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Characterization of a reversed-phase high-performance liquid chromatographic system for the determination of blood amino acids

GIUSEPPE BUZZIGOLI, LAURA LANZONE, DEMETRIO CIOCIARO, SILVIA FRASCERRA, MAURIZIO CERRI, ANGELO SCANDROGLIO, ROBERTO COLDANI and ELEUTERIO FERRANNINI*

Metabolism Unit of the CNR Institute of Clinical Physiology and 2nd Medical Clinic, University of Pisa, Via Savi 8, 56100 Pisa (Italy)

SUMMARY

High-performance liquid chromatography was used to separate physiological amino acids in perchloric acid supernatants of blood samples. Precolumn derivatization with phenyl isothiocyanate was carried out, starting with 20 μl of supernatant; 2–10 μl were injected into a 30-cm Pico Tag column, which was eluted with a gradient of two eluents in 64 min. Stock amino acid solutions prepared in water, hydrochloric acid or perchloric acid showed comparable recoveries on serial dilution (parallelism test). The recovery of crystalline amino acids added to blood in amounts ranging from normal to six times normal was generally satisfactory. The within-assay relative standard deviations were $<5\%$ for many amino acids. The performance of the system was less than satisfactory for cysteine and methionine. Glutamine and asparagine are interconverted into glutamate and aspartate, respectively, in a time-dependent fashion; a separate measurement of one member of the pair is therefore required in order to assay the other starting from the sum of both chromatographic peaks. The method is suitable for the relatively rapid, sensitive and accurate measurement of blood amino acids in perchloric acid supernatants (in which other relevant metabolites are customarily assayed) over a wide range of physiological concentrations, on very small amounts of sample.

INTRODUCTION

In metabolic studies, the determination of blood amino acid concentrations, together with those of other metabolites (*e.g.*, glucose, lactate, pyruvate, β-hydroxybutyrate, glycerol) is often important[1]. Physiological amino acids can be assayed in plasma, serum or whole blood[2]. Although plasma values are more prevalent in the literature[3], whole-blood measurements are of prime interest because red blood cells actively participate in amino acid exchange between blood and tissues. Therefore,

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when assessing the role of an organ in amino acid metabolism with the use of the net balance technique, flow-rates and blood arteriovenous amino acid concentration gradients should be used to determine the true net organ balance[4,5]. Moreover, most of the metabolites of interest in physiological studies are also measured in whole blood. Obtaining measurements of amino acids and intermediate metabolites in the same pool therefore presents an obvious advantage. Metabolites are generally unstable at room temperature, and correct processing (including deproteinization) of blood samples is critical[6]. It should also be considered that experiments are often of long duration and, customarily, many blood samples are obtained at timed intervals. For this reason, important factors for amino acid analysis in metabolism studies are, in addition to reproducibility and sensitivity, the use of micro-methods (many parameters to be measured in the same sample) with a high level of automation (many samples to be assayed).

The classical approach to amino acid analysis is separation on a sulphonate cation-exchange resin, followed by derivatization with ninhydrin and spectrophotometric detection[7]. These methods are adequate but generally time consuming; further, they require substantial amounts of sample. The use of reversed-phase high-performance liquid chromatography (HPLC) permits amino acid determinations in a relatively short time, on small samples and with good sensitivity and specificity[8,9]. In this work, we selected a method that employs precolumn derivatization with phenyl isothiocyanate[10,11] and adapted it to blood samples deproteinized with chilled perchloric acid.

EXPERIMENTAL

Materials

Acetonitrile and methanol (LiChrosolv) were obtained from Merck (Darmstadt, F.R.G.). Sodium acetate trihydrate, disodium hydrogenphosphate, sodium dihydrogenphosphate, phosphoric acid, perchloric acid (PCA), ethylenediaminetetraacetic acid, disodium salt (EDTA) and hydrochloric acid (HCl) were of analytical-reagent grade, purchased from Merck (Darmstadt, F.R.G.). Triethylamine (TEA) and phenyl isothiocianate (PITC) were obtained from Pierce (Rockville, IL, U.S.A.). High-purity water was obtained by a Milli-Q purification system (Millipore, Bedford, MA, U.S.A.), which was fed with a supply of previously deionized reversed-osmosis-purified water. Basic (product N A-6282), neutral and acid (product N A-6407) physiological amino acid standard solutions were obtained from Sigma (St. Louis, MO, U.S.A.). These standards were mixed at the time of derivatization and used for calibration. Moreover, a set of standards (20, 50, 100, 286, 400 and 667 μM) was prepared by diluting Sigma standard solutions in water, and another set (20, 50, 100, 200, 400, 600 and 800 μM) by diluting the same Sigma standards in 0.1 M HCl or 0.5 M PCA. Individual crystalline amino acids (reference substances for chromatography obtained from Merck) were used to prepare three physiological standards in 0.5 M PCA. First, concentrated stock solutions in 0.1 M HCl of 20 single amino acids were made. Then, a six times normal physiological standard (6N) in 0.5 M PCA was obtained by means of appropriate dilutions (each amino acid was brought up to six times the basal, post-absorptive value of normal humans). In the same way, three times normal (3N) and normal (N) physiological standards were prepared (to cover a wide

range of possible final concentrations in actual blood samples in PCA). Derivatization tubes were Pirex brand (Corning, Corning, NY, U.S.A.); vacuum vials were purchased from Waters Assoc. (Milford, MA, U.S.A.).

Sample preparation and derivatization

Blood samples were deproteinized immediately after drawing blood by adding an equal volume of chilled 1 *M* PCA (the final sample concentration was therfore *ca.* 0.5 *M* PCA). The samples were spun at 1500 *g* at 4°C for 20 min in a refrigerated centrifuge. Appropriate aliquots of the supernatants were passed through 0.45-μm HV Millipore filters obtained from Waters Assoc. Volumes of 20 μl were used for derivatization, and dried under vacuum (*ca.* 80 mTorr for 30 min) by using a Pico Tag work station (Waters Assoc.). Dried residues were reconstituted with 20 μl of 1 *M* sodium acetate–methanol–TEA (2:2:1) solution. Samples were re-dried (*ca.* 80 mTorr for 15 min), and reconstituted in 20 μl of derivatization solution [methanol–water–TEA–PITC (7:1:1:1)]. A period of 20 min at room temperature was allowed for the reaction of PITC with amino groups (to produce phenylthiocarbamyl amino acid residues). The samples were then dried again until a constant vacuum of 60 mTorr was obtained (*ca.* 90 min). Finally, the dried samples were reconstituted with 200 μl of phosphate buffer (pH 7.40). After vortex mixing and sonicating for a few seconds, the samples were injected. The same derivatization scheme was applied to standard solutions.

Chromatographic system

The system utilized was purchased from Waters Assoc. and consisted of two M510 solvent-delivery pumps driven by an M680 automated gradient system controller. A Pico Tag column (30 cm × 3.9 mm I.D.) was employed. The column temperature was maintained at 46 ± 1°C by means of a column heater with a temperature control module. An M441 fixed-wavelength UV detector set at 254 nm was connected to a M745 recorder/integrator. Samples were injected in volumes ranging from 2 to 10 μl using an M712 Wisp autoinjector equipped with a Wisp cooling unit (samples were kept at 4°C). The mobile phase consisted of a gradient of two eluents, kept under a blanket of helium. Eluent A was an aqueous buffer of 0.07 *M* sodium acetate containing 2.5% acetonitrile and 1 ppm EDTA titrated to pH 6.50 with 10% glacial acetic acid. Eluent B was an organic phase consisting of acetonitrile–water–methanol (45:40:15). The gradient employed in the separation started with eluent B rising from 3 to 34% in *ca.* 64 min. After a washing step of 10 min with 100% B, the column was re-equilibrated for 20 min with 100% A. A constant flow-rate of 1 ml/min was maintained throughout.

Assay procedure

Each daily derivatization set consisted of ten unknown samples, two Sigma standards containing 38 amino acids, and two N "physiological" standards. The unknown samples were run after two perfect blanks, one Sigma (125 μmol/l) and one N physiological standard. The run was terminated with another Sigma and another N physiological standard. Peak reading was done by means of a one-point calibration external standard (Sigma). The control of the procedure in terms of accuracy and precision was obtained through the N physiological standards[12].

RESULTS

The separation of an N physiological standard in 0.5 *M* PCA is shown in Fig. 1. A typical separation of an amino acid calibration standard (125 pmol injected) is shown in Fig. 2. A representative separation of a blood sample is shown in Fig. 3. Data on the between-assay precision of the retention times are given in Table I.

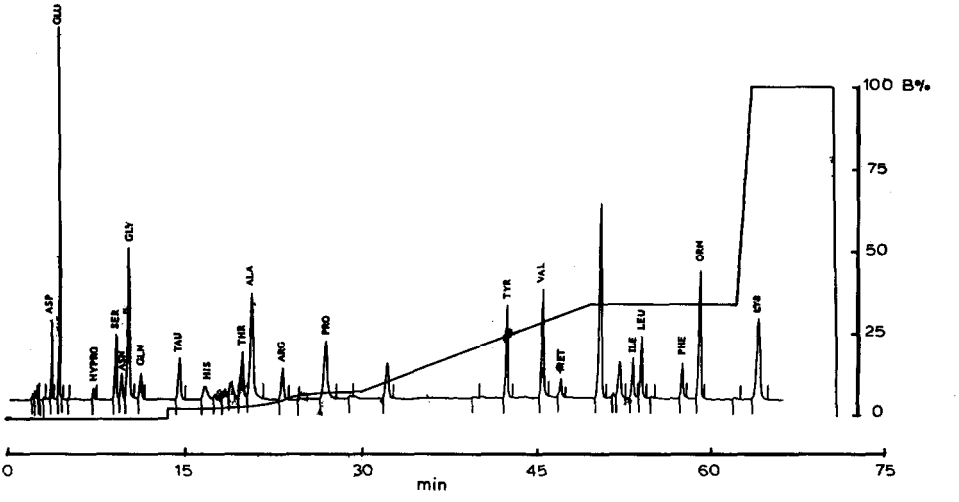

Fig. 1. Elution pattern of a mixture of physiological amino acids dissolved in 0.5 *M* PCA at concentrations close to those found in the blood of normal fasting subjects. Twenty amino acids were separated by using a gradient of two eluents: eluent A (aqueous buffer of 0.07 *M* sodium acetate containing 2.5% acetonitrile and 1 ppm EDTA titrated to pH 6.50 with glacial acetic acid) and eluent B [acetonitrile–methanol–water (45:40:15)]. The gradient is indicated as a percentage of eluent B. Flow-rate was constant at 1 ml/min.

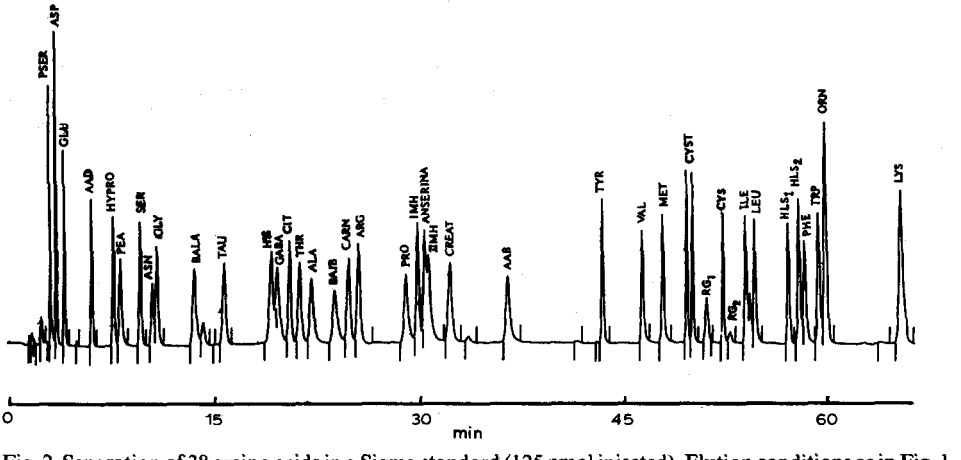

Fig. 2. Separation of 38 amino acids in a Sigma standard (125 pmol injected). Elution conditions as in Fig. 1.

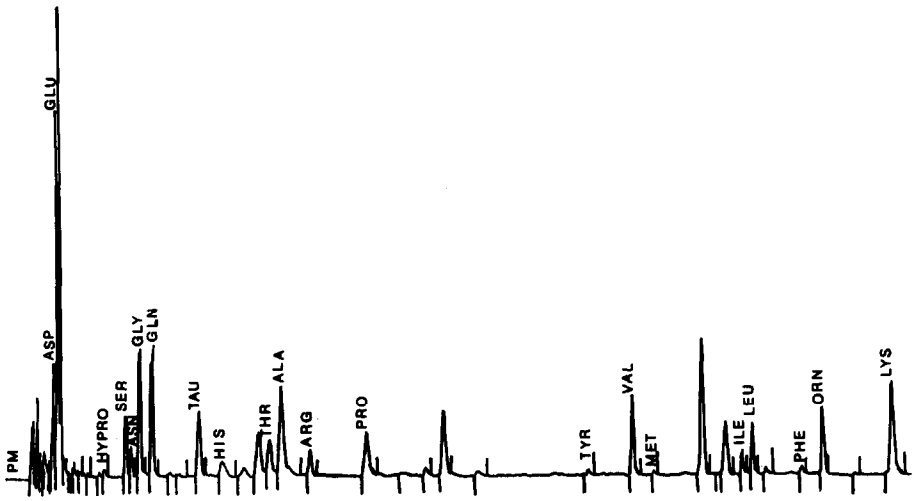

Fig. 3. Elution pattern of a blood sample obtained from a healthy subject after an overnight fast. Elution conditions as in Fig. 1.

TABLE I

RETENTION TIMES FOR VARIOUS AMINO ACIDS

Data from 30 chromatographic runs. C.V. = coefficient of variation.

Amino acid	*Retention time (min)*		*Amino acid*	*Retention time (min)*	
	Mean ± S.D.	*C.V. (%)*		*Mean ± S.D.*	*C.V. (%)*
Aspartic acid	3.48 ± 0.09	2.5	Alanine	20.93 ± 0.52	2.5
Glutamic acid	4.02 ± 0.12	3.0	Arginine	23.78 ± 1.05	4.4
Hydroxyproline	7.19 ± 0.20	2.8	Proline	27.25 ± 0.69	2.5
Serine	9.12 ± 0.21	2.3	Tyrosine	42.55 ± 0.30	0.7
Asparagine	9.67 ± 0.32	3.3	Valine	45.61 ± 0.25	0.6
Glycine	10.22 ± 0.26	2.5	Methionine	47.20 ± 0.24	0.5
Taurine	14.69 ± 0.38	2.6	Isoleucine	53.26 ± 0.28	0.5
Histidine	17.16 ± 1.06	6.2	Leucine	53.92 ± 0.28	0.5
GABA	17.71 ± 0.66	3.7	Phenylalanine	57.34 ± 0.43	0.8
Citrulline	18.48 ± 0.80	4.4	Ornitine	58.84 ± 0.50	0.9
Threonine	20.04 ± 0.52	2.6	Lysine	63.52 ± 0.95	1.5

Linearity of response in water, 0.1 M HCl and 0.5 M PCA

The use of an external standard prepared in 0.1 *M* HCl to be used against blood samples deproteinized with 0.5 *M* PCA made it necessary to check that the results were not affected by the use of different solvents. Using the solutions previously desribed, seventeen principal amino acids were measured in water, 0.1 *M* HCl or 0.5 *M* PCA (three measurements for each concentration point). No significant difference was found in either the intercepts or the regression coefficients between the three solvents: the correlation coefficients ranged from 0.963 (valine) to 0.999 (methionine) in water,

from 0.982 (phenylalanine) to 0.995 (glutamine) in 0.1 *M* HCl and from 0.977 (alanine) to 0.994 (isoleucine) in 0.5 *M* PCA. There were two exceptions. For cysteine, when run in either 0.1 *M* HCl or 0.5 *M* PCA, the slopes (0.418 and 0.379, respectively) were almost one third of those of the other amino acids, with intercepts of different sign, even though the correlation coefficients were almost identical (0.9413 and 0.9411, respectively). Methionine, on the other hand, had a very good pattern of recovery in water, with $r = 0.9985$, slope = 0.919, and an intercept not significantly different from zero; in 0.1 *M* HCl, these figures were $r = 0.9028$, slope = 0.692, with a positive intercept, and in 0.5 *M* PCA, $r = 0.891$, slope = 0.487, with a negative intercept.

Recovery under physiological conditions

Physiological amino acid standards (N, 3N and 6N) in 0.5 *M* PCA were run at least in quadruplicate for every concentration point to evaluate the recovery under conditions mimicking normal and pathological states (Table II). The recovery was also tested by adding the N physiological standard to a blood sample and then measuring the overall recovery (Table III). In addition, to determine the differential recovery along the chromatogram (the run takes *ca.* 64 min), hydroxyproline (retention time, $t_R = 7.19$ min), alanine ($t_R = 20.43$ min) and ornitine ($t_R = 58.84$ min) were added as crystalline amino acids to an unknown blood sample and their recoveries measured. The recoveries were 96, 102 and 87%, respectively.

TABLE II

RECOVERY OF VARIOUS AMINO ACIDS FROM PHYSIOLOGICAL STANDARDS IN 0.5 *M* PERCHLORIC ACID

Table entries are percent recoveries from standards having normal, 3 × normal, and 6 × normal amino acid concentrations.

Amino acid	*Recoveries (%)*	*Amino acid*	*Recoveries (%)*
Aspartic acid	69, 71, 73	Proline	93, 93, 97
Glutamic acid	93, 95, 97	Tyrosine	96, 122, 124
Hydroxyproline	91, 93, 94	Valine	93, 93, 96
Serine	96, 88, 93	Isoleucine	95, 93, 93
Glycine	94, 95, 101	Leucine	84, 99, 82
Taurine	97, 96, 100	Phenylalanine	92, 89, 93
Histidine	86, 84, 89	Ornitine	93, 89, 96
Threonine	92, 86, 91	Lysine	93, 99, 95
Alanine	91, 87, 93	γ-Aminobutyric acid	100, 102, 91
Arginine	86, 86, 91	Citrulline	94, 96, 103

Within-run and between-run precision

Forty duplicate unknowns were used to estimate the within-assay standard deviation (Table IV). The between-assay precision was determined from the N physiological standards included in every run. The results for 40 runs over a period of 6 months are given in Table IV.

TABLE III

RECOVERY OF VARIOUS AMINO ACIDS ADDED TO BLOOD SAMPLES

Amino acid	*Recovery (%)*	*Amino acid*	*Recovery (%)*
Aspartic acid	85	Arginine	104
Glutamic acid	107	Proline	101
Hydroxyproline	110	Tyrosine	97
Serine	102	Valine	102
Asparagine	104	Methionine	106
Glycine	109	Isoleucine	93
Taurine	101	Leucine	102
Histidine	102	Phenylalanine	98
Threonine	97	Ornitine	99
Alanine	100	Lysine	103

TABLE IV

WITHIN-ASSAY AND BETWEEN-ASSAY PRECISION

Amino acid	*Within-assay*			*Between-assay*		
	Mean ± S.D. (μM)		*C.V. (%)*	*Mean ± S.D. (μM)*		*C.V. (%)*
Aspartic acid	74	5	6.7	43	4	9.6
Glutamic acid	618	14	2.2	298	31	10.3
Hydroxyproline	12	3	25.2	16	14.0	
Serine	101	3	2.9	95	11	11.1
Asparagine	77	3	4.2	63	8	12.8
Glycine	251	8	3.2	325	25	7.8
Glutamine	1845	19	1.0	–	–	–
Histidine	63	9	14.6	52	8	15.4
Taurine	178	4	2.4	103	10	9.7
Threonine	89	8	9.4	126	15	11.5
Alanine	264	12	4.4	324	32	10.0
Arginine	52	2	4.2	72	7	9.8
Proline	156	5	3.4	206	16	7.7
Tyrosine	47	3	5.7	170	26	15.5
Valine	165	4	2.1	211	17	7.8
Methionine	11	2	20.5	40	6	15.2
Cysteine	331	13	4.0	–	–	–
Isoleucine	65	7	11.1	81	11	13.6
Leucine	80	3	4.0	106	12	11.5
Phenylalanine	38	5	12.8	72	8	10.6
Ornitine	62	2	3.4	137	16	11.5
Lysine	114	3	2.6	139	16	11.4

Stability and conservation of standards and samples

The stock standard solutions, when stored in PCA at −20°C, presented problems depending on the solvent used for dilution. In particular, tryptophan, cysteine and hydroxylysine disappeared over a short period of time, whereas tyrosine and phenylalanine were prone to degradation. It was possible to demonstrate that this

problem is related to the use of PCA on account of its acidic and oxidizing power. Once derivatized, samples are stable for at least 1 month if stored under vacuum at −20°C. When reconstituted in phosphate buffer (pH 7.4) after derivatization, samples are stable for at least 1 week if the autoinjector is used, because they stay protected and refrigerated. When stored at −20°C, no appreciable variations were observed over a 2-week period.

DISCUSSION

The method described is able to assay most physiological amino acids in an acidic supernatant with satisfactory accuracy, recovery and precision. The assay time (a total of 90 min for a complete run and subsequent column reconditioning) and sample volume (10 μl) are favourable. The stability of the sample after derivatization is satisfactory if a refrigerated autoinjector is used; the stability of samples reconstituted in phosphate buffer is also favourable.

There are several unresolved problems. First, it is impossible to measure tryptophan, which is unstable in 0.5 *M* PCA. Second, cysteine is lost from stored samples and methionine is assayed with insufficient accuracy. It is possible that pretreatment of samples with antioxidants will stabilize these two amino acids; however, this may distort the elution pattern of other amino acids. Third, in samples stored at −20°C, glutamine and asparagine are converted into glutamate and aspartate, respectively, in a time-dependent fashion. We attempted to overcome this problem by neutralizing samples with chilled potassium hydroxide immediately after deproteinization with PCA. Under these neutral pH conditions, glutamine and asparagine were in fact assayed, but aspartate and glutamate eluted with the eluent front and were lost. Therefore, this method is accurate only for the sums glutamine + glatamate and asparagine + aspartate. To obtain actual values, separate measurements of asparagine and glutamine (or aspartate and glutamate) by an enzymatic spectrophotometric method should be made; the values can then be subtracted from the sums determined by HPLC to obtain the concentration of the other member of the pair. In general, prompt treatment of blood samples with the deproteinizing agent and adequate storage of the resulting supernatants seem to be critical factors for the accuracy of the method. In fact, delayed deproteinization of plasma samples has been shown to change amino acid concentrations after only 30 min at room temperature[13]. Further, even storage at −20°C appears to lead to a decrease in the measurable levels of glutamine/glutamate and asparagine/aspartate[14]. We are currently evaluating the effect of storing immediately PCA-deproteinized supernatants at −20°C *vs.* −70°C.

The recovery and reproducibility data presented here refer to HPLC analyses run in the absence of internal standards. The results show that most physiological amino acids are assayed with a precision of $\leqslant 5\%$. Hydroxyproline and phenylalanine have a relative standard deviation of $> 10\%$, possibly on account of their low blood concentration. The pattern of dispersion of retention times (Table I) indicates that the inclusion of multiple internal standards may be necessary to improve the reproducibility of this chromatographic system. The recovery experiments (Tables II and III), however, suggest that variable recovery during the HPLC run may not be a major source of scatter. In a separate series of experiments, we found that the relative standard deviations for almost all amino acids decrease to 2–3% when quadruplicate

injections are made of the same sample after derivatization. This result indicates that at least half of the variability of the method may derive from the derivatization step. Accordingly, any internal standards would most suitably be added to the PCA solution before addition of blood specimens. We are currently testing the possibility of improving the precision of the method in this way.

In conclusion, the HPLC method described here demonstrates that blood amino acids can be assayed in PCA supernatants with high sensitivity and good accuracy, using very small volumes of material and in relatively short time. Precolumn derivatization of PCA supernatants remains a time-consuming (*ca.* 3 h) step, and a probable source of imprecision. Improvement of the precision seems to be feasible. Ultimately, nevertheless, the detection of small arteriovenous differences for some amino acids must rely on replicate measurements, visual selection of chromatographic peaks and statistical treatment of outlying observations.

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