Amino Acids (2012) 43:1653–1661
DOI 10.1007/s00726-012-1243-9

ORIGINAL ARTICLE

New approach for amino acid profiling in human plasma by selective fluorescence derivatization

Małgorzata Jaworska · Marta Stańczyk · Małgorzata Wilk · Gabriela Kłaczkow · Elżbieta Anuszewska · Justyna Barzał · Piotr Rzepecki

Received: 8 December 2011 / Accepted: 1 February 2012 / Published online: 18 February 2012

Abstract A new approach for the separation of 6-aminoquinolyl-carbamyl (AQC)-derivatized amino acids has been proposed. The chromatography used ion-pairing mechanism to increase the method selectivity. Mobile phase was based on triethylamine buffer containing *N*,*N*-dimethyloctylamine as a modifier. A number of factors, buffer composition and pH, counterion concentration, temperature and acetonitrile gradient profile, were optimized to achieve final chromatographic conditions. With the presented analytical method, the separation and identification of 34 AQC-amino acids and amino compounds present in human plasma is possible. The results of validation proved the applicability of the method for quantification of 27 amino acids in biological samples. The ultrafiltration proposed as deproteinization procedure gave repeatable and reliable results for the amino acids under investigation. This method introduced in routine testing can be a suitable tool for amino acid profiling in plasma including all aspects of clinical application.

Keywords Amino acids · Human plasma · Ultrafiltration · Derivatization · 6-Aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) · Ion-pair chromatography · *N*,*N*-dimethyloctylamine

M. Jaworska (✉) · M. Stańczyk · M. Wilk · G. Kłaczkow · E. Anuszewska
Department of Biochemistry and Biopharmaceuticals, National Medicines Institute, 30/34 Chełmska str, 00-725 Warsaw, Poland
e-mail: m-jaworska@il.waw.pl

J. Barzał · P. Rzepecki
Department of Oncology, Bone Marrow Transplantation Unit, Military Institute of Health Services, 128 Szaserów str, Warsaw, Poland

Introduction

Plasma amino acid (AA) profile is influenced by various factors and is often a subject of diagnostic tests. Evaluation of nutrition state or screening for metabolic disorders can be easily performed with determination of net or relative amino acid content in body fluids. The complexity of biological sample, however, requires a selective and efficient method that is suitable for reliable determination of target analytes.

As amino acids are not well detectable in their native state, the methodology generally recommends labeling of analytes with high-absorbing or fluorescent reagent. 6-Aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) was introduced by Cohen and Michaud (1994) as an efficient labeling tool for AA giving sufficiently stable, well-detectable products. The reagent is designed for both fluorescence and UV detection. The author also proposed a chromatographic method for separation of 17 common AA that was intended for analysis of protein hydrolysates. However, samples containing higher number of AA, like body fluids, could not be successfully analyzed with the method because of undesirable peak overlapping. A few authors described some improvements (Cohen 1994; van Wandelen and Cohen 1997; Liu et al. 1998; Bosch et al. 2006; Reverter et al. 1997) to the original Cohen's (1994) method, but still utilized the same or almost the same mobile phase system. Minor modifications proposed were related to a specific purpose of the study; hence, a number of separated amino acids could not be really increased. The advantages offered by AQC as labeling agent make it worth a try to expand the range of its applicability. In view of separation of approximately 30 physiological AA and other plasma amino compounds, appearing in extremely various concentrations, the selectivity of the method needed to be enhanced.

The aim of the study was to develop a new HPLC method for AA separation after selective precolumn derivatization with AQC, allowing quantification of physiological amino acids in plasma samples. The new concept of the presented method is based on improved selectivity by use of ion-pair reagent *N*,*N*-dimethyloctylamine in combination with triethylamine buffer. The optimized and validated method can be a screening tool for changes of plasma amino acids of diagnostic importance.

Materials and methods

Chemicals

The individual 20 common L-amino acid standards (Gly, Ala, Ser, Thr, Arg, Lys, Pro, Asn, Asp, Gln, Glu, His, Met, Cys (cysteine), Cst (cystine), Phe, Tyr, Trp, Ile, Leu, Val) and 8 other physiologically important amino acids [L-hydroxyproline (Hyp), L-citrulline (Cit), taurine (Tau), L-ornithine (Orn), L-α-aminobutyric acid (Aba), γ-aminobutyric acid (GABA), α-aminoadipic acid (Ada), β-alanine (b-Ala)] as well as internal standard *nor*-leucine (Nle) were purchased from Sigma (Sigma-Aldrich, Poznań, Poland). An additional seven amino compounds such as histamine (Hsm), tryptamine (Trm), sarcosine (Src), ethanolamine (Eta), *o*-phosphorylethanolamine (P-Eta), *o*-phospho-*DL*-serine (P-Ser) and hydroxylysine (Hyl) from Sigma (Sigma-Aldrich, Poznań, Poland) were used for identification purpose. His, Arg, Lys, Orn, Hsm, Eta and Hyl were obtained as hydrochlorides.

Sodium acetate from Applichem (Darmstad, Germany), triethylamine (TEA), *N*,*N*-dimethyloctylamine (DMOA) from Fluka (Sigma-Aldrich, Poznań, Poland) and acetonitrile (ACN, gradient grade) from LabScan (Dublin, Ireland) were used as mobile phase constituents. Acetic acid 99.9% and hydrochloric acid 37% (HCl) were purchased from POCH (Gliwice, Poland). AQC reagent was supplied by Waters (Waters, Milford, MA, USA) as a part of AccQ Tag Reagent Kit containing also borate buffer for derivatization and acetonitrile (ACN) as AQC diluent.

Highly purified deionized water was prepared using EASY Pure RF deionizer (Barnstead-Thermolyne, Dubuque, IA, USA). All buffers and solutions applied to chromatography were filtered through hydrophilic polypropylene 0.45-μm membrane filters (Pall Corporation, Ann Arbour, MI, USA).

Single stock solutions of all the AA were prepared at concentrations of 0.3–0.7 mg/ml in 0.05 M HCl, the internal standard solution contained 8 μg Nle in 1 ml. For method development, single AA solutions were mixed in a way to obtain a final concentration of 25 nmol/ml of each AA. For validation and quantification of plasma samples, the mixture contained AA at a level corresponding to averages of human plasma (Le Boucher et al. 1997) except GABA for which ten times higher concentration was used (Schmidt and Löscher 1982).

Sample preparation

Ten healthy volunteers were included in the study (male:female = 4:6; age 26–55 years). After at least 12 h of overnight fasting, blood samples were collected in sterile tubes containing K_2EDTA and gently mixed. Plasma was separated immediately by centrifugation at 1,000 rpm (approx. 200×*g*) for 10 min with cooling down to 10°C (Haereus Megafuge 1.0R, Thermo Scientific, Walthman, MA, USA) and stored at −70°C before use.

For the amino acids determination, plasma sample was deproteinized by ultrafiltration. Plasma was diluted fivefold with deionized water and 200 μl was placed in Microcon Ultracel YM-10 (10 kD cutoff) cartridges (Millipore, Warsaw, Poland). Before use, the ultrafiltration membrane was pretreated by subsequent centrifugation of 200 μl of 0.1 M HCl and deionized water at 8,000 rpm (approx. 5,870×*g*) for 20 min at 15°C (Sigma 3K15, Osterode am Harz, Germany). The same speed and temperature, but for 60 min, were applied for the ultrafiltration of diluted plasma sample. Ultrafiltrates were directly taken for derivatization or stored at −70°C if not analyzed immediately.

Derivatization procedure

The dilution of references and samples was adjusted to maintain fivefold molar excess of AQC reagent (Cohen 1994) compared to the total amount of subjects for derivatization—about 3,000 nmol/ml in deproteinized plasma (Nasset et al. 1979). Each plasma sample was assayed in duplicate. The results were expressed in nmol/ml of plasma.

Derivatization was done according to the supplier's procedure. AQC solution (10 mM) was prepared in ACN; 50 μl of sample solution (ultrafiltrate) or reference solution was mixed with 80 μl of borate buffer to achieve an alkaline reaction. Then, 20 μl of the internal standard solution and 350 μl of water were added. The derivatization was accomplished by use of 20 μl of AQC solution and vortexing the mixture thoroughly. Finally, a heating step (55°C, 10 min) was applied to convert tyrosine to the monolabeled product.

Liquid chromatography

The chromatographic separation was performed using Dionex Ultimate 3000 HPLC system (Dionex, Sunnyvale, CA, USA). The system consisted of a dual low-pressure gradient pump allowing a ternary gradient formation, a degasser, an autosampler and a column oven. A reverse

phase AccQ-Tag 150 × 3.9 mm 4-µm column for amino acid hydrolysates (Waters, Milford, MA, USA) protected with Nova-Pak C18 Guard Column 20 × 3.9 mm 4 µm (Waters, Milford, MA, USA) was mounted into the system. Chromeleon software v. 6.8 was used for data acquisition and analysis.

During chromatographic runs, samples were kept at 10°C and injection volume was 10 µl. Peaks were measured simultaneously at photodiode array (PDA-3000, Dionex, USA) and fluorescence (RF2000, Dionex, USA) detectors. UV signal was recorded at 248 nm with 4-nm bandwidth, and fluorescence was measured at 395 nm after excitation at 250 nm.

Chromatography was performed at 25°C in a ternary gradient mode (see Table 1). Buffer for the mobile phase contained 50 mM TEA and 2 mM DMOA, and pH was adjusted to 5.1 with acetic acid.

Results and discussion

Method development

The original method for AQC-AA separation developed by Cohen and Michaud (1994) is based on sodium acetate buffer and triethylamine (TEA) as mobile phase constituents and allows efficient separation of 17–20 AA. In order to separate approximately 30 physiological AA and other plasma amino compounds, the increase in selectivity of the method required a new approach for the mobile phase system. The 6-aminoquinoline moiety linked to the analytes does not make them extremely hydrophobic; therefore, gradient adjustment of organic modifier gave us little improvement of a peak capacity of the system. The use of different types of column (high carbon load, low silanol activity) (Choudhury and Norris 2005) became an alternative provided that the column was able to work with mobile phase of high water content. Our experience showed that stationary phases with hydrophilic end-capping (i.e., YMC-Pack ODS-AQ, Waters) offering increased retention and withstanding 100% aqueous media can be also a good choice for further development (results not shown). Nevertheless, smaller particle size (4 µm) of the dedicated AccQ-Tag column seems to be one of the factors outweighing commonly used 5-µm particle sorbents. Thus, the column was finally selected for the method.

AQC-tagged amino acids possess at least one carboxylic group that makes them suitable targets for ion-pair chromatography. To obtain hydrophobicity and increased retention, *N*,*N*-dimethyloctylamine was selected as a counterion (Vervoort et al. 2002). A series of experiments were performed in searching for optimal separation conditions that differed with basic buffer composition, concentration and pH, counterion concentration, temperature of separation and acetonitrile gradient profile. Table 2 shows the data of the studied buffers.

With both buffer systems based on sodium acetate and TEA, amino acids were washed out of the column as three groups. The first contained Thr, Arg and ammonia (NH_3) peaks, accompanied with mono-derivatized Orn and Lys if present. The remaining polar AA eluted as the second group starting from Asn, and all the acidic AA appeared at the end of the section. Hydrophobic AA including double-derivatized Orn and Lys were recorded as the last group. A few pairs of AA occurred to be difficult to separate: or few pairs of AA were found to be difficult to separate: Thr/Arg/NH_3, Ser/Gln, Gly/Cit, GABA/Tau and Asp/Pro/Glu.

Table 1 Optimized gradient program for the elution of 28 amino acids and Eta

Time	Solvent A% (H_2O)	Solvent B% (ACN)	Solvent C% (buffer)
0	56	4	40
5	53.2	4	42.8
20	42.5	6	51.5
28	36	8	56
68	0	21	79
69	0	21	79
71	40	60	0
73	40	60	0
75	56	4	40
84	Stop		

Table 2 Buffer systems tested during the method development

Buffer system	Based on sodium acetate				Based on triethylamine			
	1	2	3	4	5	6	7	8
pH	4.9	5.0	5.1	5.2	5.0	5.1	5.2	5.3
Sodium acetate (mM)	20–30	20–50	20–50	20–30				
TEA (mM)					20–75	20–75	20–50	30–50
DMOA (mM)	4–6	4–10	4–10	4–7	1–6	1–10	1–5	2–5
Temperature (°C)	30; 35	30; 35; 40	30; 35; 40	30; 35; 40	25; 30; 35; 40	25; 30; 35; 40	25; 30; 35; 40	30; 35; 40

A comparison of the two buffer systems revealed that with sodium acetate, analytes were separated within a shorter time and required lower ACN and higher DMOA concentration. However, the TEA-based buffer offered higher resolving power and finally that was used for further optimization.

The resolution depended highly on pH of the buffer. Lower pH (5.1 and below) was more suitable to resolve Thr/Arg/NH_3, GABA/Tau and Asp/Pro/Glu, while the best separation of Ser/Gln and Gly/Cit took place with higher pH (5.3). A similar tendency was observed for the temperature effect. The distance between Ser/Gln and Gly/Cit increased with increase of TEA and DMOA concentration, although it was more effective with lower pH. The separation of Thr/Arg/NH_3, GABA/Tau and Asp/Pro/Glu was favored at DMOA concentration below 4 mM. The retention and elution order of hydrophobic AA turned out relatively stable apart from cystine (Cst). Even small changes within the optimized parameters resulted in dramatic shifts in Cst retention (the peak appeared between Lys and Phe) and no clear tendency was found.

Finally, satisfactory separation was achieved using 50 mM TEA acetate buffer, pH 5.1, containing 2 mM of DMOA as the counterion with optimized gradient of both buffer and acetonitrile elution (Fig. 1). The separation of 27 AA was accomplished within 70 min. An additional 14-min step of column wash and re-equilibration was set. All the AA were separated with sufficient selectivity. The closest eluted peaks Cit/b-Ala, Asp/Pro and GABA/Tau demonstrated the resolution R_s 1.2; 1.4 and 1.6, respectively.

During method development, simultaneous UV and fluorescence detection gave an advantage in keeping trace of the weakly fluorescing amino acids like Asp, Cys and especially Trp. Despite additional searching, no other excitation/emission wavelength was found suitable for Trp detection. UV detector allowed recording higher Trp peaks than with fluorescence detector and thus the assay was more precise. For that reason, double detection was maintained taking into account slight band broadening and decreased resolution for close eluting peaks: Cit/b-Ala, GABA/Tau and Asp/Pro/Glu (Fig. 2).

Method validation

The pH of the buffer became the crucial parameter affecting the separation. The differences in resolution between several AA depending on pH (Fig. 3) were a base for defining the system suitability criteria of the method: Asp/Pro resolution $R_s \geq 1.25$ and additionally Met/Orn resolution $R_s \geq 2.4$.

Although the method is able to distinguish both Cys and Cst, the oxidation that occur in alkaline medium makes Cys not quantifiable from a practical point of view. Therefore, validation and further assays are based on Cst peak that represents the sum of both amino acids expressed as Cst.

For Lys and Orn, two peaks representing both mono- and bis-derivatized AA were observed despite the reagent excess (up to 25× molar) or sequential reaction (additional portion of the reagent) applied. It seems that it may be related to pH of the derivatization buffer (pH too low to reverse the dissociation of side-amino group of AA, and thus derivatization of Lys and Orn may not be complete). Nevertheless, validation data proved that derivatization yield was comparable and repeatable from sample to sample. The results for Lys and Orn were calculated in relation to double-labeled reference peak.

The method was validated by evaluation of the following parameters: linearity range, limits of detection (LOD) and quantification (LOQ), precision, intermediate precision and recovery. Upper level for linearity range was set up based on limited amount of AQC available for derivatization while retaining fivefold molar reagent excess (vs. total amine content). For all the amino acids, good correlation was shown for the concentration range tested. In the case of Trp, the lowest point of the linearity range lay between LOQ and LOD values as a result of UV detection used and

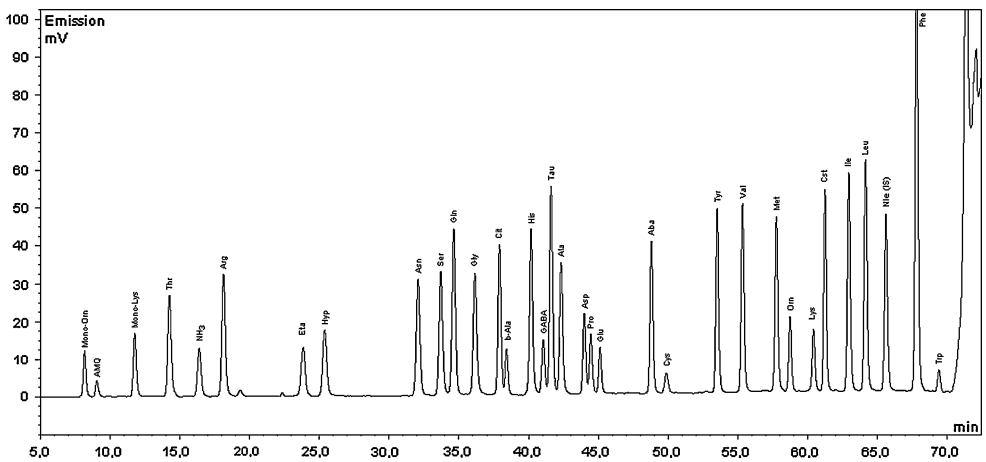

Fig. 1 Separation of mixture of AA standards intended for further validation (27 amino acids and Eta; conc. 25 nmol/ml) under optimized conditions. Signal recorded at ex/em 250/395 nm. Other visible peaks with respect to mono-Orn, mono-Lys, AMQ, NH_3 and Cys. Cys was intentionally added to the mixtute to visualize the peak location; however, it is negligible from quantification point of view as oxidation to cystine takes place very quick (see text for details)

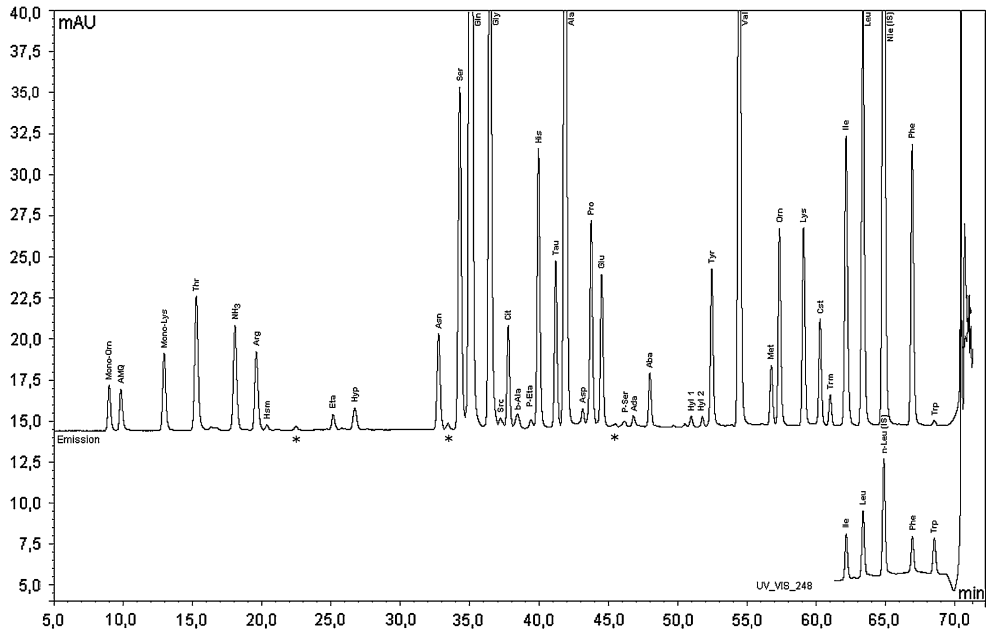

Fig. 2 Chromatogram of normal plasma sample. Overlaid signals from fluorescence and UV detector show advantages of UV signal for Trp peak acquisition and integration. Peaks related to matrix effect of ultrafiltration membrane were signed with *asterisk*

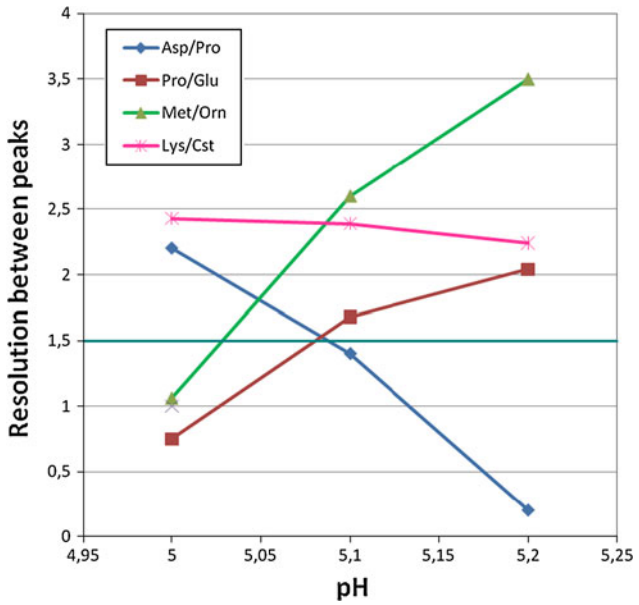

Fig. 3 Changes in resolution between critical pairs of peaks dependent on pH of mobile phase as a base for setting the system suitability criteria. A *line* at $R_s = 1.5$ represents the theoretical limit for complete separation of peaks

it was also in line with increased RSD for precision and intermediate precision. However, the correlation coefficient for Trp did not differ from other AAs.

LOD and LOQ were estimated from linearity range by extrapolation of S/N value to 3 and 10, respectively. The LOD and LOQ values given in Table 3 represent the amount of amino acid in pmol in the test solution after derivatization. The validation of the method was completed by precision and intermediate precision evaluation; the results are presented in Table 4. The precision of the method is expressed as relative standard deviation (RSD) of six consecutive assays performed on the same day. The intermediate precision is calculated as RSD of six assays performed within successive days. The RSD (%) values for method precision are generally below 1%. The result for b-Ala exceeding 1% arises from the low concentration tested and incomplete separation from Cit that produces a much higher signal. Similarly for Trp, precision greater than 1% is related to its low response factor, as mentioned above. The intermediate precision of the method is estimated below 2.5% for all the analytes except Trp with RSD (%) value of 2.83%.

A recovery of the method was checked, as it is an important validation parameter in case of complex sample pretreatment. A mixture of AA standards was subjected to ultrafiltration followed by spiking to diluted plasma ultrafiltrate as a matrix. The results of AA assay were calculated in relation to the unspiked matrix and compared with theoretical AA content. The overall recovery was very good for all analytes (mean 97.53%; range 90–104%). Our findings are in accordance with results published by other authors (Iwase et al. 1995), which proves the applicability of the method for AA quantification in plasma samples.

Analysis of plasma samples

Amino acid profiling requires initial plasma deproteinization. A few procedures are generally applied from which organic solvents (Tcherkas et al. 2001) or strong organic acids (Le Boucher et al. 1997; Teerlink et al. 1994) are commonly used. Organic solvents, however, are not recommended in case of AQC derivatization. They increase the elution power within the sample zone and strongly

Table 3 Validation data (I): linearity range, detection and quantification limits, repeatability of retention times

Amino acid		Linearity range[a] (nmol)	R^2 ($N = 8$)	LOD[a] (pmol)	LOQ[a] (pmol)	Repeatability of retention time RSD (%) ($N = 6$)
1	Thr	0.051–1.930	0.9967	6.91	11.43	0.77
2	Arg	0.020–0.742	0.9977	3.21	6.55	0.71
3	Eta	0.012–1.958	0.9994	2.08	4.62	0.63
4	Hyp	0.015–0.581	0.9961	4.47	11.23	0.50
5	Asn	0.023–0.846	0.9981	3.62	6.78	0.29
6	Ser	0.048–1.809	0.9979	1.86	3.65	0.27
7	Gln	0.119–4.446	0.9986	15.42	18.98	0.26
8	Gly	0.075–2.794	0.9982	5.26	7.63	0.23
9	Cit	0.021–0.791	0.9992	2.27	4.17	0.22
10	b-Ala	0.015–0.568	0.9980	1.56	4.42	0.21
11	His	0.043–1.630	0.9975	4.47	6.26	0.20
12	GABA	0.067–2.251	0.9971	11.56	17.31	0.20
13	Tau	0.040–1.499	0.9960	4.87	7.01	0.19
14	Ala	0.088–3.283	0.9993	9.95	12.96	0.18
15	Asp	0.009–0.349	0.9940	2.98	3.89	0.18
16	Pro	0.059–2.219	0.9998	9.88	16.75	0.17
17	Glu	0.020–0.735	0.9989	3.49	6.04	0.17
18	Aba	0.010–0.361	0.9998	2.08	4.55	0.14
19	Tyr	0.033–1.246	0.9964	3.76	5.97	0.13
20	Val	0.062–2.340	0.9999	7.05	8.64	0.13
21	Met	0.017–0.652	0.9981	1.83	3.52	0.14
22	Orn	0.046–1.730	0.9971	9.71	11.61	0.15
23	Lys	0.055–2.048	0.9957	9.13	13.85	0.16
24	Cst	0.019–0.721	0.9967	4.37	8.45	0.17
25	Ile	0.027–0.998	0.9999	3.23	4.66	0.17
26	Leu	0.041–1.537	0.9999	4.83	6.35	0.18
27	Phe	0.030–1.109	0.9967	2.91	3.85	0.19
28	Trp	0.028–1.056	0.9972	17.91	55.07	0.22

AA and Eta are listed in order of elution

[a] Amount in derivatized sample

disturb the separation that is mainly carried out with low concentration of organic modifier. The use of acid also results in some disadvantages. Labeling reaction is performed in alkaline medium that is obtained due to borate buffer addition. The acidified sample requires much more buffer for pH switching, thus increasing the ionic strength of the sample. This can interfere with the ion-pairing process during chromatography. Based on literature data (Ralston and Strein 1977) and the above-described findings, ultrafiltration was selected as a tool for sample deproteinization. The advantages to be gained from adopting this strategy concerned no manipulation affecting derivatization and separation, as well as no effect on the physico-chemical status of the free AA fraction in the plasma.

As a consequence of application of non-denaturing protein removal, it was decided that the internal standard must be added at the derivatization step. Initial tests revealed high variability of AA levels assayed in plasma when Nle appeared in a sample before ultrafiltration. It can be explained as the effect of external amino acid on the equilibrium between free and protein-bound fraction of physiological AA. Satisfactory results of the recovery during the validation confirmed that in the proposed method, internal standardization was essential for compensation of errors related to incomplete derivatization rather than sample pretreatment.

The developed chromatographic method was applied for amino acid profiling in plasma samples (Fig. 2). Each analyte was identified by comparing the retention time with reference sample. The amino acid profile of plasma samples revealed about 43 peaks, from which only 2–3 were verified to be of matrix origin (i.e., eluted from

Table 4 Validation data (II): precision, intermediate precision and recovery evaluated at a level of concentration given in third column

Amino acid		Concentration[a] (nmol/ml)	Precision RSD (%) $N = 6$	Intermediate precision RSD (%) $N = 6$	Recovery (%) $N = 3$
1	Thr	2.57	0.72	1.07	93.66
2	Arg	0.99	0.89	1.22	94.59
3	Eta	1.21	0.80	1.35	95.25
4	Hyp	0.77	0.79	1.21	93.94
5	Asn	1.13	0.61	0.96	96.78
6	Ser	2.41	0.78	2.47	103.83
7	Gln	5.93	0.48	1.04	94.10
8	Gly	3.73	0.78	1.65	99.77
9	Cit	1.05	0.42	1.21	101.07
10	b-Ala	0.76	1.19	1.82	98.60
11	His	2.17	0.39	1.13	98.53
12	GABA	3.36	0.35	1.55	90.59
13	Tau	2.00	0.49	0.96	94.23
14	Ala	4.38	0.49	1.37	98.09
15	Asp	0.47	0.61	2.03	104.01
16	Pro	2.96	0.86	0.85	99.93
17	Glu	0.98	0.85	0.68	103.40
18	Aba	0.48	0.57	0.80	92.54
19	Tyr	1.66	0.41	0.98	93.87
20	Val	3.12	0.46	0.81	99.65
21	Met	0.87	0.96	0.63	95.76
22	Orn	2.31	0.76	1.53	104.28
23	Lys	2.73	0.72	1.28	102.11
24	Cst	0.96	0.45	1.32	94.35
25	Ile	1.33	0.30	0.89	100.26
26	Leu	2.05	0.37	0.76	101.53
27	Phe	1.48	0.28	0.66	94.64
28	Trp	1.41	1.13	2.83	91.54

AA and Eta are listed in order of elution

[a] In derivatized sample

ultrafiltration membrane). The use of additional reference substances made it possible to identify other amino compounds in plasma (P-Eta, P-Ser, Hsm, Trm, Src, Ada, Hyl), which gave a total of 34 constituents distinguished in plasma; however, quantification was performed for 27 of them.

The results of 27 AA (26 AA + Eta) assayed in plasma samples from healthy donors are presented in Table 5. To evaluate the applicability of the method, our findings were compared with the results from other studies (Le Boucher et al. 1997; Iwase et al. 1995; Teerlink et al. 1994). For most amino acids, a good compliance can be seen. For others (like Ser, Gly, His, Tau, Pro), the differences probably arise from high population variability of AA concentration. Significantly lower findings for Trp are a result of the deproteinization procedure chosen. During ultrafiltration, Trp is not removed from protein binding sites, and thus only "free" amino acid is determined.

GABA was not detected in plasma samples. Although the method was optimized for GABA separation, the average plasma level close to LOQ did not allow its determination in the presence of other AA of higher concentration.

Conclusions

A new approach for the separation of AQC-derivatized amino acid has been proposed. The chromatography used ion-pairing mechanism to increase the selectivity of the method. Mobile phase was based on triethylamine buffer containing *N*,*N*-dimethyloctylamine as a counterion. With the herein presented analytical method, the separation and identification of 34 AQC-derivatized amino acids and amino compounds present in human plasma is possible, which provides substantial advantage compared to previously described methods for AQC-tagged analytes. The

Table 5 Concentrations of amino acids in human plasma (nmol/ml)—result comparison with other studies (Le Boucher et al. 1997; Iwase et al. 1995; Teerlink et al. 1994)

LC method		Current method		Ref. (Iwase et al. 1995)		Ref. (Le Boucher et al. 1997)		Ref. (Teerlink et al. 1994)	
Deproteinization		RP-AQC		IEC-ninhydrin		IEC-ninhydrin		RP-OPA	
Amino acid		Ultrafiltration		Hydroxyapatite cartridge		SSA		SSA	
		$N = 10$		$N = 3$		$N = 100$		$N = 44$	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
1	Thr	135.3	10.1	129.4	1.1	140	33	129	24
2	Arg	82.7	16.7	73.9	0.4	80	20	90	15
3	Eta	10.5	3.2	–	–	–	–	–	–
4	Hyp	11.4	3.8	–	–	13	10	–	–
5	Asn	52.2	8.1	35.5	1.0	41	10	51	10
6	Ser	137.4	32.2	108.2	1.5	114	19	112	20
7	Gln	694.8	99.8	525.4	2.3	586	84	681	95
8	Gly	300.4	127.1	186.1	4.8	230	52	224	47
9	Cit	33.5	6.7	31.3	0.1	38	8	32	9
10	b-Ala	5.4	2.0	5.21	0.0	–	–	–	–
11	His	131.0	37.9	92.4	85.4	82	10	83	12
12	GABA	nd	–	–	–	–	–	–	–
13	Tau	138.1	87.9	–	–	55	13	83	40
14	Ala	364.9	84.1	458	0.7	333	74	356	73
15	Asp	7.2	4.0	6.47	0.5	3	1	4	3
16	Pro	225.3	67.5	199.9	2.9	168	60	–	–
17	Glu	71.6	54.4	66	–	24	15	43	20
18	Aba	20.8	6.3	–	–	23	8	25	7
19	Tyr	72.9	15.1	87.8	0.4	59	12	59	11
20	Val	221.1	49.8	319.4	0.6	233	43	237	44
21	Met	28.0	4.2	55.7	0.3	25	4	24	5
22	Orn	48.8	10.9	89.8	2.3	55	16	57	18
23	Lys	159.4	24.8	183.5	3.3	188	32	163	33
24	Cst	31.1	16.2	49.2	1.0	52	11	–	–
25	Ile	65.2	16.6	92.4	0.4	62	14	68	15
26	Leu	126.4	26.6	168.9	1.0	123	25	122	23
27	Phe	59.0	10.2	77.2	0.4	57	9	55	9
28	Trp	15.8[a]	6.6	54.1	1.2	44	7	45	10

AA are listed in order of elution

nd not detected, *SSA* sulfosalicylic acid, *IEC* ion-exchange chromatography, *OPA* o-phtaldialdehyd

[a] Trp free

results of validation proved the applicability of the method for quantification of 27 amino acids in biological samples. The ultrafiltration as proposed deproteinization procedure gave repeatable and reliable results for the amino acids under investigation. This method introduced in routine testing can be a suitable tool for amino acid profiling in plasma including all aspects of clinical application.

Conflict of interest The authors declare that they have no conflict of interest.

References

Bosch L, Alegría A, Farré R (2006) Application of the 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) reagent to the RP-HPLC determination of amino acids in infant foods. J Chromatogr B 831:176–183

Choudhury SD, Norris GE (2005) Improved binary high performance liquid chromatography for amino acid analysis of collagens. In: Proceedings of The First International Conference on Sensing Technology, Palmerston North, New Zealand, pp 332–337. http://www-ist.massey.ac.nz/conferences/icst05/proceedings/ICST2005-Papers/ICST_199.pdf

Cohen SA (1994) Application of amino acid derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate. Analysis of feed grains, intravenous solutions and glycoproteins. J Chromatogr A 661:25–34

Cohen SA, Michaud DP (1994) Synthesis of a fluorescent derivatizing reagent, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate, and its application for the analysis of hydrolysate amino acids via high performance liquid chromatography. Anal Biochem 211:279–287

Iwase H, Ozawa S, Ikuta M, Ono I (1995) Determination of amino acids in human plasma by liquid chromatography with postcolumn ninhydrin derivatization using a hydroxyapatite cartridge for precolumn deproteinization. J Chromatogr B 663:15–24

Le Boucher J, Charret C, Coudray-Lucas C, Giboudeau J, Cynober L (1997) Amino acid determination in biological fluids by automated ion-exchange chromatography: performance of Hitachi L-8500A. Clin Chem 43:1421–1428

Liu H, Sañuda-Peña MC, Harvey-White JD, Kalra S, Cohen SA (1998) Determination of submicromolar concentrations of neurotransmitter amino acids by fluorescence detection using a modification of the 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate method for amino acid analysis. J Chromatogr A 828:383–395

Nasset ES, Heald FP, Calloway DH, Margen S, Schneeman P (1979) Amino Acids in Human Blood Plasma after Single Meals of Meat, Oil Sucrose and Whiskey. J Nutr 109:621–630

Ralston PB, Strein TG (1977) A study of deproteinization methods for subsequent serum analysis with capillary electrophoresis. Microchem J 55:270–283

Reverter M, Lundh T, Lindberg JE (1997) Determination of free amino acids in pig plasma by precolumn derivatization with 6-N-aminoquinolyl-N-hydroxysuccinimidyl carbamate and high-performance liquid chromatography. J Chromatogr B Biomed Sci Appl 696:1–8

Schmidt D, Löscher W (1982) Plasma and cerebrospinal fluid γ-aminobutyric acid in neurological disorders. J Neurol Neurosurg Psych 45:931–935

Tcherkas YV, Kartsova LA, Krasnova (2001) Analysis of amino acids in human serum by isocratic reversed-phase high-performance liquid chromatography with electrochemical detection. J Chromatogr A 913:303–308

Teerlink T, van Leeuwen PAM, Houdijk A (1994) Plasma Amino acids determined by liquid chromatography within 17 minutes. Clin Chem 40:245–249

van Wandelen C, Cohen SA (1997) Using quaternary high-performance liquid chromatography eluent systems for separating 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate-derivatized aminoacid mixtures. J Chromatogr A 763:11–22

Vervoort RJM, Ruyter E, Debets AJJ, Claessens HA, Cramers CA, de Jong GJ (2002) Characterisation of reversed-phase stationary phases for the liquid chromatographic analysis of basic pharmaceuticals by thermodynamic data. J Chromatogr A 964:67–76