed evacuated tube.

Derivatisation of samples

The N(O)-HFB isobutyl ester derivatives of amino acids or purified samples were prepared as previously described [28, 29]. For histidine, ethyl acetate (25 μl) and acetic anhydride (25 μl) were added and the vial was heated at 150°C for 3 min. The vial was cooled to room temperature and the appropriate amount of ethyl acetate was added to give the required concentration of amino acid derivatives.

RESULTS

The variable conditions that affected the response of the AFID were previously reported [45—47, 52]. With N-HFB alanine isobutyl ester as a test standard studies were made on the flow-rates of gases. Detector response and probe distance increased with increasing flow-rate of nitrogen and no optimum response was obtained over the range 50—100 ml/min for nitrogen. Similarly, Greenhalgh and Wilson [52] showed an increase in response as the nitrogen flow-rate was increased from 30 to 50 ml/min. Butler and Darbre [47] obtained an optimum detector response with a nitrogen flow-rate of 65 ml/min, but in their study no mention was made about the probe distance. If the probe distance is kept constant and the flow-rate of nitrogen varied, an optimum response can be obtained. An optimum response was obtained between 34 and 39 ml/min hydrogen (above 39 ml/min a decreased response was observed). The detector response was increased with decreasing probe distance and increased flow-rate of air (300—650 ml/min). Thus, if the objective when setting up the detector is maximum sensitivity, it should be used with higher flow-rates of air and nitrogen and the variables probe distance and hydrogen flow-rate adjusted to give optimum detector response. With higher flow-rates of air, consumption of rubidium chloride was fast and noise

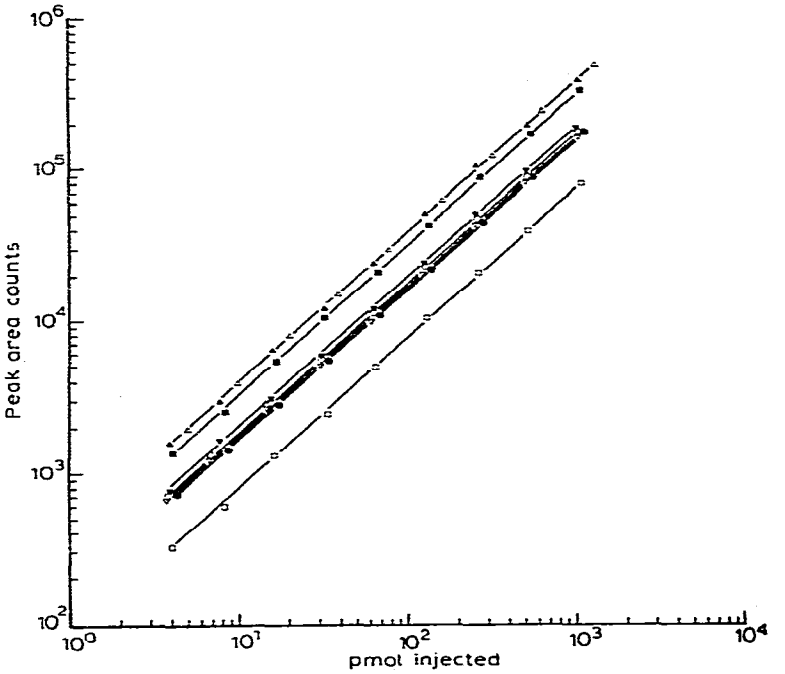


Fig. 1. Response—concentration curves for N(O)-heptafluorobutyryl isobutyl ester derivatives of amino acids with AFID and an OV-101 SCOT column. Preparation of sample: see Experimental. GLC conditions: Pye series 104 Model 24, AFID. 25 m × 0.4 mm I.D. glass column coated with 5% Chromosorb R and 15% OV-101. Carrier gas: hydrogen, 3 ml/min. Make-up gas: nitrogen, 80 ml/min. Hydrogen, 31 ml/min. Air, 350 ml/min. Temperatures: detector, 300°C; column, 90°C with programme 4°C/min. Attenuation, $2 \cdot 10^2$. Sample size, 1 μl. □ = Valine; ▽ = norleucine; ▼ = methionine; ● = tyrosine; ▲ = arginine, △ = histidine; ○ = tryptophan; ■ = cystine.

levels were high. In practice, 350 ml/min air, 35 ml/min hydrogen and 80 ml/min nitrogen were used. The discrimination of detector response between hydrocarbon, sulphur and phosphorus compounds was not studied.

Fig. 1. shows the response–concentration curves for seven N(O)-HFB isobutyl esters of amino acids determined on an OV-101 SCOT column. Twenty-nine amino acids were studied but the results of only seven are given because cystine, tryptophan, histidine, arginine, tyrosine and methionine were the only ones which either interact with the active sites present on the column or were difficult to derivatise. The linear dynamic range was approximately 300 for each compound under the conditions quoted and no molecular breakdown of the amino acid derivatives was observed within the experimental concentration range studied. The linear dynamic range for the other amino acids was identical to that for the amino acids quoted above.

Fig. 2 shows the separation of N(O)-HFB isobutyl ester derivatives of amino acids with FID, and Fig. 3 with AFID, where 29 amino acids were separated in approximately 35 min.

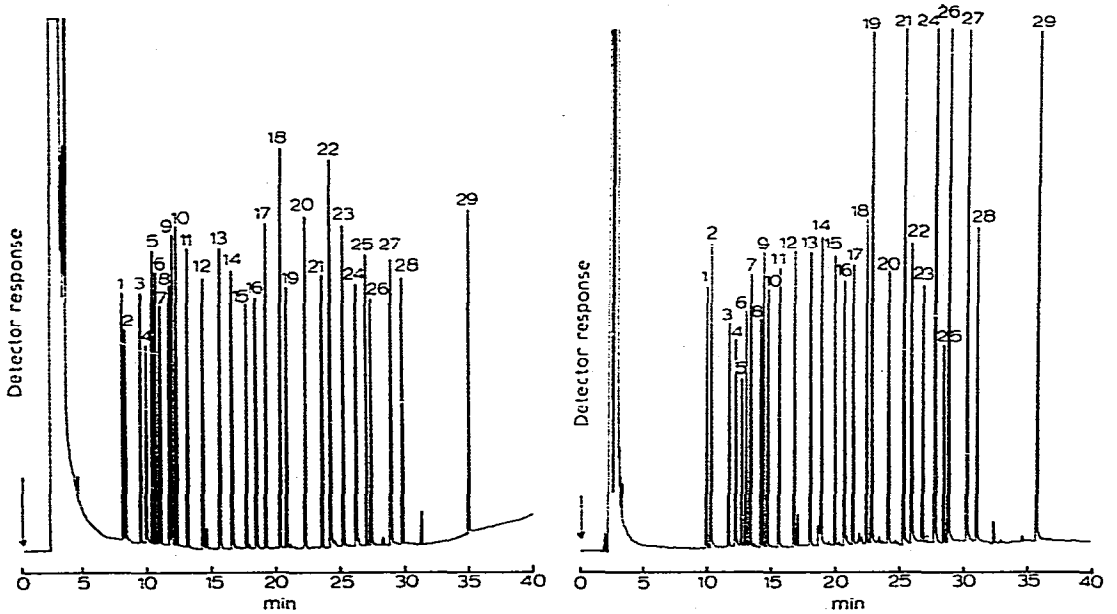


Fig. 2. Separation of N(O)-heptafluorobutyryl isobutyl ester derivatives of amino acids with FID. Preparation of sample: see Experimental. GLC conditions: Pye series 104 Model 24, FID. 25 m × 0.4 mm I.D. glass column coated with 5% Chromosorb R and 15% OV-101. Carrier gas: hydrogen, 3 ml/min. Make-up gas: nitrogen, 30 ml/min. Hydrogen, 27 ml/min. Air, 350 ml/min. Column temperature: 90°C with programme 4°C/min. Attenuation, $2 \cdot 10^2$. Sample size, 1 μl containing approximately 250 pmol of each amino acid. Peaks: 1 = alanine; 2 = glycine; 3 = 2-aminobutyric acid; 4 = β-alanine; 5 = valine; 6 = threonine; 7 = serine; 8 = allothreonine; 9 = leucine; 10 = isoleucine; 11 = norleucine; 12 = proline; 13 = pipecolinic acid; 14 = hydroxyproline; 15 = methionine; 16 = ϵ-aminocaproic acid; 17 = aspartic acid; 18 = phenylalanine; 19 = ornithine; 20 = glutamic acid; 21 = lysine; 22 = tyrosine; 23 = 3,4-dihydroxyphenylalanine; 24 = arginine; 25 = carboxymethylcysteine; 26 = histidine; 27 = homoarginine; 28 = tryptophan; 29 = cystine.

Fig. 3. Separation of N(O)-heptafluorobutyryl isobutyl ester derivatives of amino acids with AFID. Preparation of sample: see Experimental. GLC conditions: as in Fig. 1. Sample size, 1 μl containing approximately 50 pmol of each amino acid. Peaks as in Fig. 2.

TABLE I

LITERATURE RMR VALUES FOR N(O)-HEPTAFLUOROBUTYRYL ISOBUTYL ESTER DERIVATIVES OF AMINO ACIDS

Amino acid	MacKenzie and Tenaschuk [28] Packed column	Pearce [35] Packed column	Siezen and Mague [30] Packed column	Pearce [35] SCOT column	Desgres et al. [34]* WCOT column
Alanine	0.64	0.61	0.55	0.66	1.00
Glycine	0.59	0.45	0.50	0.63	0.83
Valine	0.90	0.87	0.83	0.88	1.08
Threonine	0.97	1.02	0.93	0.98	0.89
Serine	0.88	0.89	0.84	0.88	0.83
Leucine	1.00	1.09	1.00	1.05	0.90
Isoleucine	1.01	1.04	0.98	1.00	1.06
Norleucine	1.00	1.00	1.00	1.00	—
Proline	0.90	0.65	0.85	0.91	0.88
Pipecolinic acid	0.97	—	0.94	—	—
Methionine	0.89	0.79	0.86	0.77	0.56
Aspartic acid	1.19	1.11	1.08	1.11	0.91
Phenylalanine	1.36	1.32	1.28	1.33	1.10
Glutamic acid	1.23	1.09	1.10	1.19	0.96
Lysine	1.12	0.98	0.97	1.04	0.73
Tyrosine	1.40	1.38	1.28	1.21	0.96
Arginine	1.17	0.72	2.20	0.86	0.62
Histidine	0.94	—	0.60	0.55	0.47
Cystine	1.04	0.21	0.70	0.28	0.48

*RMR values determined against cycloleucine = 1.00.

Table I shows published relative molar response (RMR) values for HFB isobutyl esters of amino acids. They are listed in order of increasing retention time. There was little variation in most of the RMR values down to tyrosine, but arginine, histidine and cystine give variable results. For the packed column variation seems to be due to residual chemical activity of the support [53]. Capillary column activity is usually considered to be less than with packed columns but no improvement is shown by Pearce [35] and Desgres et al. [34]. Injection technique is important for quantitative work with capillary columns [54].

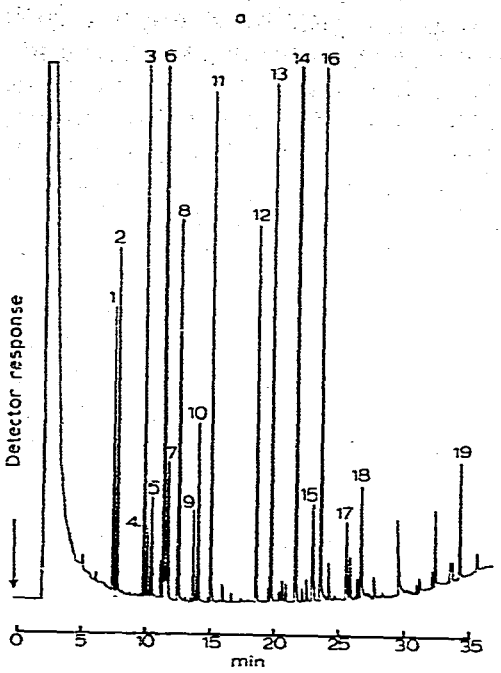
Table II shows the RMR values of N(O,S)-HFB isobutyl ester derivatives of amino acids with two detectors (FID and AFID) and with packed and SCOT columns. The packed column was prepared with Chromosorb W HP, 100—120 mesh, coated with 5% (w/w) OV-101 stationary phase. Using less than 5% stationary phase, the elution of histidine and cystine was not satisfactory. The RMR values obtained with this packed column are given in Table II, column 2. They agree reasonably well with published RMR values (MacKenzie and Tenaschuk [28], Table I), but there are discrepancies, with a difference of more than 10% being shown by serine, arginine and histidine.

Fig. 4a—c shows the amino acids obtained from insulin hydrolysate and analysed with FID, AFID and ECD, respectively. The chromatogram obtained with the AFID was cleaner than with the FID. The ECD chromatogram showed many extraneous peaks because of the high sensitivity of detection and due to derivatisation products from the breakdown of amino acids during acid hydrolysis with 6 *M* HCl. This breakdown of amino acids was not studied further.

Serum and tissue extracts are much "cleaner" to analyse by GLC than urine samples which are very complex. The worst samples from which to obtain good chromatograms were those prepared from urine and analysed with ECD, as shown in Fig. 5. It was difficult to remove excess HFBA in the final drying procedure of the sample. HFBA caused particular difficulty with ECD. Traces of HFBA did not interfere unduly with FID and AFID.

Fig. 6a and b shows the amino acid profiles obtained from a urine sample using FID and AFID, and Table III gives the analysis of one urine sample using both FID and AFID. From both the profiles and the quantitative results it is apparent that, although many of the amino acids gave values of the same order of magnitude with both FID and AFID, there were some discrepancies. Proline, hydroxyproline and 3,4-dihydroxyphenylalanine gave higher values with the AFID. Tyrosine with FID gave a single peak on the chromatogram, but with AFID there was an interfering peak.

Table IV gives the amino acid values for 16 urine samples from one deep-sea diver (dive 9B). The values were calculated as μg/mg creatinine, mg/day and nmol/ml so that full comparison could be made with published results. The results giving most meaningful information are given as μg/mg creatinine excreted (Table IV), where the coefficients of variation and ranges are less than when the results are given as mg/day or nmol/day. Some amino acids such as 2-aminobutyric acid, valine, leucine and phenylalanine, were excreted in relatively small amounts (mg/day). The largest range for all the amino acids was for valine (1.1—11.1 mg/day), showing a tenfold change in daily excre-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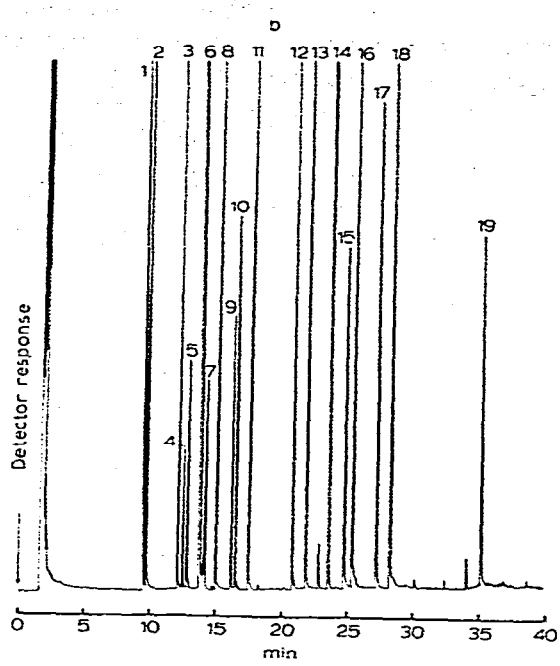
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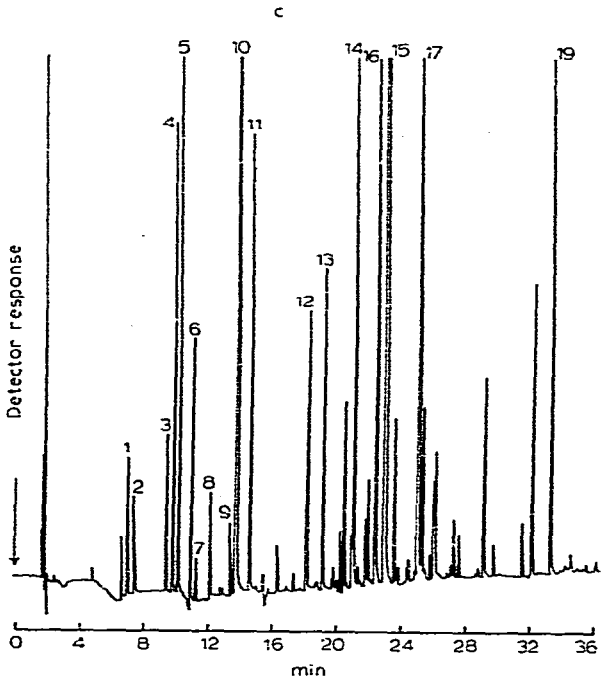
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TABLE II

RELATIVE MOLAR RESPONSE (RMR) VALUES OF N(O,S)-HEPTAFLUOROBUTYRYL ISOBUTYL ESTER DERIVATIVES OF AMINO ACIDS DETERMINED AGAINST NOR-LEUCINE (= 1)

Preparation of sample: see Experimental. GLC conditions for packed column: Pye series 104 Model 24, FID. 2.25 m × 2.5 mm I.D. glass column packed with Chromosorb W HP, 100—120 mesh, coated with 5% (w/w) OV-101. Carrier gas: nitrogen, 30 ml/min. Hydrogen, 30 ml/min. Air, 350 ml/min. Column temperature, 90°C with programme 4°C/min. GLC conditions for SCOT column FID: as in Fig. 2. GLC conditions for SCOT column AFID: as in Fig. 1.

Amino acid	FID Packed column (RMR ± S.D., n = 8)	FID SCOT column (RMR ± S.D., n = 10)	AFID SCOT column (RMR ± S.D., n = 10)
Alanine	0.69 ± 0.01	0.68 ± 0.01	0.82 ± 0.01
Glycine	0.55 ± 0.04	0.56 ± 0.01	1.03 ± 0.03
β-Alanine	nd*	0.69 ± 0.01	0.99 ± 0.01
Valine	0.92 ± 0.01	0.89 ± 0.01	0.45 ± 0.01
Threonine	0.92 ± 0.03	0.92 ± 0.03	0.77 ± 0.01
Serine	0.78 ± 0.02	0.79 ± 0.02	0.85 ± 0.01
Leucine	1.02 ± 0.01	1.03 ± 0.02	1.04 ± 0.01
Isoleucine	0.99 ± 0.02	0.99 ± 0.01	0.88 ± 0.02
Proline	0.92 ± 0.03	0.91 ± 0.02	1.05 ± 0.02
Cysteine	nd	0.76 ± 0.05	0.81 ± 0.01
Pipecolinic acid	1.02 ± 0.02	1.02 ± 0.02	1.08 ± 0.02
Hydroxyproline	nd	1.07 ± 0.04	1.04 ± 0.01
Methionine	0.92 ± 0.03	0.93 ± 0.04	1.14 ± 0.03
Aspartic acid	1.11 ± 0.01	1.14 ± 0.01	0.96 ± 0.01
Phenylalanine	1.35 ± 0.01	1.35 ± 0.02	1.15 ± 0.01
Ornithine	nd	1.00 ± 0.02	1.86 ± 0.03
Glutamic acid	1.21 ± 0.01	1.23 ± 0.02	0.98 ± 0.02
Lysine	1.12 ± 0.03	1.14 ± 0.01	1.99 ± 0.04
Tyrosine	1.38 ± 0.01	1.38 ± 0.02	0.98 ± 0.02
Arginine	0.96 ± 0.06	1.06 ± 0.03	2.32 ± 0.14
Histidine	0.83 ± 0.03	0.85 ± 0.03	2.31 ± 0.14
Tryptophan	nd	0.85 ± 0.05	1.01 ± 0.02
Cystine	0.97 ± 0.06	1.33 ± 0.04	1.92 ± 0.05

*nd = not determined.

Fig. 4. Separation of N(O)-heptafluorobutyryl isobutyl ester derivatives of amino acids obtained from insulin hydrolysate and analysed with FID, AFID and ECD. Preparation of sample: see Experimental. GLC conditions: (a) for FID as in Fig. 2; (b) for AFID as in Fig. 1; (c) Hewlett-Packard Model 7620A gas chromatograph fitted with a 2-mCi ^{63}Ni ECD. 25 m × 0.4 mm I.D. glass column coated with 5% Chromosorb R and 15% OV-101. Carrier gas: hydrogen, 3 ml/min. Make-up gas, argon—methane (90:10), flow-rate 50 ml/min. Temperatures: detector, 320°C; column, 90°C with programme 4°C/min. Pulse interval, 15 μsec. Attenuation, $2 \cdot 10^2$. Peaks: 1 = alanine; 2 = glycine; 3 = valine; 4 = threonine; 5 = serine; 6 = leucine; 7 = isoleucine; 8 = norleucine (internal standard, I.S.); 9 = proline; 10 = cysteine; 11 = pipecolinic acid (I.S.); 12 = aspartic acid; 13 = phenylalanine; 14 = glutamic acid; 15 = lysine; 16 = tyrosine; 17 = arginine; 18 = histidine; 19 = cystine.

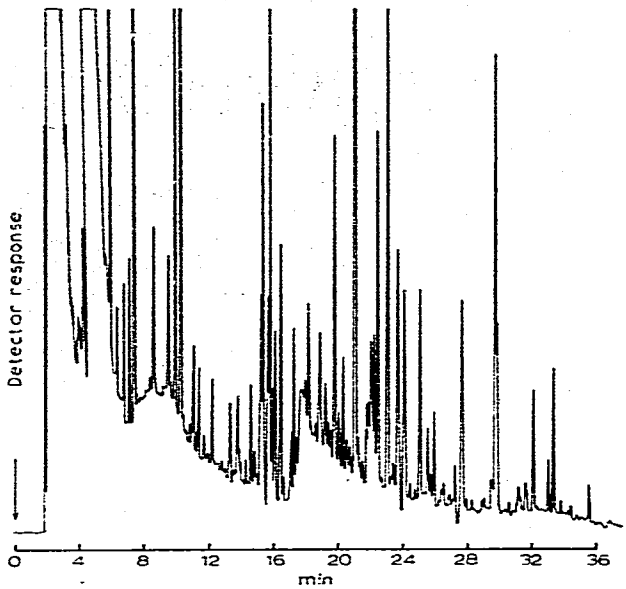

Fig. 5. Separation of N(O)-heptafluorobutyryl isobutyl ester derivatives of amino acids obtained from a urine sample and analysed with ECD. Preparation of sample: see Experimental. GLC conditions: as in Fig. 4c.

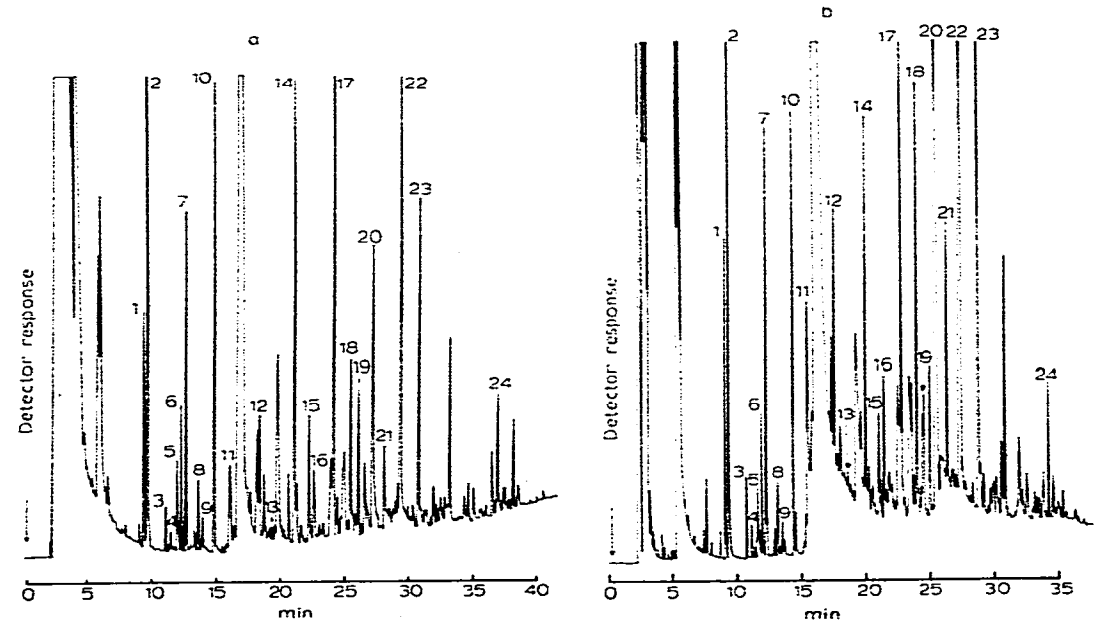

Fig. 6. Separation of N(O)-heptafluorobutyryl isobutyl ester derivatives of amino acids obtained from a urine sample and analysed with FID and AFID. Preparation of sample: see Experimental. GLC conditions: (a) for FID as in Fig. 2; (b) for AFID as in Fig. 1. Peaks: 1 = alanine; 2 = glycine; 3 = 2-aminobutyric acid; 4 = β-alanine; 5 = valine; 6 = threonine; 7 = serine; 8 = leucine; 9 = isoleucine; 10 = norleucine (I.S.); 11 = proline; 12 = hydroxyproline; 13 = methionine; 14 = aspartic acid; 15 = phenylalanine; 16 = ornithine; 17 = glutamic acid; 18 = lysine; 19 = tyrosine; 20 = 3,4-dihydroxyphenylalanine; 21 = arginine; 22 = histidine; 23 = homoarginine (I.S.); 24 = cystine.

TABLE III

AMINO ACID ANALYSIS OF A URINE SAMPLE BY GLC

Preparation of sample: see Experimental. GLC conditions: for FID as in Fig. 2; for AFID as in Fig. 1.

Amino acid	nmol/ml (n = 3)	
	FID	AFID
Alanine	179.8	181.3
Glycine	610.2	661.7
2-Aminobutyric acid	60.5	55.9
β-Alanine	5.3	5.8
Valine	47.8	50.1
Threonine	87.1	94.9
Serine	301.4	286.7
Leucine	30.3	34.7
Isoleucine	8.5	8.9
Proline	36.7	57.2
Hydroxyproline	74.6	120.2
Methionine	2.4	2.8
Aspartic acid	215.4	206.1
Phenylalanine	50.1	48.2
Ornithine	25.4	27.8
Glutamic acid	217.9	207.1
Lysine	118.7	124.7
Tyrosine	63.9	—
3,4-Dihydroxyphenylalanine	45.6	110.1
Arginine	42.3	44.6
Histidine	397.8	411.1
Cystine	28.6	24.7

tion. Adams [38] analysed 15 urine samples by GLC and showed large ranges of excretion values, but these overlap to a large extent with results presented here, with four main exceptions — our values are lower for threonine, serine and leucine but higher for hydroxyproline.

In dive 9B the subject was exposed to normal conditions in the diving chamber, known as the pre-dive period (2 days). This was followed by a period of steady compression (120 m/day) over a period of four days. The subject remained at a maximum depth of 540 metres sea water for two days and decompression at about 28 m/day proceeded for 20 days. In this dive (dive 9B) the subject was on a controlled diet and exposed to an atmosphere of oxygen—helium (20:80, v/v).

Figs. 7—10 show the variation of amino acid excretion during the period of the dive. Most of the amino acids showed an increased excretion for day 19 during the decompression period. Otherwise all the amino acids showed a fairly consistent drop in excretion (μg/mg creatinine) during the last few days of decompression.

TABLE IV

AMINO ACID CONTENT OF 16 URINE SAMPLES FROM ONE DEEP-SEA DIVER (DIVE 9B, RED SUBJECT)

Each urine sample was analysed in triplicate using both the FID and AFID. Hydroxyproline, tyrosine and 3,4-dihydroxyphenylalanine values are from FID.

Amino acid	μg/mg creatinine			mg/day			nmol/ml		
	Mean	C.V. (%)	Range	Mean	C.V. (%)	Range	Mean	C.V. (%)	Range
Alanine	11.8	40.9	5.8–25.4	20.8	49.4	6.1–44.3	165.7	39.9	41.3–306.3
Glycine	34.3	34.0	16.6–69.8	59.4	43.4	27.5–121.8	563.9	35.9	284.1–999.3
2-Aminobutyric acid	3.1	42.0	1.3–5.8	5.2	43.1	1.6–8.7	36.3	44.4	12.7–78.6
Valine	2.3	48.5	0.8–4.2	4.0	62.6	1.1–11.1	25.2	60.9	6.8–50.3
Threonine	6.8	30.2	3.3–9.7	11.9	42.7	4.5–22.1	71.4	35.7	28.2–100.5
Serine	14.7	35.7	5.6–20.7	25.3	46.4	11.1–47.2	173.3	41.8	69.8–286.7
Leucine	2.5	33.7	0.9–3.7	4.3	50.5	1.8–9.9	23.4	43.4	9.7–42.6
Hydroxyproline*	18.7	53.4	5.4–38.2	32.9	64.0	7.0–70.3	177.1	53.0	43.1–291.4
Aspartic acid	16.2	24.2	8.8–22.7	28.0	38.8	13.2–51.8	151.7	33.1	69.8–228.0
Phenylalanine	4.8	27.2	2.9–7.8	8.3	40.7	3.2–16.6	35.9	34.0	15.8–57.6
Glutamic acid	27.5	33.7	14.1–48.1	48.1	46.6	18.1–99.3	227.1	32.9	99.7–366.8
Lysine	11.8	28.6	5.6–17.4	20.7	46.4	8.0–46.0	101.1	37.1	34.3–166.6
Tyrosine	8.5	59.8	2.5–24.2	14.9	65.1	2.6–42.2	59.3	57.7	8.8–143.4
3,4-Dihydroxyphenylalanine*	10.5	28.6	5.4–15.7	18.2	44.6	7.5–41.5	66.1	38.2	30.7–111.4
Histidine	66.8	34.2	28.8–126.3	116.6	48.1	47.1–243.3	430.7	36.8	168.0–708.5

*Mixed peak.

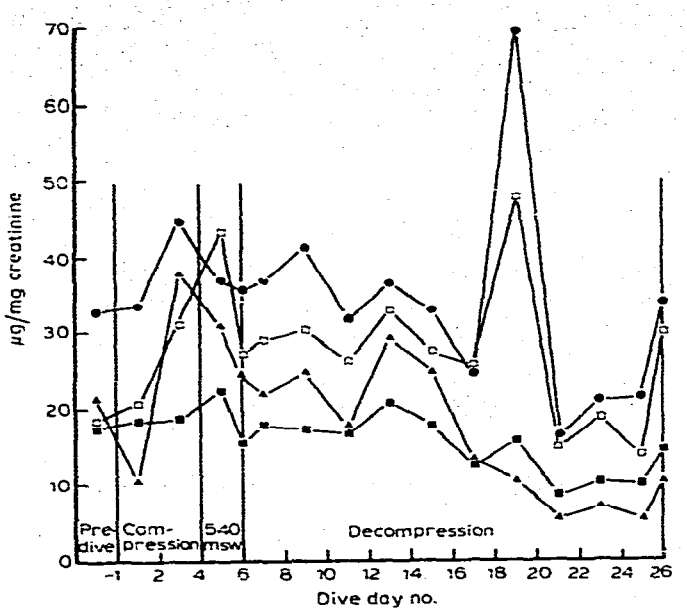

Fig. 7. Changes in urinary glycine, hydroxyproline, glutamic acid and aspartic acid during dive 9B. ● = Glycine; ▲ = hydroxyproline; □ = glutamic acid; ■ = aspartic acid. msw = metres sea water.

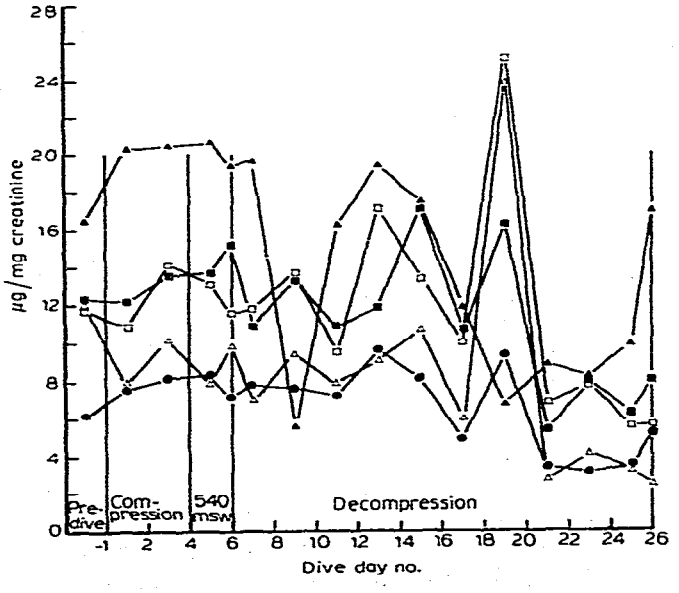

Fig. 8. Changes in urinary serine, threonine, tyrosine, lysine and alanine during dive 9B. ▲ = Serine; ● = threonine; △ = tyrosine; ■ = lysine; □ = alanine.

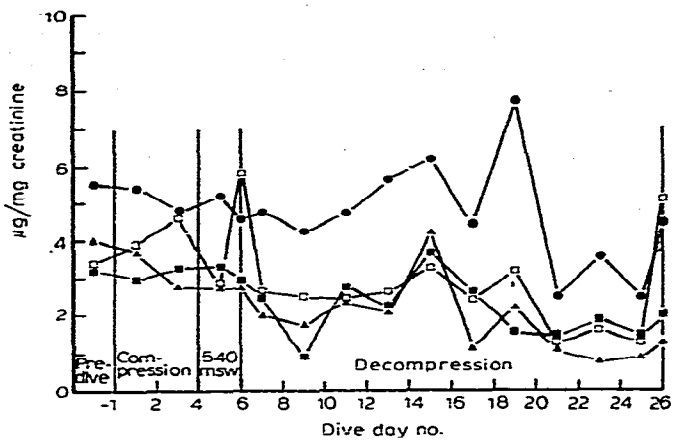

Fig. 9. Changes in urinary phenylalanine, 2-aminobutyric acid, valine and leucine during dive 9B. ● = Phenylalanine; □ = 2-aminobutyric acid; ▲ = valine; ■ = leucine.

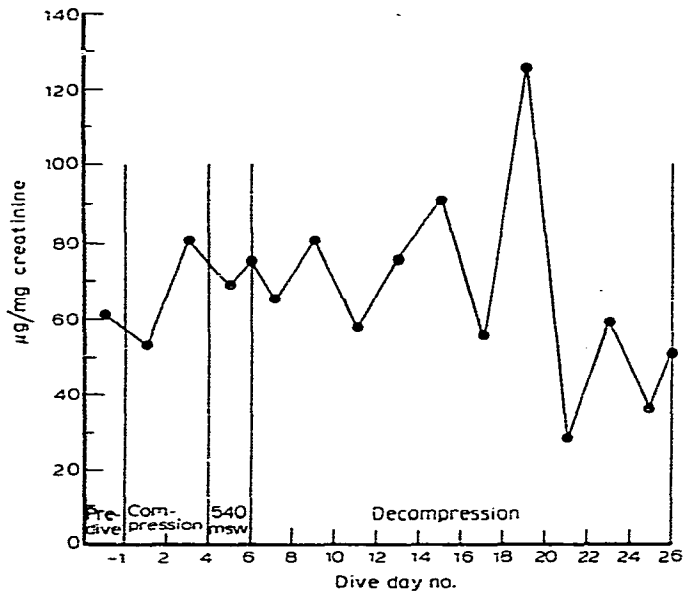

Fig. 10. Changes in urinary histidine during dive 9B.

DISCUSSION

The advantages of using N(O)-HFB isobutyl ester derivatives of amino acids have been described [26—29]. However, a packed column does not offer a sufficiently high number of theoretical plates to enable complete separation of closely similar metabolites present in urine samples. The resolution obtainable from capillary columns is in general restricted by relatively inefficient injection techniques. Due to the large differences in the volatility of different amino acid derivatives, the injection technique plays an important role in the quantitation of amino acids with glass capillary GLC. Recent ad-

vances in injection techniques, especially on-column injection [55] and direct injection [51], largely overcome the problems associated with injection technique.

Two types of injection system (with and without inlet heater) were used [51] and both gave the same RMR values for amino acids. It was necessary to use 300°C as the inlet temperature for the quantitative recovery of all amino acids, although direct injection without the inlet heater was preferred, because this allowed large sample volumes (up to 2 μl) to be injected. Pearce [35] used a SCOT column with SE-30 as stationary phase and with an injection system similar to the one used in this study but with 260°C inlet temperature. At this inlet temperature lower recoveries of arginine, histidine and cystine were observed which partially explains the lower recoveries obtained by Pearce for these amino acids (Table I, column 5). Desgres et al. [34] used a WCOT column and showed similar RMR values for alanine (1.00), glutamic acid (0.96), isoleucine (1.06), phenylalanine (1.10) and tyrosine (0.96). They used the falling-needle method for the solid injection of sample [56]. The falling-needle method was not studied here, but it seems from the RMR values obtained by Degres et al. [34] that discrimination must have occurred (Table I, column 6). Moodie and Burger [57] have compared different modes of sample injection (split and on-column) and concluded that the on-column system is a prerequisite for quantitative amino acid analysis by glass capillary GC.

The FID RMR values obtained from packed and SCOT columns (Table II, columns 2 and 3) are not significantly different; the correlation coefficient was 0.93. Arginine and cystine gave higher RMR values on the SCOT column (1.06 and 1.33) than on the packed column (0.96 and 0.97). Active sites on the packed column probably cause some breakdown of the arginine derivative but on a SCOT column active sites may not be so numerous. Histidine gave a similar value on the packed column (0.83) and on the SCOT column 0.85). MacKenzie and Tenaschuk [28] (Table I, column 2) gave a value of 0.94 for histidine; their on-column derivatisation of histidine with acetic anhydride proved satisfactory as long as the inlet heater temperature was greater than 250°C. Direct injection without the inlet heater [51] was employed with histidine being derivatised in an additional stage with acetic anhydride and subsequently injected onto the column with the inlet heater at the ambient oven temperature of 60°C.

Theoretically, with AFID those compounds having one nitrogen atom per molecule should give equal responses. The fact that the responses are not equal may be due to the way the compounds burn in the flame. This is seen in Table II, column 4. Valine showed a surprisingly low RMR value (0.45). Ornithine, lysine and cystine (two nitrogen atoms per molecule) showed approximately twice the response given by many amino acids with one nitrogen atom, such as norleucine. Histidine, with three nitrogen atoms, shows a higher response (2.31) than ornithine (1.86) and lysine (1.99). However, arginine with four nitrogen atoms only gives an RMR value of 2.32. Attempts were made to change the reaction conditions to improve the arginine response but these were not successful. The low RMR value for tryptophan (1.01) may reflect its breakdown during esterification. When small amounts ($<$ 25 nmol)

were derivatised, tryptophan gave a much lower RMR value. It was observed that elution of arginine depends on acylation conditions [29] but histidine and cystine recovery depended on the activity of the column. When a SCOT column coated with 5% Chromosorb R and 5% OV-101 was used, the histidine peak emerged only when amounts greater than 250 pmol were injected, and the recovery of cystine was only 75%. With higher concentrations of OV-101 (15%) recovery for these two amino acids was improved. There are no comparable published RMR values that were obtained with AFID. Adams et al. [33] have used a WCOT column in conjunction with AFID but no RMR values were given. They used a splitter system (which was not used here) and discrimination may have occurred against these low volatility compounds [54].

Most of the publications for the separation of amino acid derivatives indicate use of an FID. Due to the complexity of urine samples, a single peak may be due to several compounds [50, 58]. Although specific detection of amino acids can be obtained using gas chromatography—mass spectrometry, the required instrumentation is expensive and not available to everyone. However, if the peaks are detected by different detectors some specificity can be obtained (Table III). There are further advantages for using a multi-detection technique, especially for the peaks that have a differential response on different detectors. For example, arginine and histidine have a low response with FID (Fig. 4a) but an adequate response was obtained with AFID (Fig. 4b); similarly, threonine and serine have a low response with FID (Fig. 4a), but a reasonable response was obtained with ECD (Fig. 4c).

Upon increasing the hydrostatic pressure of the diver's environment to about 180 m of sea water and beyond, he will be subject to a variety of ill-effects collectively termed high-pressure neurological syndrome (HPNS); a detailed description of these effects has been reported [59]. No reports on the quantitation of urinary amino acids have as yet been published. Most of the amino acids (of special interest is the increment in the putative neurotransmitters glycine and glutamic acid) increased for one day (day 19) during the decompression period. On this particular day the subject reported an attack of the "bends". This increase could be due either to the "bends" or to a "leakage" from some tissue. Further analysis of plasma samples would confirm this. Otherwise all amino acids were excreted with a day-to-day variation, which did not appear to correlate in any way with the hyperbaric conditions to which the diver was exposed, except for a fairly consistent drop in excretion during the last few days of the decompression.

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