Journal of Chromatography, 528 (1990) 9–23
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 5238

Amino acid analysis of physiological fluids by high-performance liquid chromatography with phenylisothiocyanate derivatization and comparison with ion-exchange chromatography

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(First received October 31st, 1989; revised manuscript received January 18th, 1990)

SUMMARY

The suitability of pre-column derivatization with phenylisothiocyanate followed by high-performance liquid chromatography was investigated as a means of analyzing free amino acids in plasma and other physiological fluids. A comparison was made between this method and a conventional ion-exchange method. The correlation coefficient for all the amino acids tested was greater than 0.9, except for proline and tryptophan. Various forms of sample preparation were tried for plasma and amniotic fluid; it was finally decided that protein precipitation with acetonitrile was most suitable. Ultrafiltration was used for cerebrospinal fluid preparation while urine was treated the same as a standard mixture. The retention times relative to the internal standard (nor-leucine) are given for over 90 compounds. Some of these were chromatographed underivatized because they are known to be present in some physiological fluids and absorb at 254 nm because of their aromaticity. The imprecision for this method compared favourably with the standard ion-exchange method although each had specific amino acids for which the imprecision was poor. The technique is suitable for the same routine clinical analysis purposes as high-resolution ion-exchange chromatography. It also offers the advantages of speed of analysis, sensitivity and equipment versatility over the conventional ion-exchange methods.

INTRODUCTION

In the past decade high-performance liquid chromatographic (HPLC) methods for derivatized amino acids have replaced cation-exchange chroma-

0378-4347/90/$03.50

tography with post-column detection for the analysis of protein hydrolysates as they offer reduced analysis times and high sensitivity [1]. They have not gained such wide acceptance for the analysis of amino acids in physiological fluids mainly because of the increased demands of sample preparation and chromatographic separation [2]. Additionally few derivatization reagents react with as wide a range of naturally occurring amino compounds as ninhydrin.

Pre-column derivatization of free amino acids in physiological fluids followed by reversed-phase HPLC has been described with *o*-phthalaldehyde, dansyl chloride, 4-dimethylaminoazobenzene-4-sulphonyl chloride, 4-fluoro-7-oxo-1,3-diazole [3,4] and phenylisothiocyanate (PITC) [1–6]. However, none of these methods have so far been adequately compared with the traditional ion-exchange method using post-column detection with ninhydrin [7], which is accepted as the standard by which any method proposed as an alternative must be judged [5].

Previous studies on phenylthiocarbamyl (PTC) derivatives of amino acids present in physiological fluids [1,2,8] did not include several clinically important compounds [3] and reported quantitative results for only a few amino acids [2,8].

The purpose of this investigation was (i) to characterise the chromatographic behaviour of a larger number of PTC derivatives of clinically important amino acids, (ii) to compare both the chromatographic resolution and the quality of quantitative data obtained with an established ion-exchange method and (iii) to assess its performance in the routine clinical laboratory.

EXPERIMENTAL

Reagents and materials

Amino compounds, organic solvents and other HPLC reagents were purchased from BDH (Dagenham, U.K.). PITC was obtained from Sigma (Poole, U.K.)

Bond Elut SCX (propylbenzenesulphonic acid bonded to silica, H^+ form) ion-exchange columns (100 mg) and a Vac Elut work station manufactured by Analytichem International (Harbor City, CA, U.S.A.) were purchased from Jones Chromatography (Hengoed, U.K.).

The Centrifree micropartition system for ultrafiltration of protein-containing samples was obtained from Millipore (U.K.) (Harrow, U.K.). All samples were evaporated to dryness in small glass tubes (50 mm × 6 mm, BDH) using a Waters Pico-Tag work station (Millipore, Waters Chromatography Division, Milford, MA, U.S.A.) and a JVAC vacuum pump (JAVAC (U.K.), Farnham, U.K.). Multistix were purchased from Ames Division, Miles Labs. (Slough, U.K.).

Sample preparation

Protein removal. For protein removal by ultrafiltration, 10 000 M_r cut-off membranes were used. Volumes of physiological fluid from 50 to 250 μl were diluted 1:1 with the internal standard (250 μM norleucine in 0.1 M hydrochloric acid) and centrifuged at 2200 g until at least 50 μl of filtrate was obtained. An aliquot was then taken and dried under vacuum ready for derivatization.

SCX columns were prepared for use by washing sequentially with 1 ml of 0.5 M hydrochloric acid, 1 ml of methanol, 2 ml of water and 1 ml of 0.05 M hydrochloric acid using a Vac Elut work station [9]. Plasma (100 μl) was added to an equal volume of internal standard and protein was precipitated by adding 10 mg solid sulphosalicylic acid or 800 μl of either acetone or methanol. An aliquot (50 μl) of plasma supernatant was then applied to the SCX column and unabsorbed material washed through with 2 ml water. The fraction containing the amino acids was then eluted with 2×500 μl of a reagent containing water–methanol–triethylamine (TEA) (2:2:1, v/v). This eluate was vacuum-dried in the usual manner. Three common organic solvents were tested as protein precipitants. These were methanol, acetone and acetonitrile. Protein-containing physiological fluid (50 μl) was added to an equal volume of internal standard followed by 400 μl of organic solvent. After mixing and settling the protein precipitate was centrifuged for 5 min and 125 μl of the clear supernatant were taken for analysis. In all cases it was possible to derivatize directly but mostly it was preferable to vacuum-dry first.

A 6-mm disc punched from blood dried out on filter paper [8] was wetted by adding 25 μl internal standard followed by 100 μl of acetonitrile to precipitate protein. After standing for 30 min, samples were centrifuged and 75 μl of the clear supernatant taken for derivatisation.

Urine samples were diluted in water to give creatinine of about 1 mM. Multistix were used to test for the presence of protein. If protein was present the urine was mixed with an equal volume of internal standard, ultrafiltered and 50 μl were taken for vacuum-drying. When no protein was detected 25 μl of urine and 25 μl of internal standard were directly dried. Underivatized urine was prepared by drying 25 μl in the usual manner.

Derivatization of amino acids with phenylisothiocyanate (PITC). Standards or sample preparations which had been evaporated to dryness were treated with 10 μl of a mixture of methanol–1 M sodium acetate–TEA (2:2:1, v/v) and the drying process was repeated under vacuum until a pressure of 70 mTorr was reached. This redrying step ensured that any residual acid was neutralized and that the alkaline pH which favoured derivatization was attained. The derivatization reagent consisted of methanol–TEA–water–PITC (7:1:1:1, v/v) and was freshly prepared each time. The dried sample was dissolved in 20 μl of derivatizing reagent and left to react for 20 min at ambient temperature. Ex-

cess reagent, TEA and other volatile products were then removed by evaporation under vacuum.

Derivatized samples could be stored dry at 4°C for 24 h or frozen at −20°C for a week or more. The derivatives were dissolved either in 50 μl [cerebrospinal fluid (CSF), blood spots] or 100 μl (plasma, amniotic fluid, urine) of a diluent composed of 5 m*M* sodium phosphate buffer (pH 7.4) containing 50 ml/l acetonitrile. Samples were transferred to limited-volume insert tubes suitable for use in the WISP 712 automatic sample injection system. Usually 20 μl of sample solution were injected onto the column.

Chromatography

Chromatographic separations were performed using a Waters Pico-Tag amino acid analysis system (Millipore Corporation, Waters Chromatography Division). This consisted of a temperature control module (set at 46°C), a Model 440 multi-wavelength absorbance detector with 254-nm filter, two Model 510 solvent delivery systems, a Model 680 automated gradient controller and an application-specified Pico-Tag free amino acid column (300 mm × 3.9 mm I.D.). Samples were injected onto the column using the WISP 712 sample processor. The data generated were processed on a Tandon computer using Drew Scientific Interface and Roseate Chromatography software (Drew Scientific, London, U.K.). Derivatized amino acids were eluted from the column using a gradient (Table I). The gradient was formed from eluent A (70 mmol sodium acetate, pH 6.55, and 25 ml acetonitrile per l) and eluent B (acetonitrile–methanol–water, 450:150:400, v/v). The eluent flow-rate was 1.0 ml/min. The UV detector was normally set at 0.1 absorbance units full scale.

TABLE I

GRADIENT CONDITIONS FOR THE HPLC SEPARATION

Time (min)	Eluent A (%)	Eluent B (%)	Curve No.[a]
Initial	100	0	Isocratic
13.5	97	3	11
24	94	6	8
30	91	9	5
50	66	34	6
62	66	34	6
62.5	0	100	6
66.5	0	100	6
67	100	0	6

[a]This refers to the optional gradient curves available on a Waters Model 680 gradient controller. Curve 11 is a step, curve 8 is slighly concave, curve 5 is slightly convex and curve 6 is linear.

Ion-exchange chromatography of free amino acids in physiological fluids was performed on a Hilger Chromaspek M amino acid analyser [10]. Sulphosalicylic acid was used for protein precipitation and norleucine was the internal standard. Amino acids were separated on a heated (42–56°C) column (350 mm×3 mm, cation-exchange resin, 7 μm, 8% DVB) in the Li^+ form using a pH gradient of 2.8–11.5. Post-column reaction was by heating (95°C) with strongly buffered (pH 6) reduced ninhydrin, and the derivatives were detected at 570 and 440 nm. Quantitative data were obtained with an Epson computer using Drew Scientific software.

Imprecision

A plasma pool was prepared by precipitation with acetonitrile as described above and the clear supernatant divided into aliquots of 125 μl. These were vacuum-dried and derivatized as before. The tubes were sealed with parafilm and stored dry at −20°C until needed. In a similar way a BDH amino acid calibration mixture at a concentration of 250 μ*M* was prepared.

Intra-assay imprecision was assessed both by using the control plasma which had a wide variation in amino acid concentration and the equimolar calibration mixture. The inter-assay imprecision was assessed by a control plasma preparation placed at random in each new batch of samples which were analysed.

Recovery

A plasma pool was prepared and sample preparation carried out by the acetonitrile method. As well as the base pool in duplicate three other duplicates were prepared by adding a concentrate of an amino acid standard mixture in order to raise the level by 50, 100 and 200 μ*M*, respectively.

RESULTS AND DISCUSSION

Sample preparation

Ultrafiltration was the recommended method for protein removal from biological fluids for analysis by the Pico-Tag system [6]. The method was suitable for some plasma samples but for the lipemic samples the time required to filter sufficient sample was very variable and occasionally no filtrate could be obtained, presumably because the membrane pores got blocked. Consequently other methods of sample preparation were examined.

A strong cation-exchange resin (Dowex 509-8X) had been used to partially purify physiological samples before forming derivatives with PITC [2]. As these resins need quite careful handling it was decided instead to use Bond Elut SCX which is a strong cation exchanger bonded to silica. It is possible to

apply proteinaceous material directly onto an SCX column but if the protein is removed beforehand the column can be reused several times. Methanol, acetone and sulphosalicylic acid were each suitable as protein precipitants and did not affect the performance of the SCX. It was critical to wash the SCX with dilute hydrochloric acid before applying the sample and to wash with sufficient eluting reagent (water–methanol–TEA) to ensure that all amino acids were displaced. Very acidic and neutral material did not adhere to the column but came straight through the initial wash. This meant that taurine, cysteic acid and phosphoethanolamine were not retained. The retention of aspartic acid by the SCX was also somewhat variable. All the other amino acids were readily retained and quantitatively removed with 500 μl of the eluting reagent except for arginine, which required an extra volume of the reagent for total removal. Samples prepared in this way could be derivatized directly but the time taken to dry a suitable volume of this dilute solution limited its value for routine use.

The organic solvents methanol, acetone and acetonitrile are commonly used protein precipitants. At neutral pH all of these work very well but under the acidic conditions necessary when dealing with free amino acids in a complex matrix, methanol and to some extent acetone were inefficient. Acetonitrile functions well under the acidic conditions and forms a firm pellet of protein precipitate, leaving clear supernatant. However, with a large excess of acetonitrile present, PITC reacts to form some derivative with a retention time similar to citrulline. This did not occur if acetonitrile was evaporated prior to derivatization and therefore this was the procedure routinely adopted for plasma-like fluids instead of ultrafiltration.

Chromatographic separation

Any reference to amino acid separation implies the PTC derivative unless otherwise stated.

A standard mixture of 37 PTC amino acids should be routinely resolved by a Waters Pico-Tag package [6]. This was verified on three columns with different batch numbers. A simpler mixture (Fig. 1A) relevant to clinical analysis

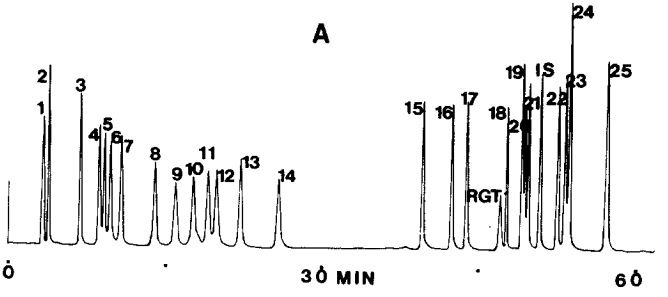

Fig. 1.

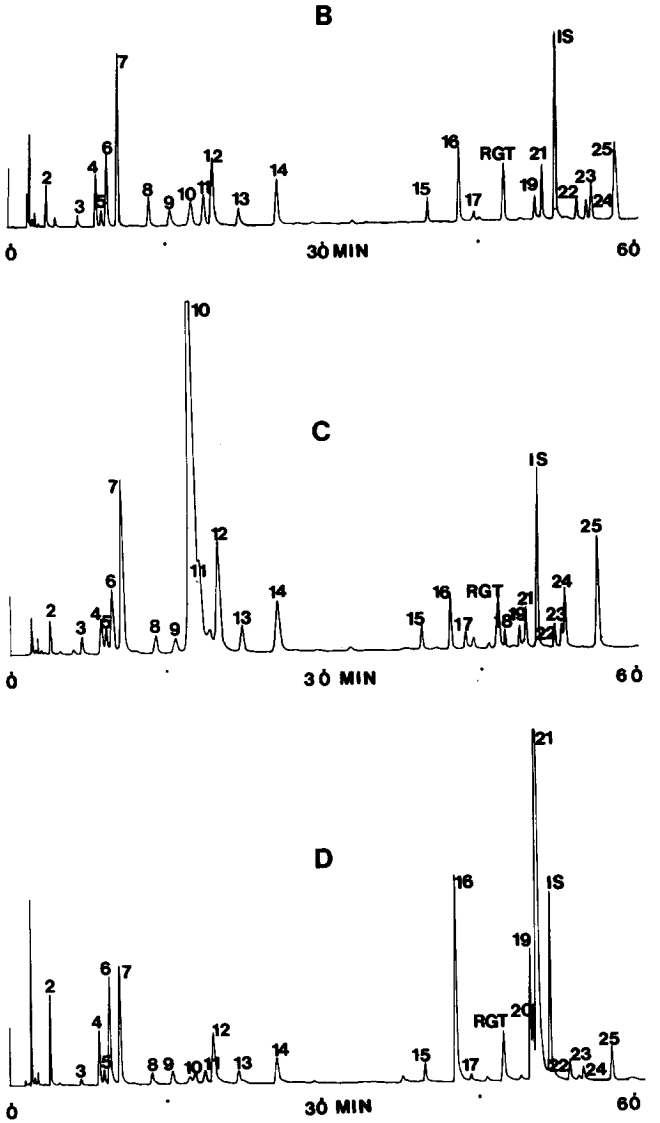

Fig. 1. Chromatographic separation of the amino acids of (A) 250 μM amino acid standard mixture, (B) normal human plasma (equivalent to 2.5 μl), (C) plasma from a subject with citrullinemia and (D) plasma from a subject with maple syrup urine disease (MSUD). Peaks: 1 = aspartic acid; 2 = glutamic acid; 3 = hydroxyproline; 4 = serine; 5 = asparagine; 6 = glycine; 7 = glutamine; 8 = taurine; 9 = histidine; 10 = citrulline; 11 = threonine; 12 = alanine; 13 = arginine; 14 = proline; 15 = tyrosine; 16 = valine; 17 = methionine, reagent I; 18 = cystine, reagent II; 19 = isoleucine; 20 = alloisoleucine; 21 = leucine, IS = internal standard, norleucine; 22 = phenylalanine; 23 = tryptophan; 24 = ornithine; 25 = lysine; RGT = reagent.

TABLE II

RELATIVE RETENTION TIMES FOR A SELECTION OF DERIVATIZED AND UNDERIVATIZED COMPOUNDS BY THE HPLC METHOD

Compound	Relative retention time	Compound	Relative retention time
Orotic acid[a]	0.039	Threonine	0.342
Uric acid[a]	0.039	Ampicillin II[b]	0.344
L-DOPA[a]	0.044	Alanine	0.361
Creatinine[a]	0.045	β-Aminoisobutyric acid	0.386
Homovanillic acid[a]	0.050	Carnosine	0.402
Phosphoserine	0.054	β-Amino-*n*-butyric acid	0.404
Cysteic acid	0.056	Arginine	0.419
Aspartic acid	0.061	Methionine sulfone	0.425
Glutamic acid	0.069	Proline	0.474
Thymine[a]	0.074	δ-Amino-*n*-valeric acid	0.485
Benzoic acid[a]	0.084	1-Methylhistidine	0.499
Arginosuccinic acid	0.087	Anserine	0.508
Nicotinamide[a]	0.091	Homocitrulline	0.514
α-Aminoadipic acid	0.095	3-Methylhistidine	0.515
Ampicillin I[b]	0.108	4-Aminobenzoic acid	0.533
4-Hydroxyproline	0.124	Ethanolamine	0.563
Hippuric acid[a]	0.129	Homoarginine	0.579
Phosphoethanolamine	0.131	Cysteine	0.610
Salicylic acid[a,b]	0.139	γ-Amino-*n*-butyric acid	0.618
Serine	0.158	Glutathionine (oxidized)	0.618
Galactosamine	0.163	L-DOPA	0.620
Aspartylglucosamine	0.164	Tris	0.626
Asparagine	0.165	4-Aminophenylacetic acid	0.700
Indole-3-acetic acid	0.165	4-Aminohippuric acid	0.733
Paracetamol[a,b]	0.168	Glycyltyrosine	0.739
Glycine	0.177	Tyrosine	0.768
Glucosamine	0.180	Valine	0.824
Glutamine	0.190	Methionine	0.853
2-Hydroxyhippuric acid[a]	0.200	3-Hydroxyanthranilic acid	0.862
β-Alanine	0.215	Cystathionine	0.885
Homoserine	0.220	3-Hydroxykynurenine	0.888
Sarcosine	0.220	Reagent I[c]	0.894
Glycylglycine	0.228	Ethylamine	0.913
Taurine	0.251	Ampicillin III[b]	0.915
Histidine	0.289	Cystine	0.923
Penicillamine I[b]	0.292	α-Aminophenylacetic acid	0.927
γ-Aminobutyric acid	0.302	Reagent II[c]	0.933
Ammonia	0.309	Glycylleucine	0.939
Citrulline	0.312	3-Amino-3-phenylpropionic acid	0.940
Glycylhistidine	0.331	Isoleucine	0.965

TABLE II (continued)

Compound	Relative retention time	Compound	Relative retention time
Alloisoleucine	0.970	Homocystine	1.029
Leucine	0.977	5-Hydroxylysine II[d]	1.032
Cysteine-homocysteine (mixed disulphide)	0.980	Phenylalanine	1.037
		Tryptophan	1.053
Penicillamine II[b]	0.981	Ornithine	1.061
Ethionine	0.984	Penicillamine IV[b]	1.061
Glycylphenylalanine	0.995	Ampicillin IV[d]	1.103
Norleucine (internal standard)	1.000	Lysine	1.142
Kynurenine	1.019	Ampicillin V[b]	1.172
Penicillamine III[b]	1.019	Serotonin	1.197
5-Hydroxylysine I[d]	1.020		

[a]Underivatized compounds.
[b]Drug or drug metabolite.
[c]Structure unknown.
[d]Stereoisomers.

was routinely used in this study supplemented with other compounds when appropriate.

The absolute retention time of the internal standard was 55.07 ± 0.14 min (mean ± 1 S.D., $n=14$) on a Pico-tag column after approximately 600 sample runs. A similar retention time was obtained with a new column. Retention times expressed relative to that of the internal standard were extremely stable. The S.D. for the relative retention times of 17 common amino acids ($n=14$) was less than 0.002 in all cases.

The relative retention times for a selection of compounds are listed in Table II. While not totally comprehensive it includes the amino acids commonly found in clinical specimens, drug preparations and some of their metabolites, compounds which may be dietary in origin and compounds which even if they do not form a PTC derivative still absorb at 254 nm. Table II also indicates the high resolving power of this chromatographic system; a difference in relative retention time of 0.005 is sufficient to separate two compounds.

Certain intermediary metabolites such as creatinine, phenylacetylglutamine, pipecolic acid and hippuric acid do not form PTC derivatives. Other compounds such as orotic acid, uric acid and salicyluric acid, which structurally look amenable to derivatization, do not seem to do so. Sarcosine forms an unstable derivative which decomposes to another product which elutes at a much later time in the chromatographic run. Some drugs and their metabolites (Table II), either through their aromatic character or due to conjugate formation, can produce substantial peaks on a chromatogram which can interfere

with some of the common amino acids. These are only very occasionally present in measurable amounts in plasma but can be a significant problem in urine analysis.

Some compounds which should definitely have formed PTC derivatives, such as 2-phenylethylamine and cadaverine (1,5-diaminopentane) were not detected on the chromatogram. It was assumed that they were sufficiently hydrophobic to be retained longer than lysine (Fig. 1) and probably chromatographed with the miscellaneous material eluted in the final column wash.

Cystine can be problematical, not only through its chemical instability but also because it elutes close to a reagent peak with which it may be confused. This is not exceptional as other reports with both pre- and post-column derivatization methods have mentioned difficulties in measuring cystine [3].

A chromatographic trace of a normal plasma is shown in Fig. 1B. In Fig. 1C a chromatographic trace of a plasma from a subject with citrullinemia is shown. In this case the citrulline concentration was greater than 3 mmol/l which is 100 times the concentration normally found in plasma. Fig. 1D shows a chromatographic trace of a plasma from a subject with maple syrup urine disease (MSUD). This is an example of the need for very good resolution, as the unusual amino acid L-*allo*-isoleucine elutes in a very tight space between isoleucine and leucine.

A chromatographic trace of the amino acids present in normal amniotic fluid is shown in Fig. 2A. This pattern is very similar to that of a normal plasma (Fig. 1B) except lysine is always present in higher concentrations than normally found in plasma. In Fig. 2B the pattern of amino acids present in CSF is shown. In this case the glutamine may be up to 600 times greater than the amino acid present at the lowest concentration which can be detected by this system. Fig. 2C shows a trace obtained from a chromatogram of a blood spot obtained from a subject with phenylketonuria (PKU).

Fig. 3 is a trace obtained from a chromatogram of urine obtained from a subject on high-dose paracetamol. The trace in Fig. 3A shows the pattern obtained with PTC derivatives and Fig. 3B is a trace obtained without derivatization except for the internal standard. Not all of the peaks are labelled because their identity has not yet been established. The peak marked X is present in Fig. 3B in the underivatized form whereas in Fig. 3A it has virtually disappeared and is replaced most likely by the derivatized peak Y. As shown by Fig. 3 it can sometimes be extremely difficult to interpret the early portion of a urine chromatogram and when this occurs it is essential to perform an underivatized run for comparison or isolate amino acids on an SCX column.

Other ways of fractionating urine samples before HPLC are being examined and will form part of a separate study.

Linearity and recovery

Individual amino acids gave a linear response over initial concentration up to 2 mmol/l. However, only those amino acids which are known to increase dramatically in pathological conditions were tested at the highest concentra-

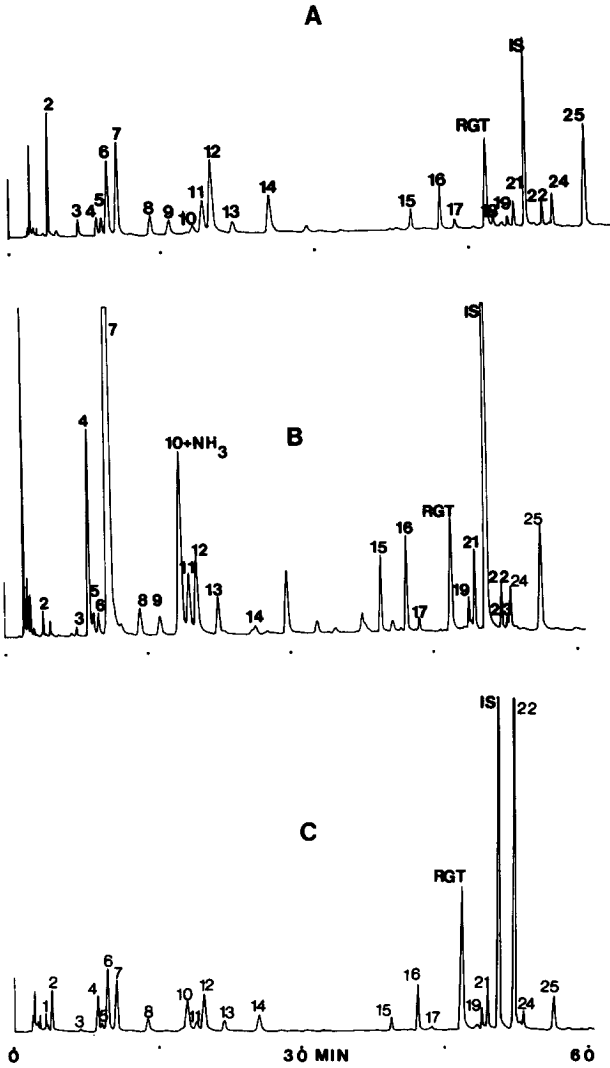

Fig. 2. Chromatographic separation of the amino acids from (A) normal amniotic fluid, (B) CSF from a subject with cerebral palsy and (C) an eluted blood spot from a PKU subject. Identification of the peaks is the same as for Fig. 1.

tion. The mean recovery for all the amino acids added to plasma was 89–117%. Only aspartic acid was consistently less than 90%.

Comparison with ion-exchange chromatography

Over a period of two months, 21 samples which had been quantitated by the ion-exchange method were simultaneously analysed by this HPLC method. The subjects from whom the samples were obtained are as follows: two with

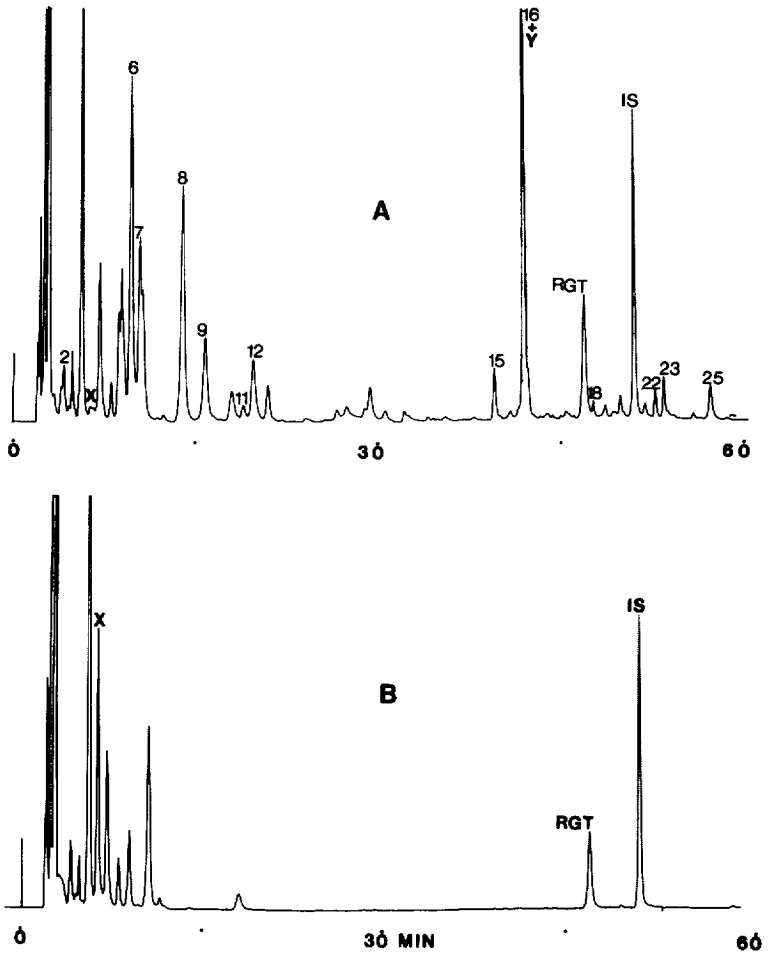

Fig. 3. Chromatographic separation of the amino acids in a urine from a subject on high-dose paracetamol: (A) PTC-derivatized; (B) underivatized except for internal standard. Peaks where identified are labelled the same as for Fig. 1.

methylmalonic acidemia, three with tyrosinemia, three with MSUD, five with urea cycle defects, one with propionic acidemia, two with PKU, one with non-ketotic hyperglycinemia, one with homocystinuria, one with Wilson's disease and two which were normal. These samples which are representative of the requests received by laboratories treating patients with inborn errors of metabolism were considered the most valid to use for a working method comparison. Because of the metabolic disorder and also due to dietary restriction or non-compliance, the concentration of many amino acids varied widely from sample to sample.

The intra- and inter-assay imprecision for the HPLC method and the intra-

assay imprecision for the ion-exchange method are given in Table III. Where the amino acids are of a fairly high and relatively similar concentration the precision is good as with a 250 μM standard mixture. The anomaly here is leucine, where the imprecision is exaggerated due to interference from the cysteine–homocysteine mixed disulfide (see Table II). It seems that PTC derivatives of these sulphur amino acids can rearrange even under low-temperature storage conditions to form the mixed disulphides. Consequently homocystine was omitted from the usual calibration mixture.

In a normal plasma sample there can be a 60-fold difference between the lowest and highest amino acid concentration which is usually measured. Con-

TABLE III

INTER- AND INTRA-ASSAY IMPRECISION FOR THE HPLC METHOD AND CORRELATION COEFFICIENTS FOR THE COMPARISON WITH AN ION-EXCHANGE METHOD

Amino acid	Coefficient of variation (%)				Correlation coefficient[a]
	Inter-assay	Intra-assay			
	HPLC ($n=18$)	HPLC ($n=11$)		Ion-exchange ($n=18$)	
	Control plasma	Control plasma	Standard 250 μM mixture	Control plasma	
Glutamic acid	10.87	1.08	1.3	1.22	0.910
Hydroxyproline	14.47	14.27	4.35	N.D.[b]	N.D.
Serine	4.0	2.25	2.1	1.23	0.955
Asparagine	4.87	3.11	2.34	8.13	N.D.
Glycine	4.76	4 79	3.66	1.43	0.980
Glutamine	5.37	3.5	2.27	8.02	0.964
Taurine	7.81	5.5	3.69	5.3	0.963
Histidine	N.D.	4.79	1.6	1.94	0.994
Citrulline	N.D.	15.7	5.0	18.7	N.D.
Threonine	5.3	1.73	2.04	2.55	0.989
Alanine	5.44	1 21	2.0	3.81	0.975
Arginine	5.79	3.24	2.19	7.4	0.992
Proline	5.87	5.5	3.74	7.9	0.885
Tyrosine	6.73	1.46	1.48	0.81	0.985
Valine	4.69	5.37	2.27	1.19	0.977
Methionine	12 23	20 51	3.57	4.55	0.997
Isoleucine	9.9	2.07	1.55	0.45	0.962
Leucine	6.96	0.5	9.92	0.43	0.992
Phenylalanine	5.97	1.47	4.54	1.44	0.898
Tryptophan	9.1	6.5	1.91	2 65	0.884
Ornithine	11.61	3.87	1.29	0.86	0.968
Lysine	5.57	1 41	0.95	1.48	0.994

[a]HPLC versus ion-exchange.
[b]N.D. = not determined.

sequently the imprecision will be variable as is shown in the case of hydroxyproline, citrulline and methionine. This is the real situation which confronts the clinical analyst and it is quite misleading to assume that a physiological fluid will behave in the same way as an equimolar standard mixture.

The results in Table III indicate that the performance of the HPLC system and the ion-exchange analyser are very similar for the majority of amino acids examined. Each method has different amino acids with poor precision. Apart from citrulline by both methods and methionine and ornithine by HPLC the imprecision is acceptable.

The correlation coefficient for all the amino acids tested was greater than 0.9 with the exception of proline and tryptophan (Table III). The reason for low correlation with proline may reside in the poor reaction with ninhydrin by the ion-exchange method; with tryptophan the reason may be partly due to the manner of sample preparation by both methods as this amino acid is well known to bind to proteins [11].

The chromatographic separation of PTC derivatives using the Pico-Tag system is comparable to the high-resolution ion-exchange procedures used for physiological fluids [3] but analysis cycle times are significantly shorter. While both profiles contain several areas of incomplete resolution [3] the amino acids involved vary with the method. Apart from the exceptions previously mentioned PITC reacts with the same group of amino compounds as ninhydrin with an advantage in detection sensitivity of at least ten-fold. This is considerably greater for non-α compounds such as hydroxyproline, β-aminoisobutyric acid and aspartylglucosamine which react poorly with ninhydrin whereas their PTC derivatives have molar responses equivalent to those for α-amino acids.

The technique is therefore suitable for the same routine clinical analysis purposes as high-resolution ion-exchange chromatography. While the capital equipment costs for either a dedicated ion-exchange amino acid analyser or an HPLC system are similar, the latter is more attractive to those with small workloads, as it can more readily be converted to the analysis of other groups of compounds. For those with high workloads, HPLC of PTC derivatives offers greater instrumental capacity and some reduction in cost per sample, due to the reduced analysis times. The higher sensitivity of HPLC of derivatized amino acids allows more efficient use of the small volumes of sample frequently presented to paediatric departments.

Amino acid derivatives which are fluorescent offer greater sensitivity by comparison to PITC [3] but the reagents either do not react with all relevant amino compounds or the derivatives are sufficiently unstable as to require sophisticated mechanisation in order to obtain quantitative data. The time for complete reaction with PITC is longer than for alternative reagents and the necessity for vacuum evaporation at two stages during the derivatization procedure further delays the analysis of a clinically urgent sample. The time from

reception to reporting for a single sample is similar to that for ion-exchange methods. Samples can, however, be conveniently prepared in batches of up to twenty without complex automation and stored frozen for over a week until analysed, which is attractive for routine application to large numbers of specimens.

Routine cleaning and repair to the proximal end of the column and correct equilibration of the column with mobile phase prior to analysis were found to be the two factors which most influenced maintenance of the high resolution.

ACKNOWLEDGEMENT

We are very grateful to Dr. J.W.T. Seakins for helpful advice and encouragement.

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