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Note

Determination of amino acids in rat muscle by gas chromatography

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A fully evaluated procedure for the quantitative determination of amino acids is a prerequisite for obtaining data for a meaningful interpretation of fluctuations in free amino acid levels in muscle. Several publications [1–7] have appeared in which muscle amino acid levels have been reported and important conclusions have been drawn from the data. Few present details of the analytical procedure beyond indicating the source of the automatic amino acid analyser used. Furthermore, no statistical evaluation (e.g. reproducibility and recovery) of the chromatography was reported thus precluding an assessment of the reliability of these data. The need for a fully tested technique was, therefore, evident.

In a recent report [8], we have described a complete methodology, incorporating a sample clean-up technique, for quantitative determination of plasma amino acids as their N-heptafluorobutyryl isobutyl ester (HBB) derivatives [9] using packed-column gas chromatography (GC). We now report on the development of a procedure for quantitative analysis of amino acids in rat muscle, using a fused-silica open-tubular (FSOT) capillary column since the resolution obtained using packed and wide-bore columns proved inadequate for this purpose.

EXPERIMENTAL

Amyloglucosidase solution

Purified amyloglucosidase (EC 3.2.1.3.), as a crystalline ammonium sulphate suspension (14 U/mg), was obtained from Boehringer-Mannheim (Mannheim, F.R.G.). Before use, aliquots (0.2 ml) were centrifuged (1350 *g*) at 4°C for 20 min. The supernatant was discarded, the pellet redissolved in distilled water (0.4 ml) and this solution applied to a column (160 mm × 10 mm) of Sephadex G-25

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(100–300 μm particle size) in water (dry Sephadex swollen in water before use). Elution was performed at ca. 0.5 ml/min and, after discarding the first 7 ml of eluate, the following 2.4 ml containing desalted amyloglycosidase were collected. These volumes were confirmed by monitoring the start and finish of protein elution with methanolic sodium hydroxide solution. The final volume (2.4 ml) contained amyloglucosidase (28 U) and was stored at 4 °C before use.

Hexokinase solution

Purified hexokinase (Type C-300; EC 2.7.1.1.), as a crystalline ammonium sulphate suspension, was obtained from Sigma (St. Louis, MO, U.S.A.) and prepared for use as previously described [8].

ATP solution (0.08 *M*), barium acetate solution (1.96 *M*), amino acid standard solution and esterifying reagent were prepared as described elsewhere [8]. Heptafluorobutyric anhydride (HFBA) was obtained from Fluka (Buchs, Switzerland). Glass crimp-top vials (Type 03-CV3; 300 μl capacity) were obtained from Chromacol (London, U.K.).

Muscle extraction

Fresh quadriceps, obtained from rats sacrificed under diethyl ether anaesthesia, were immediately placed in screw-capped vials, rapidly frozen in liquid nitrogen and stored at −20 °C until required. Thawed muscle was finely chopped at 4 °C using a scalpel, at the same time removing excess connective tissue. Using a pestle and mortar, a fine powder was obtained by grinding a mixture of the muscle frozen in liquid nitrogen. The muscle material was rapidly weighed, avoiding moisture uptake, and transferred to a tissue grinder with sufficient sodium chloride solution (0.9%) to give a final solid-to-liquid ratio of 1:10 (w/v). The mixture was homogenised for 3 min and centrifuged (2190 *g*), the resulting supernatant being further centrifuged (16 460 *g*) for 30 min. All preparative steps were carried out at 4 °C. The final supernatant was analysed or stored in aliquots (ca. 1.2 ml) at −20 °C prior to use.

Glycogen and glucose removal

To the muscle extract (1 ml), internal standard solution (25 μl) and methanol (4 ml) were added. The stirred mixture was allowed to stand at room temperature for 3 min and centrifuged (2190 *g*) for 10 min. The supernatant was filtered through a Millex PTFE filter (25 mm diameter; 0.5 μm pore size) into a screw-capped culture tube (120 mm × 16 mm) and acidified with 12 *M* hydrochloric acid (40 μl). The solution was then taken to dryness on a Virtis freeze-drier (Virtis, New York, NY, U.S.A.). The residue was redissolved in water (500 μl), transferred to a smaller culture tube (100 mm × 16 mm) and freeze-dried. To the freeze-dried residue was added amyloglucosidase solution (390 μl; 4.5 U) and the pH was adjusted to 5.0 with 0.5 *M* sodium hydroxide. The mixture was incubated in a water bath for 30 min at 30 °C, then allowed to cool for 10 min before addition of ATP (0.08 *M*; 40 μl) and hexokinase solution (400 μl; 200 U). After readjustment of the pH to 7.4 with 0.5 *M* ammonium hydroxide, the mixture was incubated for 15 min at 30 °C whereupon methanol (2 ml) was added to precipitate

enzymic protein. After vigorous stirring (Vortex mixer) for 20 s, barium acetate solution (7 μl) was added to precipitate excess ATP. Further vigorous stirring for 20 s followed by centrifugation (1160 g) at room temperature for 10 min gave a clear supernatant. The latter was filtered through a Millex PFTE filter into a screw-capped culture tube (120 mm$\times$16 mm) and acidified with 12 M hydrochloric acid (20 μl). The solution was freeze-dried and the residue redissolved in 0.25 M hydrochloric acid (500 μl) followed by extraction with chloroform (3 ml). After centrifugation (3 min) to separate the phases, a portion of the aqueous phase (300 μl) was transferred to an esterification tube, taken to dryness (freeze-drier) and azeotroped with methylene chloride (100 μl).

Derivatisation

Esterification and acylation were carried out using procedures described elsewhere [8]. The derivatives in acetic anhydride (50 μl) were sealed under nitrogen in a small crimp-capped vial and heated (by immersion of the lower half) in an oil bath at 150°C for 3 min after which the vial was rapidly chilled (ice–water) and stored at −20°C until required. For analysis on the FSOT column, aliquots (2 μl) were diluted with ethyl acetate (18 μl) and chromatographed immediately.

Chromatography

GC was carried out using a Carlo Erba Model 5360 Mega series gas chromatograph equipped with a cold on-column injector and a flame ionisation detector.

Analysis was performed on a cross-linked apolar PS-225 FSOT column, 25 m$\times$0.32 mm I.D., 0.7 μm film thickness (obtained from Professor B.V. Burger, Department of Chemistry, University of Stellenbosch).

Chromatographic conditions were: detector temperature, 295°C; carrier gas (hydrogen) velocity, 47 cm/s; air flow-rate, 300 ml/min; hydrogen flow-rate, 30 ml/min. After injection, the secondary cooling was immediately switched off and the initial oven temperature of 110°C was maintained for 5 min before being increased to 160°C at 4°C/min. After a 3-min isothermal period, the temperature was increased to 265°C at 4°C/min and held for 25 min before cooling to 110°C. Aliquots (0.3 μl) of the diluted derivatives were injected.

RESULTS AND DISCUSSION

In common with other physiological fluids (e.g. plasma), muscle extracts contain major components which may cause interference in determination of amino acid levels by GC. An approach to the pre-chromatographic clean-up of plasma, which differs fundamentally from the frequently employed cation-exchange procedure, has been described elsewhere [8]. Identification of components which are responsible for chromatographic interference, and their removal, are the key steps in this alternative strategy.

In the present study, implementation of this approach similarly confirmed that the glucose component leads to major interference in the determination of muscle amino acids. By applying the methodology used in the case of plasma, attempts were made to remove glucose by enzymic breakdown in the presence of hexoki-

TABLE I

REPRODUCIBILITY OF AMINO ACID RESPONSE FACTORS FOLLOWING DERIVATISATION AND ADDITIONAL HEATING IN ACETIC ANHYDRIDE FOR 3 MIN AT 150°C USING AN FSOT COLUMN

Amino acid	R_F (mean ± S.D.)[a]	C.V. (%)
Alanine	1.035 ± 0.008	0.7
Glycine	0.958 ± 0.011	1.1
Valine	1.025 ± 0.011	1.1
Threonine	1.018 ± 0.007	0.7
Serine	1.028 ± 0.019	1.9
Leucine	0.986 ± 0.005	0.6
Isoleucine	1.018 ± 0.007	0.7
Proline	0.994 ± 0 008	0.8
Hydroxyproline	0.974 ± 0.016	1 6
Methionine	1.536 ± 0.025	1.7
Aspartic acid	0.896 ± 0.018	2.1
Phenylalanine	0 956 ± 0.007	0.7
Glutamic acid	0.908 ± 0.008	0.9
Lysine	1.087 ± 0.013	1.2
Tyrosine	1.012 ± 0.018	1.8
Arginine	1.343 ± 0.025	1.9
Histidine	1.370 ± 0.015	1.1

[a]Mean of six separately derivatised and chromatographed standard samples.

TABLE II

AMINO ACID LEVELS IN UNSPIKED AND SPIKED SAMPLES ($n=6$) OF RAT MUSCLE EXTRACT DETERMINED WITH AN FSOT COLUMN

Amino acid	Unspiked sample		Level added (μmol/g)	Spiked sample		Mean recovery (%)
	Mean level (μmol/g)	C.V. (%)		Mean level (μmol/g)	C.V. (%)	
Alanine	1.67	1.9	2.24	4 06	0.9	107
Glycine	3 24	2 3	1.33	4.58	1.0	101
Valine	0.26	2.9	0.85	1.14	0.7	104
Threonine	0.49	2.4	0.84	1.31	0.7	96
Serine	1.02	1.2	0.19	1.20	1.3	95
Leucine	0.47	1.8	0.76	1.13	2.4	87
Isoleucine	0.16	7.2	0.76	0.90	2.0	97
Proline	0.34	1.2	0.87	1.19	0.5	98
Hydroxyproline	0.25	2.5	0.15	0.40	1.3	100
Methionine	0.16	6.6	0.13	0.29	4.0	100
Aspartic acid	0.22	3.7	0.15	0.31	4.1	60
Phenylalanine	0.22	4.6	0.61	0.82	3.2	98
Glutamic acid	4.13	0.7	1.36	5.28	1.8	85
Lysine	0.48	3.5	1.37	1.63	5 7	84
Tyrosine	0.25	4.7	0.55	0.75	2.9	91
Arginine	0.26	3 3	0.57	0.75	5.2	86
Histidine	0.66	4.9	0.64	1.29	3 1	98

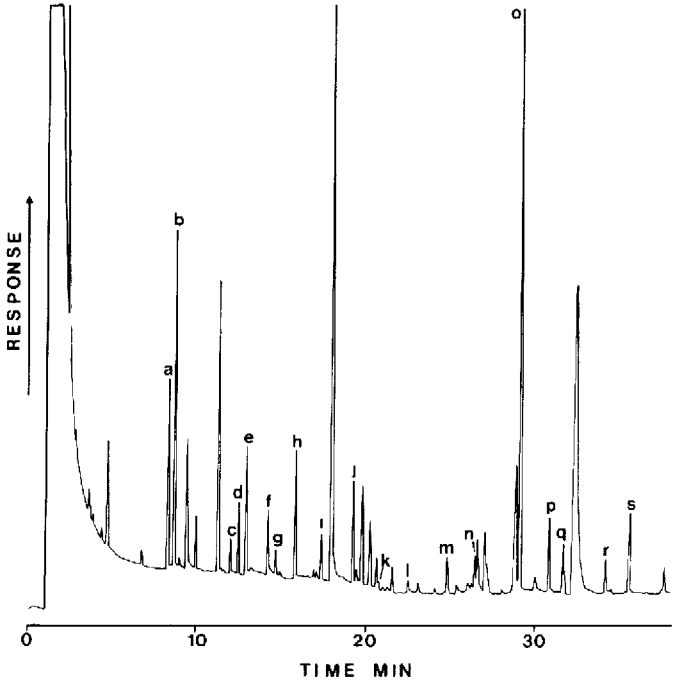

Fig. 1. Chromatogram of derivatised product from rat muscle extract, after the complete pre-chromatographic treatment. Chromatogram obtained using the FSOT column. Peaks: a=alanine; b=glycine; c=valine; d=threonine; e=serine, f=leucine; g=isoleucine, h=norleucine (internal standard); i=proline; j=pipecolic acid (internal standard); k=hydroxyproline; l=methionine; m=aspartic acid; n=phenylalanine; o=glutamic acid; p=lysine; q=tyrosine; r=arginine; s=histidine.

nase; however, the interfering glucose pattern was observed to have been reduced but not eliminated. The compound responsible for this remaining interference was found to be a non-dialysable glucose polymer, which was acid-hydrolysed to glucose during esterification, yielding the usual glucose-type interference. Glycogen, a glucose polymer and major component in muscle, was considered to be the likely source of the interference. Chemical and physical techniques for the removal of glycogen were explored but found to be unsatisfactory. The enzyme, amyloglucosidase, is reported [10,11] to cleave both the $\alpha(1\rightarrow4)$ and $\alpha(1\rightarrow6)$ linkages found in glycogen. A procedure was developed in which a glycogen solution, pretreated with methanol and freeze-dried, was incubated with purified amyloglucosidase at 30°C for 15 min to bring about conversion of the glycogen to glucose at pH 5.0. Hexokinase and ATP at pH 7.4 were then used to remove the glucose as previously described [8]. Earlier attempts to shorten the protocol, by promoting both enzymic reactions simultaneously, failed due to the inability of both enzymes to function satisfactorily at a common pH [10,12]. Sequential use of the enzymes on rat muscle extract followed by derivatisation resulted in successful removal of this glucose-type interference. Despite this improvement, however, the secondary interference that still persisted could not be eliminated by the enzymatic procedure described.

Creatine, another major component of muscle, is readily converted to creatinine at the low pH used during sample manipulation. This latter compound has previously been shown [13] to cause major interference in the GC analysis of urinary amino acids. By heating the final acetic anhydride solution of derivatives at 150°C for a period of 3 min, the creatinine peak pattern was modified to remove this interference.

Amino acid standard derivatives were prepared in the usual manner and subjected to the additional heating step. Quantitative analysis (Table I) showed that FSOT results were reproducible, values for coefficient of variation (C.V.) being 2.1% or less. These figures are similar to previously reported data [8,14].

A protocol incorporating sequential incubation with two enzymes and post-derivatisation heating in acetic anhydride followed by GC analysis was carried out on muscle extract (Fig. 1). The C.V. was less than 7% with a mean recovery of 93% (Table II). Although the results for levels of aspartic acid are reproducible, the cause of the low recovery has not been established but is believed to be related to the pre-chromatographic methodology.

In conclusion glucose, glycogen and creatine causing major interference in the quantitative determination of amino acids in rat muscle have been successfully removed resulting in a methodology of good reproducibility and recovery.

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