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# DETERMINATION OF PLASMA AMINO ACIDS BY GAS CHROMATOGRAPHY

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## SUMMARY

A complete methodology, incorporating a novel clean-up technique, for quantitative determination of amino acids in plasma by gas chromatography is described. Glucose, a component causing major analytical interference, is removed by an enzymic reaction included in the pre-chromatographic clean-up. The procedure for derivatisation of amino acid standards is shown to be reproducible down to a level of 2.5 $\mu$g for each amino acid, relative standard deviations for all amino acids except arginine and histidine being 3% or lower. For the entire procedure applied to plasma, relative standard deviations for most amino acids are below 5% with recoveries ranging from 90 to 120%. Normal values, obtained using eighteen plasma samples, are in reasonable agreement with published data. Plasma amino acid values were determined simultaneously by gas chromatographic and ion-exchange chromatographic techniques. Statistical evaluation shows there to be no significant difference between corresponding values for eleven amino acids. Values for tyrosine, histidine and particularly phenylalanine show significant differences ($p < 0.001$).

## INTRODUCTION

Amino acid analysis is assuming an increasingly important role in biochemical and clinical research. In particular, the field of metabolic studies stands to derive considerable benefit from improvements in both accuracy and precision of determination of amino acids in physiological fluids.

The disadvantages of determining amino acids in plasma using the ion-exchange chromatography (IEC) technique are becoming more widely recognised and, as appears from a recent review on the subject, a number of research groups [1] have adopted alternative procedures based on gas chromatography

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(GC). In common with the IEC technique, most GC methodologies include treatment of the plasma with a protein precipitant. The deproteinised plasma then undergoes a purification step prior to the synthesis of volatile amino acid derivatives. The method of choice for such purification has over the years been that of cation-exchange chromatography, though the latter has been used alone to avoid protein precipitation and directly clean-up a plasma sample pretreated with acetic acid [2, 3]. The limitations and disadvantages of the cation-exchange clean-up step have also recently been reviewed [1]. Of particular relevance in this regard is the non-specific nature of the procedure since it removes all anionic and non-charged moieties present in the plasma irrespective of whether or not they contribute to interference in the subsequent analysis. Furthermore, a wide diversity of protocols has been described for this procedure, some of which involve conditions that have been shown to be not only sub-optimal but also detrimental to accurate analysis. Thus considerable doubt is thrown on the validity of quantitative results obtained from protocols involving cation-exchange clean-up. That these difficulties have not been widely recognised and addressed is evident from the apparent absence of effort to standardise procedures for pre-chromatographic purification of amino acids from physiological fluids and also a striking lack of data on accuracy and precision of clean-up steps along with most published methodologies [1].

Our previous investigations [4, 5] into GC analysis of amino acids in deproteinised plasma have shown that, although most amino acids can be successfully resolved, there is a major component responsible for a number of interfering chromatographic peaks which elute over a broad area precluding the resolution of hydroxyproline, methionine, aspartic acid and phenylalanine. We have further demonstrated [4] that this component is glucose and have attempted to explain how its presence may give rise to this interference.

The present study centres on the quantitative documentation and modification or our previously published method [5] of eliminating glucose as an interfering substance in the analysis of plasma amino acids by GC.

## EXPERIMENTAL

### *Materials and reagents*

*ATP solution (0.08 M).* A 48-mg amount of ATP disodium salt (Boehringer-Mannheim) was dissolved in distilled water (1 ml) and stored at 4°C for no longer than four days before use.

*Hexokinase solution.* Purified hexokinase, EC 2.7.1.1 (Type C-300), as a crystalline ammonium sulphate suspension, was obtained from Sigma (St. Louis, MO, U.S.A.). Before use, 0.24-ml aliquots of this suspension were centrifuged at 1350 *g* and 4°C for 15 min. The supernatant was discarded, the pellet redissolved in approximately 0.4 ml distilled water and this solution applied to a column (160 mm × 10 mm) of Sephadex G-25 (100—300 μm particle size) in water (dry Sephadex G-25 swollen in water before use). Elution was performed at ca. 0.5 ml/min and after discarding the initial 7—8 ml of eluate, the following 3—3.5 ml containing desalted hexokinase were collected. These volumes were confirmed by monitoring the start and finish of protein elution with 10% trichloroacetic acid solution. The final volume, made to 4 ml

with distilled water, contained 2000 U of hexokinase and was stored at 4°C for no longer than four days before use.

*Plasmalyte-B.* Plasmalyte-B, a balanced solution of electrolytes (1 l contained 6.0 g sodium chloride, 2.3 g sodium bicarbonate, 0.3 g potassium chloride and 0.3 g magnesium chloride; pH approximately 7.4), was obained from SABAX (South Africa). This solution was used in the dialysis of aliquots (5 ml) of plasma contained in bags made from Visking dialysis tubing (Union-Carbide). Dialysis was carried out at 4°C over a period of 24 h. During this time, the Plasmalyte-B (800 ml) was changed at hourly intervals over the first 3 h and a further three times at 2-h intervals prior to ending the dialysis.

*Barium acetate solution (1.96 M).* A 500-mg amount of barium acetate purchased from Merck (Darmstadt, F.R.G.) was dissolved in distilled water (1 ml). The reagent was prepared fresh for each batch of analyses.

*Amino acid standard solution.* Amino acid standards were obtained as solids from Sigma. The standard solution of amino acids in 0.25 *M* hydrochloric acid (25 ml) consisted of: alanine, glutamic acid and lysine (20 mg of each); glycine, valine, threonine, leucine, isoleucine, proline, pipecolic acid (internal standard), phenylalanine, tyrosine, arginine and histidine (10 mg of each); serine, hydroxyproline, methionine, aspartic acid and tryptophan (2 mg of each). This solution was then subdivided into smaller aliquots (0.5 ml) and stored frozen (−20°C) for up to twelve months before renewal.

*Internal standard solution.* Pipecolic acid (50 mg) was dissolved in 0.25 *M* hydrochloric acid (50 ml). This solution was subdivided into smaller aliquots (10 ml) and stored at 4°C for up to twelve months before renewal.

*Esterifying reagent (3 M hydrogen chloride in isobutanol).* Dry hydrogen chloride gas (11.0 g), generated from ammonium chloride and sulphuric acid as described previously [6], was dissolved in chilled redistilled isobutanol (Merck) and made to volume (100 ml). The reagent was then subdivided into convenient smaller aliquots (2.6 ml) and stored under nitrogen at 4°C for up to four months before renewal. After use, any remaining reagent from an aliquot was discarded.

*Acylating reagent.* Heptafluorobutyric anhydride (HFBA) was obtained from Sigma and stored in small aliquots (0.6 ml) under nitrogen in sealed vials at 4°C. Any remaining reagent in an opened vial was discarded.

*Procedure*

To plasma (0.5 ml) were added internal standard solution (10 μl) or amino acid standard solution (25 μl), 0.08 *M* ATP solution (40 μl) and hexokinase solution (200 U; 400 μl); 0.5 *M* ammonium hydroxide solution was then added to adjust the pH to 7.4 and the mixture incubated for 15 min at 30°C whereupon methanol (2 ml) was added to accomplish deproteinisation. After stirring vigorously (vortex mixer) for 20 s, barium acetate solution ( 6 μl) was added to precipitate excess ATP. Further vigorous stirring for 20 s, followed by centrifugation at 2100 *g* for 10 min, yielded a yellow—white pellet and clear supernatant. The latter was carefully removed, 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 by addition of 12 *M* hydrochloric acid (20 μl). This solution was taken to dryness using a Virtis freeze dryer. The residue

was redissolved in 0.25 *M* hydrochloric acid (500 μl) and the solution stirred vigorously with chloroform (3 ml). After centrifugation to separate the layers, a portion of the aqueous phase (300 μl) was transferred to an esterification tube, taken to dryness using the freeze dryer and azeotroped with methylene chloride (100 μl).

Esterifying reagent (250 μl) was added to the dry residue. The tube was then briefly stirred (vortex mixer), flushed for 30 s with a current of dry nitrogen gas, capped and placed in an oil bath at 100 ± 2°C. The reaction was continued for 45 min during which time the tube was removed and stirred at the 5-, 20- and 30-min elapsed periods. On completion of esterification, the tube was chilled (ice—water mixture), centrifuged for ca. 5 min at 2100 *g*, the supernatant then being transferred to an acylation tube. Esterifying reagent was removed in vacuo (freeze dryer) and the residue azeotropically dried using three successive additions and evaporations of methylene chloride (100 μl). After addition of HFBA (100 μl), the tube was flushed with dry nitrogen (30 s), stirred and the acylation carried out at 150°C for 10 min after which the mixture was chilled (ice—water). Acylating reagent was removed (freeze dryer) care being taken not to allow over-exposure of the residue to vacuum conditions [7]. The latter was then dissolved in acetic anhydride (50 μl), the resulting solution being used for chromatography or stored at −20°C until required.

GC was carried out using a Varian 3700 gas chromatograph equipped with on-column injection, a flame ionisation detector and a silanised coiled glass column (3 m × 2.7 mm I.D.). Packings were prepared by coating 3.5% OV-101 on resilanised Supelcoport* (100—120 mesh). Columns were conditioned overnight by programming from 100 to 275°C at 1°C/min followed by holding at the latter temperature for about 16 h. Flow-rate of nitrogen carrier gas for conditioning and analysis was 19 ml/min. Chromatographic conditions were: injector temperature, 210°C; detector temperature, 250°C; air flow-rate, 300 ml/min; hydrogen flow-rate, 30 ml/min. After sample injection, the oven was held at initial temperature of 100°C for 5 min, then programmed from 100 to 240°C at 5°C/min and held for 1 min, 240 to 295°C ballistically and held for 16 min at 295°C, followed by cooling to 100°C. The latter heating ramp removes high-boiling compounds from the column. A Spectra-Physics 4100 electronic integrator/chart recorder was used for data acquisition and processing for quantitative analysis.

*Statistics*

Statistical evaluation of results was performed using the two-tailed Student's *t*-test.

## RESULTS AND DISCUSSION

At the outset of this investigation, an examination of the literature for a reliable and rapid procedure for quantitative chromatographic analysis of

*The quality of Supelcoport has recently become variable. Use of alternative support material of the high-performance type is recommended [I.M. Moodie, G.S. Shephard and D. Labadarios, J. Chromatogr., 362 (1986) 407].

amino acids in plasma revealed there to be a surprising absence of a comprehensively proven methodology [1]. None of the techniques, based on either IEC or GC, reported to date, seek to identify substances which interfere with chromatographic analysis of plasma amino acids and to establish steps for their removal although protein precipitation using, for example, perchloric, picric, sulphosalicylic or trichloroacetic acids has been widely employed for many years. There appeared to be little potential for novel and significant development in this area and, for this reason, rather than try to improve any of the existing techniques, it was decided to approach the problem in a different manner from that hitherto considered.

The decision to adopt the use of GC analysis rather than the more conventional IEC technique was based upon arguments discussed in detail elsewhere [1]. Macro methods for amino acid analysis using GC have been established and well reviewed [8]. Moreover, the reproducibility of a macro technique with relative standard deviation (R.S.D.) generally better than 1% for standards and less than 5% on samples such as protein hydrolysates has been fully reported [6]. However, typical quantities of amino acids (ca. 10 mg) used in

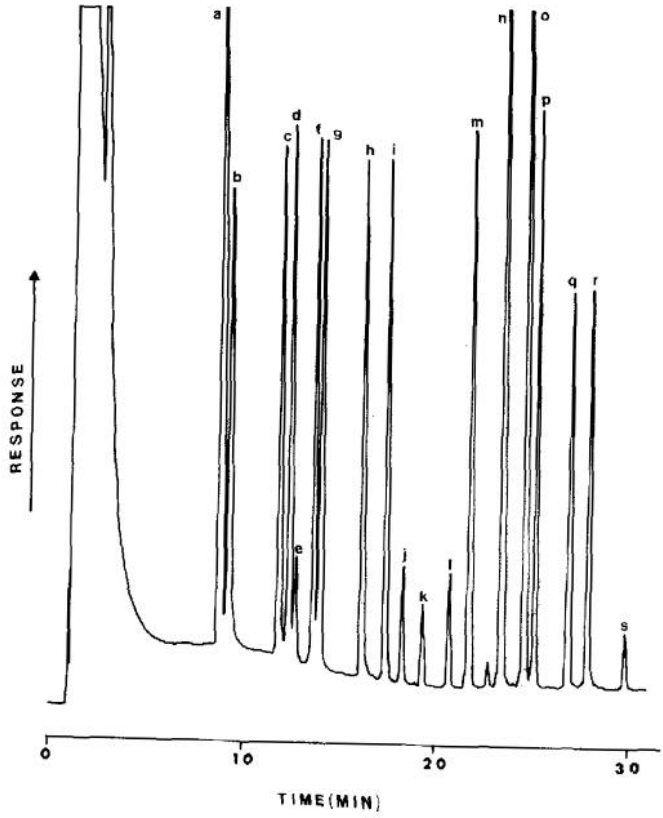


Fig. 1. Chromatogram of amino acid heptafluorobutyryl isobutyl ester (HBB) derivatives prepared from a 25-$\mu$l aliquot of amino acid standard solution (for composition see *Materials and reagents*). Peaks: a = alanine; b = glycine; c = valine; d = threonine; e = serine; f = leucine; g = isoleucine; h = proline; i = pipecolic acid, internal standard; j = hydroxyproline; k = methionine; l = aspartic acid; m = phenylalanine; n = glutamic acid; o = lysine; p = tyrosine; q = arginine; r = histidine; s = tryptophan.

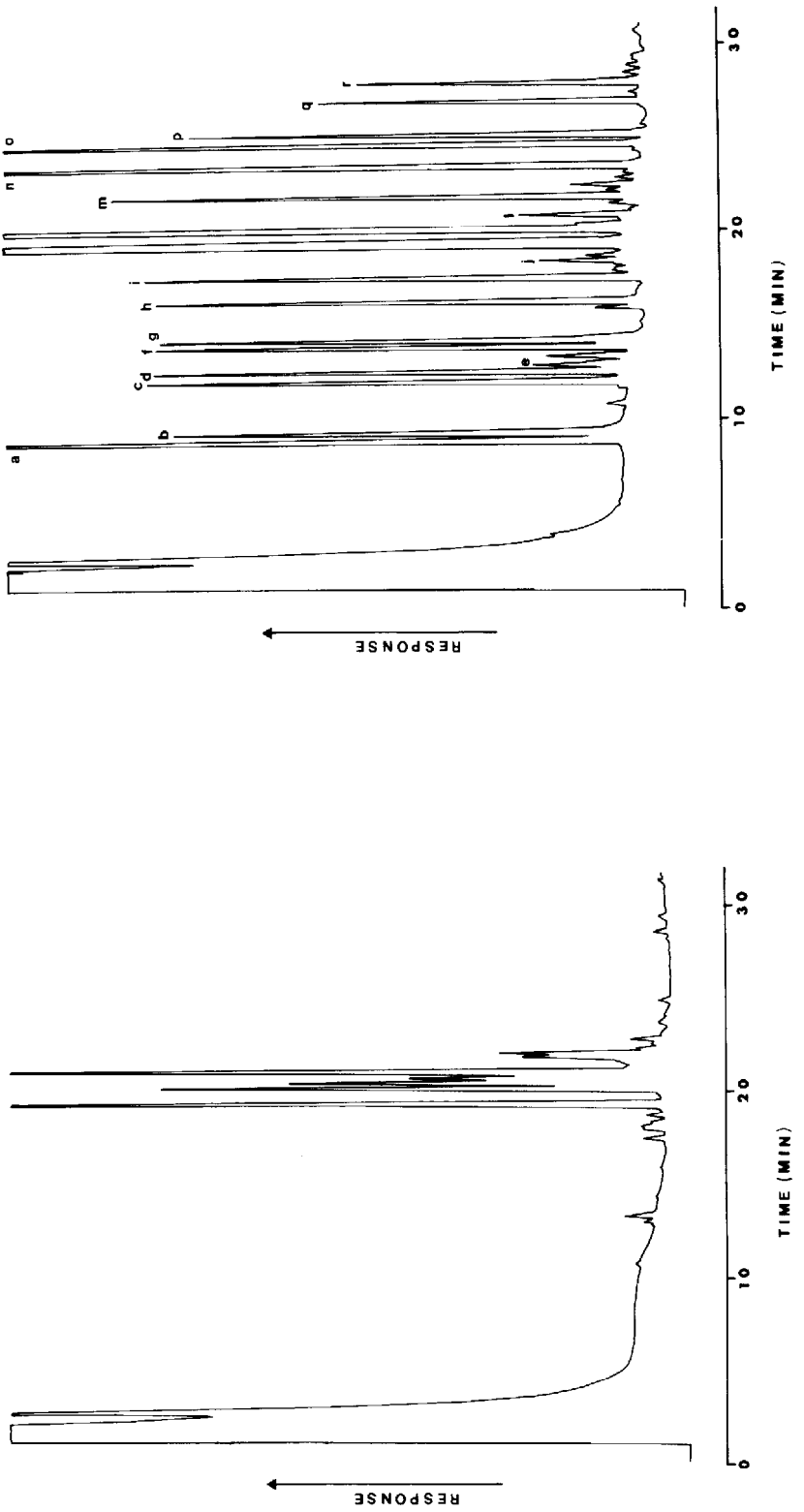


Fig. 2. Chromatogram of the product from glucose subjected to the derivatisation procedure.
Fig. 3. Chromatogram of HBB amino acid derivatives together with simultaneously derivatised glucose. Peak identification as in Fig. 1.

this procedure are high in relation to levels in biological fluids such as human plasma or cerebrospinal fluid in which typical concentrations are in the region of 0.3 mg total free amino acids per millilitre. This macro method therefore required modification to suit manipulation of such small quantities. There have been several reports of microtechniques applied to small volumes of plasma and serum [9—12]. We decided, however, to develop a micro version of the macro-procedure [6] which detailed a number of important improvements on previously described methodologies. The micro technique has been successfully developed [7] and gave reproducible results for an aliquot of standard solution containing 25 μg of each amino acid; R.S.D. values generally better than 1% were obtained. Furthermore, and in order to minimise errors during quantitation of recoveries, a standard of similar amino acid concentrations to those found in plasma was prepared and used for spiking plasma (see *Materials and reagents* for composition). Aliquots (25 μl) were derivatised and a typical chromatogram is illustrated (Fig. 1). Relative response factors for each amino acid were determined using pipecolic acid as internal standard. The R.S.D. values for all amino acids were better than 4% (Table I).

In view of the limitations of the rapid pre-chromatographic cation-exchange clean-up procedure [1], it was decided that it might be beneficial to obviate its use by identifying and removing specific interfering plasma components. We have demonstrated for the first time that the only major obstacle to successful quantitative analysis of amino acids in plasma was the presence of glucose [4], which resulted in the occurrence of several large chromatographic peaks (Figs.

TABLE I

REPRODUCIBILITY OF AMINO ACID RESPONSE FACTORS FOLLOWING DERIVATISATION OF 25-μl ALIQUOTS OF STANDARD SOLUTION

| Amino acid | $R_F$ (mean ± S.D.) * | R.S.D. (%) |
|---|---|---|
| Alanine | 1.056 ± 0.016 | 1.6 |
| Glycine | 1.000 ± 0.010 | 1.0 |
| Valine | 1.014 ± 0.010 | 1.0 |
| Threonine | 0.951 ± 0.011 | 1.1 |
| Serine | 0.923 ± 0.009 | 1.0 |
| Leucine | 0.999 ± 0.005 | 0.5 |
| Isoleucine | 0.973 ± 0.007 | 0.7 |
| Proline | 0.982 ± 0.003 | 0.4 |
| Internal standard | 1.000 | |
| Hydroxyproline | 0.902 ± 0.017 | 1.9 |
| Methionine | 1.350 ± 0.021 | 1.6 |
| Aspartic acid | 0.947 ± 0.004 | 0.4 |
| Phenylalanine | 0.944 ± 0.004 | 0.5 |
| Glutamic acid | 0.899 ± 0.011 | 1.2 |
| Lysine | 0.997 ± 0.004 | 0.5 |
| Tyrosine | 0.929 ± 0.006 | 0.7 |
| Arginine | 1.096 ± 0.028 | 2.6 |
| Histidine | 1.321 ± 0.044 | 3.3 |

*Mean of six separately derivatised standard samples (see *Materials and reagents* for composition).

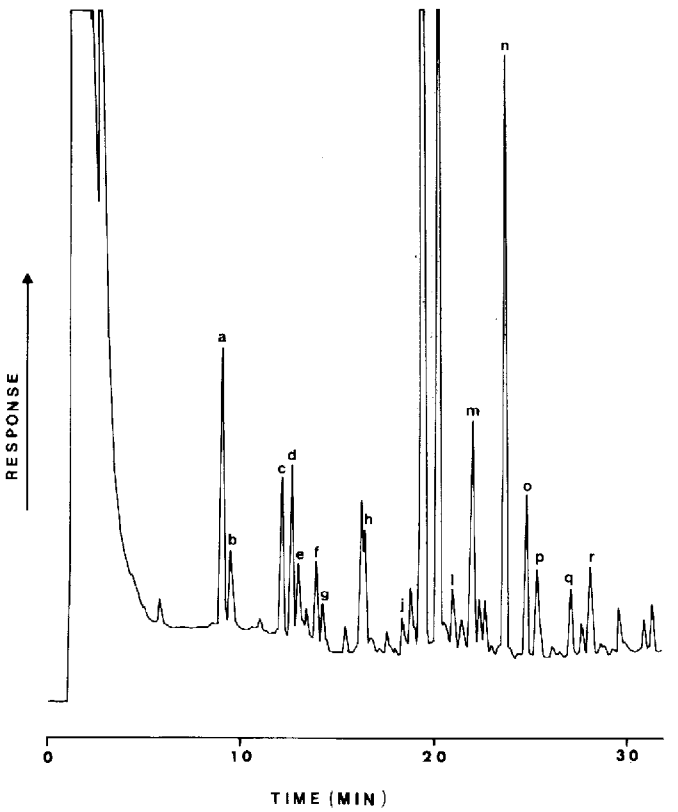


Fig. 4. Chromatogram of HBB derivatives of deproteinised plasma showing the glucose interference. Peak identification as in Fig. 1.

2—4) obliterating the region in which methionine and aspartic acid are found; hydroxyproline and phenylalanine also suffer considerable interference.

Various chemical procedures for the removal of glucose were investigated but found to be unsatisfactory. A novel approach to the clean-up process was perceived in the principle of using a specific enzyme technique. Glucose oxidase, the initial enzyme system investigated, produced gluconic acid during glucose removal. This approach could not, however, be used because of the interference of gluconic acid and the presence of a phosphate buffer which was responsible for major decomposition of the basic amino acids. The use of hexokinase to eliminate glucose was then considered. A procedure was developed in which a mixture of plasma, purified hexokinase [13] and the necessary co-factor ATP at pH 7.4 was incubated at 30°C to bring about the phosphorylation of glucose. Deproteinisation was then conveniently accomplished by addition of methanol. Centrifugation yielded a clear supernatant. After acidification and evaporating to dryness in vacuo, to remove methanol from the system, the residue was dissolved in dilute acid and extracted with chloroform. This latter step was found to contribute to reduction of small background peaks found in the subsequent glucose-free chromatograms (Fig. 5). However, a tailing interference in the region of, and sometimes overlayering, isoleucine appeared to be due to the presence of excess ATP.

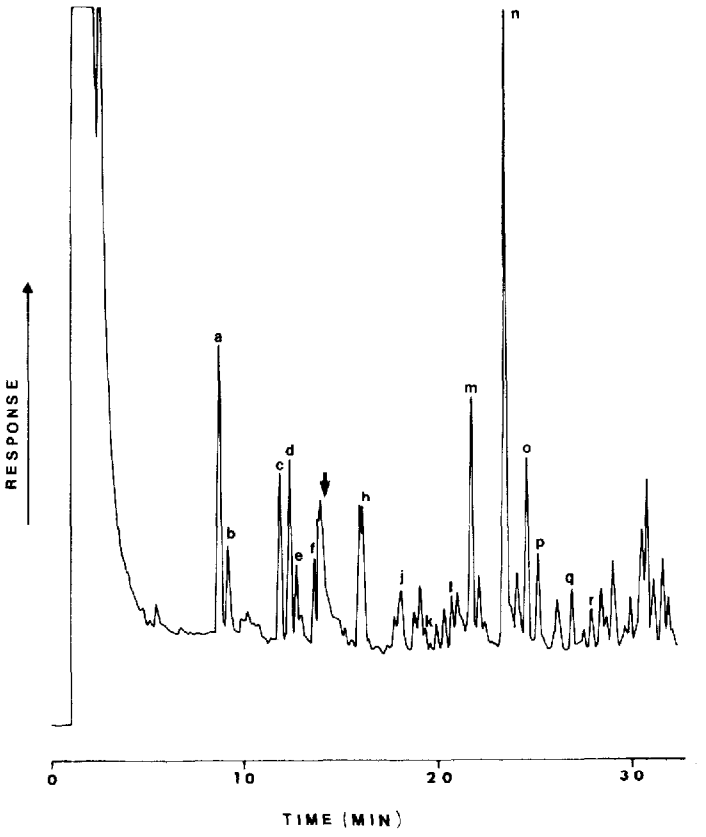


Fig. 5. Chromatogram of HBB derivatives of ATP/hexokinase treated plasma showing the absence of glucose interference and presence of ATP interference arrowed. Peak identification as in Fig. 1.

In order to study individually this and other effects in a plasma-like environment, several experiments were carried out using plasma which had been previously dialysed against Plasmalyte-B in order to remove low-molecular-weight components (e.g. amino acids). In this way, it was possible to confirm that, even when its level is optimised, ATP causes an interfering peak (Fig. 6) and leads to reduction of chromatographic response of the basic amino acids. An earlier practice of adding magnesium ion, a co-factor necessary for the phosphorylation, to the incubation mixture, over and above the level inherent in plasma, does not improve the efficiency of glucose removal but also leads to a similar deterioration of lysine, arginine and histidine. The use of additional magnesium was therefore discontinued. Apart from allowing confirmation of the ATP interference, this system also offered a valuable means of monitoring subsequent attempts to accomplish the removal of ATP. Furthermore, by adding a known amount of amino acid standard solution to dialysed amino acid-free plasma, any influence which this clean-up procedure might have on the ability to recover amino acid levels could be determined. Recoveries of added amino acids to dialysed plasma were greater than 90% for all amino acids except aspartate (77%) and histidine (120%).

Precipitation was considered the most convient means of ATP removal

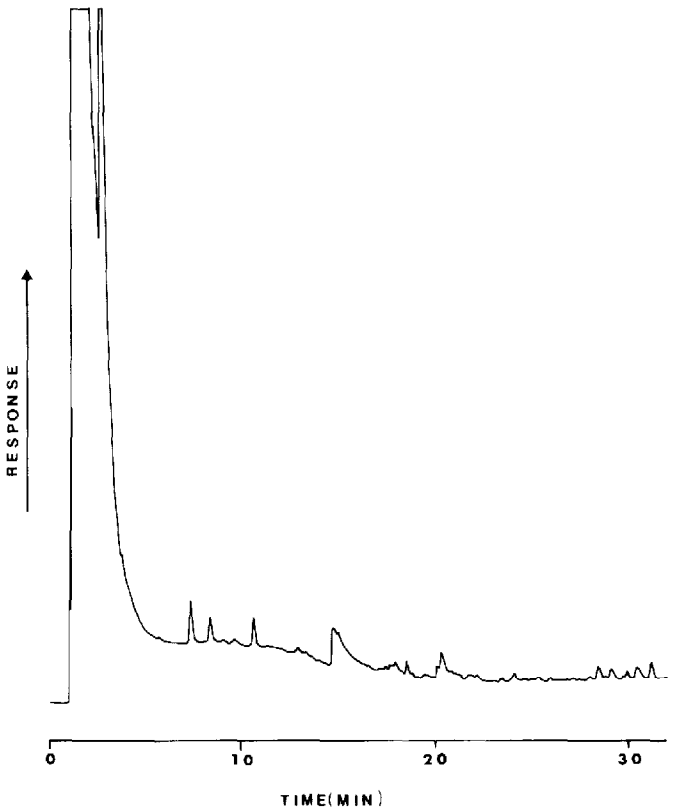


Fig. 6. Chromatogram of HBB-derivatised product from ATP/hexokinase treatment of dialysed plasma showing the ATP interference.

which could be integrated into the current methodology. ATP is insoluble in water-miscible organic solvents such as acetone and methanol. By using carefully controlled levels of the former in aqueous acid, ATP may be successfully rendered insoluble. Such a medium, however, appeared to be selective in its dissolution of amino acids and its further use was therefore discontinued. Use of barium salts in procedures for fractionating organic phosphates has been reported [14], ATP being amongst those which are precipitated at pH between 7 and 8. To test the efficiency of barium ion in the removal of ATP in the present context, dialysed plasma containing hexokinase and ATP was incubated at pH 7.4 and, after deproteinisation, a small excess of barium acetate was added to the methanolic solution. After removal of the precipitate, subsequent examination of the supernatant revealed absence of the tailing ATP interference (Fig. 7, cf. Figs. 5 and 6). We also showed that the product from reaction of hexokinase with glucose, namely glucose-6-phosphate, was also effectively removed by precipitation with barium ion (Fig. 8). The small spurious peaks observed (Fig. 7) were ignored since they did not coelute with any of the amino acids.

Quantitative data were then obtained so as to assess the reliability of the complete technique. Firstly, we established the minimum level of amino acids which, after derivatisation, led to acceptable reproducibility. Derivatisa-

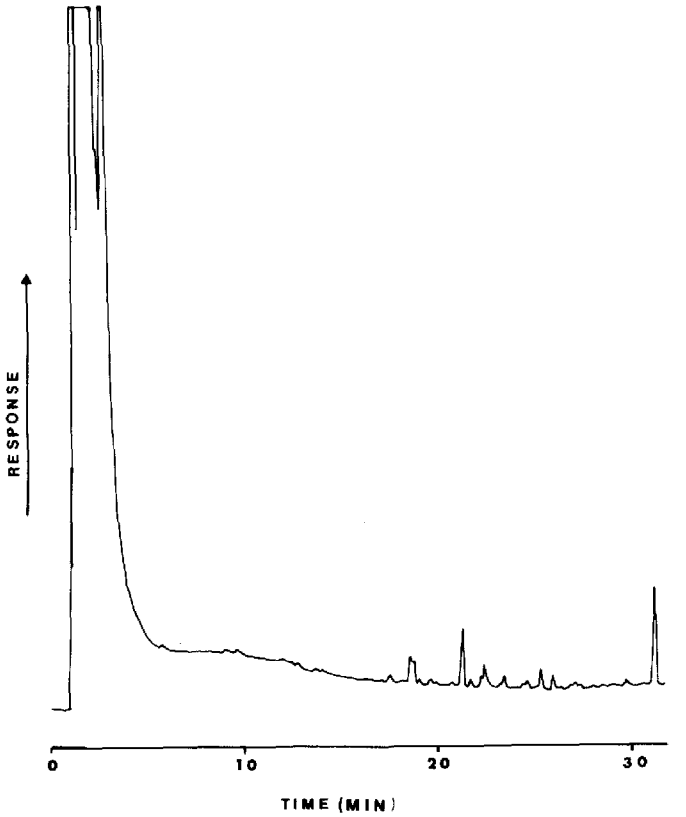


Fig. 7. Chromatogram of HBB-derivatised product from dialysed plasma following sequential treatment with ATP/hexokinase and barium acetate. ATP interference is absent.

tion of six aliquots of standard solution at each of four levels (i.e. 2.5, 5.0, 10 and 15 $\mu$g) yielded the data shown in Table II, which indicate that at or above levels of 2.5 $\mu$g of each amino acid in the sample R.S.D. values were below 5% with the exception of arginine at the 2.5- and 5.0-$\mu$g levels. Furthermore, only 77% of the expected arginine value was obtained at the 2.5-$\mu$g level. It has been reported [15], however, that this amino acid is particularly sensitive to derivatisation conditions. At these levels, the effect will be more pronounced and may be expected to result in reduced precision and accuracy.

The method was then tested on six spiked and unspiked aliquots of the same plasma sample. These were taken simultaneously through the procedure in order to evaluate reproducibility and provide recovery data under typical analytical conditions. Most R.S.D. values were below 5% and all except one, namely histidine, were less than 10% (Table III). Recoveries ranged from 90 to 120%.

Eighteen fasting plasma samples were obtained from healthy volunteers and analysed in duplicate using this procedure, the resulting normal values being compared with literature values (Table IV). In general, amino acid levels are in reasonably good agreement with the expected pattern as judged by previously published values [16, 17]. In addition, spiked samples were analysed and recoveries determined, mean values ranging from 85 to 122%.

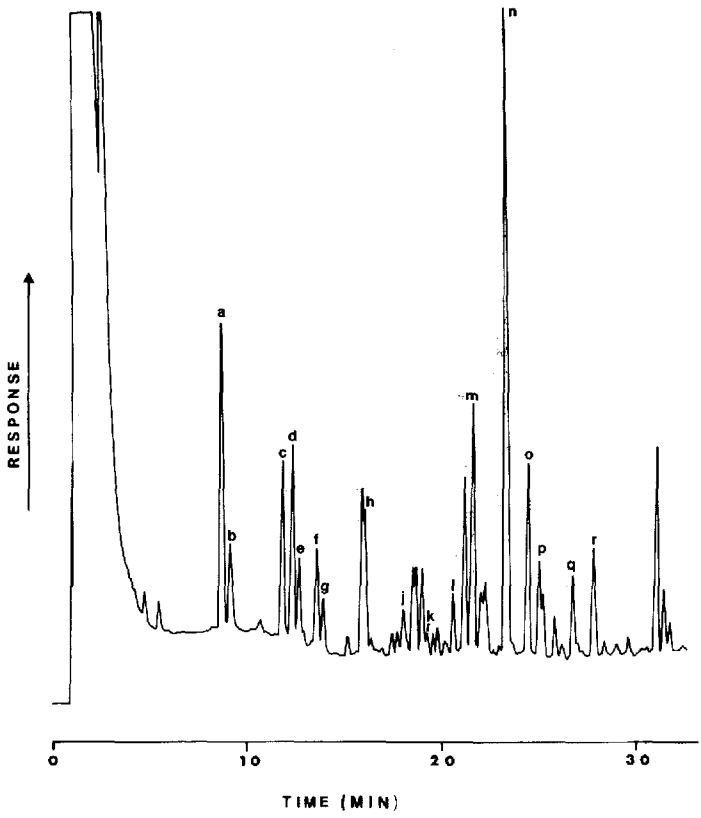


Fig. 8. Chromatogram of HBB derivatives of amino acids from plasma after sequential treatment with ATP/hexokinase, barium acetate and methanol followed by extraction with choroform. Peak identification as in Fig. 1.

Finally, ten plasma samples were analysed by GC and IEC in a parallel exercise, which was conducted blind, the sample code being revealed only on completion of the study. Results are compared in Table V. Values for tyrosine, histidine and phenylalanine only are significantly different ($p < 0.001$). The enhanced GC values for phenylalanine and tyrosine are attributed to insufficient resolution between the amino acid peaks and background components. No explanation was found for the disparity between histidine values. GC data are again similar to published plasma levels [16]. Lewis et al. [17] reported on a similar exercise in which seven plasma samples were analysed using IEC and GC, the latter involving different amino acid derivatives from the present study. Results for only ten amino acids (i.e. alanine, glycine, valine, threonine, serine, leucine, isoleucine, proline, phenylalanine and tyrosine) were compared and show closer agreement than that reflected in Table V. Furthermore, their observation that "values for leucine and serine were consistently higher by GC whereas values for glycine and tyrosine were lower by GC" is not supported by our data. However, in the same paper, values reported for fifteen amino acid levels (excluding arginine and histidine) derived from GC analyses of eighteen different fasting samples are in reasonable agreement with our data (Table IV).

TABLE II

REPRODUCIBILITY AND ACCURACY OF AMINO ACID DETERMINATIONS AFTER DERIVATISATION AT FOUR DIFFERENT LEVELS ($n$=6)

| Amino acid | Amount of each amino acid derivatised | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 2.5 μg | | 5.0 μg | | 10.0 μg | | 15.0 μg | |
| | Mean (μg) | R.S.D. (%) | Mean (μg) | R.S.D. (%) | Mean (μg) | R.S.D. (%) | Mean (μg) | R.S.D. (%) |
| Alanine | 2.22 | 4.1 | 4.76 | 1.3 | 9.78 | 2.3 | 14.99 | 0.5 |
| Glycine | 2.33 | 2.2 | 4.69 | 2.0 | 9.67 | 1.6 | 14.84 | 0.3 |
| Valine | 2.33 | 2.6 | 4.93 | 1.0 | 9.92 | 1.7 | 15.14 | 0.3 |
| Threonine | 2.47 | 1.1 | 5.14 | 0.8 | 10.24 | 1.1 | 15.38 | 0.7 |
| Serine | 2.50 | 0.8 | 4.98 | 0.5 | 10.06 | 1.3 | 15.25 | 0.8 |
| Leucine | 2.46 | 0.7 | 5.06 | 0.3 | 10.05 | 0.3 | 15.07 | 0.3 |
| Isoleucine | 2.43 | 1.5 | 4.93 | 0.5 | 9.87 | 0.7 | 15.01 | 0.5 |
| Proline | 2.50 | 0.2 | 4.99 | 0.2 | 9.96 | 0.4 | 14.91 | 0.2 |
| Hydroxyproline | 2.57 | 0.6 | 5.12 | 0.3 | 10.22 | 1.1 | 15.22 | 0.7 |
| Methionine | 2.50 | 1.1 | 5.07 | 1.5 | 10.02 | 1.5 | 15.38 | 2.5 |
| Aspartic acid | 2.56 | 0.5 | 5.08 | 0.1 | 10.11 | 1.4 | 15.12 | 0.4 |
| Phenylalanine | 2.55 | 1.5 | 5.13 | 0.4 | 10.16 | 1.0 | 15.20 | 1.2 |
| Glutamic acid | 2.57 | 1.1 | 5.10 | 1.2 | 10.14 | 1.5 | 15.09 | 0.7 |
| Lysine | 2.62 | 1.4 | 5.22 | 1.8 | 10.25 | 1.0 | 15.30 | 1.1 |
| Tyrosine | 2.59 | 0.8 | 5.09 | 1.1 | 10.14 | 1.2 | 15.17 | 0.8 |
| Arginine | 1.92 | 8.5 | 4.23 | 7.9 | 10.05 | 2.9 | 14.92 | 1.6 |
| Histidine | 2.20 | 4.0 | 4.94 | 3.9 | 10.62 | 3.1 | 16.02 | 2.6 |

TABLE III

AMINO ACID LEVELS IN UNSPIKED AND SPIKED LEVELS OF THE SAME PLASMA SAMPLE

| Amino acid | Unspiked sample | | Level added (μmol/l) | Spiked sample* | | Mean recovery (%) |
|---|---|---|---|---|---|---|
| | Mean level** (μmol/l) | R.S.D. (%) | | Mean level** (μmol/l) | R.S.D. (%) | |
| Alanine | 491 | 3.8 | 449 | 1012 | 2.5 | 116.0 |
| Glycine | 159 | 3.0 | 266 | 451 | 3.2 | 109.7 |
| Valine | 187 | 5.8 | 171 | 376 | 1.6 | 110.5 |
| Threonine | 166 | 3.7 | 168 | 345 | 2.9 | 106.6 |
| Serine | 87 | 3.4 | 38 | 133 | 3.9 | 121.1 |
| Leucine | 89 | 4.0 | 152 | 246 | 1.7 | 103.3 |
| Isoleucine | 45 | 4.2 | 152 | 196 | 1.1 | 99.3 |
| Proline | 164 | 3.6 | 174 | 369 | 1.6 | 117.8 |
| Hydroxyproline | 17 | 8.4 | 31 | 51 | 7.3 | 109.7 |
| Methionine | 18 | 7.0 | 27 | 44 | 8.5 | 96.3 |
| Aspartic acid | 42 | 7.4 | 30 | 69 | 4.1 | 90.0 |
| Phenylalanine | 140 | 3.3 | 121 | 268 | 2.8 | 105.8 |
| Glutamic acid | 396 | 3.6 | 272 | 673 | 1.4 | 101.8 |
| Lysine | 135 | 4.9 | 274 | 416 | 7.2 | 102.6 |
| Tyrosine | 58 | 8.0 | 110 | 167 | 3.8 | 99.1 |
| Arginine | 74 | 7.4 | 115 | 203 | 8.0 | 112.2 |
| Histidine | 106 | 10.9 | 129 | 259 | 8.1 | 118.6 |

*Aliquots (500 μl) of plasma were spiked with 25 μl of amino acid standard solution.
**Levels represent the mean of six separate analyses of the same sample.

TABLE IV

AMINO ACID VALUES (μmol/l) DETERMINED ON PLASMA FROM EIGHTEEN FASTING ADULTS

| Amino acid | Present study | | | | | Ref. 16* | | Ref. 17 (mean) |
|---|---|---|---|---|---|---|---|---|
| | Mean | Range | S.D. | Recovery (%) | | Mean | S.D. | |
| | | | | Mean | Range | | | |
| Alanine | 337 | 227—405 | 72 | 98.8 | 89.5—106.6 | 396 | 84 | 321 |
| Glycine | 197 | 112—270 | 47 | 100.2 | 88.5—111.0 | 268 | 99 | 166 |
| Valine | 215 | 159—314 | 45 | 99.4 | 88.3—120.4 | 230 | 31 | 216 |
| Threonine | 126 | 81—168 | 26 | 93.0 | 83.7—110.0 | 150 | 34 | 109 |
| Serine | 92 | 66—144 | 22 | 109.1 | 33.0—153.5 | 121 | 29 | 97 |
| Leucine | 120 | 85—188 | 27 | 95.9 | 84.9—110.5 | 141 | 26 | 112 |
| Isoleucine | 68 | 40—125 | 20 | 93.4 | 86.0—106.7 | 74 | 16 | 68 |
| Proline | 179 | 97—273 | 50 | 104.3 | 95.0—117.7 | 203 | 63 | 114 |
| Hydroxyproline | | | | | | 18 | 10 | |
| Methionine | 19 | 9— 29 | 6 | 122.3 | 93.5—156.0 | 29 | 5 | 29 |
| Aspartic acid | 45 | 35— 65 | 8 | 88.2 | 44.5—151.5 | 48** | 8 | 49 |
| Phenylalanine | 150 | 130—169 | 13 | 100.1 | 89.8—111.5 | 61 | 8 | 60§ |
| Glutamic acid | 481 | 396—627 | 60 | 85.2 | 49.3—118.2 | 665*** | | 229 |
| Lysine | 148 | 96—196 | 28 | 85.0 | 79.2— 97.5 | 191 | 31 | 150 |
| Tyrosine | 83 | 54—116 | 16 | 100.3 | 86.9—112.3 | 67 | 13 | 115 |
| Arginine | 69 | 42—116 | 22 | 93.8 | 55.2—119.4 | 82 | 24 | N.D.§§ |
| Histidine | 78 | 63—103 | 11 | 95.2 | 84.0—112.8 | 86 | 12 | N.D. |

*Values were calculated from those reported by Armstrong and Stave [16] for between 86—91 adult males and 93—103 adult females (fasting).
**Literature value reported for asparagine only.
***Literature value reported separately as glutamine and glutamic acid.
§This value for phenylalanine includes hydroxyproline.
§§N.D. = not determined.

TABLE V

PARALLEL COMPARISON OF AMINO ACID LEVELS (μmol/l) IN TEN PLASMA SAMPLES DETERMINED BY GC AND IEC

| Amino acid | GC | | | IEC | | | *P* | Ref. 16* | |
|---|---|---|---|---|---|---|---|---|---|
| | Mean | Range | S.D. | Mean | Range | S.D. | | Mean | S.D. |
| Alanine | 369 | 271—402 | 54 | 309 | 206—473 | 82 | | 396 | 84 |
| Glycine | 202 | 146—299 | 42 | 164 | 118—199 | 41 | | 268 | 99 |
| Valine | 227 | 154—346 | 56 | 177 | 125—245 | 37 | | 230 | 31 |
| Threonine | 142 | 107—202 | 29 | 125 | 80—169 | 31 | | 150 | 34 |
| Serine | 117 | 74—174 | 25 | 130 | 86—153 | 24 | | 121 | 29 |
| Leucine | 117 | 67—170 | 29 | 138 | 68—206 | 35 | | 141 | 26 |
| Isoleucine | 66 | 40—103 | 17 | 67 | 47— 97 | 18 | | 74 | 16 |
| Proline | 176 | 128—239 | 36 | 141 | 118—155 | 21 | | 203 | 63 |
| Hydroxyproline | 60 | 42— 91 | 15 | N.D.** | | | | 18 | 10 |
| Methionine | 28 | 16— 39 | 8 | 32 | 25— 36 | 4 | | 29 | 32 |
| Aspartic acid | 48 | 34— 59 | 8 | N.D. | | | | 48*** | 8 |
| Phenylalanine | 168 | 132—188 | 19 | 53 | 39— 63 | 7 | <0.001 | 61 | 8 |
| Glutamic acid | 475 | 333—605 | 75 | N.D. | | | | 665§ | |
| Lysine | 160 | 93—208 | 37 | 163 | 98—227 | 42 | | 191 | 31 |
| Tyrosine | 91 | 64—118 | 16 | 64 | 42— 88 | 14 | <0.001 | 67 | 13 |
| Arginine | 81 | 46—138 | 27 | 71 | 50— 88 | 16 | | 82 | 24 |
| Histidine | 109 | 78—133 | 19 | 52 | 39— 66 | 10 | <0.001 | 86 | 12 |

*Values were calculated from those reported by Armstrong and Stave [16].
**N.D. = not determined.
***Literature value reported for asparagine only.
§Literature value reported separately as glutamine and glutamic acid.

## CONCLUSION

A new pre-chromatographic clean-up procedure has been successfully developed and, coupled with packed column GC determination of amino acids, has been more comprehensively evaluated than similar previous methodologies. Resulting data from analyses of plasma amino acids were very reproducible (most R.S.D. values are below 5%) and the overall profile for fasting plasma samples reflects expected levels, as judged by previously reported data. Statistical comparison of results from a parallel GC and IEC exercise on ten plasma samples reveals that values for eleven of the fourteen amino acids do not differ significantly.

The new clean-up step, designed to remove specific interference, permits simultaneous handling of multiple samples which can be rapidly analysed on account of the short chromatographic and instrument turn-around time. The complete procedure is more reproducible than those previously published and can readily be adopted by most clinical laboratories.

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