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Application of amino acid analysis by high-performance liquid chromatography with phenyl isothiocyanate derivatization to the rapid determination of free amino acids in biological samples

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ABSTRACT

An amino acid analysis by reversed-phase high-performance liquid chromatography after precolumn derivatization with phenyl isothiocyanate was adapted to the determination of free amino acids in plasma or other biological fluids and in tissue homogenates. Preparation of samples included deproteinization by 3% sulphosalicylic acid, and careful removal under high vacuum of residual phenyl isothiocyanate after derivatization. A Waters Pico-Tag column (15 cm long) was used, immersed in a water-bath at 38°C. In rat or human plasma, separation of 23 individual amino acids, plus the unresolved pair tryptophan and ornithine, was obtained within 13 min. Including the time for column washing and re-equilibration, samples could be chromatographed at 23-min intervals. Variability was tested for each amino acid by calculating the coefficients of variation of retention times (less than 1% in the average) and peak areas (less than 4% for both intra-day and inter-day determinations). The linearity for each standard amino acid was remarkable over the concentration range 3–50 nmol/ml. The mean recovery of amino acid standards added to plasma prior to derivatization was 97 ± 0.8%, except for aspartate (82%) and glutamate (81%). This method is rapid (almost three samples per hour can be analysed, more than in any other reported technique), with satisfactory precision, sensitivity and reproducibility. Therefore, it is well suited for routine analysis of free amino acids in both clinical and research work.

INTRODUCTION

Quantitative high-performance liquid chromatographic (HPLC) determination of amino acids from protein hydrolysates has been performed using precolumn derivatization with phenyl isothiocyanate (PITC), reversed-phase separation and UV detection (254 nm) of the resulting phenylthiocarbamyl derivatives [1–3]. This method has been proved to be sensitive and reproducible [4,5] and has been applied to the separation of free amino acids in physiological fluids and tissues [5–11]. For this purpose, most authors have been using amino acid analysis columns of 25–30 cm in length, requiring elution times of 60–80 min [5–7,9,11].

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This paper describes the application of this method to determination of free amino acids in biological fluids and tissue homogenates, suitable for rapid, accurate and reproducible routine analysis, using a standard 15-cm-long column. In rat or human plasma, 23 individual amino acids could be separated by this method within 13 min.

EXPERIMENTAL

Apparatus

Analyses were performed on a Waters gradient HPLC system (Waters Assoc., Milford, MA, USA), consisting of two Model 501 high-pressure pumps, a Model 680 automated gradient controller, a Model 7010 Rheodyne sample injector and a Model 740 computing integrator. A variable-wavelength spectrophotometer (LC-75 Perkin-Elmer) was used for UV detection.

Reagents

PITC and triethylamine (TEA) were from Pierce (Rockford, IL, USA). The amino acid standard solution and the individual crystalline amino acids were from Sigma (St. Louis, MO, USA). HPLC-grade solvents were used. High-purity water was produced with a Milli-Q purification system (Millipore, Bedford, MA, USA).

Sample preparation

Aliquots of plasma (or urine or ascitic fluid) were deproteinized by the addition of an equal volume of cold 6% sulphosalicylic acid (the final sample concentration was therefore close to that considered optimal for amino acid analysis [12]. Aliquots of deproteinized supernatant collected after centrifugation at 4°C (1800 *g* for 12 min) were used for derivatization. When an internal standard was used, the standard mixture of amino acids and the deproteinized samples were diluted 1:1 with methionine sulphone (0.4 m*M* in 0.1 *M* hydrochloric acid) before the derivatization step.

Liver fragments were homogenized in five volumes of ice-cold 3% sulphosalicylic acid; aliquots of the supernatant obtained by centrifugation (2000 *g* for 15 min) were used for derivatization.

Chromatographic procedure

To vacuum-dried deproteinized standards or samples were added 20 μl of ethanol–water–TEA (2:2:1, v/v) solution and vacuum-dried again. The derivatization was carried out on the dried residues with 20 μl of a 7:1:1:1 (v/v) solution of ethanol–TEA–water–PITC for 20 min at room temperature. The derivatization reagent was freshly made each time before use. After careful removal of the excess of reagents under vacuum (*ca.* 70 mTorr for 90–120 min), the chromatographic separation of the phenylthiocarbamyl amino acid derivatives, reconsti-

tuted in 250 μl of phosphate buffer (pH 7.4), was performed on a C_{18} Pico-Tag column (15 cm × 3.9 mm I.D.) immersed in a thermostatic water-bath at 38°C.

The elution solvent consisted of eluent A (0.14 *M* sodium acetate in water containing 0.5 ml/l TEA, titrated to pH 6.40 with glacial acetic acid, with the addition of 60 ml/l acetonitrile), and eluent B (60% acetonitrile in water). A gradient with a convex curve (Waters No. 5) was assessed, usually going from 2 to 46% of eluent B in 10 min (flow-rate 1.0 ml/min). After a washing step of 5 min with 100% eluent B, the column was re-equilibrated for 7 min with 98% eluent A. Including the time required for these steps, samples could be chromatographed at 23-min intervals.

RESULTS AND DISCUSSION

The elution profile of an amino standard containing 23 amino acids (Fig. 1) shows a good separation of each component. In addition, citrulline can be analysed under the same conditions, its peak appearing well resolved between those of histidine and taurine. A linear correlation between concentration and peak area was found for each amino acid, in the range 3–50 nmol/ml.

The reproducibility was determined by calculating the coefficient of variation (C.V.) of several analyses with respect to both retention time and peak area for each amino acid, according to the formule C.V. = (standard deviation/mean) × 100. The mean C.V. of the retention time was 0.49 ± 0.09% (n = 9) for an amino

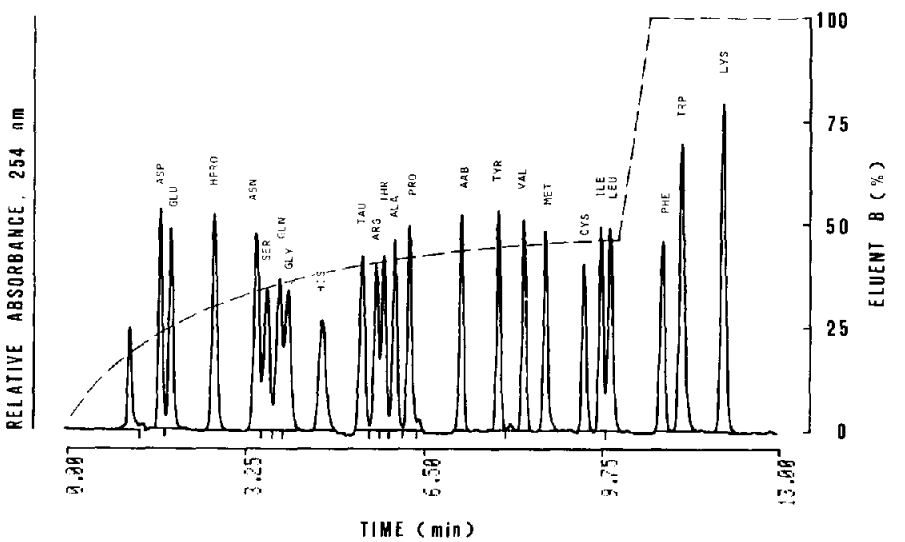

Fig. 1. Elution profile of standard mixture containing 23 amino acids by HPLC analysis using precolumn derivatization with phenyl isothiocyanate. Usually, 25 nmol of each amino acid were derivatized and 250 pmol chromatographed. The eluent gradient indicated by the dashed line was used, going from 2 to 46% of eluent B in 10 min, with a convex curve.

acid standard, and 0.73 ± 0.10% in another series of runs including standard (n = 4), plasma (n = 3) and liver homogenate (n = 2) samples. The mean C.V. of the peak area of an amino acid standard solution chromatographed either eight times on the same day or three times on different days was 3.54 ± 0.27 and 3.63 ± 0.20%, respectively.

The recovery of amino acid standards added to plasma before derivatization was good, with the partial exception of aspartate and glutamate (Table I). This test demonstrates that precolumn derivatization of sulphosalicylic acid supernatants is a reliable procedure, even if it remains a potential source of variability. Therefore, the addition of an internal standard, such as methionine sulphone, to both standards and samples prior to derivatization in recommended (Fig. 2A and B). The methionine sulphone peak appears after and well separated from the proline peak, thus making it possible to check the derivatization procedure.

As it can be seen in Fig. 2B, plasma analysis by this method allows the separation and quantitation of 23 amino acids plus the doublet tryptophan–ornithine. To determine the actual values of each of these two amino acids, separate quantitation of tryptophan by a fluorimetric method [13] should be performed, the ornithine concentration then being calculated by the subtraction of this value from the value of the doublet.

TABLE I

RECOVERY OF AMINO ACID STANDARDS ADDED TO PLASMA

Amino acid	Amino acid in sample (nmol)			Recovery (%)
	Plasma alone	Plasma + 6.25 nmol (added)	Plasma + 6.25 nmol (found)	
Asp	1.02	7.27	5.96	82.0
Glu	2.73	8.98	7.27	81.0
Ser	4.00	10.25	10.55	103.0
Gly	16.78	23.03	23.01	99.9
His	2.70	8.95	8.16	91.1
Arg	9.35	15.60	15.55	99.7
Thr	8.33	14.58	13.88	95.2
Ala	19.00	25.25	24.61	97.5
Pro	15.87	22.12	21.14	95.6
Tyr	6.24	12.49	11.91	95.3
Val	6.69	12.94	12.52	96.7
Met	4.43	10.68	9.90	92.8
Ile	2.68	8.93	8.77	98.3
Leu	4.80	11.05	10.99	99.4
Phe	2.99	9.24	9.06	98.0
Lys	16.35	22.60	22.41	99.2

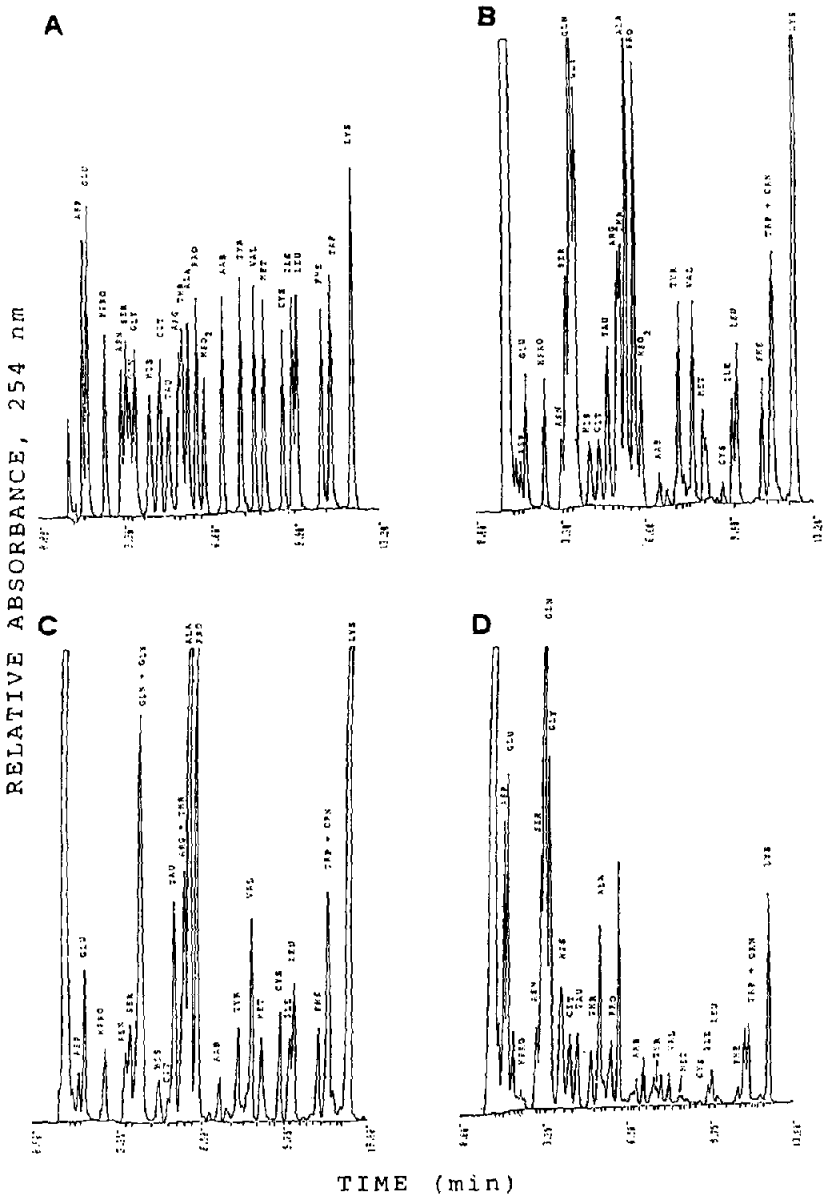

Fig. 2. Representative chromatographic profiles of amino acid PITC derivatives in a standard mixture and various biological samples. (A) Standard mixture of 24 amino acids to which the internal standard methionine sulphone (MSO_2) was added prior to derivatization. (B) Rat plasma sample with MSO_2 as an internal standard. (C) Ascitic fluid from rats with implanted Yoshida AH-130 ascite hepatoma cells, withdrawn seven days after implantation. (D) Rat liver homogenate.

A major advantage of the method described here is the short time required for analysis (12–13 min for the chromatographic run plus 10–11 additional minutes to make the column ready for the next analysis). This time compares favourably

TABLE II

FASTING PLASMA AMINO ACID CONCENTRATION IN HEALTHY SUBJECTS AND IN TYPE I DIABETIC PATIENTS WITH POOR METABOLIC CONTROL

Amino acid	Concentration (mean ± S.E.M.) (nmol/ml)	
	Controls (n = 4)	Diabetics (n = 7)
Asp	24 ± 5	13 ± 3
Glu	49 ± 7	70 ± 6
HPro	30 ± 7	34 ± 7
Asn	71 ± 1	68 ± 5
Ser	127 ± 23	123 ± 12
Gln	450 ± 31	405 ± 44
Gly	202 ± 14	198 ± 26
His	93 ± 9	75 ± 6
Tau	63 ± 6	60 ± 5
Arg	90 ± 12	85 ± 9
Thr	106 ± 9	115 ± 9
Ala	285 ± 14	284 ± 31
Pro	202 ± 17	167 ± 10
Tyr	64 ± 7	70 ± 5
Val	187 ± 18	277 ± 16[a]
Met	40 ± 5	35 ± 6
Cys	32 ± 3	68 ± 11
Ile	67 ± 5	97 ± 7[a]
Leu	106 ± 10	149 ± 10[a]
Phe	65 ± 12	67 ± 5
Lys	180 ± 10	199 ± 27

[a] $P < 0.05$ *versus* controls (Student's t-test).

with that required in other methods for HPLC separation of amino acids in biological fluids [3]. The length of the chromatographic run may vary slightly, according to small changes either in the temperature of analysis (± 0.5°C) or in the initial proportions of eluents A and B in the solvent mixture (± 1%). These adjustments are sometimes necessary to improve the separation of glutamine from serine. It should be emphasized that the critical points of this method are the separation of the doublets glutamine—serine and taurine–arginine. To obtain sufficient resolution of these pairs, good column efficiency is required. As the column deteriorates or ages, either the first or the second doublet is no longer separated into its individual components. In this event, however, it is usually possible to separate the unresolved doublet by rechromatographing the sample with suitable variation of the conditions of analysis, as indicated above.

We found that the column life was satisfactory, because several hundred chromatographic runs could usually be made on the same column, with the only major problem being that of the progressive worsening of the separation of one of

the two amino acid pairs discussed above. In this respect, we believe that great care in vacuum-drying of derivatized samples, to remove as much residual PITC as possible, is particularly beneficial.

The method described here has been successfully applied to the study of amino acid metabolism in diabetic patients [14] and in rats of various ages [15]. An example of the application of the method to the analysis of plasma amino acid concentrations in diabetics is given in Table II.

It has also been possible to separate quantitatively as many as twenty amino acids in other physiological or pathological fluids, such as urine or ascitic fluid (Fig. 2C), and in tissue homogenates, such as brain or liver (Fig. 2D).

This method of amino acid analysis appears to be particularly useful whenever a large number of samples must be analysed in short time, without any major loss in sensitivity and accuracy.

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