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Short communication

Analysis of amino acids in human serum by isocratic reversed-phase high-performance liquid chromatography with electrochemical detection

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Abstract

A simple, sensitive and reproducible isocratic high-performance liquid chromatography (HPLC) method has been developed for the determination of amino acids in human serum. The method involves precipitation of the serum proteins with methanol followed by pre-column derivatization of amino acids with *o*-phthalaldehyde–2-mercaptoethanol or *o*-phthalaldehyde–sodium sulfite. HPLC separation of the derivatives was performed using an ODS column with an isocratic mobile phase system and electrochemical detection (+0.75 V). The response was linear over the range 5–300 μ*M* for all amino acids. The method allows quantitative determination of glutamic acid, asparagine, serine, glutamine, histidine, taurine, alanine, arginine, methionine, isoleucine, ornithine, leucine, phenylalanine, lysine and tryptophan at concentrations as low as 0.5–5.0 pmol (signal-to-noise ratio=2). Using this method, the levels of amino acids in serum from healthy donors and patients with ischemic stroke were determined.

Keywords: Derivatization, LC; Amino acids

1. Introduction

The determination of amino acids in biological fluids is an important problem in clinical biochemistry and analytical chemistry. Changes in the concentrations of these compounds in the serum and other physiological fluids is thought to be, in part, correlated with several neurological disorders such as Alzheimer's disease [1] and ischemic stroke [2] as well as with a number of metabolic disorders such as phenylketonuria [3], argininemia [4], maple syrup urine disease [5] and others.

Because of these reasons, methods for determination of amino acids such as pre-column derivatization with phenylisothiocyanate, dansylchloride, 9-fluorenylmethyl chloroformate, and *o*-phthalaldehyde (OPA) followed by separation of the corresponding derivatives by gradient reversed-phase high-performance liquid chromatography (HPLC) have been developed (for reviews, see Refs. [6,7]). HPLC methods using pre-column derivatization with OPA have been the most widely used for the determination of primary amino acids in plasma because of their high sensitivity, simplicity, speed

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and reliability. The derivatives formed by the reaction of OPA with amino acids in the presence of a sulfhydryl components such as 2-mercaptoethanol (2-ME) [8,9], 3-mercaptopropionic acid [7,10] or sodium sulfite (Na_2SO_3) [11,12] are fluorescent and/or electroactive.

However, the published HPLC methods for determination of amino acids have some limitations: gradient elution results in a non-stable baseline when using electrochemical detection and leads to loss of analysis sensitivity. Isocratic HPLC is more reproducible, sensitive and simple in comparison with gradient systems. Besides, stability of the OPA amino acid derivatives during the whole cycle of chromatographic separation has not been studied sufficiently.

Only a few studies were devoted to the determination of amino acids by isocratic HPLC. In the method suggested by Lottspiech [13] analysis of 17 amino acids after derivatization with phenylisothiocyanate was performed within 16 min. Donzanti and Yamamoto [14] described a method for the determination of amino acids in different structures of rat brain after derivatization with OPA–2-ME. Aspartic (Asp) and glutamic (Glu) acids, serine (Ser), glutamine (Gln), taurine (Tau), alanine (Ala), and γ-aminobutyric acid (GABA) were separated within 15 min. In a previous study, we have separated Asp, Glu, Gln, asparagine (Asn), Ser, histidine (His), arginine (Arg), Tau, and Ala in human cerebrospinal fluid within 55 min [15]. However, all these studies were devoted to separation of the hydrophilic amino acids by isocratic HPLC. They are not suitable for separation of hydrophobic amino acids such as methionine (Met), leucine (Leu), isoleucine (Ile), phenylalanine (Phe), tryptophan (Trp) and others.

In this paper we report a sensitive and reproducible isocratic HPLC method using electrochemical detection for the determination of amino acids in human serum and demonstrate its clinical application.

2. Experimental

2.1. Chemicals

Individual amino acids were purchased from Sigma (St. Louis, MO, USA). OPA and sodium sulfite were purchased from Fluka (Buchs, Switzerland). 2-ME was obtained from Merck (Darmstadt, Germany). All other reagents and solvents were of analytical or HPLC grade purchased from Riedel-de Haen (Seelze, Germany).

2.2. Chromatographic instrumentation

HPLC was performed using a Gilson HPLC system (Gilson, Villiers le Bel, France) equipped with a Gilson 305 pump, Gilson 805 manometric module, a column heater LC101 (Ecom, Prague, Czech Republic), a Rheodyne 7125 injection valve (Rheodyne, Cotati, CA, USA) fitted with a 20-μl sample loop and a LC-4B electrochemical detector (BAS, West Lafayette, IN, USA). The glassy carbon electrode was used at a potential of +0.75 V. Peak areas and sample concentrations were calculated with a Gilson 714 chromatography data system. A stainless steel Spherisorb ODS-2 column (250.0×4.0 mm I.D., 5 μm) protected by guard column Spherisorb ODS-2 (10.0×4.0 mm I.D., 5 μm) (Pharmacia, Uppsala, Sweden) was used. The mobile phase consisted of 0.01–0.02 *M* sodium dihydrogenphosphate, 0.01–0.02 *M* disodium hydrogenphosphate, 10–19% (v/v) methanol, 2 m*M* Na_2EDTA, pH 7.0 and was delivered at a flow-rate of 0.7 ml/min and a temperature of 26°C.

2.3. Standards and reagents

Stock solutions of individual amino acids at concentration of 20 m*M* were prepared in double distilled water. Working concentrations of amino acids at 200 μ*M* were prepared from the stock solutions by appropriate dilution before use. All the standards were stored at −70°C and showed no measurable degradation over a period of 3 months.

The OPA–2-ME reagent was prepared according to the previously published method [14]. A 27-mg amount of OPA was dissolved in 1 ml of methanol (0.2 *M*), 10 μl of 2-ME was mixed with 0.5 ml of methanol. Both solutions were stored at −20°C. The OPA–2-ME working solution was prepared by mixing 10 μl of 0.2 *M* OPA with 20 μl of 2-ME in methanol followed by dilution to 500 μl with 0.1 *M* sodium borate buffer (pH 9.65).

The preparation of the OPA–Na_2SO_3 reagent was performed by the earlier suggested method [12] with minor modifications. A 25-μl volume of the 0.2 *M* OPA solution in methanol was mixed with 25 μl of a 1 *M* Na_2SO_3 and diluted to 1 ml with 0.1 *M* sodium borate buffer (pH 9.65). Both reagents were kept in the dark at +4°C and prepared freshly every 3 days.

2.4. Sample preparation and derivatization

Venous blood samples were collected between 8 a.m. and 10 a.m. after overnight fast and centrifuged at 3000 *g* for 15 min. Serum was taken for analysis and stored frozen at −70°C before use. For the amino acid determination, 100 μl of serum sample was deproteinized by mixing with 400 μl of methanol. After centrifugation at 6000 *g* for 5 min, 20 μl of the supernatant was derivatized or stored at −70°C if not analyzed immediately. A 100-μl volume of standard amino acid solution was added to 400 μl of methanol and 20 μl of the mixture was used for the derivatization.

For OPA–2-ME derivatives, a 20-μl aliquot of serum methanol supernatant or 20 μl of amino acid standard was mixed with 30 μl of OPA–2-ME reagent and 150 μl of 0.1 *M* $Na_2B_4O_7$ at room temperature, and after exactly 3 min 20 μl of the reaction mixture was injected onto the HPLC system.

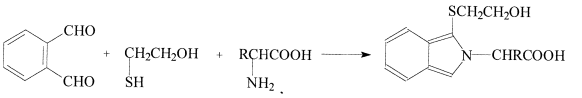
For OPA–Na_2SO_3 derivatives, a 20-μl aliquot of the serum methanol supernatant or amino acid standard was added to 170 μl of 0.1 *M* $Na_2B_4O_7$ and 10 μl of OPA–Na_2SO_3, vortex-mixed and after exactly 25 min, 20 μl was injected onto the HPLC system.

3. Results and discussion

The objective of the present study was to develop an isocratic HPLC method for the quantitative analysis of amino acids in human serum.

At preliminary steps OPA with different sulfhydril substances (2-ME, sodium sulfate) as the reagent for derivatization was used. The OPA–2-ME derivatives of the hydrophilic amino acids had suitable retention factors for HPLC analysis: $k'_{Glu}=1.8$, $k'_{Asn}=5.4$, $k'_{Ser}=6.3$, $k'_{Gln}=10.7$, $k'_{Gly}=14.9$. However, OPA–2-ME derivatives of the compounds that are less hydrophilic than Gln, such as GABA, Tyr, Met, Orn, Lys and others, were strongly retained and their retention factors were very high. OPA–Na_2SO_3 sulfite adducts of the hydrophilic amino acids (Glu, Asn and others) were eluted in the free volume of the column: $k'_{Glu}=0$; but GABA and other hydrophobic amino acids had suitable retention: $k'_{GABA}=0.7$; $k'_{Val}=2.4$; $k'_{Met}=4.0$; $k'_{Ile}=6.5$; $k'_{Orn}=8.2$. A comparison of the retention factors of the OPA derivatives showed that amino acids have to be divided into groups for their subsequent determination using different types of the OPA reagents. In this case the isocratic elution mode can be used.

The amino acids were divided into two groups, which were converted to derivatives using different reagents: Glu, Asn, Ser, Gln, Gly, Thr, Tau, Ala and Arg (group 1) by their reaction with OPA–2-ME:



and GABA, Val, Tyr, Met, Ile, Orn, Leu, Phe, Lys and Trp (group 2) by their reaction with OPA–Na_2SO_3:

There are only a few studies on the stability of OPA adducts. Some authors [14,16] noted that the half-life period of the OPA–2-ME derivatives of different amino acids were 7–40 min. Smith and Sharp showed that OPA–Na_2SO_3 derivatives were stable for 3 h [12]. Turiak and Volicer [17] found that the detector response to OPA–Na_2SO_3 derivatives of Ala, Arg, Glu, Ser, and Tyr decreased by 6% within 15 h after the reaction.

We investigated the stability of the OPA–amino acid adducts. The time necessary for separation of each group of amino acid derivatives (25 min for OPA–Na_2SO_3 and 55 min for OPA–2-ME) was the key factor. As shown in Figs. 1 and 2, respectively, the signal decreased from 4% (Gln) to 54% (Tau) for the OPA–2-ME adducts 60 min after derivatization and from 2% (Ile) to 15% (Orn) for the OPA–Na_2SO_3 adducts 30 min after derivatization.

A standard solution of 20 amino acids including

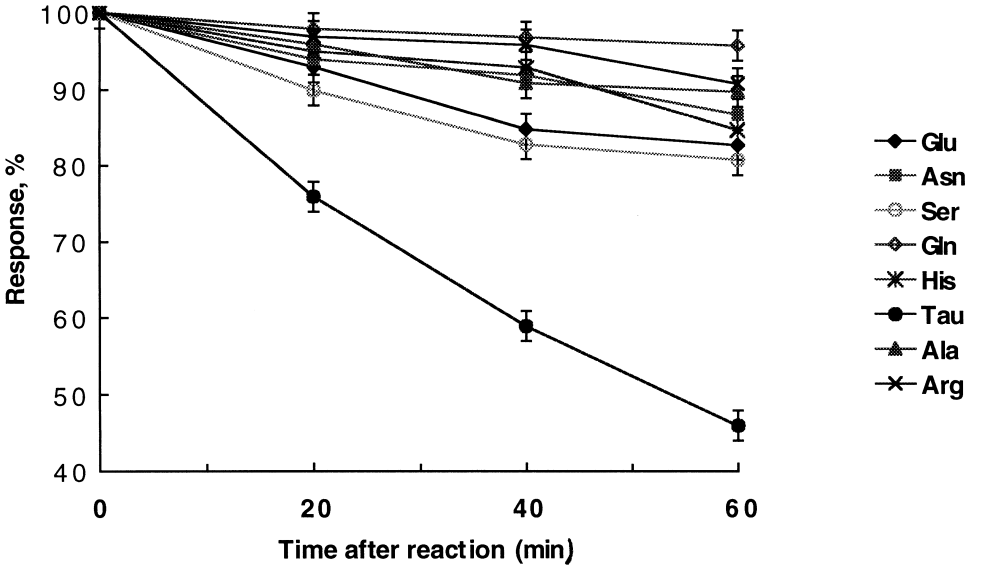


Fig. 1. The stability of the OPA–2-ME amino acid adducts (response after 3 min was taken as 100%).

17 protein compositional amino acids, as well as GABA, Orn and Tau was prepared and derivatized by the procedure described in Section 2. To carry out the separation of these compounds in the isocratic elution mode, several parameters, such as concentration of methanol and phosphate salts in the eluent were optimized. We used the systems that contained methanol (10–19%, v/v), Na_2HPO_4 (0.01–0.02 *M*), NaH_2PO_4 (0.01–0.02 *M*), and Na_2EDTA (2 m*M*). The optimal pH of the mobile phase was 6.6–7.0. The OPA derivatives of amino acids are unstable in acidic solutions; the stationary phase of the column decomposes in alkaline solutions, leading to rapid column deterioration. The best separation of the

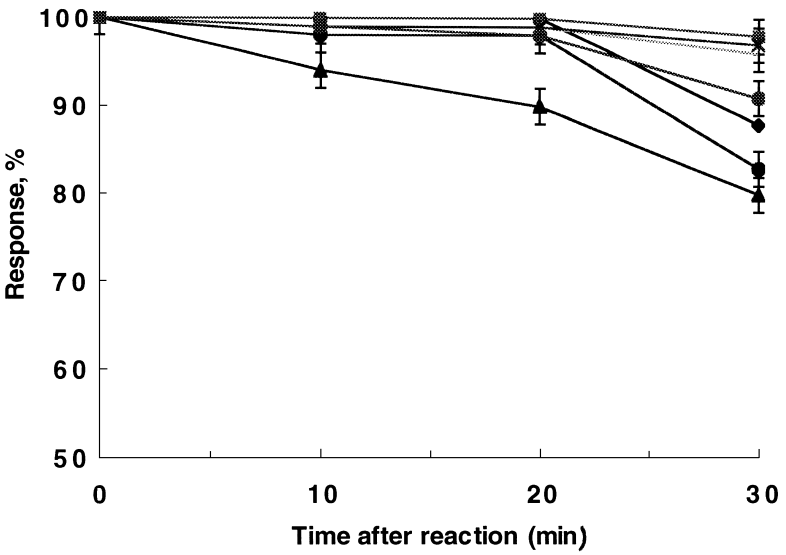


Fig. 2. The stability of the OPA–sulfite amino acid adducts (response after 25 min was taken as 100%).

amino acids was achieved using the eluent containing 18% (v/v) methanol, 0.01 *M* Na_2HPO_4, 0.01 *M* NaH_2PO_4, and 2 m*M* Na_2EDTA (pH 7.0). Under these conditions, Glu, Asn, Ser, Gln, His, Tau, Ala, and Arg can be determined as OPA–2-ME derivatives within 55 min, and GABA, Met, Ile, Orn, Leu, Phe, Lys, and Trp can be determined as OPA–Na_2SO_3 derivatives within 25 min. On the other hand, Gly and Thr as well as Tyr and Val were eluted simultaneously. Figs. 3a and 4a show typical chromatograms of a standard mixture of 15 amino

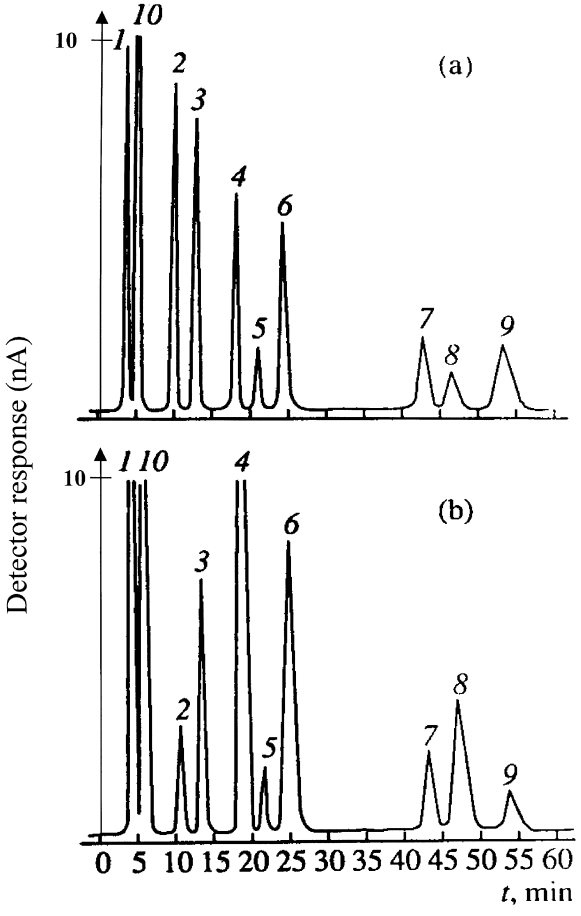


Fig. 3. Elution profiles of (a) an amino acid standard and (b) human serum after derivatization with OPA–2-ME. (1) Glu, (2) Asn, (3) Ser, (4) Gln, (5) His, (6) Gly+Thr, (7) Tau, (8) Ala, (9) Arg and (10) OPA–2-ME reagent. Chromatographic conditions: column, Spherisorb ODS II (5 μm) (250×4.0 mm I.D.); eluent, 18% methanol in 0.01 *M* NaH_2PO_4, 0.01 *M* Na_2HPO_4, 2 m*M* EDTA (pH 7.0); flow-rate, 0.7 ml/min; temperature, 26°C; electrochemical detection, +0.75 V; injection volume, 20 μl.

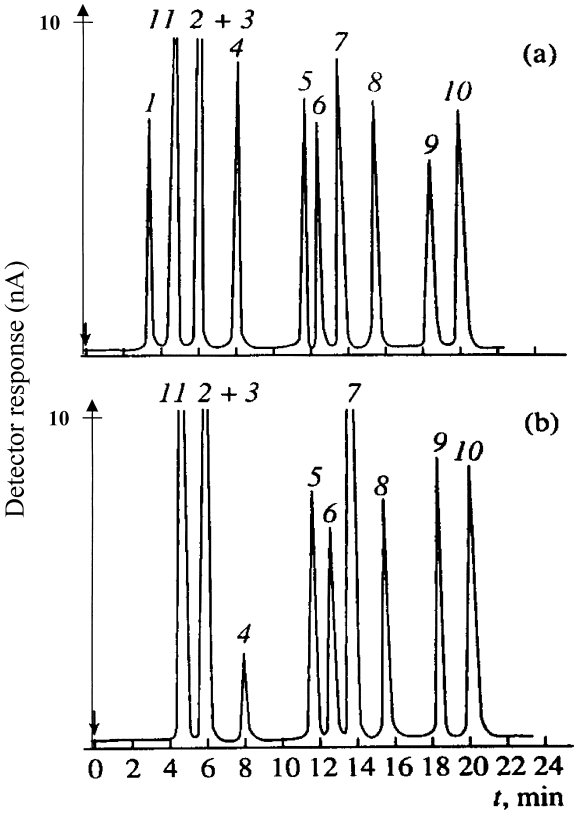


Fig. 4. Elution profiles of (a) an amino acid standard and (b) human serum after derivatization with OPA–Na_2SO_3. (1) GABA, (2) Val, (3) Tyr, (4) Met, (5) Ile, (6) Orn, (7) Leu, (8) Phe, (9) Lys, (10) Trp, and (11) OPA–Na_2SO_3 reagent. Chromatographic conditions as in Fig. 3.

acids. A linear response between the peak area and concentrations of amino acids was obtained over the concentration range 5–300 m*M* (correlation coefficients for all amino acids >0.995).

Typical chromatograms of human serum are shown in Figs. 3b and 4b. The analysis reproducibility was determined by the replicate analysis of the amino acid standard mixture ($n=18$) and serum sample ($n=5$). Relative standard deviations (RSDs) were 1.5–5.4% for the standard mixture of amino acids and 2.6–5.8% the for the serum samples. The detection limits (signal-to-noise ratio=2) were 0.5 pmol for Glu, Asn, GABA, and Met; 1 pmol for Ser, Gln, Ile, Leu, Phe, Lys, and Trp; 4 pmol for Tau, Ala, and Arg; and 5 pmol for Orn and His.

We determined the concentrations of amino acids

Table 1
Concentrations of amino acids in human serum (nmol/ml) of healthy donors and patients with ischemic stroke (mean±S.E.M.)

Amino acid	Healthy subjects ($n=16$)	Patients with progressing ischemic stroke ($n=9$)
Glu	79.9±3.5	172.1±20.6**
Asn	40.3±4.5	48.3±2.8
Ser	116.7±9.0	135.3±5.4*
Gln	516.4±25.6	408.9±26.1*
His	85.7±5.4	78.4±4.9
Tau	102.5±17.3	146.5±10.1*
Ala	397.6±50	357.9±55.2
Arg	80.0±6.8	60.0±7.3*
Met	18.5±2.1	21.5±1.9
Ile	60.2±8.5	91.5±5.0**
Orn	272.0±33.2	382.4±44.6*
Leu	100.5±11.7	146.2±9.1**
Phe	59.5±5.6	99.4±7.2***
Lys	156.0±11.4	168.9±11.1
Trp	70.9±6.8	54.6±4.4*

*, Significantly different from healthy donors, $P<0.05$.
**, Significantly different from healthy donors, $P<0.01$.
***, Significantly different from healthy donors, $P<0.001$.

in serum obtained from 16 healthy donors and nine patients with ischemic stroke (Table 1). The mean concentrations of free amino acid in healthy subjects are in agreement with those obtained by other authors [7,9,10,18]. GABA could not be detected because of its lower physiological concentrations [7,9,10,18]. It has been found that concentrations of Glu, Tau, Ser, Ile, Orn, Leu and Phe were significantly higher, while those of Gln, Arg and Trp were significantly lower in patient with ischemic stroke in comparison to healthy donors.

4. Conclusions

In this study we have developed a simple and reliable procedure for the quantitative determination of Glu, Asn, Ser, Gln, His, Tau, Ala, Arg, Met, Ile, Orn, Leu, Phe, Lys and Trp in human serum by isocratic HPLC with electrochemical detection. OPA in combination with 2-ME or Na_2SO_3 was used for pre-column amino acids derivatization. The method requires only a small sample volume and needs minimal manual sample preparation. Although we primarily focused on the analysis of amino acids in serum, the method could also be applied to the determination of these compounds in other physiological fluids.

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