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Analysis of plasma amino acids by HPLC with photodiode array and fluorescence detection

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Abstract

Background: Plasma amino acids are usually analyzed by ion-exchange chromatography (IEC), a reproducible but time consuming method. Here, we test whether plasma amino acids can be analyzed using reverse-phase high performance liquid chromatography (HPLC).
Methods: Filtered plasma, with *S*-carboxymethyl-L-cysteine as the internal standard, was derivatized and analyzed by an Agilent 1100 HPLC system. Primary amino acids were derivatized with *o*-phthalaldehyde 3-mercaptopropionic acid (OPA) and detected by a diode array detector. Secondary amino acids were derivatized with 9-fluorenylmethyl chloroformate (FMOC) and detected fluorometrically. Chromatographic separation is achieved by two gradient elutions (two injections per sample), starting at different pHs, on a reverse phase Agilent Zorbax Eclipse C_{18} column AAA (4.6×150 mm).
Results: The HPLC method evaluated correlated well with IEC ($0.89 \leq r \leq 1.00$) with linearity up to 2500 μmol/l. The between- and within-run CVs were <6.0%. In addition, this method is able to separate argininosuccinic acid, homocystine and allo-isoleucine, rare but clinically significant amino acids.
Conclusion: This HPLC method was comparable to IEC and could represent an alternative for amino acid analysis.

Keywords: Amino acid analysis; HPLC

1. Introduction

Analysis of amino acids in physiological fluids is extensively used for diagnosis and monitoring of inherited disorders of metabolism. The reference method for amino acid analysis is ion-exchange chromatography (IEC) with ninhydrin detection because of its ability to resolve in one analysis all clinically important amino acids, its precision and minimal sample preparation [1,2]. Several methods have been developed for amino acid analysis using high performance liquid chromatography (HPLC) [3–14]. They offer the advantage of shorter analysis time, but do not separate all the amino acids detectable by

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IEC. This is especially relevant when screening for metabolic disorders. For example, diagnosis and monitoring of therapy of inborn errors of homocysteine metabolism utilizes quantitation of plasma methionine, free homocystine and total homocysteine. While total plasma homocysteine requires a separate method for quantitation, methionine and free homocystine can be both detected and quantitated using IEC [15–18]. Nevertheless, existing HPLC methods can only quantitate methionine [3–6]. Allo-isoleucine, an isomer of leucine and isoleucine, detected in patients with maple syrup urine disease (MSUD) and pathognomonic for this disease, cannot be separated from isoleucine by conventional HPLC methods, but allo-isoleucine can be separated and accurately quantitated by IEC [19–21]. The purpose of this study was to develop an HPLC method that would be comparable to ion-exchange chromatography and useful for clinical applications. The combination of the two chromatographic separations, starting at two different pHs, described in this paper allows separation of physiological amino acids in plasma including free homocystine, allo-isoleucine and argininosuccinic acid when they are present in high concentration.

2. Materials and methods

2.1. Equipment

The analysis was performed using two identical Agilent 1100 HPLC systems (Agilent Technologies, Palo Alto, CA). Each system consisted of a binary pump, a photodiode array detector, a fluorescence detector and an autosampler. A reverse phase Agilent Zorbax Eclipse C_{18} column AAA (4.6×150 mm, 3.5 micron) was used for the chromatographic separation. Two Phenomenex C_{18} security guard columns (4.0×3.0 mm), from Phenomenex, Torrance, CA, were used to protect the column. Chemstation Plus Family for LC software was used for data acquisition and analysis.

Ion-exchange chromatography analyses were performed using a Biochrom 20 plus amino acid analyzer system (Biochrom, Cambridge, UK), equipped with a type 830 Midas autosampler, a high pressure PEEK column of Ultropac 8 cation-exchange lithium resin and a photometer unit. EZchrom Elite ver. 2.0 software was used for data acquisition and analysis.

2.2. Chemicals

Acidic and neutral, basic (with tryptophan) and basic (with homocystine) physiological amino acid standards solutions were obtained from Sigma Chemical, St. Louis, MO. Glutamine, argininosuccinic acid, *S*-carboxymethyl-L-cysteine, *S*-(2-aminoethyl)-L-cysteine, sodium phosphate monobasic and 5-sulfosalicylic acid were also from Sigma. 9-Fluorenylmethyl chloroformate (FMOC), *o*-phthalaldehyde 3-mercaptopropionic acid (OPA-3MPA) and borate buffer were from Agilent. OPA-3MPA was stored at 2–8 °C in small vials crimped with silicon rubber, PTFE-coated cap [9]. A fresh aliquot of OPA was used for each set of samples [3,5]. All other solvents were HPLC grade from Fisher Scientific, Pittsburgh, PA. The mobile phases, A1 (40 mmol/l Na_2HPO_4 at pH 7.8), A2 (40 mmol/l Na_2HPO_4 at pH 6.8), B (45% acetonitrile, 45% methanol, 10% water) and were filtered through a 0.22-micron Millipore Durapore PVDF membrane filter. To prevent algae growth in mobile phase, sodium azide (5 mg/l) was added.

A mixture of acidic, neutral and basic (with tryptophan) amino acids standard was used as calibrator for the separation with mobile phase A1 (pH=7.8). A mixture of acidic, neutral and basic (with homocystine) amino acids standard was used as calibrator for the separation with mobile phase A2 (pH=6.8). The amino acids concentrations of the two standard mixtures were identical. Glutamine and internal standard (*S*-carboxymethyl-L-cysteine) were added to both standard mixtures.

Lithium buffers (buffer A pH 2.80, 0.20 mol/l Li^+; buffer B pH 3.00, 0.30 mol/l Li^+; buffer CII pH 3.15, 0.50 mol/l Li^+; buffer DII pH 3.50, 0.90 mol/l Li^+; buffer pH 3.55, 1.65 mol/l Li^+; lithium hydroxide solution, 0.30 M), ninhydrin kit and lithium citrate loading buffer pH 2.2 were from Biochrom, Cambridge, UK and used for ion-exchange chromatography.

2.3. Sample preparation for reverse phase chromatography

Twenty microliters of internal standard (500 μmol/l *S*-carboxymethyl-L-cysteine) were added to 200 μl of plasma. The mixture was deproteinized by ultrafiltration, using millipore Ultrafree-MC 10,000 NMWL filters, at 20,800×*g* for 15 min 10 °C [12]. The filtrate

Table 1
Injector program

Injector program for mobile phase A1 (pH7.8) and B			
Line	Function	Amount	Reagent
1	borate buffer		
2	draw	0.5 µl	sample
3	mix	3.0 µl in air, 400 µl/min speed, 2 times	
4	wait	0.50 min	
5	draw	0.0 µl	water—needle wash, use uncapped vial
6	draw	1.0 µl	OPA-3MPA
7	mix	3.5 µl in air, 400 µl/min speed, 6 times	
8	draw	0.0 µl	water—needle wash, use uncapped vial
9[a]	draw	0.5 µl	FMOC
10[a]	mix	4.0 µl in air, 400 µl/min speed, 6 times	
11	draw	32.0 µl	water—use capped vial
12	mix	18.0 µl in air, 400 µl/min speed, 2 times	
13	inject	(0.5 µl)	

[a] Omit this step for injector program with mobile phase A2 (pH=6.8) and B.

was transferred into vials and placed in the refrigerated autosampler of the HPLC system at 2–8 °C. Automatic pre-column derivatization with OPA-3MPA and FMOC was performed at room temperature, according to the injector programs listed in Table 1, using 1 µl of filtrate [4]. After the derivatization, 0.5 µl of the mixture were injected for each chromatographic separation. Primary amino acids were derivatized with OPA-3MPA and detected by photodiode array UV detector at 338 nm, with a reference=390 nm, band width=10, slit of 4 nm, peak width of >0.1 min. Hydroxyproline and proline were derivatized by adding FMOC to OPA-3MPA (Table 1) and detected by fluorescence detector with excitation=266 nm, emission=305 nm and PMT gain of 10.

2.4. Sample preparation for ion-exchange chromatography

Two hundred microliters of plasma were deproteinized with an equal volume of 10% (w/v) sulfosalicylic acid and centrifuged at 20,800×*g* for 3 min at room temperature. Fifty microliters of internal standard (500 µmol/l *S*-(2-aminoethyl)-L-cysteine) were added to 200 µl of supernatant and 250 µl of loading buffer pH 2.2. Fifty microliters of this mixture were injected into the amino acid analyzer.

2.5. Method for reverse phase chromatography

The two chromatographic separations were obtained using two gradient elutions (Table 2) and a column temperature of 40 °C. Each sample was injected on two separate HPLC systems and each HPLC system was set to run one chromatographic separation, starting with either mobile phase A1 (pH 7.8±0.05) or A2 (pH 6.8±0.05). Column conditioning before analysis is a critical step to obtain good recovery and separation of the amino acids. Each column was initially conditioned as follows: mobile phase B was pumped for 25 min at a flow rate of 2 ml/min, followed by 15

Table 2
Mobile phases and gradient conditions

Gradient for pH 7.8		
Time	Mobile phase A1, 40 mmol/l Na_2HPO_4, pH 7.8	Mobile phase B, 45% acetonitrile, 45% methanol, 10% water
0.00	100.0	0.0
1.90	100.0	0.0
8.00	79.7	21.3
19.30	44.8	55.2
19.80	0.0	100.0
23.50	0.0	100.0
24.40	100.0	0.0
25.20	100.0	0.0

Gradient for pH 6.8		
Time	Mobile phase A2, 40 mmol/l Na_2HPO_4, pH 6.8	Mobile phase B, 45% acetonitrile, 45% methanol, 10% water
0.00	100.0	0.0
1.90	100.0	0.0
8.00	79.7	21.3
15.50	56.2	43.8
16.00	0.0	100.0
19.70	0.0	100.0
20.60	100.0	0.0
21.40	100.0	0.0

min of 10% methanol at 1.0 ml/min and 20 min of mobile phase A1 (pH 7.8) or A2 (at pH 6.8) at 2 ml/min. The flow rate was kept at 2.0 ml/min throughout the analysis. The run time, injection to injection, was 32 min for mobile phase A1 (pH=7.8) and 27 min for mobile phase A2 (pH=6.8). In order to maintain column integrity, a mixture of 90% isopropanol and 10% methanol was run at the end of each set of samples for 30 min and/or every 10–15 injections. This column washing step was necessary for both chromatographic separations.

The linearity and accuracy of the method were assessed using seven different concentrations between 5 and 2500 μmol/l (2.5–1250 μmol/l for cystine and homocystine) of amino acid standard mixtures, injected in duplicate. Linearity and accuracy were evaluated with EP Evaluator Release 4-CLIA software (David G. Rhoads Associates, Kennett Square, PA).

Aliquots of normal plasma (nine parts) spiked with 2500 μmol/l amino acid standards (one part), kept at −70 °C until analysis, were used to evaluate the within-run and between-run imprecision. Within-run imprecision was determined using replicate analyses of eight spiked plasma aliquots run with both chromatographic separations. Cystine, taurine, hydroxyproline and proline were determined using the chromatographic separation starting with mobile phase A1 (pH 7.8). The other amino acids studied were determined using the chromatographic separation starting with mobile phase A2 (pH 6.8). Linearity, between-run imprecision and comparison studies were done under the same conditions.

To evaluate between-run imprecision, aliquots of the same spiked plasma were injected in different days for a total of 23 analyses over a 1-month period, on two HPLC instruments with both mobile phases. Two medical technologists performed these analyses using three chromatographic columns.

Surplus plasma samples, obtained from heparinized blood and submitted for amino acid analysis, were de-identified according to a protocol approved by the Institutional Review Board of the University of Utah Health Science Center, aliquoted, stored at −70 °C until analysis and used for methods comparison. Results were evaluated using EP Evaluator Release 4-CLIA software.

2.6. Method for ion-exchange chromatography

Amino acids were separated by varying temperature, ionic strength and pH according to the instrument manufacturer recommendation. The amino acids eluted were mixed in a high temperature reaction coil with ninhydrin to form colored compounds. The color produced was measured photometrically at 570 nm (amino acids) and 440 nm (imino acids). The run time, injection to injection, for each sample was 130 min.

3. Results and discussion

Fig. 1 shows the separation of amino acid standards with mobile phase A1 starting at pH 7.8. Separation of taurine and tryptophan can only be achieved with this mobile phase. FMOC was added to OPA in the derivatization mixture to detect and quantitate hydroxyproline and proline using fluorescence detector [5] (Fig. 1b). Hydroxyproline, proline and taurine are separated and quantitated with mobile phase A1 (pH 7.8). Cystine was separated with either mobile phases (A1 or A2), but baseline separation was obtained only with mobile phase A1 (pH 7.8). This is why we have chosen to quantitate cystine using mobile phase A1.

Fig. 2 shows the separation of amino acid standards with mobile phase A2 starting at pH 6.8. Separation and quantitation of alanine and phenylalanine can only be achieved with this mobile phase. Lysine and ornithine can be separated with both pHs (7.8 and 6.8), but since their recovery is affected by FMOC; they are quantitated with mobile phase A2 (at pH 6.8) where FMOC is not added. Homocystine and argininosuccinic acid are also identified with mobile phase A2 (pH 6.8).

All other amino acids studied can be separated and quantitated with either mobile phases A1 (pH 7.8) or A2 (pH 6.8). Although most of the amino acids separations are not at baseline, comparison data with IEC (Table 4) show that this level of separation is sufficient for accurate quantitation. The method performance for both chromatographic separations is comparable. The use of two mobile phases with different pHs facilitates the identification of rare, but clinically significant amino acids, such as free

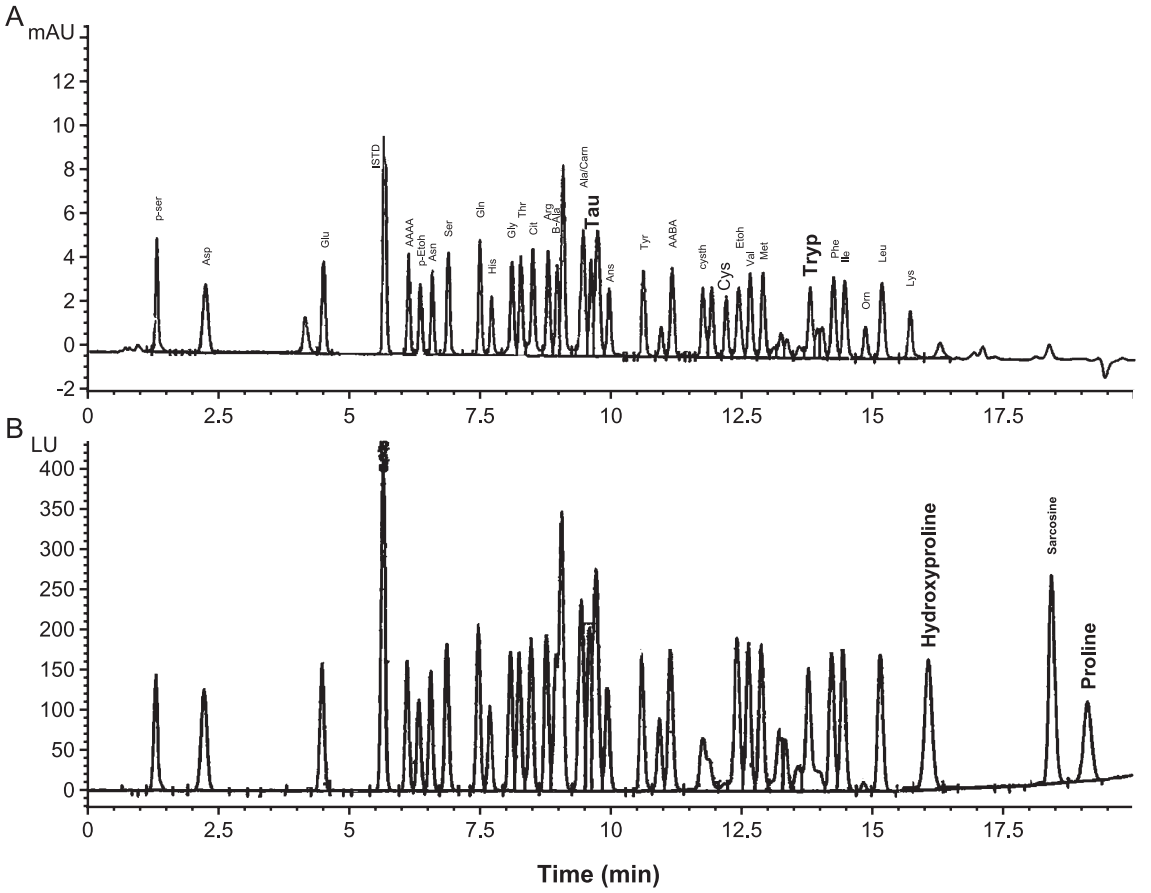

Fig. 1. Chromatograms of a mixture of 250 μmol/l amino acids standards separated using HPLC with mobile phase A1 (pH=7.8): (A) photodiode array UV detection at 338 nm, (B) fluorescence detection. The amino acids separated only using chromatographic separation starting with mobile phase A1 (pH7.8) are in bold characters (taurine, tryptophan, hydroxyproline and proline). Abbreviations: p-Ser=phosphoserine, Asp=aspartic acid, Glu=glutamic acid, ISTD=internal standard, AAAA=alpha amino adipic acid, p-Etoh=phosphoethanolamine, Asn=asparagine, Ser=serine, Gln=glutamine, His=histidine, Gly=glycine, Thr=threonine, Cit=citrulline, Arg=arginine, β-Ala=beta alanine, Ala=alanine, Carn=carnosine, Tau=taurine, Ans=anserine, Tyr=tyrosine, AABA=alpha amino butyric acid, Cysth=cysthathionine (2 peaks), Cys=cystine, Etoh=ethanolamine, Val=valine, Met=methionine, Tryp=tryptophan, Phe=phenylalanine, Ile=isoleucine, Orn=ornithine, Leu=leucine, Lys=lysine.

homocystine (Fig. 2), argininosuccinic acid (Fig. 2) and allo-isoleucine (Fig. 3).

Linearity studies were done to evaluate the analytical measurement range of each amino acid. The method was linear within allowable systematic error of 10% up to 2500 μmol/l for all the amino acids studied (1250 μmol/l for cystine and homocystine; 500 μmol/l for proline and hydroxyproline). The wide analytical measurement range for all the amino acids studied could only be achieved using photodiode array UV detection, with the exception of hydroxyproline and proline. Since these imino acids were detected using fluorescence detector, a lower linearity range was obtained. The accuracy of the method, evaluated by the highest maximum deviation from the theoretical value of the measured amino acid concentration was 10% and observed for glycine, taurine, threonine and valine at their lowest measured concentration of 5 μmol/l. For all other amino acids the accuracy was <10%.

The within-run CVs for all amino acids studied was <5% (Table 3). The between-run coefficient of variation (CV) was <10% for all amino acids studied (Table 3). A critical step for the reproducibility of the method was the addition of column washes, performed every 10–15 injections, with a mixture of isopropanol/water (90/10) to regenerate the column.

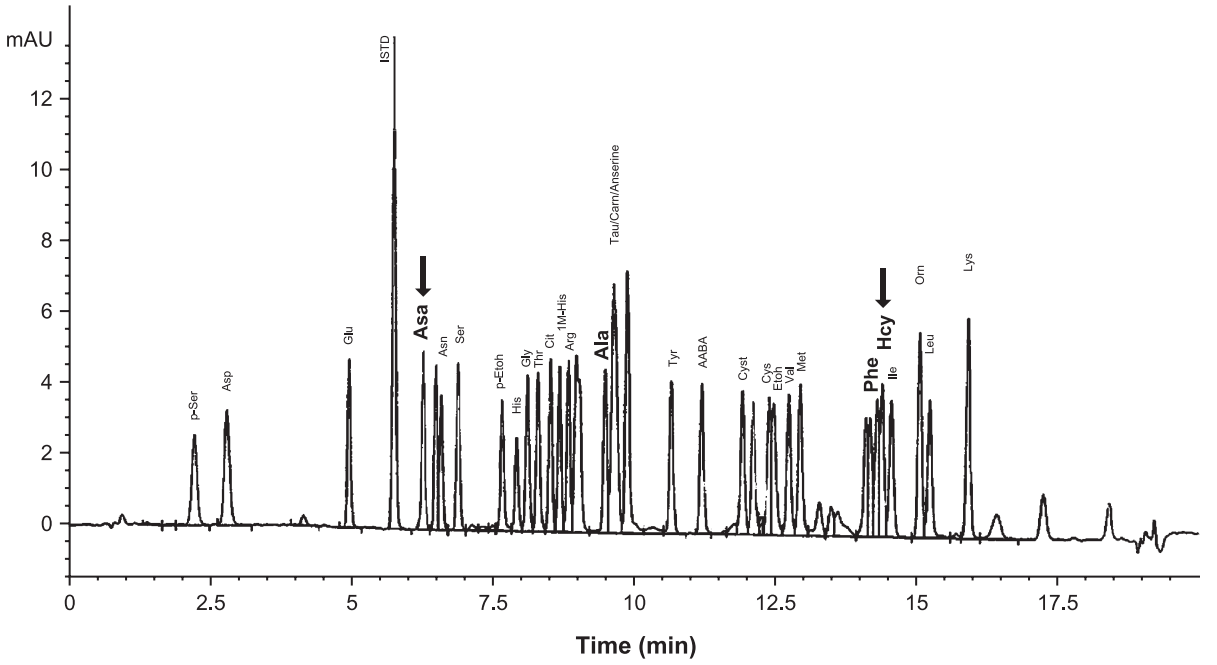

Fig. 2. Chromatograms of a mixture of 250 μmol/l amino acids separated using mobile phase A2 (pH=6.8) and photodiode array UV detection at 338. The amino acids separated only using HPLC starting with mobile phase A2 are in bold characters (argininosuccinic acid, alanine, phenylalanine and homocystine). Abbreviation as in Fig. 1, plus ASA=argininosuccinic acid, 1M-his=1 methyl histidine, Hcy=free homocystine.

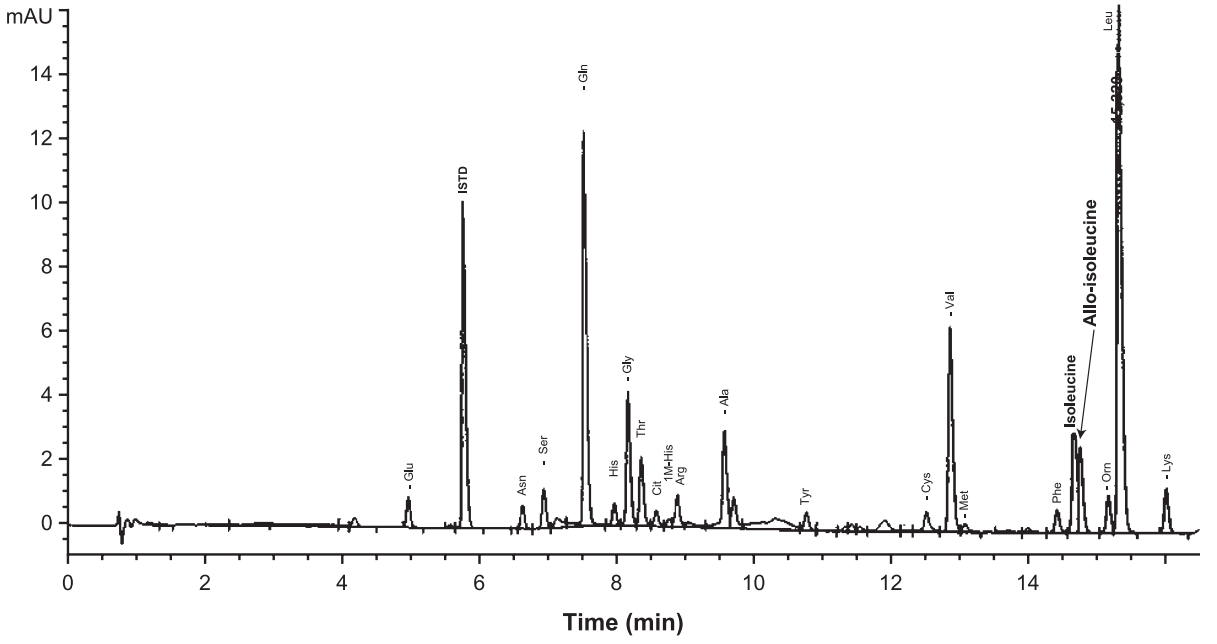

Fig. 3. Chromatogram of a plasma sample obtained from a known MSUD patient. The arrow indicates allo-isoleucine. Abbreviation as in Fig. 1, plus Allo-ile=alloisoleucine.

Table 3
Between-run and within-run imprecision for standard amino acids

Amino acid	Within-run %CV	Between-run %CV
Alanine[+++]	1.6	1.8
Aspartic acid	1.4	5.6
Arginine	1.0	4.1
Asparagine	0.4	4.3
Citrulline	0.9	1.9
Cystine	3.6	5.6
Glutamic acid	0.7	5.0
Glutamine	1.0	1.6
Glycine	2.7	1.9
Histidine	0.7	2.7
Hydroxyproline[a]	3.4	5.9
Isoleucine	3.2	1.9
Leucine	3.1	2.1
Lysine	4.5	4.7
Methionine	3.0	2.0
Ornithine	4.4	3.3
Phenylalanine[+++]	3.5	1.6
Proline[a]	1.5	3.6
Serine	1.2	4.0
Taurine[a]	4.0	5.9
Threonine	1.3	1.8
Tyrosine	1.4	1.9
Valine	2.2	2.0

All other amino acids were separated and quantitated with both mobile phases and the within-run and between-run imprecision were comparable for both.

[a] Amino acids separated and quantitated using chromatographic separation method starting with mobile phase A1 (pH 7.8).

[+++] Amino acids separated and quantitated using chromatographic separation method starting with mobile phase A2 (pH 6.8).

Ion-exchange chromatography with post-column derivatization with ninhydrin has always been considered the reference method for amino acid analysis in biological fluids. We compared the results obtained by our HPLC method with those obtained by ion-exchange chromatography for a batch of 16 plasma samples. The results were compared by Deming regression, with ion-exchange chromatography data on the *x*-axis and HPLC data on the *y*-axis (Table 4). The correlation between the two methods was >0.90 for most of the amino acids. Ornithine and tyrosine showed a negative bias. Ornithine had a slope of 0.88 with a concentration range on ion-exchange of 30–511μmol/l and on HPLC of 23–429 μmol/l. Tyrosine had a slope of 0.86 with a concentration range on ion-exchange of 29–128 μmol/l and on HPLC of 32–104 μmol/l. Lower concentration of tyrosine measured using HPLC and OPA-3MPA as the derivatizing reagent were also reported previously by Fürst et al. [5]. Reference intervals with this method should be established to account for this bias.

Fig. 3 shows the chromatogram obtained from a plasma sample of a patient with Maple Syrup Urine Disease (MSUD) using mobile phase A2 (pH 6.8). At this sample concentration of isoleucine=200 μmol/l, allo-isoleucine=160 μmol/l and leucine=930 μmol/l, the amino acids are well separated. Using diode array detection, the method was linear over a wide range of concentrations. This method, therefore, represents a good approach to amino acid analysis for screening for metabolic disorders. The major disadvantage of this method is its dependency from a perfectly equilibrated column [22]. The pH of the mobile phase is critical for achieving separation of all amino acids and even minimal variations in the pH of the mobile phase (0.05 pH units), will result in co-elution of amino acids. The derivatizing agent also affects the

Table 4
Correlation of amino acid concentration in plasma determined by HPLC and by ion-exchange chromatography

Amino acid	Slope	Intercept (μmol/l)	$S_{Y/X}$ (μmol/l)	*r*	Concentration range (μmol/l)
Alanine	1.12	−39.4	46.6	0.96	230–846
Arginine	1.00	7.1	11.7	0.98	43–334
Asparagine	1.01	−0.6	12.3	0.92	28–140
Citrulline	0.96	9.4	5.0	1.00	12–254
Cystine	0.95	−4.5	1.9	0.98	37–69
Glutamate	1.02	−8.3	17.5	0.98	423–724
Glutamine	1.04	23.1	41.2	0.94	419–1008
Glycine	0.93	22.5	20.5	0.96	178–440
Histidine	1.01	−1.3	9.0	0.97	58–185
Hydroxyproline	0.9	−2.8	2.7	0.89	38–287
Isoleucine	0.9	9.7	5.9	0.96	34–121
Leucine	0.97	5.9	8.2	0.98	61–225
Lysine	0.92	0.0	15.0	0.91	84–227
Methionine	0.98	3.2	2.2	0.95	14–60
Ornithine	0.88	−2.8	11.4	0.99	23–429
Phenylalnine	1.07	−0.6	13.7	1.00	33–1393
Proline	1.00	−1.2	18.0	0.99	98–546
Serine	0.99	1.1	9.6	0.96	58–185
Taurine	1.02	−0.3	6.0	0.98	35–157
Threonine	0.92	14.4	14.8	0.93	35–250
Tyrosine	0.86	9.9	6.8	0.95	39–114
Valine	0.96	14.1	15.2	0.96	127–295

Sixteen heparinized plasma samples were analyzed by HPLC and IEC. Results for the amino acids studied were compared by Deming regression with data obtained by ion-exchange chromatography on the *x*-axis and data obtained by HPLC on the *y*-axis.

performance of the column; therefore, the washing and re-equilibration steps are necessary every 10–15 samples in order to preserve column integrity and to obtain consistent results [22]. With this maintenance the column can be used for 300–350 injections.

In summary, we described an HPLC method for quantifying plasma amino acids. The advantages of this method are its ability to separate all amino acids present in plasma in a short time, although two injections per sample are required, and the wide analytic measurement range obtained using a photodiode array detector. The only disadvantages of this method are the column washes needed to maintain column integrity and the fact that it requires two injections per sample in order to achieve separation of all amino acids. This method, however, represents an alternative to ion-exchange chromatography for analysis of amino acids in plasma.

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