Journal of Animal and Feed Sciences, 7, 1998, 453 – 463

Determination of free amino acids in blood plasma by high-performance liquid chromatography with fluorescence detection

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(Received 11 August 1998; accepted 22 September 1998)

ABSTRACT

A high-performance liquid chromatography method with pre-column derivatization for separation and quantification of free amino acids in blood plasma samples is described. Plasma samples after deproteinization with 5-sulphosalicylic acid are used for derivatization with o-phthaldialdehyde (OPA) in the presence 2-mercaptoethanol (reagent 1) or ethanethiol (reagent 2). Primary amino acid derivatives were separated on a C-18 Nova-Pak column (4 μm, 250 x 4.6 mm I.D., Waters) by gradient elution and fluorescence detection. Cystine, cysteine and proline are oxidized with sodium hypochlorite prior to derivatization with OPA in the presence of ethanethiol. Separations of derivatives of oxidized amino acids are carried out using a C-18 Symmetry column (5 μm, 250 x 4.6 mm I.D., Waters) by gradient elution and fluorescence detection. All OPA amino acid derivatives are detected with a monochromator set at 338 nm with a 425 nm cut-off filter. Clear resolution of at least seventeen amino acids and taurine was obtained in about 42 min. Low values of within and between run coefficients of variation, rapid derivatization and high sensitivity (in femtomole range) render this HPLC method suitable for routine free amino acid determinations in a large number of blood plasma samples.

KEY WORDS: free amino acids, blood, derivatization, determination, HPLC

INTRODUCTION

The use of high-performance liquid chromatography (HPLC) with fluorescence detection for the determination of free amino acids in blood plasma has received wide attention due to its satisfactory sensitivity, accuracy and reliability of assays (Qureshi et al., 1984; Sarwar et al., 1993; Peters et al., 1998). Moreover, the use of

reversed-phase HPLC with pre-column derivatization is becoming established as a cheaper and more reliable alternative to commercial amino acid analysers or gas chromatography techniques (Jones et al., 1983; Qureshi et al., 1984). For routine analyses of primary amino acids (except cystine) in biological materials, the use o-phthaldialdehyde (OPA) as the reagent for pre- or post-column derivatization has been preferred (Roth, 1971; Rattenbury, 1981; Cooper et al., 1984; Lindroth et al.,1985; Sarwar et al., 1993). Indeed, in the presence of a thiol compound, OPA reacts with primary amines to form highly fluorescent isoindoles (Roth, 1971; Lee et al., 1978), with a sensitivity in the femtomole range, a broad linear range for most OPA-derivatized amino acids and well-suited for HPLC separation (Davies et al., 1992). Moreover, derivatization of amino acids with OPA was preferred because the reagent itself does not fluoresce and the derivatization procedure is rapid. Lack of direct reactivity with secondary amino acids is a certain limitation, however, this problem can be overcome by the addition of an oxidizing agent prior to derivatization with OPA. So, when it was necessary to measure proline, cystine or cysteine, sodium hypochlorite for oxidation of these amino acids is used (Rattenbury, 1981). Sodium hypochlorite oxidizes proline to a product that reacts with OPA giving a highly fluorescent compound. Moreover, this oxidizing agent oxidizes cystine or cysteine to cysteic acid that reacts with OPA to form highly fluorescent tioalkyl substituted isoindoles (Lee et al., 1978; Lindroth et al., 1985).

The main purpose of this study was to develop and improve the method of Umagat et al. (1982) and the procedure of proline, cystine or cysteine determination (Rattenbury, 1981). Thus, in the present study the separation of OPA primary amino acid derivatives by binary gradient HPLC was examined. This derivatization procedure is based on conversion of amino acids with OPA and 2-mercaptoethanol (Et(OH)SH). Furthermore, attention has been paid to simultaneous quantitative analysis of proline, cystine and cysteine. Thus, attempts were made to use oxidation with sodium hypochlorite followed by derivatization with OPA in the presence of ethanethiol (EtSH) (Hill et al., 1979; Jones et al., 1984; Lindroth et al., 1985) for the determination of proline, cystine or cysteine in blood plasma. The developed method allowed rapid reversed-phase HPLC separation and fluorescence detection of amino acids as their OPA derivatives, including proline and cysteine or cystine.

MATERIAL AND METHODS

Reagents

Tetrahydrofuran and 2-mercaptoethanol (Et(OH)SH) were purchased from Merck (Darmstadt, Germany), while o-phtaldialdehyde (OPA) and the amino acid

standards were obtained from Sigma (St. Louis, MO, USA). Ethanethiol (EtSH) was a product of Aldrich (Germany) and sodium hypochlorite water solution (10% available Cl) was from Flucka. Methanol and all other chemicals were of analytical reagent grade and purchased from POCh (Gliwice, Poland). Water used for the preparation of eluent buffers and for dissolving standards or chemical reagents was prepared using an Elix™ water purification system (Millipore, Toronto, Canada), and then deionized using a Milli-Q-system (Millipore, Toronto, Canada). The mobile phase (solvents A and B) was filtered through a 0.2 μm membrane filter (Millipore). Solvents A and B were degassed for 10 min in vacuum with ultrasonication prior to use.

Chromatographic equipment

The instrument used consisted of a Waters 625LC system that includes a controller for gradient elution and two Waters Model 501 pumps. The apparatus is coupled to a Waters 712 WISP autosampler, Waters 474 fluorescence detector, and computer data handling system. The OPA derivatives were detected with the monochromator set at 338 nm and a 425 nm cut-off filter. Development of the analytical method, collection and data integration were performed using Millennium 2001 software and a Pentium 5P60 computer.

The analytical column used was a Nova-Pak C-18 column (4 μm, 250 x 4.6 mm I.D., Waters) or a Symmetry C-18 column (5 μm, 250 x 4.6 mm I.D., Waters) in conjunction with a guard column (Waters) of 10 x 6 mm I.D. containing reversed-phase C-18 (30-40 μm) pellicular packing material.

Analytical solvents and gradient composition

Two HPLC grade solvents were used in this study. Solvent A was tetrahydrofuran-0.05M sodium acetate (pH was adjusted to 6.6 with phosphoric acid) (1:99 v/v). Solvent B was methanol.

For analyses of amino acids in blood plasma or standards, two binary gradient programs (Waters curvilinear program) were used. When solvent B rose from 15% to 80%, the system pressures decreased from 38±1 to 24±1 MPa. The gradient compositions are shown in Tables 1 (system I) and 2 (system II). All separations were performed at 24°C (system I) and 29°C (system II).

Preparation of the borate buffer

Boric acid, 2.473 g, was dissolved in 80 ml of reagent grade water and the pH adjusted to 9.8-9.9 with 4 M KOH. The resulting solution was filtered and then diluted to a total volume of 100 ml to make a 0.4 M borate buffer.

TABLE 1

Gradient composition: the System I (flow rate – 1.5 ml/min)

Time, min	Composition,%	
	solvent A	solvent B
0	85	15
3	72	28 (linearly increased from 2 min)
20	56	44 (linearly increased from 17 min)
26	44	56 (linearly increased from 25 min)
32.4	20	80 (linearly increased from 32 min)
54	24	76 (linearly decreased from 36.5 min)
60	24	76

TABLE 2

Gradient composition: the System II

Time, min	Composition[1], %	
	solvent A	solvent B
0	85	15
3	72	28 (linearly increased from 2 min)
20	56	44 (linearly increased from 17 min)
26	44	56 (linearly increased from 25 min)
34	20	80 (linearly increased from 32.5 min)
55	79	21 (linearly decreased from 54.7 min)

[1] the initial flow-rate was 1.3 ml/min, then 1.5 ml/min for 20 to 60 min

Preparation of derivatizing solutions

Seventy-five milligrams of OPA were dissolved in 4.5 ml of methanol and 0.5 ml of borate buffer. Seventy microliters of Et(OH)SH (reagent 1) or EtSH (reagent 2) were added and the resulting solution was mixed. It is recommended that the derivatizing solutions (reagents 1 and 2) be protected from light and stored refrigerated (-28°C) when not in use. The reagents' strength was maintained by addition of 20 µl of a respective thiol (i.e.: Et(OH)SH or EtSH) every 2 days.

Preparation of NaClO oxidizing solution

To 0.97 ml of deionized water, 30 µl of sodium hypochlorite solution (10% available Cl) were added. It is recommended that the oxidizing solution be stored refrigerated (-28°C) when not in use. As a guide, a fresh solution should be used.

Sample preparation

Whole blood was obtained from the jugular vein of a cow using heparinized tubes and centrifuged at 2000 *g* for 10-15 min at 0-4°C. The plasma was stored at -20°C. Five milliliters of the plasma were deproteinized with 250 mg of 5-sulphosalicylic acid and then centrifuged at 2000 *g* for 10-15 min at 4°C.

The supernatants were filtered through 0.20 μm nylon filters (Cole Parmer). The filtrates were stored refrigerated at -20°C used for the derivatization as below.

Derivatization procedure A

To an autosampler vial were added 43 μl of deproteinized plasma sample (1-4°C), 8 μl of 2 M NaOH and 200 μl of OPA/Et(OH)SH (reagent 1). The contents were mixed and reacted for 2 min at room temperature. All mixtures were protected from the light. At the end of the 2 min period, 10-20 μl of the derivatized sample were injected onto the column. The derivatizing procedure for standards was the same as for plasma samples.

Derivatization procedure B

To an autosampler vial were added 43 μl of deproteinized plasma sample (1-4°C), 8 μl of 2 M NaOH, 200 μl NaClO solution and 10 μl of 1 M NaOH. The contents were mixed and reacted for 24 min at room temperature. At the end of the 24 min period, 200 μl of OPA/EtSH (reagent 2) were added to the vial and allowed to react for 2 min. All mixtures were protected from the light. Injections of 10-20 μl of the mixtures were made onto the HPLC system immediately after derivatization. The standards were processed in the same way as the plasma samples.

RESULTS AND DISCUSSION

Although high-performance liquid chromatography of amino acids derivatized with OPA has been reported, the separation and quantification of cysteine, cystine and secondary amino acids such as proline necessitated the development of new HPLC methods that would exhibit improved specificity and reliability. Recent works (Umagat et al., 1982; Krishnamurti et al., 1984; Sedgwick et al., 1991; Sarwar et al., 1993) have demonstrated that OPA amino acid derivatives can be successfully resolved by reversed-phase gradient HPLC with fluorimetric detection.

All amino acid peaks (Figures 1 and 2) were identified on the basis of the retention time (t_R) of amino acid standards and by separately adding the amino acid standards (spiking) to processed plasma samples.

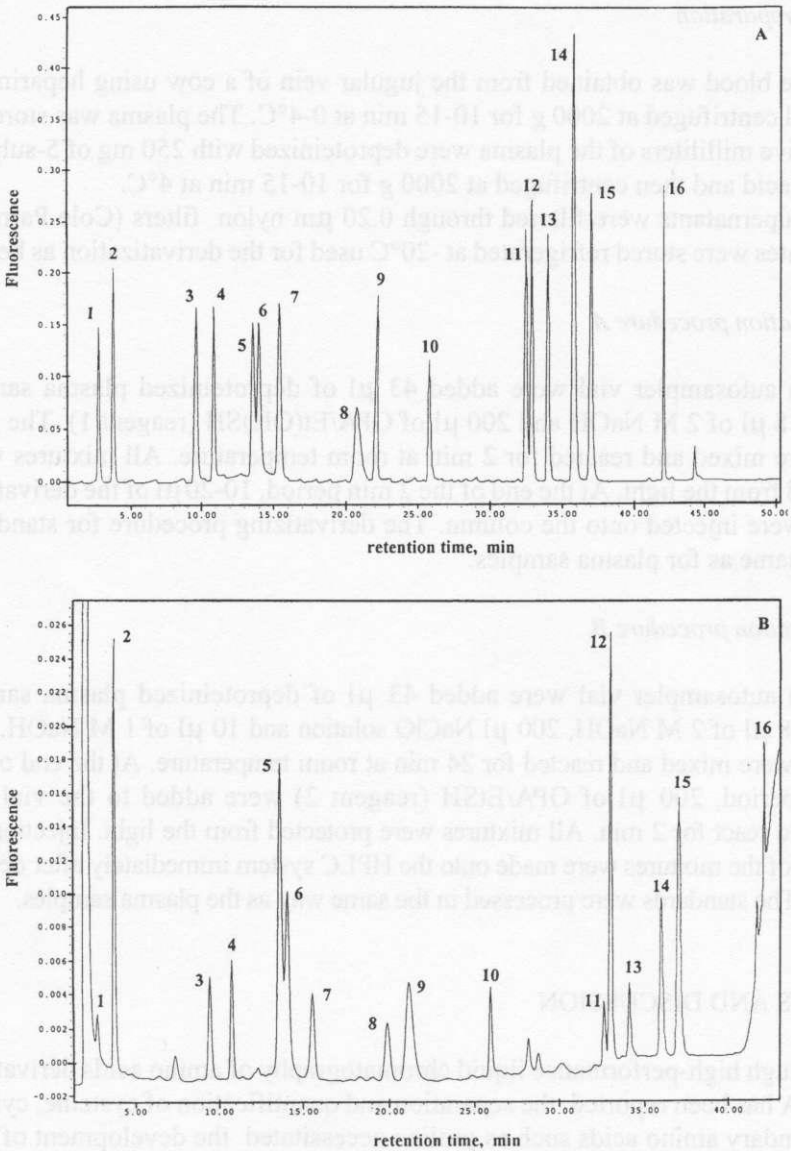

Figure 1. Chromatograms of OPA amino acids derivatives on a Nova Pak C-18 column (at 24°C). The chromatograms for (A) – an amino acids standard; (B) – free amino acids in a plasma sample. Peaks: 1 – aspartic acid (2.72±0.08 min); 2 – glutamic acid (3.71±0.03 min); 3 – serine (9.76±0.15 min); 4 – histidine (11.01±0.13 min); 5 – glycine (13.83±0.13 min); 6 – threonine (14.48±0.23 min); 7 – arginine (16.08±0.31 min); 8 – taurine (20.15±0.30 min); 9 – alanine (21.60±0.29 min); 10 – tyrosine (26.06±0.11 min); 11 – methionine (32.68±0.19 min); 12 – valine (33.15±0.20 min); 13 – phenylalanine (34.24±0.16 min); 14 – iso-leucine (36.13±0.20 min); 15 – leucine (37.16±0.20 min); 16 – lysine (42.09±0.07 min)

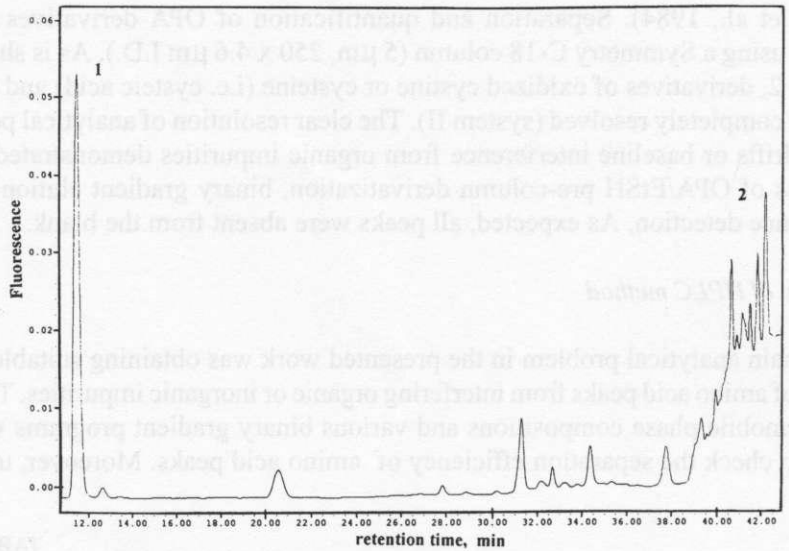

Figure 2. Separation of proline, cystine or cysteine using oxidation prior to derivatization with OPA/ethanethiol reagent. Operating conditions: a Symmetry C-18 column; column temperature: 29°C; gradient elution: the system II. The chromatogram of spiked (with cysteine and proline) plasma sample. Peaks: 1 – cysteine or cystine oxidized and separated as the OPA cysteic acid adduct (t_R = 11.11±0.26 min); 2 – proline oxidized and separated as OPA derivative (t_R = 42.28±0.09 min)

Determination of amino acids by the procedure A

In the present studies, a reversed-phase a C-18 column with 4 μm particle size and 250 mm in length was used for separation of OPA primary amino acids. Elution profiles of amino acids derivatized with OPA/Et(OH)SH and their retention times (t_R – the mean ± SD of 30 processed plasma samples) are given in Figure 1. As is shown in Figure 1, in the gradient elution system I developed in this study, all OPA amino acid derivatives are well-separated from background interference and unidentified peaks. As expected, all peaks were absent from the blank. In general, the elution orders are comparable to those reported by Umagat et al. (1982) who obtained a similar gradient profile. Of at least the fifteen amino acids and taurine in the standards (Figure 1A) or plasma samples (Figure 1B) were clearly resolved.

Determination of proline, cystine or cysteine by procedure B

To measure proline, cystine or cysteine, these amino acids were first oxidized with sodium hypochlorite. The oxidized compounds react in an alkaline medium with the OPA/EtSH reagent to form highly fluorescent products (Rattenbury 1981;

Lindroth et al., 1984). Separation and quantification of OPA derivatives was achieved using a Symmetry C-18 column (5 μm, 250 x 4.6 μm I.D.). As is shown in Figure 2, derivatives of oxidized cystine or cysteine (i.e. cysteic acid) and proline were completely resolved (system II). The clear resolution of analytical peaks without drifts or baseline interference from organic impurities demonstrated the usefulness of OPA/EtSH pre-column derivatization, binary gradient elution and fluorescence detection. As expected, all peaks were absent from the blank.

Reliability of HPLC method

The main analytical problem in the presented work was obtaining suitable separation of amino acid peaks from interfering organic or inorganic impurities. Thus, different mobile phase compositions and various binary gradient programs were applied to check the separation efficiency of amino acid peaks. Moreover, using

TABLE 3

The fluorescent response of OPA amino acids derivatives and precision of the determination

Amino acid	Regression equation[1]	Coefficient of correlation r	Precision, %	
			C.V. within run	C.V. between run
Cysteic acid[2]	$y=3.29x10^{-4}S$	0.995	3.37[3]	6.284
Aspartic acid	$y=3.96x10^{-5}S - 0.01$	0.999	0.33±0.06	3.99
Glutamic acid	$y=4.39x10^{-5}S - 0.01$	0.999	2.36±1.75	3.72
Serine	$y=3.04x10^{-5}S - 0.06$	0.999	1.72±1.00	4.12
Histidine	$y=4.13x10^{-5}S - 0.01$	0.999	1.74±0.99	3.88
Glycine	$y=3.93x10^{-5}S - 0.07$	0.999	1.99±1.35	3.64
Threonine	$y=2.68x10^{-5}S - 0.02$	0.999	1.64±0.82	4.23
Arginine	$y=3.32x10^{-5}S - 0.05$	0.999	0.80±0.54	2.07
Taurine	$y=2.56x10^{-5}S - 0.01$	1.000	1.27±0.78	1.42
Alanine	$y=4.13x10^{-5}S - 0.05$	0.999	0.98±0.31	3.98
Tyrosine	$y=3.22x10^{-5}S - 0.10$	0.999	2.10±0.38	4.02
Methionine	$y=3.46x10^{-5}S - 0.01$	0.999	1.43±1.02	4.45
Valine	$y=2.82x10^{-5}S$	0.999	0.41±0.32	3.96
Phenylalanine	$y=3.52x10^{-5}S - 0.05$	0.999	2.27±0.97	4.02
iso-Leucine	$y=3.08x10^{-5}S - 0.02$	0.999	1.25±0.63	3.76
Leucine	$y=3.67x10^{-5}S - 0.01$	0.999	1.97±1.23	4.83
Lysine	$y=4.79x10^{-5}S - 0.11$	0.999	2.21±1.09	3.51
Proline	$y=4.58x10^{-5}S + 0.19$	0.999	1.90[3]	3.59[5]

[1] S and y are the peak areand amino acid concentration (nmol/ml) in standards, respectively

[2] cystine or cysteine oxidized and separated as the OPA cysteic acid adduct

[3] the within run C.V. based on a sample injected three times

[4] the between run C.V. based on processing three samples

[5] the between run C.V. based on processing four samples

TABLE 4

Free amino acids concentrations in blood plasma of cows, nmol of amino acid/ml of plasma

Amino acid	Retention time[1] min	Concentration[1] ± standard deviation	Precision, % the between run C.V.
		System I	
Aspartic acid	2.84	20.03±0.62	3.27
Glutamic acid	3.66	224.2 ±8.7	3.91
Serine	9.99	70.1 ±2.4	3.43
Histidine	10.99	114.2 ±3.9	3.58
Glycine	13.83	261.6 ±6.8	2.61
Threonine	14.59	175.4 ±2.7	1.55
Arginine	16.16	101.1 ±2.1	2.11
Taurine	20.33	32.3 ±0.5	1.50
Alanine	21.69	194.4 ±4.5	3.88
Tyrosine	26.00	43.8 ±1.3	3.05
Methionine	32.71	24.13±0.97	4.05
Valine	33.09	203.9 ±7.7	3.82
Phenylalanine	34.18	33.1 ±1.28	3.88
iso-Leucyne	36.14	86.7 ±3.1	3.77
Leucyne	37.21	105.8 ±4.3	4.11
Lysine	42.04	72.4 ±2.2	3.10
		System II	
Cysteic acid[2]	10.98	16.41±0.87	5.33
Proline	42.21	115.2 ±3.79	3.27

[1] the mean calculated from three measurements

[2] cystine or cysteine oxidized and separated as the OPA cysteic acid adduct

UV (at short and medium wave lengths, e.g. 205, 210, 254 or 330 nm) and fluorescence detection, the lack of interference and „purity" of amino acid peaks were confirmed.

The system reproducibility and the reliability of presented HPLC method for assaying amino acids was evaluated by analysing the within and between run coefficients of variation (C.V., %) calculated from the measurements of amino acid concentrations in plasma or standard samples. The within run C.V. was based on four samples each with two or three injections, while the between run C.V. was based on five or six processed samples (derivatization and injection). A summary of the results obtained from the quantitative analysis can be seen in Table 3. The low values of within and between run coefficients of variation, rapid derivatization and analysis, the lack of interference from substances in the plasma render this HPLC method suitable for routine amino acid determination in a large number of plasma samples.

The feasibility of the HPLC method for the determination of free amino acids was also evaluated using blood plasma. The results obtained for the amino acids analysis are summarised in Table 4. The determined compositions are in good agreement with reported amino acid concentrations in blood plasma (Kowalczyk, 1973). It can be concluded that the HPLC method described in this study enables rapid analysis of free amino acids in biological samples with a sensitivity in the femtomole range.

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STRESZCZENIE

Oznaczanie wolnych aminokwasów we krwi krów metodą HPLC

Aminokwasy w odbiałczonym kwasem 5-sulfosalicylowym osoczu krwi przeprowadzano w związki pochodne używając o-dwualdehyd ftalowy w obecności 2-merkaptoetanolu.

Pochodne aminokwasów rozdzielano na kolumnie C-18 z odwróconą fazą (Nova-Pak 4 μm, 250 x 4,6 mm) poprzez elucję gradientową. Cystynę, cysteinę i proline utleniano podchlorynem sodowym przed przeprowadzeniem w pochodne o-dwualdehydem ftalowym w obecności etanotiolu. Pochodne tych aminokwasow rozdzielano wykorzystując kolumnę C-18 z odwróconą fazą (Symmetry, 5 μm, 250 x 4.6 mm) poprzez elucję gradientową. Wszystkie pochodne oznaczano stosując detekcję fluorescencyjną (wzbudzanie 338 nm, pomiar 425 nm). Czas rozdziału wszystkich pochodnych wynosił około 42 min. Niskie wartości współczynnika zmienności w obrębie przeprowadzania w pochodne i iniekcji prób, szybkość analizy oraz wysoka czułość (na poziomie femtomoli) sprawia, że metoda ta może być z powodzeniem wykorzystana w badaniach rutynowych.