Plasma Amino Acids Determined by Liquid Chromatography Within 17 Minutes

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We present an HPLC method for the determination of amino acids in plasma. The method is based on automated precolumn derivatization of amino acids with o-phthalaldehyde, separation of the derivatives by reversed-phase chromatography, and quantification by fluorescence detection. Complete separation was achieved within 12 min. Total analysis time, including derivatization, chromatography, and reequilibration of the column, was 17 min. The assay was linear from 5 to 800 μmol/L for all amino acids. Recovery of amino acids added to plasma samples was 96–106%, except for tryptophan (89%). Within-run precision (CV) was 1.8–6.4%, and between-run precision was 2.1–7.2%. The method can be used for determining primary amino acids in plasma and cerebrospinal fluid. The simple sample preparation and short analysis time make the method particularly suitable for routine analysis of large series of samples.

Indexing Terms: chromatography, reversed-phase/fluorometry

Determination of plasma amino acids has become increasingly important in evaluating the nutritional requirements of patients. This has resulted in supplementation of nutrition with amino acids such as glutamine, arginine, and branched-chain amino acids (1–3). The beneficial effects of these feedings on the nutritional status of patients has stimulated clinicians and researchers to study amino acid metabolism in different disease states (4–6). These developments have led to an increase in the number of plasma amino acid determinations and the need for a rapid determination method.

Physiological amino acids are traditionally determined by ion-exchange chromatography in combination with postcolumn ninhydrin detection. These analyses, usually performed on dedicated instruments, are somewhat laborious, costly, and time-consuming. Alternatively, amino acids can be analyzed by reversed-phase HPLC after precolumn derivatization. Various reagents for the precolumn derivatization of amino acids can be used, e.g., dabsylchloride (7), phenylisothiocyanate (8–11), naphthylisocyanate (12), 9-fluorenylmethyl chloroformate (13, 14), and o-phthalaldehyde (OPA) (15–23). Of these reagents, OPA is most widely used. In the presence of a thiol compound, OPA reacts readily with primary amino acids, forming highly fluorescent derivatives. Although the isoindole derivatives formed are not very stable (24), accurate results can be obtained by automation of the derivatization reaction. Stability of the derivatives depends on the choice of the sulphydryl reagent added to the derivatization mixture (24). 3-Mercaptopropionic acid (MPA) reportedly results in more stable products than does 2-mercaptoethanol (18, 23).

Many of the published methods that include OPA derivatization require analysis times close to 1 h, limiting the number of samples that can be analyzed in a single run. Furthermore, most reports indicate that not all amino acids in plasma are adequately separated. We have developed a method for the analysis of all major primary amino acids in plasma, using automated precolumn derivatization with OPA in combination with MPA. Separation is achieved in <12 min with a cycle time of 17 min.

Materials and Methods

Chemicals

We obtained OPA and MPA from Fluka (Buchs, Switzerland). 5-Sulfosalicylic acid (SSA), boric acid, potassium dihydrogen phosphate, and HPLC-grade methanol were supplied by Merck (Amsterdam, The Netherlands). HPLC-grade acetonitrile was obtained from Westburg (Leusden, The Netherlands). Tetrahydrofuran was supplied by J. T. Baker (Deventer, The Netherlands). We prepared HPLC-grade water from demineralized water by using a Milli-Q UF Plus water purification system (Millipore, Milford, MA). Triethylamine was obtained from Aldrich (Brussels, Belgium). Individual amino acids were obtained from Sigma (St. Louis, MO), Merck, and BD Chemicals (Poole, UK). Amino acid standard solutions were prepared in 100 mmol/L HCl. A combined standard solution, prepared by mixing individual standard solutions, was stored in small aliquots at −20°C. A separate standard solution of glutamine dissolved in water was prepared and stored at −20°C.

Equipment

The HPLC system consisted of a Model 480 pump from Gynkotek (Germering, Germany) and a Model 232-401 autosampler from Gilson (Villiers le Bel, France). The injection valve (Rhodeyne, Cotati, CA) was equipped with a 20-μL sample loading loop. To minimize pressure pulses during switching of the injection valve, we fitted it with a pressure-bypass circuit, as described by Dolan and Snyder (25). Eluents were degassed on-line with a Gastorr GT-103 degasser from Lab Quatex (Tokyo, Japan). Peak monitoring was performed with a Model 980 fluorescence detector from Applied
Bioanlytical Systems (Ramsey, NJ). A Baseline 810 chromatography workstation from Millipore, running on a M230S personal computer (Olivetti, Rotterdam, The Netherlands) was used for data processing. Analyses were performed on a 3-μm Microsphere C18 column (10 cm × 4.6 mm (i.d.)) from Chrompack (Middelburg, The Netherlands), protected by a reversed-phase guard column (1 cm × 2.0 mm (i.d.)) from the same supplier.

Subjects
To determine reference values for amino acids, we analyzed plasma samples from 44 healthy volunteers (mean age 38 years, range 19–77 years). Blood samples were obtained after an overnight fast. The protocol was conducted with approval of the Ethics Committee of the Free University Hospital and with oral informed consent of each subject.

Collection and Processing of Samples
Tubes containing 20 mg of SSA were prepared as follows: A solution of 200 g SSA in absolute ethanol was divided into 0.1-mL aliquots in 1.0-mL tubes. After evaporation of the ethanol in an oven (overnight at 50°C), the tubes were capped and stored at room temperature. These SSA tubes contain sufficient SSA to deproteinize 0.5 mL of plasma. We also prepared tubes containing 8 mg of SSA, sufficient to deproteinize 0.2 mL of plasma. Venous blood was collected in heparin-containing tubes. After centrifugation (2000g for 10 min at 4°C), 0.2 or 0.5 mL of plasma was pipetted into a SSA tube. The tubes were vigorously mixed in a vortex-type mixer, snap-frozen in liquid nitrogen, and then stored at −70°C. On the day of analysis the tubes were thawed at room temperature and centrifuged (15 min at 3000g and 4°C) to remove precipitated protein. The clear supernatants were transferred to polypropylene vials and stored in a refrigerated (4°C) sample rack of the autosampler.

Preparation of Reagents
The OPA–MPA reagent stock solution was prepared every 3 days. We dissolved 25 mg of OPA in 0.5 mL of methanol, after which we added 4.5 mL of borate buffer (100 mmol/L, pH 10.0) and 25 μL of MPA. OPA–MPA working solution was prepared on the day of analysis by adding one part of stock solution to 20 parts of 100 mmol/L borate, pH 10.0. Final concentrations of OPA and MPA were 1.77 and 2.72 mmol/L, respectively. An internal standard stock solution (10 mmol/L norvaline in 50 mmol/L HCl) was prepared every 2 months and stored at room temperature. Internal standard working solution was prepared by diluting the stock solution with water to a final concentration of 1 mmol/L norvaline. Neutralizing buffer consisted of 400 mmol/L potassium dihydrogen phosphate and 10 mL/L triethylamine and was prepared each week.

Derivatization and Chromatography
The buffer used for the preparation of the mobile phases consisted of 9 mmol/L potassium dihydrogen phosphate and 0.5 mL/L triethylamine, adjusted to pH 6.9 with KOH. Mobile phase A was prepared by mixing 1000 mL of this buffer, 1000 mL of water, and 4 mL of tetrahydrofuran. Mobile phase B was a mixture of the buffer, methanol, and acetonitrile (50/35/15 by vol). Both mobile phases were filtered through a 0.45-μm filter by suction filtration. Fully automated precolumn derivatization was performed by using the Gilson autosampler. The sample tray contained three racks. One rack contained up to 96 samples in capped vials. A second rack contained an equivalent number of empty reaction vials. Internal standard solution, OPA–MPA reagent, and neutralizing buffer were each stored in multiple capped vials in the third rack. Each set of vials was used for the derivatization of six consecutive samples. Derivatization was started by the transfer of 20 μL of water to an empty reaction vial, followed by the addition of 5 μL of sample, 5 μL of internal standard solution, and 90 μL of OPA–MPA reagent. Sample and reagent were mixed by five cycles of aspirating the incubation mixture from the bottom of the tube and dispensing it from a height of 5 mm. After a 3-min incubation at room temperature, 50 μL of neutralizing buffer was added; after mixing as described above, 3 μL of the reaction mixture was injected. The needle of the autosampler was washed with water between all fluid-transfer steps. During chromatography of one sample, the next sample was being derivatized.

Chromatographic separation was performed at ambient temperature. Table 1 shows the composition of the eluent during analysis. Detection was performed fluorometrically by using an excitation wavelength of 230 nm and an emission cutoff filter of 389 nm. We calculated amino acid concentrations by using the peak area relative to the area of the internal standard peak.

Assay Performance
Linearity of the analysis was determined from 5 to 800 μmol/L by plotting peak response (area of the amino acid peak divided by the area of the internal standard peak) vs concentration. At concentrations ≤10 μmol/L, a mixture of all amino acids evaluated was used. At higher concentrations, mixtures of only five amino acids were analyzed, to ensure that the OPA–MPA reagent was present in sufficient excess for complete derivatization.

Within-run reproducibility was evaluated by replicate analysis of a standard solution (n = 33) and a plasma pool (n = 28) in a single run. Between-run reproducibility was

<p>| Table 1. HPLC gradient conditions. a |
|-------------------------------|-----------|</p>
<table>
<thead>
<tr>
<th>Time, min</th>
<th>% mobile phase B in eluent</th>
</tr>
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<tbody>
<tr>
<td>0.0</td>
<td>2</td>
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<tr>
<td>3.5</td>
<td>25</td>
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<tr>
<td>5.2</td>
<td>44</td>
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<tr>
<td>6.9</td>
<td>52</td>
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<tr>
<td>10.0</td>
<td>100</td>
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<tr>
<td>11.0</td>
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a Flow rate was 1.5 mL/min.
tested by analyzing a plasma pool stored at \(-70^\circ\text{C}\) on different days over 4 months \((n = 24)\).

Recovery was evaluated by mixing equal volumes of plasma and amino acid standard mixture or water. After mixing, the samples were deproteinized with SSA-coated tubes and analyzed immediately. Recovery was calculated as the difference between samples containing amino acid standards and samples without the mixture, expressed as a percentage of the amount of amino acid added.

**Results**

Fig. 1 shows the chromatographic separation of an amino acid standard and a plasma sample. Eluent composition and gradient shape were optimized to obtain baseline resolution of all amino acids. Some critical

![Typical chromatograms of amino acid standard (A) and human plasma sample (B).](image)

Concentrations of amino acids in the standard solution are 400 \(\mu\text{mol/L}\), except His, 1-mh, and 3-mh (100 \(\mu\text{mol/L}\)) and Tau (200 \(\mu\text{mol/L}\)). 1-mh, 1-methylhistidine; 3-mh, 3-methylhistidine; Aba, \(\alpha\)-aminobutyric acid; Norval, norvaline.
pairs of amino acids are difficult to separate. To obtain baseline separation of glycine and threonine we found it essential to include a small percentage of tetrahydrofuran in mobile phase A. Separation of tryptophan and phenylalanine was accomplished by optimizing the ratio of methanol and acetonitrile in mobile phase B. Inclusion of triethylamine in mobile phase A allowed separation of aspartic acid from SSA used to deproteinize the plasma samples. The elution position of histidine and arginine relative to the other amino acids can be shifted by adjusting the ionic strength of the mobile phase buffer. Separation of 1-methylhistidine and 3-methylhistidine was optimized by varying the pH of the mobile phase buffer. After determining the optimal composition of the eluents, fine-tuning of the separation was performed by modifying the gradient shape. By using a segmented gradient (Table 1), resolution was improved with a concurrent reduction of analysis time.

There was a linear relation between peak response (ratio of areas of amino acid and internal standard) and concentration from 5 to 800 μmol/L for all amino acids (correlation coefficients all >0.999). Within-run precision was determined by replicate analysis of an amino acid standard solution (n = 33) and a plasma sample (n = 28). For the standard solution the CV was 1.0–1.8%. For the plasma sample the CV was 1.8–4.9% with the exception of aspartic acid (6.4%). For quality-control purposes a plasma pool was stored at −70°C in small aliquots; this sample was analyzed in each run. Between-run CV, assessed from the results from 24 consecutive runs performed over 4 months, was <5% with the exceptions of aspartic acid (7.2%), taurine (5.8%), and tryptophan (6.1%).

Accuracy of the method was evaluated by determining analytical recovery. Recovery of amino acids added to plasma (n = 12) was 96–103% for all amino acids, with the exception of tryptophan (89%) and aspartic acid (106%). Amino acid concentrations determined in plasma of 44 healthy volunteers are summarized in Table 2.

**Discussion**

The objective of this study was to develop a fast and reliable method for the analysis of plasma amino acids. Precolumn derivatization with OPA was used because the derivatization can be easily automated. To minimize problems due to oxidation of the reagent (26), we kept it in multiple capped vials in the autosampler. Each vial was used for the derivatization of six consecutive samples. A potential source of analytical variation may be the instability of the isoindole derivatives formed by reaction with OPA (24). We used MPA as thiol reagent because it has been shown to form more stable products than mercaptoethanol (18, 23). OPA concentration in the reagent solution was kept rather low (1.76 mmol/L), but excess OPA during derivatization was ensured by adding 18 volumes of reagent to one volume of sample. Assuming a total amino acid concentration of ~3 mmol/L for plasma, we used OPA at a 10-fold molar excess over total amino acids. In initial experiments with higher OPA concentrations, we observed that some amino acid derivatives (i.e., glycine, histidine, and taurine) degraded, and some additional unidentified peaks probably representing degradation products appeared in the chromatograms. This is in accordance with reports that excess OPA accelerates degradation of isoindole derivatives (24). On-column degradation of the derivatives was minimized by using a short column in combination with a high flow rate, resulting in an analysis time of only 12 min. To obtain sufficient resolution, we used a column containing 3-μm particles. An additional advantage of these small-diameter particles is that resolution is only marginally decreased at high flow rates, due to a more efficient mass transfer.

To obtain reliable values for plasma amino acids, we paid special attention to sample pretreatment. After collection of blood, plasma was immediately separated from blood cells by centrifugation, and plasma proteins were then precipitated by addition of SSA. In a comparison of various deproteinizing agents, Qureshi and Qureshi showed SSA to be superior to other agents (16). We used special tubes containing solid SSA to avoid errors due to sample dilution. After mixing within the tubes, the samples were quickly frozen in liquid nitrogen and then stored at −70°C. In our experience, samples prepared in this way can be stored for at least 1 year without change in amino acid concentrations.

This method is characterized by high precision and reproducibility for all amino acids evaluated. With one exception, the recoveries of amino acids added to plasma were close to 100%. The lower recovery of tryptophan...
(89%) probably reflects the fact that this amino acid is tightly bound to albumin (19).

Plasma amino acid concentrations of healthy subjects determined by the present method (Table 2) are in good agreement with values obtained by other investigators (16–18, 20, 23). Although we are primarily interested in plasma amino acids, the method can also be applied to the determination of amino acids in cerebrospinal fluid. The method is characterized by speed of analysis and minimal manual sample handling. With proper care the column can be used for at least 500 analyses. If the separation efficiency decreases, column performance can be restored by flushing the column with 0.2 mol/L phosphate buffer (pH 2.5).

References