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Ethyl-bridged hybrid column as an efficient alternative for HPLC analysis of plasma amino acids by pre-column derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate

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Abstract

Conventional C18 silica columns have proven to be useful for the analysis of amino acids (AA) from protein hydrolysates but undesirable peak overlapping is usually found when analyzing body fluids given that a large number of AAs are present in the samples. As an alternative to silica packings, an ethyl-bridged packing for reversed-phase liquid chromatography of derivatized AAs with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) has been evaluated. The new packing material improves the separation efficiency allowing better separations when analyzing biological fluids. Moreover, this packing has advantages for routine AA analysis, such as a decrease in the total running time and an increase in the life-time of the columns. The pH of the mobile phase has a significant effect on the elution behavior of the AQC hydrolysis product (AMQ) and on the AA derivatives. It is not possible to elute AMQ before detecting the first AA derivative, which requires an accurate adjustment of the pH in the range of 5.30-5.35 to obtain good separation and resolution for the most polar compounds. Under the conditions proposed, it is possible to separate all AAs except the Gly-Gln pair, which is not a problem when hydrolyzed samples are analyzed. The AMQ-Ser pair requires either the use of a different mobile phase pH for its baseline separation or the use of fluorescence detection. Two different procedures for protein removal from plasma samples have been evaluated, solvent precipitation and ultrafiltration (UF) and it has been found that UF gives better results as no significant losses of AAs were observed. The validation of the proposed method with UV detection gives method detection limits in the range of 8-12 μM, with repeatability values <8% (n=6) and inter-day precision in plasma samples ranging from 4 to 13% (n=4).

Keywords: Amino Acids, Ethyl-bridged Packing, Human Plasma, Ultrafiltration

1. Introduction

Amino acids (AAs) are not only the basic structural units of proteins and peptides; they are also a source of energy and serve as precursors for the biosynthesis of neurotransmitters, porphyrins, polyamines, and nitric oxide [1]. The determination of physiological (free) AAs in biological fluids has become commonplace in metabolic processes. Some of these AAs are regulators of key metabolic pathways that are necessary for maintenance, growth, reproduction, and immunity in organisms, and are also important in evaluating the nutritional requirements of subjects [2,3]. In healthy adults, the levels of free AAs in plasma remain relatively constant. However, circulating levels of most AAs undergo marked changes during the course of some diseases, and elevated levels and their products are pathogenic factors for neurological disorders, oxidative stress and cardiovascular disease [3].

Traditionally, ion-exchange chromatography followed by post-column derivatization (e.g., with ninhydrin, the “classical” method) was the most common method for AA determination [4]. However, over the last few decades, methods employing pre-column derivatization followed by reversed-phase liquid chromatography (RPLC) have become more common than those employing post-column derivatization procedures [4,5]. Hydrophilic interaction chromatography (HILIC) is an attractive alternative due to the elimination of the sample derivatization step, but it suffers from several drawbacks (e.g., column care, long equilibration times and poor separation efficiency compared to RPLC) [6] and requires mass spectrometric detectors to analyze all AAs due to the weak UV absorbance of some non-derivatized AAs.

There are many different pre-column derivatizing reagents used for HPLC analysis of AAs [4,5], but *o*-phthaldehyde (OPA), 9-fluoroenylmethyl chloroformate (FMOC-Cl) and phenylisothiocyanate (PITC) are probably the most widely used. However, various shortcomings have been described for all of them [5,7]. OPA does not react with secondary AAs, some derivatives are unstable and some AAs provide more than one derivative [8]. FMOC reacts with water, yielding the interfering FMOC-OH, and the excess of reagent must be removed before chromatography [9]. PITC yields unstable derivatives and has a complex sample preparation [10]. Cohen and Michaud introduced 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) for pre-column derivatization in 1993 [11]. Since then, this compound has shown interesting features and seems to solve many of the previous indicated shortcomings. AQC reacts with primary and secondary AAs in a simple and fast process and yields derivatives that are stable at room temperature for some days. The only disadvantages described are the long analysis time, peak interference by AQC hydrolysis product (6-aminoquinoline, AMQ) with UV detection and intramolecular quenching [11-13].

When analyzing biological fluids, the limiting step in any pre-column derivatization method is the sample preparation. Firstly, it is necessary to perform a clean-up of the biological matrix to remove interfering proteins. Deproteinization can be achieved by precipitation (using organic solvents, lowering the pH with acids, addition of salts, and heat) and by ultrafiltration [14-22]. Precipitation of proteins with the addition of solvents or acids followed by centrifugation is the most common and routine methodology but it presents certain limitations, such as a decrease in the sensitivity of the method due to sample dilution, the addition of acids and solvents can

have a significant effect on the derivatizing process and the chromatographic separation, and the possible loss of metabolites by co-precipitation.

Ultrafiltration (UF) is based on separation by the size of the molecules without any chemical reaction. The major advantages are that UF completely removes the lipoprotein and the lipid resonances that are still present after the use of precipitation methods, it allows separation without destruction of either fraction, it does not dilute the sample, and it does not introduce new reagents that can affect the derivatization reaction required for the analysis of AAs with HPLC [18]. Quantitative AA recoveries have been obtained with ultrafiltration of plasma samples through 10-kDa cut-off [15,19-22].

Another key parameter in the HPLC analysis of AAs is the selection of the chromatographic column. The derivatization of AAs reduces their polarity but some derivatives are still polar and gradient separations starting with highly or totally aqueous mobile phases are required for a good separation of the most polar compounds. Given this, exhaustive and long procedures are required to re-equilibrate the columns after each run as the use of highly aqueous mobile phases can lead to the phase collapse of conventional octyl or octadecyl alkyl chains commonly used in RPLC, resulting in poor retention and selectivity for polar analytes as well as poor reproducibility, reduced column efficiency, changes in retention, and enhanced opportunity for unsymmetrical peaks [23]. New packing materials known as organic-inorganic hybrids have been created and the best characterized are those packings prepared by sol-gel synthesis using organosilanes (ethylene-linked triethoxysilane) [24,25]. The bridging ethylene groups are embedded into the silica matrix and nearly one-third of the surface silanols are removed, which results in a significant reduction in secondary retention mechanisms on these surfaces. These particles also have greater chemical stability than silica particles that enlarges the life-time of the columns.

This study has focused on two main objectives. First, the comparison of silica and hybrid columns for the analysis of plasma AAs using a pre-derivatization reaction with AQC. The effect of the pH mobile phase has also been investigated with hybrid columns. Second, the evaluation of two protein removal methodologies (solvent precipitation and UF) for the treatment of plasma samples in AA analysis.

2. Experimental

2.1. Reagents and Solutions

For the identification of each peak in the chromatograms, individual solutions of each L-amino acid were prepared from the following salts (all analytical reagent grade with >97% purity): L-glutamic acid monosodium salt hydrate, L-arginine, L-aspartic acid, L-serine, glycine, L-histidine, L-threonine, L-cysteine, L-tyrosine, L-lysine, L-glutamine, L-phenylalanine, and L-asparagine were from Sigma-Aldrich (Germany); L-alanine, L-proline, L-valine, L-tryptophan were from Merck (Germany); L-isoleucine was from Fluka (Switzerland); L-leucine was from Panreac (Spain); and L-methionine was from BDH (Spain).

A standard solution containing 17 AAs at 2.5 mM in 0.1 M HCl (except L-cysteine at 1.25 mM) was used for quantification (Sigma-Aldrich). The standard three other AAs (Gln, Asn, and Trp) were prepared from the previously indicated salts in 0.1 M HCl. γ-Aminobutiric acid (GABA) was used as the internal standard (IS). Stock solutions containing the 20 AAs at 1 mM and the IS were prepared weekly in 0.1 M HCl and stored at 4ºC. Calibration working solutions were freshly prepared before use.

The AQC derivatization reagent (AccQ·Tag^{TM} derivatization kit) was purchased from Waters Corporation (Milford, MA, USA). Methanol for HPLC was from Scharlab (Spain) and Acetonitrile for HPLC was from Fisher Chemical (Fisher Scientific, Loughborough, UK). Deionized water was from a Milli·Q Ultrapure water system (Millipore Iberica, Spain).

Mobile phases were prepared following the procedure described by Cohen and Michaud [11]. They consist of (i) a buffer solution containing 140 mM sodium acetate (Fluka, Switzerland), 17 mM trimethylamine (Sigma, Germany), 1mg·L^{-1} of ethylendiaminetetraacetic acid (EDTA) (Sigma), and *o*-phosphoric acid 85% (Sigma) to adjust the pH to the desired value (between 5.0 and 5.6), and (ii) 60% acetonitrile solution. All mobile phase solutions were filtered through 0.45 μm filters (Whatman, Germany) and degassed before use.

2.2. Instrumental

The chromatographic experiments were performed with a SpectraSYSTEM (Thermo Scientific, USA) liquid chromatograph equipped with a vacuum membrane degasser (SCM1000), a gradient pump (P4000), an autosampler with a column oven and a Rheodyne injection valve (AS3000), and a diode array detector (PDA Plus). 10 μL samples were injected for each analysis. A preliminary evaluation of the injection volume showed that an injection volume of 20 μL resulted in excessive band broadening for the most polar AA derivatives that leads to incomplete baseline separation of some pair of compounds, such as Glu and Asn. Detection was performed at 254 nm. The acquisition of chromatographic data was performed by means of ChromQuest software (v. 5.0, Thermo Electron).

Three different reversed-phase columns were evaluated for the separation of the AA derivatives: (i) Ascentis C18 (150 mm x 2.1 mm, 5 μm) (Supelco, USA), (ii) TRACER EXCEL 120 ODS-B C18 (150 mm x 4 mm, 5 μm) (Teknokroma, Spain), and (iii) Gemini - NX C18 110A (250 mm x 4.6 mm, 5 μm) (Phenomenex, Torrance, CA, USA). All columns were protected with guard columns.

2.3. Plasma samples and treatment

Blood samples were obtained from the Stroke Unit at the Dr Josep Trueta University Hospital (Girona, Spain). Samples were collected in Vacutainer tubes containing EDTA as the anticoagulant. After mixing, the tubes were centrifuged at 1500 x g for 12 min in under 30 minutes. The supernatant plasma was transferred to clean polypropylene tubes and stored

at -80ºC until analysis. The local ethical committee approved the study and all participants gave written informed consent.

Two different procedures were evaluated for the elimination of proteins before the derivatization of free AAs. First, slow protein precipitation with methanol (1:4 plasma:methanol ratio) at 4ºC, and centrifuged for 10 min at 7600 x g . Second, ultrafiltration with Amicon Ultra-0.5mL centrifugal filters with a 3-kDa Ultracel membrane (Merck Millipore, Ireland): 200-250 μL of plasma samples were added in a centrifugal filter and centrifuged for 20 min at 1200 x g.

2.4. AA derivatization

AA derivatization was conducted according to the manufacturer's instructions. Briefly, 20 μL of amino acid stock mixture or a plasma extract were mixed with 140 μL of borate buffer and 40 μL of AQC (AccQ·Tag reagent, previously dissolved in 1.0 mL of diluent). The reaction was performed for 10 min at 55ºC in a dry heating block.

The manufacturer indicates that the resulting derivatives are stable at room temperature for up to one week. However, storing at 4ºC we found that derivatives were stable for at least 15 days (p>0.50 when the results obtained for each AA derivative from the same standard and the same plasma sample were compared over a 15 day period).

2.5. Quality controls

Method blank controls were prepared following the same derivatization procedure as plasma and standard samples using milli·Q water as the blank matrix. Some standards and duplicate plasma samples were used as control samples.

Before proceeding with the HPLC procedure, a standard was analyzed sequentially until stable retention times were obtained for all AAs (<0.1 min variation in retention times in three consecutive analyses). Once the stability of the system was obtained, a method blank was injected twice to confirm that no system contamination took place. Calibration using six standards in duplicate was performed at the beginning of each sequence of batches. Series of 15 samples were analyzed in each batch: 2 method blanks, 2 quality controls (1 standard and 1 plasma duplicate) and 10 plasma samples. Three calibration standards (low, medium and high level) were analyzed between each batch to confirm that retention times and calibration parameters were maintained.

3. Results and Discussion

3.1. Selection of the analytical column

The preliminary work developed by Cohen and Michaud [11] was intended for the analysis of protein hydrolysates where only 17 out of 20 proteinogenic AAs can be detected due to the

degradation of Gln, Asn and Trp during acidic hydrolysis [26]. When analyzing body fluid samples, a larger number of AAs are present in the samples, which results in undesirable peak overlapping [5,22,27]. Some improvements have been described using conventional C18 columns, which are mainly based on modifications of the mobile phase gradient and the use of longer columns [5,27]. However, the resolution was always incomplete when analyzing free AAs instead of protein hydrolysates. The best results have been obtained through modification of the column dimensions [5,22] or by using different types of stationary phase [28].

Given that C18 silica columns are so widely used, we first tested two of these columns with the same length (150 mm) and particle diameter (5 µm) but different internal diameters (2.1 and 4 mm). As the two columns had different internal diameters, the linear velocity was taken into account instead of the volumetric flow (f_{vol}) [29]. The diameter ratio between the two columns was:

$$R = \left(\frac{4.6\ mm}{2.1\ mm}\right)^2 = 4.8$$

Therefore, as f_{vol} for the 4 mm id column was set at 1 mL·min^{-1}, f_{vol} for the 2.1 mm i.d. column had to be:

$$f_{vol} = \frac{1\ mL \cdot min^{-1}}{4.8} = 0.21\ mL \cdot min^{-1}$$

The analysis of standard samples at the indicated f_{vol} for each column confirmed that linear velocity did not vary and that the retention times and resolutions for AAs were almost the same in the two columns. Although the use of the column with the smaller i.d. does not solve the problem of resolution with non-hydrolysates, it does result in a significant reduction in the volume of eluent solvents required (up to 5 times) for the analysis. Unfortunately, the equilibration of the lower i.d. column was not satisfactory. The use of an equilibration procedure between injections that gave constant retention times (<0.2 min variation) between runs with the 4 mm i.d. column was unsuitable with the column with the smaller i.d. It was not possible to obtain reproducible retention times for those derivatives that eluted with percentages of aqueous buffer in the mobile phase >90%. This suggests that the most suitable parameter for conditioning the column is the total conditioning volume regardless of the flow used. This will lead to a significant increase in the total run time required between samples with the lower i.d. column.

As the reduction in the i.d. column did not give practical improvements, a different type of packing was evaluated. Choudhury and Norris [28] found that the higher surface area and carbon loading of a Gemini column with respect to C18 columns resulted in better selectivity of AAs and critical separations usually found with the C18 columns, such as His from Gly, were easily achieved. In this study, we chose a new generation of Gemini columns containing bridged ethylene groups embedded into the silica matrix (Gemini - NX C18 110A).

Some advantages were found with this new type of column: (i) critical separations shown in previous studies were easily solved, except for the Gly-Gln pair; (ii) total analysis time (run + equilibration) with the Gemini NX column was shorter (65+20 min) than with the standard C18 column (95+35 min); and (iii) although the Gemini NX column was 1.67 times longer than the

C18 column, the backpressure was reduced by more than two times resulting in the Gemini column having a longer life-time. As indicated in the introduction, this type of column is expected to have a longer life-time as these particles are more stable than conventional silica particles. In our study, the conventional C18 column was able to give adequate chromatographic efficiency for about 100-150 injections. When the Gemini column was used, more than 500 injections were performed with the same column without changes in the efficiency being recorded.

Given the results we obtained, we selected the Gemini column as being the most appropriate.

3.2. Temperature and gradient elution

Increased column temperatures improve the resolution of some pairs of compounds with C18 columns and the best results have been obtained using thermostated ovens at 37 [11] and 34ºC [27]. Therefore, the effect of oven temperature was evaluated with the Gemini column in the 27 to 40ºC range. As with C18 columns, sharper peaks are obtained for less retained compounds when the column temperature was increased. Moreover, a 10-15% decrease in the retention time for the most polar compounds was also observed. A temperature of 40ºC was therefore selected as the working temperature because no improvement in the selectivity and resolution was observed at higher temperatures.

Different gradients were evaluated with the Gemini column and the best separation was obtained with the conditions indicated in Table 1. Shorter cleaning and equilibration times than those indicated in Table 1 allowed reproducible retention times to be obtained when analyzing standard samples. However, a cleaning time of around 10 min and an equilibration time with 100% buffer solution for another 10 min were required when plasma samples were analyzed.

3.3. The effect of mobile phase buffer pH

It is already known that the separation of most polar AA-AQC derivatives and the hydrolyzed excess of reagent (AMQ) depends on the pH of the aqueous buffer with silica-based columns [11,22,27,28,30] and the compound that has a greater variation in the retention time with the pH is AMQ. In general, acidic pH is needed for the complete resolution of AA derivatives and AMQ, and pH values of around 5.0 are required to displace the AMQ peak at the beginning of the separation before any AA derivative can be detected [11]. Moreover, variations of less than 0.2 pH units can cause significant modifications in the chromatographic resolution [22,27].

We evaluated the effect of mobile phase pH on separation with the Gemini NX column and a significant dependence on the pH was also found although behavior varied depending on the compound. Figure 1 shows the evolution of the retention times with the pH for those compounds that eluted at the beginning of the chromatograms (AMQ, Asp, Glu, Asn, Ser, and His).

The compound that showed the greatest variation in its retention time is AMQ and a pH<5.0 was required to elute AMQ at the beginning of the chromatograms, before the first AA derivative (Asp). However, at pH<5.1 there was an overlap between Glu, Asn and Ser. In our case, this is important as Glu is one of the main target compounds in the studies developed in our laboratory. When the buffer pH was increased, AMQ always appeared between the different AAs.

In general, AAs can be classified into three groups according to the different tendencies observed. A first group corresponds to the AA derivatives that appear at the beginning of the chromatograms (Asp and Glu), which did not give significant differences in their retention times in the pH range evaluated (p>0.25, ANOVA test, three replicates at each pH). A second group is formed by Asn, Ser, His, Gly, Gln, and Arg. In this group, all the compounds had longer retention times with increased pH (p<0.05). The third group was composed by Thr, Ala, Pro, Cys, Tyr, Val, Met, Lys, Ile, Leu, Phe, and Trp. These AAs did not show significant variations in retention times in the 5.0-5.35 pH range but a significant increase was found for all these AAs when the pH was 5.45.

The assessment of the co-elutions that took place at different pH values indicated that pH<5.3 always resulted in the overlapping of AMQ with Glu, Asn or Ser. Moreover, Glu and Asn co-eluted at pH<5.2 and Glu, Asn and Ser at pH≤5.05. Therefore a pH≥5.3 is required to separate these compounds. With regards to the other compounds, the Thr-NH_3 pair showed baseline separation when the pH was <5.35 but overlapping was observed at pH≥5.4. The same behavior was observed with the His-Gly and Cys-Tyr pairs. The first pair started to overlap at pH=5.40 and the second at pH=5.45. Separation of Gly and Gln was never achieved in the pH conditions evaluated with the Gemini NX column. In order to improve the efficiency for this pair of AAs, the same column with 3μm particle size could be used. This will lead to an increase in the back pressure, but the significant decrease in the back pressure obtained with this type of coating when compared with conventional silica based columns suggests that the pressure obtained may be affordable with a conventional HPLC system.

Taking into account these results, the best pH conditions for the separation of the AAs is pH=5.30-5.35 (we chose pH=5.33±0.02), although at this pH Ser cannot be analyzed with UV detection as it co-elutes with AMQ. It is important to note that slight variations in the pH (of only 0.05 units) have a significant effect on the separation and so the buffer solution has to be prepared precisely. Figure 2 shows the chromatogram obtained in the analysis of a standard sample prepared from the 17 AA standard solution.

3.4. Removal of proteins

As indicated in the introduction, preliminary sample treatment for the removal of proteins is required when analyzing plasma samples. Two different procedures were evaluated in the present study: (i) a conventional protein precipitation with methanol and (ii) ultrafiltration with 3-kDa membrane filters. To perform the study a pooled plasma sample was prepared and three different portions of the pooled sample were evaluated with each method. The values obtained were evaluated for each AA using the statistical t-test.

Results (Table 2) indicate that non-significant differences in the recoveries were obtained for eight AAs (p>0.05). For a further eight AAs, the levels detected with the UF procedure were significantly higher than with solvent precipitation (p<0.05). Thr and Trp gave larger recoveries with the solvent precipitation procedure.

Slow protein precipitation with solvents is required to minimize losses of small molecules by co-precipitation [16,17]. Although we tried to precipitate the proteins at low temperature (4ºC) and with a slow addition of solvent, there was still co-precipitation of some AAs, which indicates that this process is highly dependent on the test procedure used. Moreover, two other significant drawbacks were found with solvent precipitation of proteins. First, some of the impurities remaining in the supernatant gave peaks that co-eluted with the AAs, as in the case of Thr, which resulted in an excessive recovery for this AA with solvent precipitation. Second, the mixing of the plasma sample with solvent resulted in a five times dilution of the samples. This resulted in those AAs present at smaller concentrations in plasma samples (Asp and Met) not being detected with the solvent precipitation procedure.

The best results were obtained with the UF procedure. Other studies also evaluated UF for protein removal from plasma samples [15,20,22] with the same results. The only AA that did not give good recoveries with UF was Trp, a finding that has also been observed in previous studies [15,22]. The low recovery of Trp has been associated to the equilibrium between the free and protein-bound fraction of physiological Trp. With UF, those compounds that are strongly bound to proteins, as in the case of Trp, are also retained in the filter. Therefore, only "free" Trp can be analyzed [22].

Given the results obtained, the UF procedure was selected for the analysis of plasma samples.

3.5. Validation of the method

The efficiency of the conditioning procedure and the stability of the chromatographic column was evaluated from the retention times of controls over a three day period (n=2 each day). Residual standard deviations (RSD) <0.55% were obtained for all AAs when standard controls were evaluated. In the case of plasma controls, RSD<1% were obtained for all AAs except for Asp and Glu, which gave values in the range of 1-2%.

Table 3 shows the quality parameters obtained for the proposed procedure. Statistical evaluation of all calibrations showed that none of the compounds gave intercept values that were significantly different from zero. Moreover, no significant differences between the slopes of the calibration curves obtained between all calibrations performed over a four month period, with variations in the slopes <6%. Linearity was confirmed for all compounds from the evaluation of the distribution of residuals and the application of the lack-of-fit F test (p>0.56, n=6, three replicates at each level). Determination coefficients (R^2) greater than 0.996 were obtained for all analytes with direct calibration (without IS correction) and using IS correction. The method detection limits (MDL) were estimated from the linearity parameters by taking the standard deviation of the intercept as the standard deviation of the blank. MDLs ranged from 8 to 14 μM, which are low enough to analyze AAs at the expected levels in plasma samples.

Inter-day precision and repeatability was measured with standards and plasma samples without significant differences being found between the two types of matrix. RSD<6% were obtained for repeatability (n=6) and <12% for inter-day precision (Table 3), which were considered acceptable.

For sample analysis, the quality control procedure described in the experimental section was applied. No AA was detected in any of the blanks. Deviations <10% in the concentrations found and <1% in the retention times from control standards and <2% control samples were applied as control limits.

3.6. Analysis of plasma samples

The developed method was applied for amino acid profiling in forty plasma samples from different individuals. Figure 3 shows the chromatogram obtained for one of the samples. The quantitative results of 15 AAs are presented in Table 4. Ser was not analyzed as it coelutes with AMQ when UV detection is used. The Gly-Gln pair was determined as a single compound as these two analytes coeluted with the Gemini NX column. In the case of Asp, Cys and Trp, their levels were practically always below their corresponding quantification limits and so these AAs were not included in the calculations. The distribution of the values obtained for each analyte was evaluated applying the Shapiro-Wilk test. Non-normal distribution was found for all AAs (p<0.05) except for Tyr and Lys.

Different non-identified compounds (marked as unknowns, u, in Figure 3) were detected in all the plasma samples evaluated, which may be associated with other non-proteinogenic AAs or derivatives containing amino groups that have also been detected in human plasma samples, such as taurine, ornithine, cystine and homocysteine [5,22,31,32].

The levels detected for most of the AAs agree with those reported in previous studies for healthy controls [5,22,31-37]. Only two of the AAs that were quantified, Arg and Glu, had mean values that were different from those reported for healthy patients. However, plasma samples evaluated in this study were obtained from stroke patients on arrival at a stroke unit and it is known that plasma levels for these two AAs are altered as a result of this condition. Arg levels in stroke patients tend to be lower than for healthy patients [38,39] as Arg is consumed during the synthesis of nitric oxide, which is involved in ischemic brain damage. The levels found in this study agree with those reported in samples from patients suffering a stroke [38,39]. Glu behaves differently and increased levels are detected in stroke patients [35,37,40-43]. The levels reported in these studies also agree with the results obtained in the present study.

4. Conclusions

The use of reversed-phase liquid chromatography with an ethyl-bridged packing for analyzing AAs in biological fluids results in improved separation efficiency when compared with conventional silica packings. All proteinogenic AAs, except the Gly and Gln pair, can be

effectively separated with the ethyl-bridged packing. Ser can be separated from AMQ using a different mobile phase pH but this would lead to AMQ overlapping with another AA. Moreover, other AAs also present in biological fluids can also be separated and determined with this type of packing. The buffer pH is the most important parameter affecting the separation selectivity and slight differences of just 0.05 units can have a significant effect on the chromatographic efficiency for the most polar derivatives.

The comparison of a conventional protein removal procedure, such as solvent precipitation, with ultrafiltration indicates that ultrafiltration gives better results as there are no losses through co-precipitation. Moreover, ultrafiltration does not result in plasma dilution before the analysis (improving the sensitivity of the method) and does not introduce new substances that modify the matrix composition.

On analyzing a large cohort of plasma and control samples with a single column, the stability of the stationary phase was not found to be problematic and results were reproducible. This confirms the workability of this column packaging for the routine analysis of amino acids in complex biological fluids.

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Figure Captions

Figure 1. Effect of mobile phase A pH on the retention time for the derivatized AAs and the hydrolyzed excess of reagent (AMQ).

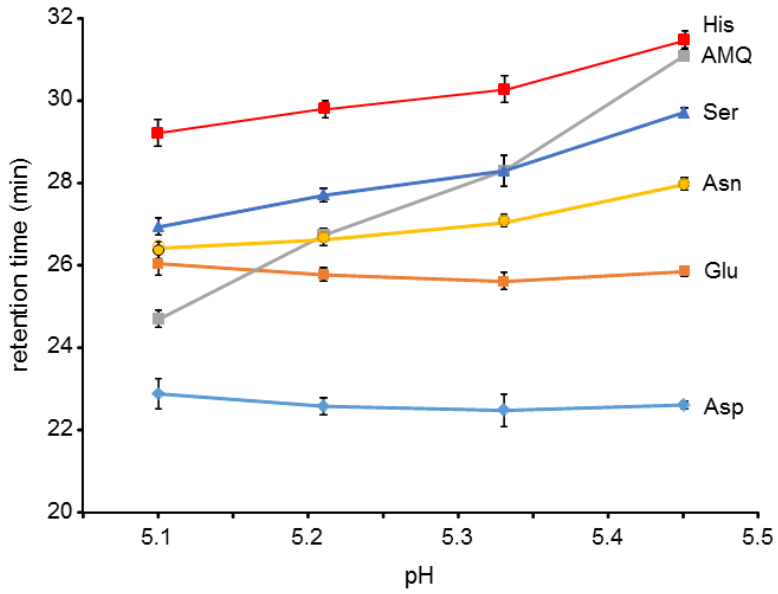

Figure 2. Chromatogram obtained in the analysis of a calibration solution at 100 μM level prepared from the 17 AA standard solution.

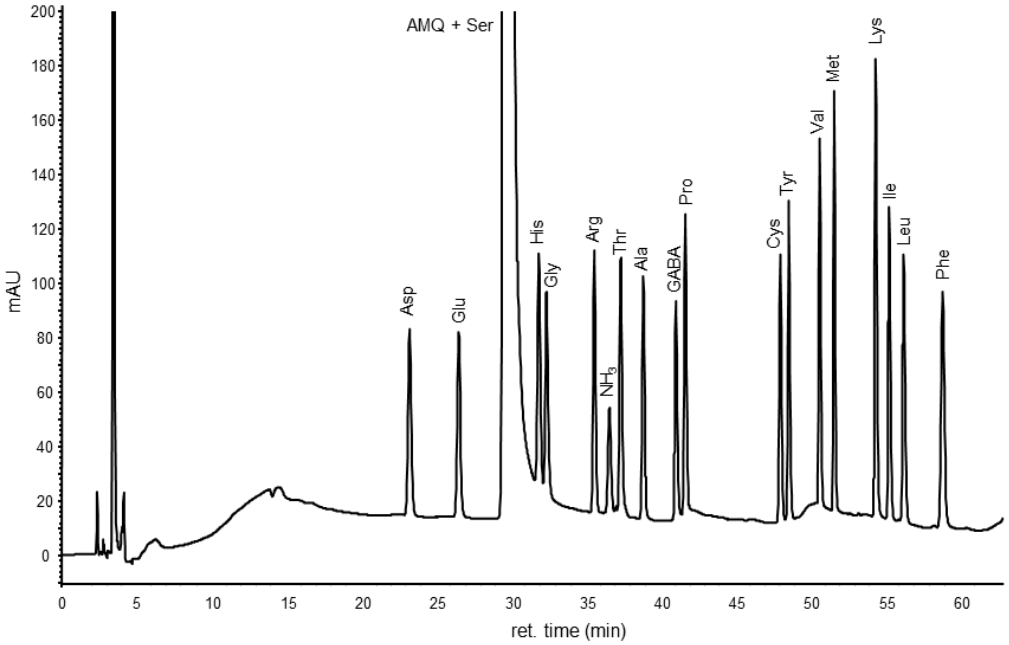

Figure 3. Chromatogram corresponding to a plasma sample.

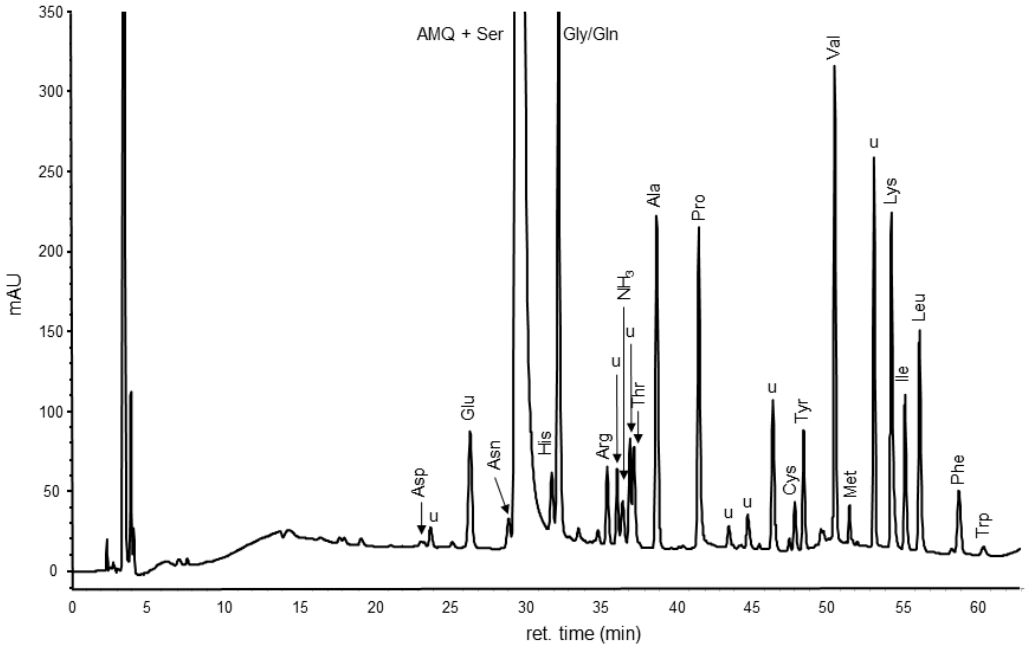

Table 1. Gradient selected for the separation of the amino acids in plasma samples. Mobile phase A is the buffer aqueous solution and mobile phase B is acetonitrile 60%

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	100	0
0.5	98	2
15	93	7
25	90	10
45	74	26
48	67	33
63	0	100
70	0	100
75	100	0
85	100	0

Table 2. Comparison between mean concentrations (μM) detected applying the two protein removal methods from a pooled plasma sample (n=3 for each method). Tabulated $t_{value}(\alpha=0.05, \nu=4)$ is 2.776. rsd: relative standard deviation. nd: non-detected. MQL: method quantification limit

AA	MeOH precipitation		UF		Experimental
	Mean (μM)	rsd	Mean (μM)	rsd	t_{value}
Asp	nd	--	<MQL	--	--
Glu	124	3	147	2	6.94
His	<MQL	--	41	6	--
Gly+Gln	156	3	157	3	**0.24**
Arg	157	10	152	5	**0.48**
Thr	250	2	128	6	24.59
Ala	143	5	192	4	8.62
Pro	145	8	164	3	**2.32**
Cys	<MQL	--	55	5	--
Val	85	7	136	4	10.21
Met	nd	--	<MQL	--	--
Lys	113	11	101	5	**1.32**
Ile	46	9	47	2	**0.285**
Leu	98	8	84	3	**2.39**
Phe	36	7	39	2	**1.68**

Table 3. Quality parameters of the developed method. *b*=slope (without IS correction), s_b=standard deviation of the slope, R^2= determination coefficient (without IS correction). Calibration: 20-600 μM. MDL: method detection limit

AA	b (s_b)	R^2	MDL	Interday precision (RSD)		Repeatability (RSD)
			(μM)	Standard (n=5)	Plasma (n=4)	Standard (n=6)
Asp	10913 (250)	0.9984	10	9.1	12.5	1.5
Glu	11119 (218)	0.9988	9	8.4	10.3	2.7
Asn	11098 (234)	0.9986	12	7.6	9.1	4.5
His	11198 (258)	0.9979	11	6.4	9.2	3.1
Gly	10553 (302)	0.9967	13	6.3	7.4	4.1
Arg	10786 (265)	0.9976	10	5.8	5.2	5.2
Thr	11317 (312)	0.9970	8	7.5	5.4	4.8
Ala	10913 (250)	0.9984	10	8.9	4.8	7.2
Pro	12359 (274)	0.9985	9	8.5	4.1	3.1
Cys	20676 (396)	0.9985	8	9.0	8.7	1.7
Tyr	12682 (313)	0.9976	9	6.2	4.6	3.8
Val	12521 (276)	0.9985	10	9.9	4.9	3.4
Met	12360 (305)	0.9982	11	7.4	5.4	2.7
Lys	18219 (383)	0.9987	10	7.1	8.2	2.2
Ile	13029 (284)	0.9986	10	10.0	5.5	3.2
Leu	12528 (278)	0.9985	14	7.8	8.9	3.5
Phe	13374 (197)	0.9991	10	7.5	8.2	3.8
Trp	13277 (282)	0.9978	9	6.9	7.8	5.4

Table 4. Amino acid concentrations (μM) and statistics found in the analysis of plasma samples (n=40).

AA	Mean	Median	sd	Min	Max
Glu	98	81	62	22	294
Asn	30	28	8	17	53
His	55	53	12	30	83
Gly+Gln	588	563	182	107	959
Arg	57	52	20	12	107
Thr	99	93	33	32	178
Ala	311	297	126	101	603
Pro	185	174	68	66	316
Tyr	59	55	22	<MQL	117
Val	191	180	49	114	345
Met	19	16	12	<MQL	77
Lys	132	124	71	63	205
Ile	60	56	19	27	117
Leu	110	102	38	65	240
Phe	50	48	16	38	90