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Determination of amino acids in human plasma by liquid chromatography with postcolumn ninhydrin derivatization using a hydroxyapatite cartridge for precolumn deproteination

Hiroshi Iwase*, Shinichi Ozawa, Makiko Ikuta, Ichiro Ono

Ajinomoto Co., Central Research Laboratories, 1-1 Suzuki-cho, Kawasaki-ku, Kawasaki, 210, Japan

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Abstract

Amino acids in human plasma were determined by liquid chromatography with postcolumn ninhydrin derivatization using a hydroxyapatite cartridge for precolumn deproteination. S-Carboxymethyl-L-cysteine, D-phenylglycine and S-aminoethyl-L-cysteine were found to be suitable internal standards. The proposed method is simple, rapid (deproteination time less than 1 min) and reproducible [relative standard deviation below 3% except for low-level aspartic acid ($n = 3$)]. The average recovery of 25 amino acids was above 90%. The elution time of amino acids in human plasma was approximately 2 h. Protein binding of tryptophan was also determined by the proposed method. The analytical data for amino acids in human plasma deproteinated using the proposed and published methods (5-sulphosalicylic acid and ethanol) were compared.

1. Introduction

The determination of amino acids in biological fluids is considered to be one of the most important indicators in the diagnosis and monitoring of inherited disorders of the amino acid metabolism and in nutritional studies. Numerous methods are available for the measurement of amino acids in biological fluids using pre- and postcolumn derivatization and the classical methods of ion-exchange chromatography (IEC) [1] and high-performance liquid chromatography (HPLC) [2–19] after deproteinization of plasma (100–1000 μl) with organic solvents or acids. Automated postcolumn derivatization with ninhydrin is commonly used for routine determinations of amino acids in IEC [2–4].

Deproteinization of biological fluids, including chemical and physical methods, has been reviewed by Deyl et al. [20] and Sarwar and Boung [21]. Deproteination of plasma or serum should be carried out within 30 min after sample collection and the deproteinated supernatant should be analysed as soon as possible or stored at low temperature (−20 to −40°C) [20]. Among the chemical deproteination methods, precipitation, elution and centrifugation procedures, which are tedious and time consuming, are most frequently used. Usually, the protein is removed by addition of organic solvents or acids to biological samples prior to

* Corresponding author.

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analysis. During the formation of a precipitate, a fraction of the amino acids is trapped in the protein precipitate. The protein precipitate must be washed several times.

The method used to prepare plasma and to remove the protein has a marked effect on the final results [20,21]. The most widely used deproteination method is precipitation with 5-sulphosalicylic acid (SSA) [2–4,10,11], followed by centrifugation to remove the precipitated protein. SSA appears to be a suitable deproteinating agent, because it does not need to be removed from the filtrate and it does not interfere with the IEC of amino acids. In addition, the pH value of 1.0–2.0 of the supernatant obtained after centrifugation of protein precipitates is almost ideal for the IEC of most amino acids, except for tryptophan, which is in completely recovered [8,9].

In previous papers [22–24], we reported a novel precolumn deproteination method of human plasma using a PCPure hydroxyapatite cartridge for the HPLC determination of theophylline, diazepam and ascorbic acid using an internal standard and amino acids without an internal standard. By using a PCPure cartridge, human plasma was simply and rapidly deproteinated within 1 min by a dilution method or an injection method [22] without the need for centrifugation.

In this paper, we report a method using deproteination of human plasma on a PCPure cartridge by an injection method followed by elution and determination of amino acids using an automated high-speed amino acid analyser with postcolumn ninhydrin derivatization using an internal standard. This paper deals with the study of internal standards and the effect of the eluent on the recoveries of standard amino acids and internal standards from a PCPure cartridge. The validity of the determination of amino acids in human plasma by postcolumn ninhydrin derivatization using a PCPure cartridge for precolumn deproteination was checked. Further, this paper also compares analytical data for amino acids in human plasma using the proposed and published methods.

2. Experimental

2.1. Reagents and materials

Amino acids were obtained from Ajinomoto (Tokyo, Japan) and Beckman (Fullerton, CA, USA). The internal standards S-carboxymethyl-L-cysteine (SCM) and D-phenylglycine (PG) were obtained from Ajinomoto and S-aminoethyl-L-cysteine (SAC) from Sigma (St. Louis, MO, USA). Ninhydrin solution was purchased from Wako (Osaka, Japan). Other reagents were of analytical-reagent grade. PCPure cartridges were obtained from Moritex or Koken (Tokyo, Japan). The cartridges were used without further purification.

2.2. Preparation of amino acid standard solutions

To an aliquot (2 ml) of freshly prepared asparagine, glutamine and tryptophan aqueous solution (400 μg/ml each) was added an aliquot (2 ml) of an aqueous solution of other standard amino acids (400 μg/ml). This combined solution was further diluted with 0.2 *M* hydrochloric acid (4 ml).

2.3. Preparation of internal standard solution

The internal standards (SCM 45 mg, PG 30 mg and SAC 50 mg) were diluted to 100 ml with deionized water in a volumetric flask and then this solution (2 ml) was further diluted to 20 ml with deionized water in a volumetric flask (SCM 45 μg/ml, PG 30 μg/ml and SAC 50 μg/ml).

2.4. Preparation of mixed standard amino acid and internal standard solution

The amino acid solution (2 ml) and internal standard solution (2 ml) were finally diluted to 20 ml with 0.2 *M* hydrochloric acid in a volumetric flask prior to use.

2.5. Plasma collection

Blood was freshly collected from healthy individuals in a heparinized vacutainer tube and plasma was obtained by centrifugation at 1700 *g* for 15 min at 5°C. The plasma obtained was then used immediately for the determination of amino acids.

2.6. Sample preparation for determination of amino acids

Volumes of 150 μl of plasma and 10 μl of mixed aqueous solution of internal standards were injected directly into a PCPure cartridge and then 0.9% NaCl was passed through the cartridges. The protein-free eluate (elution of the first 1000 μl) [22] was used for the test solution after adjusting the pH to ca. 2 with 20 μl of 1 *M* hydrochloric acid. An aliquot of 50 μl was injected into the chromatograph.

2.7. Apparatus and conditions

A Model L-8500 high-speed amino acid analyser (Hitachi, Tokyo, Japan) was used. The detection wavelengths were set at 440 and 570 nm. Amino acid analysis was carried out on a 6 × 0.46 cm I.D. ion-exchange resin 2622 column (3 μm) (Hitachi) with a guard column of 1 × 0.46 cm I.D. ion-exchange resin 2622 (5 μm). The analytical temperature was set at 28–70°C. Table 1 shows the eluents used. The gradient elution method was adopted. The flow-rates of the eluent and ninhydrin solution were 0.30 and 0.35 ml/min, respectively.

3. Results and discussion

3.1. Internal standard

The first effort was focused on the examination of the internal standard for the determination of amino acids in human plasma. Various ninhydrin-positive compounds were examined (Table 2). It was found that SCM, PG and SAC were useful internal standards, because they have suitable retention times and were well separated. The amino acids and the internal standard were well separated. It took about 2 h to elute the amino acids in human plasma completely. A typical chromatogram of amino acids in human plasma obtained with the proposed method is illustrated in Fig. 1. The amino acid analyzer gradient conditions are shown in Table 3.

Table 1
Preparation of eluent solutions for amino acid analysis

Parameter	Eluent				
	PF-1	PF-2	PF-3	PF-4	PF-RG
Distilled water (ml)	700	700	700	700	700
Lithium citrate tetrahydrate (g)	5.73	9.80	8.79	9.80	–
Lithium chloride (g)	1.24	6.36	26.62	38.15	–
Citric acid monohydrate (g)	19.90	12.0	11.27	3.30	–
Lithium hydrate (g)	–	–	–	–	8.40
Ethanol (ml)	30.0	30.0	100.0	–	30.0
Thiodiglycol (ml)	5.0	5.0	–	–	–
Benzyl alcohol (ml)	–	–	3.0	–	–
Brij-35[a] (ml)	4.0	4.0	4.0	4.0	4.0
pH	2.8	3.7	3.6	4.1	–

[a] Used as dissolved at a ratio of 25 g per 100 ml (dissolved by heating).

Table 2
Retention times of amino acids and suitability for internal standard (I.S.)

Amino acid	Retention time (min)	Suitability for I.S.[a]
S-Carboxymethyl-L-cysteine	10.40	Yes
4-Thiazolidine-4-carboxylic acid	21.68	No (Asn)
Azetidine-2-carboxylic acid	23.36	No (Glu)
Norvaline	46.77	No (cystathionine)
tert.-Leucine	44.32	No (Cys)
Norcleucine	52.34	No (Tyr)
D-Phenylglycine	44.98	Yes
Hydroxphenylglycine	45.62	No (Met)
Ethionine	49.41	No (Ile)
Homophenylalanine	67.30	No (Aba)
S-Aminoethyl-L-cysteine	86.05	Yes
Aminoguanidopropionic acid	101.5	No (arserine, carnosine)
trans-4-(Aminomethyl)-cyclohexanecarboxylic acid	122.8	No (Arg)

[a] Compounds in parentheses indicate overlapped peak.

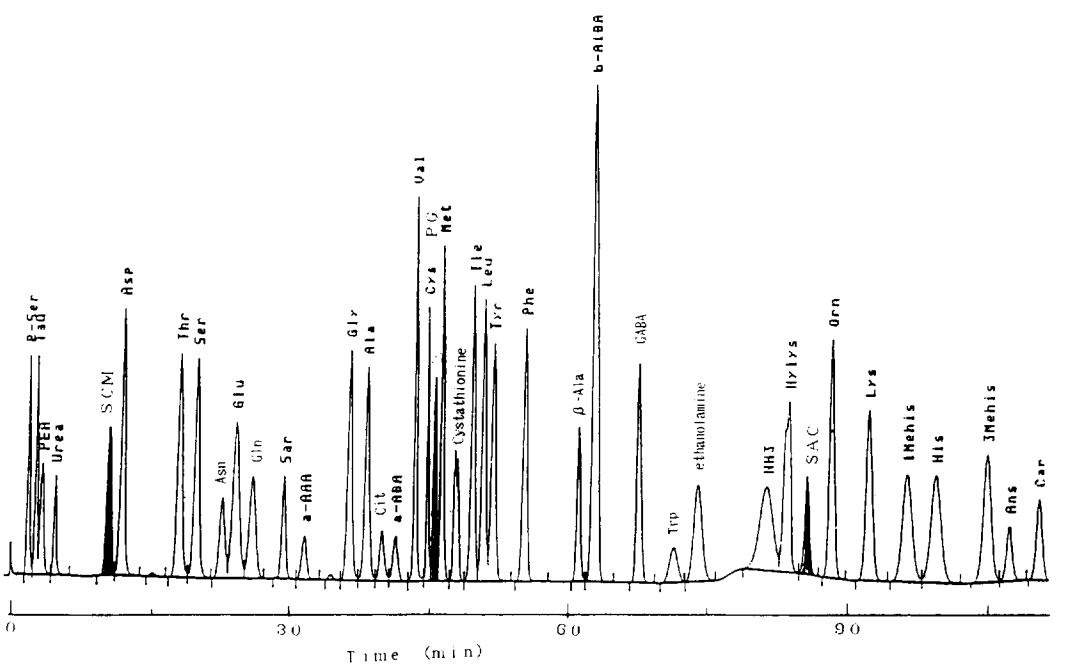

Fig. 1. Chromatogram of amino acids in human plasma using three internal standards. For amounts of amino acids, see Table 4. Amino acids were separated on a 6 × 0.46 cm I.D. column of ion-exchange resin 2622 (3 μm). Flow-rates of the eluent and ninhydrin solution were 0.30 and 0.35 ml/min, respectively. Column temperature, programmed from 28 to 70°C. The amino acid analyser gradient conditions are shown in Table 3. Abbreviations: P-Ser = phosphoserine; Tau = taurine; PER = phosphoethanolamine; Sar = sarcosine; a-AAA = α-aminoadipic acid; a-ABA = α-aminobutyric acid; b-AIBA = β-amino-isobutyric acid; HyLys = hydroxylysine; 1Mehis = 1-methylhistidine; 3Mehis = 3-methylhistidine; Ans = anserine; Car = carnosine; SCM (I.S.) = S-carboxymethyl-L-cysteine; PG (I.S.) = D-phenylglycine; SAC (I.S.) = S-aminoethyl-L-cysteine.

Table 3
Amino acid analyzer gradient conditions

Step	Time (min)	Eluent (total 100%)					Column temperature (°C)
		PF-1	PF-2	PF-3	PF-4	PF-RG	
1	0.0	100					40
2	8.0						28
3	10.0	100					
4	12.0	70	30				
5	23.0						40
6	29.0	70	30				
7	31.0						52
8	33.0	10	90				
9	38.0	10	90				
10	45.0		100				
11	48.0	10		90			
12	49.0						70
13	73.0			100			40
14	74.0				100		
15	74.1		10		90		
16	95.0		10		90		
17	95.1				100		
18	97.0						70
19	117.0				100		
20	117.1					100	
21	126.0						
22	127.0					100	
23	127.1	100					40
24	147.0						
25	157.0	100					

3.2. Elution of amino acids and internal standard

The second effort was focused on the rapid elution of both standard amino acids and three internal standards from a PCPure cartridge with several eluents.

A 0.9% NaCl solution, 10 m*M* sodium phosphate buffer (pH 6.8), 10% aqueous acetonitrile solution and 10% aqueous methanol solution were tested. Standard amino acids (5 μg) and SCM (4.5 μg), PG (3.0 μg) and SCA (5.0 μg) were injected into the cartridge followed by elution with the above eluents. Amino acids and internal standards in fractions 1–5 (300 μl each) were determined. Lower recoveries in the fractions 1–3 were found for aspartic acid (80%), cystine (58%), SAE (48%) and lysine (50%) with 10% aqueous methanol solution and cystine (80%), SAE (33%), ornithine (62%) and lysine (50%) with 10% aqueous acetonitrile solution. These amino acids might be considered to be slightly soluble in the eluents. The recoveries of the other amino acids in fractions 1–3 were over 90% with both eluents. On the other hand, the recoveries in fractions 1–3 were over 90% for the amino acids and 100% for the three internal standards with both 0.9% NaCl solution and 10 m*M* sodium phosphate buffer (pH 6.8). As described previously [22], a protein-free eluate was obtained in fractions 1–3 with 0.9% NaCl solution but was not obtained in fractions 1–3 with 10 m*M* sodium phosphate buffer (pH 6.8).

From the above results, 0.9% NaCl solution was chosen as the eluent, because both amino acids and the three internal standards were eluted in the eluate and a protein-free eluate was obtained within 1 min, as described previously

[22]. Hydroxyproline, asparagine and tryptophan were rapidly eluted in fraction 1. Acidic and basic amino acids were not eluted rapidly. Complete elution of the three internal standards was obtained in fractions 1–3. The recoveries of hydroxy, aromatic, acidic and basic amino acids and the internal standards are shown in Fig. 2.

Usually, SSA solution was used to precipitate proteins for the determination of amino acids as described above. However, the PCPure cartridge was unstable in acidic solution, so SSA was not used in this study.

3.3. Determination of amino acids in human plasma

The third effort was focused on the determination of amino acids in human plasma. The determination of amino acids was performed by plotting the peak-height ratio of the amino acids to the three internal standards (SCM, PG and SAC) against the amount of amino acids.

The results in Table 4 show the amino acid concentrations in human plasma obtained using three internal standards. A comparison of the analytical data for the amino acids and the relative standard deviations (R.S.D.s) showed that the analytical data for the amino acids were almost identical with all three internal standards and the variation in the R.S.D. obtained with SAC was larger than that with SCM and PG, and the R.S.D.s in the latter instance were small (below 4%) except for low-level aspartic acid. In this study, PG was adopted as the internal standard because of its retention time (ca. 45 min).

Further effort was focused on the recoveries of amino acids in human plasma. A known amount of amino acids was added to human plasma and the overall recoveries were calculated. In a previous study [22], drug samples were incubated at 37°C for 30 min for the study of plasma protein binding, and the recoveries of the drugs were good. To examine plasma protein binding, plasma samples were also incubated at 37°C for 30 min and allowed to stand at room temperature for 10 s. As shown in Table 5, the recovery of each amino acid except aspartic acid was over

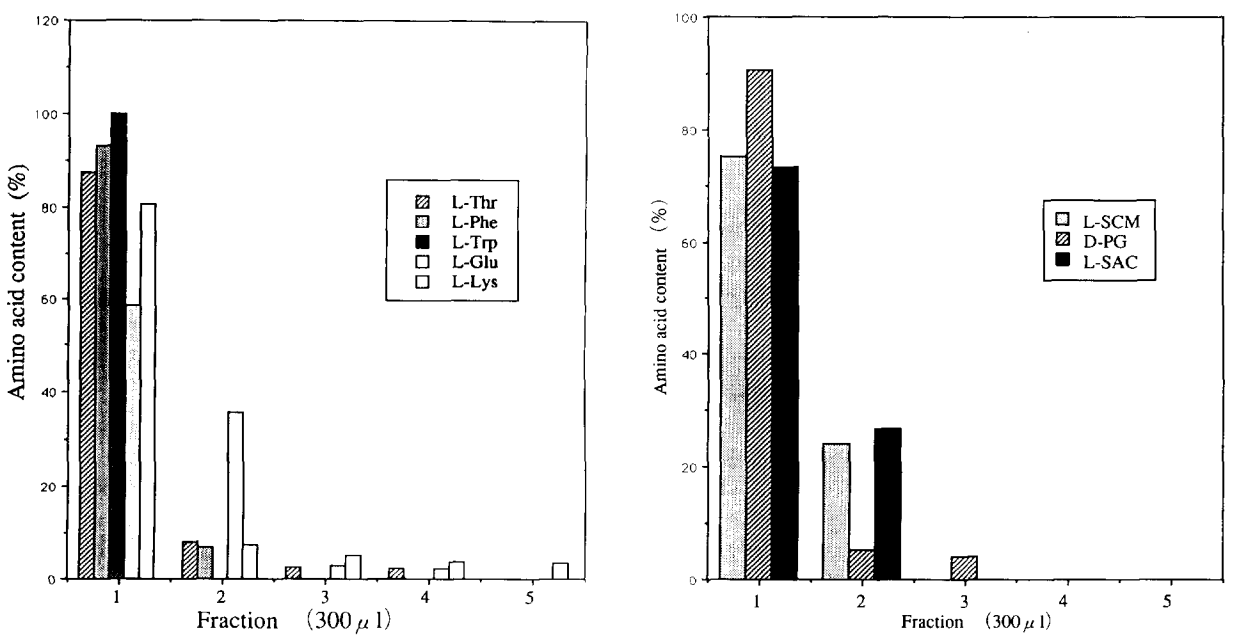

Fig. 2. (a) Content of standard amino acids in fraction (300 μl). Amount of each amino acid injected, 5 μg; eluent, 0.9% aqueous NaCl solution. (b) Content of internal standards in fraction (300 μl). Amount of each internal standard injected, SCM 4.5, PG 3.0 and SAC 5.0 μg; eluent, 0.9% aqueous NaCl solution.

Table 4
Analytical data for amino acids in human plasma by using three I.S.

Amino acid	SCM		PG		SAC	
	Mean (nmol/ml)	R.S.D.[a] (%)	Mean (nmol/ml)	R.S.D.[a] (%)	Mean (nmol/ml)	R.S.D.[a] (%)
Asp	6.53	9.09	6.47	8.38	6.67	4.40
Thr	130.0	1.04	129.4	0.87	132.7	3.63
Ser	108.9	1.74	108.2	1.39	6.8	1.17
Asn	35.6	2.67	35.5	2.70	36.4	4.34
Glu	66.7	0	66.0	0	67.9	4.66
Gln	527.4	0.07	525.4	0.44	537.9	4.98
Pro	200.6	1.20	199.9	1.46	204.6	5.17
Gly	186.8	2.72	186.1	2.58	190.1	2.44
Ala	460.0	0.30	458.0	0.16	469.3	4.59
Cit	31.3	0.67	31.3	0.36	31.9	4.87
Val	320.7	0.47	319.4	0.20	327.4	4.69
$(Cys)_2$	49.4	2.15	49.2	1.97	50.4	3.99
Met	55.8	0.84	55.7	0.47	56.9	4.30
Ile	93.1	0.81	92.4	0.41	95.1	5.36
Leu	69.6	0.59	168.9	0.59	173.6	5.34
Tyr	87.8	0.43	87.8	0.43	89.8	5.38
Phe	77.8	0.48	77.2	0.49	79.2	4.67
β-Ala	5.21	0.60	5.21	0.56	6.07	0.40
Trp	54.3	3.70	54.1	2.22	55.4	5.04
Orn	90.4	2.56	89.8	2.58	91.7	1.48
Lys	184.1	1.88	183.5	1.80	187.4	2.96
His	92.4	1.22	92.4	0.71	94.4	4.02
Arg	73.9	1.02	73.9	0.51	75.2	4.05

[a] Relative standard deviation ($n = 3$).

90% with incubation at 37°C and at room temperature. Only aspartic acid present at very low levels in plasma gave a recovery of below 90%.

Although the recovery of tryptophan was incomplete with SSA [7,8], the proposed method gave an excellent recovery of tryptophan in agreement with that reported by Uhe et al. [11]. The above data suggest that PCPure cartridges could be applied to protein-binding compounds such as tryptophan.

3.4. Comparison of analytical data for amino acids using the proposed and published methods

Analytical data for amino acids in human plasma obtained using the proposed and published methods (SSA [3] and ethanol [10]) are compared in Table 6.

Qureshi and Qureshi [10] reported the effects of 30% SSA, 1 *M* perchloric acid (PCA), 20% trifluoeoacetic acid, acetonitrile, ethanol and acetone as precipitation agents on the levels of free amino acids in human plasma. After deproteination of plasma, amino acids were determined by HPLC of on-line precolumn derivatives with *o* phthalaldehyde–mercaptoethanol. Among the six deproteination reagents, the use of SSA and PCA gave similar results with high recoveries for most amino acids. The variation in recovery was large with different organic solvents. Only threonine, phenylalanine, isoleucine and ornithine were stable towards deproteination agents. The degradation of asparagine and glutamine was lower in PCA and SSA than in organic solvents. Uhe et al. [11] also studied the effects of acetonitrile, trichloroacetic acid (TCA) and SSA as precipitation agents on the levels of free amino acids in human plasma.

Table 5
Recoveries of amino acids added to human plasma (internal standard PG)

Amino acid	Added (nmol/ml)	RT[a], 10 s		37°C, 30 min	
		Found (nmol/ml)	Recovery[b] (%)	Found (nmol/ml)	Recovery[b] (%)
Asp	274.6	230.3	83.9	231.6	84.3
HPro	274.6	270.6	98.5	267.3	97.4
Thr	274.6	255.4	93.0	257.4	93.8
Ser	274.6	256.7	93.5	256.7	93.5
Asn	293.0	286.4	97.8	291.1	99.4
Glu	274.6	258.7	94.2	262.0	95.4
Gln	300.9	279.8	93.1	279.2	92.8
Pro	274.6	254.8	92.8	253.4	92.3
Gly	274.6	249.5	90.9	252.2	91.8
Ala	274.6	258.7	94.2	260.0	94.7
Cit	67.9	64.0	94.2	64.0	94.2
Val	274.6	250.1	91.1	250.1	91.1
$(Cys)_2$	137.3	120.8	88.0	134.7	98.1
Met	274.6	254.8	92.8	254.8	92.8
Ile	274.6	259.4	94.5	260.0	94.7
Leu	274.6	259.4	94.5	261.4	95.2
Tyr	274.6	260.0	94.7	260.0	94.7
Phe	274.6	257.4	93.8	258.1	94.0
β-Ala	274.6	258.7	94.2	260.0	94.7
GABA	274.6	258.1	94.0	260.0	94.7
Trp	215.2	208.6	96.9	210.6	97.9
Orn	274.6	258.7	94.2	262.0	95.4
Lys	274.6	258.7	94.2	262.7	95.7
His	274.6	258.1	94.0	259.4	94.5
Arg	274.6	256.7	93.5	260.0	94.7
Average			93.5		94.3

[a] Room temperature.
[b] $n = 3$.

They reported a higher recovery of tryptophan with acetonitrile compared with acidic agents, in agreement with others [7–9]. The concentrations of both arginine and lysine were lower with acetonitrile in comparison with both acidic agents. Although Qureshi and Qureshi [10] also reported the degradation of amino acids in organic solvents compared with SSA and increased aspartic acid and glutamic acid levels due to hydrolysis of asparagine and glutamine in acetonitrile compared with SSA, Uhe et al. [11] did not observe such effects.

In the present study, the analytical data for most of amino acids were similar to those for published methods (SSA and ethanol). Relatively higher recoveries of asparagine, ornithine and lysine were found with SSA compared with ethanol and the PCPure cartridge. Relatively higher recoveries of aspartic acid, glutamic acid and glutamine were found with ethanol compared with SSA and the PCPure cartridge. A higher recovery of tyrosine and a lower recovery of tryptophan were observed with the PCPure cartridge in comparison with other deproteination agents. A lower recovery of arginine was found with ethanol. The R.S.D.s for most of

Table 6
Comparison of analytical data for amino acids in human plasma using the propsoed and published methods (internal standard PG)

Amino acid	PCPure		SSA [3]		Ethanol [10]	
	Mean (nmol/ml)	R.S.D.[a] (%)	Mean (nmol/ml)	R.S.D.[a] (%)	Mean (nmol/ml)	R.S.D.[a] (%)
Asp	6.47	8.38	7.66	7.57	7.78	7.60
Thr	129.4	0.87	133.3	0	132.7	0.28
Ser	108.2	1.39	114.2	1.65	112.9	0.58
Asn	35.5	2.70	42.3	1.40	32.7	2.21
Glu	66.0	0	67.9	0.55	70.6	0.53
Gln	525.4	0.44	500.9	0.33	532.6	0.52
Pro	199.9	1.46	201.3	0.50	201.9	1.66
Gly	186.1	2.58	190.7	0.39	192.1	0.20
Ala	458.0	0.16	464.6	0.06	465.3	0.16
Cit	31.3	0.36	30.5	1.09	31.5	0.56
Val	319.4	0.20	321.4	0.15	322.7	0.23
$(Cys)_2$	49.2	1.97	55.2	0.46	51.9	3.09
Met	55.7	0.47	55.4	0.38	57.6	0.36
Ile	92.4	0.41	93.1	0.41	93.7	0.40
Leu	168.9	0.59	169.6	0.22	170.9	0.22
Tyr	87.8	0.43	79.2	0.76	87.1	0.44
Phe	77.2	0.49	78.5	0.48	77.9	0.41
β-Ala	5.21	0.56	5.17	0.55	5.15	0.59
Trp	54.1	2.22	61.1	0.71	61.6	2.19
Orn	89.8	2.58	93.7	1.06	87.8	0.43
Lys	183.5	1.80	188.1	0.61	172.9	0.38
His	92.4	0.71	93.1	0.82	93.1	0.41
Arg	73.9	0.51	73.9	0.51	67.9	0.56

[a] Relative standard deviation ($n = 3$).

amino acids were below 3% except for aspartic acid (over 7%) with the three deproteination methods.

During the precipitation, the protein-bound hydroxyproline and tryptophan may be liberated. Therefore, there is no agreement between the data obtained after deproteination by precipitation and by ultrafiltration [20]. The use of an acid precipitation reagent leads to overestimation of free tryptophan owing to the release of protein-bound tryptophan from the plasma albumin [21]. In the present study, the tryptophan level was almost identical with both precipitation agents (SSA and ethanol). However, the PCPure cartridge method used in this study gave a low tryptophan level.

4. Conclusion

The use of a PCPure cartridge for simple and rapid deproteination of human plasma seems useful for the determination of amino acids in human plasma using postcolumn ninhydrin derivatization and a high-speed amino acid analyser.

After human plasma was applied to the cartridge followed by suitable elution, the protein-free amino acids were obtained within 1 min in the eluate without the need for centrifugation.

The proposed method is satisfactory with respect to simplicity, rapidity and accuracy in comparison with other published methods [3,10]. It is simple and convenient, and therefore applic-

able to automated routine analysis of human plasma for amino acids. It is probably possible to use an on-line PCPure cartridge pretreatment for amino acid determinations by HPLC.

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