ELSEVIER

Journal of Chromatography A, 979 (2002) 227–232

JOURNAL OF
CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Rapid method for the determination of amino acids in serum by capillary electrophoresis

Zuojun Shen[a,b,*], Zimin Sun[b], Lin Wu[b], Kui Wu[b], Siqin Sun[b], Zunbo Huang[a,b]

[a]*Department of Clinical Chemistry, Anhui Provincial Center for Clinical Laboratory, 17 Lujiang Road, Hefei 230001, Anhui Province, China*

[b]*Anhui Provincial Hospital, Hefei 230001, Anhui Province, China*

Abstract

A rapid method for the determination of amino acids in serum is presented. The derivatization of amino acids with 2,4-dinitrofluorobenzene was performed in 0.5 *M* sodium borate (pH 9.5). The complete separation of derivatives of 16 amino acids and an internal standard (D-norleucine) was achieved within 8 min by capillary zone electrophoresis. The running buffer consisted of 30 m*M* sodium tetraborate (pH 9.8)–isopropanol–30% Brij 35 (825:150:25, v/v). The capillary used had an internal diameter of 75 μm and an effective length of 300 mm. A voltage of 28 kV was applied. Temperature was maintained at 15 °C. Detection was 360 nm. The assay was linear from 10 to 700 μ*M*. The minimal detection limit was 2.5–7.9 μ*M*. The recovery of amino acids added to serum samples was 86.3–107.4%. Within-run precision was 2.8–10.3%, and between-run precision was 3.5–11.6%. The concentrations of amino acids in serum of 32 patients with chronic renal failure were measured. Among them, the levels of serine, isoleucine and valine were significantly lower than those of healthy volunteers ($P<0.01$), but the concentrations of cystine, tryptophan and phenylane were significantly higher than those of healthy volunteers ($P<0.01$). The result showed that the method could be used for determining amino acids in clinical practice and scientific research.
© 2002 Elsevier Science B.V. All rights reserved.

Keywords: Amino acids; Dinitrofluorobenzene

1. Introduction

Amino acids, the basic units of proteins, play an important role in the metabolic processes of a living organism. The determination of physiological amino acids is of importance not only to the clinical diagnosis and treatment of hereditary diseases, kidney diseases, liver diseases and neuropathy [1], but also to the evaluation of the nutritive status of the patient [2]. Because analysis of amino acids is of broad interest, a rapid method for the determination of amino acids in biological samples is often required.

Common methods for the determination of physiological amino acids, such as classic ion-exchange chromatography [3–9] and reversed-phase high-performance liquid chromatography (RP-HPLC) [10–22], are characteristically expensive and time-consuming.

High-performance capillary electrophoresis (HPCE) is an emerging technology that has generated considerable attention because of its ease of use, high flexibility, high resolution, and ability to provide information complementary to classic separation

*Corresponding author. Tel.: +86-551-2652797; fax: +86-551-2647212.
E-mail address: shenzuojun@yahoo.com (Z. Shen).

0021-9673/02/$ – see front matter © 2002 Elsevier Science B.V. All rights reserved.
PII: S0021-9673(02)01251-7

techniques. It has become a rapidly expanding area of analytical chemistry. In 1997 Smith reviewed the developments in amino acid analysis using capillary electrophoresis [23]. It is reported that micellar electrokinetic chromatography (MEKC) [24,25] and capillary electrochromatography (CEC) [26] have been adopted for the measurement of amino acids. The techniques of time-of-flight mass spectrometry (TOF-MS) [27] and electrospray ionization mass spectrometry (ESI-MS) [28–31] have been used for the detection of amino acids in HPCE. Higashijima et al. [32] developed a method for determining amino acids by capillary zone electrophoresis based on semiconductor laser fluorescence detection. Chen et al. [33] optimized micellar capillary electrophoresis for separation of phenylthiohydantoin amino acids. Zhou and Lunte [34] explored a direct determination of amino acids by capillary electrophoresis/electrochemistry using a copper microelectrode and zwitterionic buffers. Zahou et al. [35] reported that HPCE using SDS in phosphate buffer provided high resolution and shout time for peptide and protein hydrolysate amino acids after derivatization with phenylisothiocyanate. In order to increase the sensitivity a laser-induced fluorescence (LIF) detector has been introduced into the detection of amino acids by HPCE [36–43]. However, only a few CE protocols have been established for biological amino acid analysis to date. The methods reported need to be improved in resolution, running time, and number separated in a run.

The aim of the present paper is to establish a rapid method for the determination of amino acids in serum, which can be used for determining amino acids in clinical practice and scientific research.

2. Experimental

2.1. Chemicals

All chemicals were of analytical reagent grade unless stated otherwise. The standard amino acids (Ala, Arg, Asp, Cys, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Val, and Gly), D-Norleucine (internal standard), 2,4-dinitrofluorobenzene (DNFB), and 30% Brij 35 were purchased from Sigma (St. Louis, MO, USA). Sodium tetraborate, acetonitrile, 2-isopropanol and methanol were from Beijing Chemicals Factory (Beijing, China).

2.2. Apparatus

Experiments were performed with a P/ACE 5500 capillary electrophoresis system equipped with an on-column diode-array detection (DAD) system, operated under System Gold software for control, data acquisition, and analysis (Beckman, Fullerton, CA, USA). The column used for the determination of 16 amino acids was a fused-silica capillary of 370 mm (300 mm effective length)×75 μm I.D. (Yongnian, Hebei, China).

2.3. Collection and preparation of samples

The blood samples were collected at the Department of Nephrology of the Anhui Provincial Hospital and originated from 32 patients with renal failure. The control group was from 40 healthy volunteers. The blood samples were taken from the veins by means of plastic syringes, and after 1 h at room temperature, centrifuged at 2000 *g* for 10 min. Then the sera were immediately frozen and stored at −20 °C until analysis. An aliquot of 0.5 ml of serum was added into a 2-ml Eppendorf tube. Protein precipitation was carried out by adding 1.5 ml of acetonitrile. Eppendorf tubes were vortex-mixed for 30 s and after standing for 10 min, the tubes were centrifuged at 15 000 *g* for 10 min. The supernatant (1 ml) was transferred into another tube.

2.4. Derivatization of amino acids with DNFB

The method of derivation reported by Morton and Gerber [44] was modified. To a 1-ml aliquot of 0.5 *M* sodium tetraborate (pH 9.5), 1 ml of acetonitrile, 1 ml of standard amino acid mixtures or 1 ml of the supernatant made above (including internal standard) and 20 μl of DNFB were added. The solution was vortex-mixed for 30 s. Derivatization was carried out at 50 °C for 40 min.

2.5. Capillary electrophoresis procedure

A new capillary was activated by washing with 1 *M* and then 0.1 *M* NaOH for 30 min each, with distilled water for 30 min and then rinsing for 30 min with running buffer. The capillary was prewashed with 1.0 *M* NaOH for 10 min, 0.1 *M* NaOH for 10 min, water for 10 min and running buffer for 20 min at the beginning of each working day, and with 0.1 *M* NaOH for 2 min and running buffer for 8 min prior to each analysis. Samples were loaded onto the column by pressure injection for 5 s at 50 mbar (anode at the inlet and cathode at the outlet).

The running buffer consisted of 30 m*M* sodium tetraborate (pH 9.8)–isopropanol–30% Brij 35 (825:150:25, v/v). Temperature was maintained at 15 °C. A constant voltage of 28 kV was applied during analysis. Detection at 300 mm from the point of sample introduction was set at a wavelength of 360 nm.

3. Results

3.1. A rapid capillary electrophoretic method for determination of 16 amino acids

A rapid method for the determination of amino acids was developed. The method is based on pre-column derivatization of amino acids and an internal standard (D-norleucine) with DNFB, separation of the derivatives by capillary zone electrophoresis, and quantification by ultraviolet detection. The complete separation of 16 kinds of standard amino acids (Fig. 1) or physiological amino acids (Fig. 2) was achieved within 8 min through the optimization of separation conditions which included the value of pH, ionic strength, detergent, organic modifier and temperature.

3.2. Evaluation of methodology

The method was linear from 10 to 700 μ*M*. The minimal detection limit was 2.5–7.9 μ*M*. Within-run precision was 2.8–10.3%, and between-run precision was 3.5–11.6% (Table 1). Recovery of L-amino acids added to samples was 86.3–107.4% (Table 2).

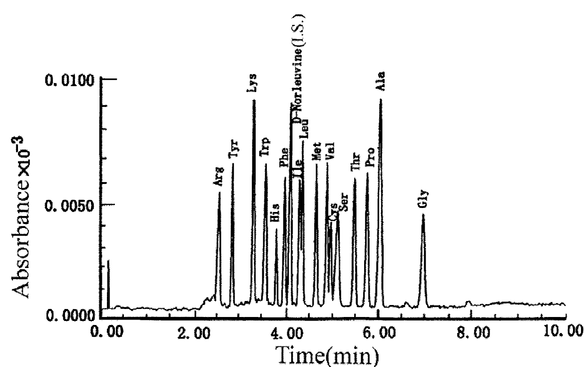

Fig. 1. Electropherograms of 16 standard amino acids and internal standard. HPCE was performed in normal polarity at 28 kV and 15 °C, with detection at 360 nm. Column size was 370 mm (300 mm effective length to the detector)×75 μm I.D. Sample was loaded into the column by hydrodynamic injection for 5 s. The concentration of the standard solution of amino acids and of the internal standard was 100 μ*M*. Running buffer: 30 m*M* pH 9.8 tetraborate–2-isopropanol–30% Brij 35 (825:150:25, v/v).

3.3. The concentrations of amino acids in serum of the healthy subjects and the patients with renal failure

The concentrations of amino acids in serum of 40 healthy subjects and 32 patients with chronic renal failure were measured. Among them, the levels of serine, isoleucine and valine were significantly lower than those of healthy volunteers ($P<0.01$), but the

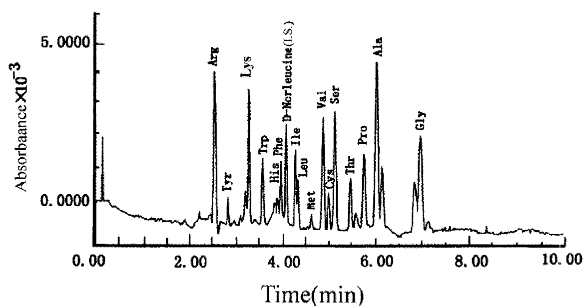

Fig. 2. Electropherograms of human serum amino acids and internal standard. HPCE was performed in normal polarity at 28 kV and 15 °C, with detection at 360 nm. Column size was 370 mm (300 mm effective length to the detector)×75 μm I.D. Sample was loaded into the column by hydrodynamic injection for 5 s. The concentration of the internal standard was 50 μ*M*. Running buffer: 30 m*M* pH 9.8 tetraborate–2-isopropanol–30% Brij 35 (825:150:25, v/v).

Table 1
Reproducibility of measurement of 16 standard amino acids

Amino acid	Within-run RSD (%) $n=12$	Between-run RSD (%) $n=10$
Arg	4.2	6.8
Tyr	5.7	8.3
Lys	5.3	5.4
Trp	3.6	4.5
His	5.8	8.6
Phe	2.6	4.9
Ile	5.6	5.3
Leu	4.0	3.7
Cys	4.3	6.7
Met	5.2	7.9
Val	1.4	1.7
Ser	4.8	4.9
Thr	2.9	3.5
Pro	3.1	4.2
Ala	1.9	2.8
Gly	4.3	6.1

concentrations of cystine, tryptophan and phenylane were significantly higher than those of healthy volunteers ($P<0.01$) (Table 3).

4. Discussion and conclusion

Capillary electrophoresis is rapidly becoming an accepted, routine analytical technique, characterized by short run time and high efficiency. It is applicable to small molecules, ions and biomacromolecules. The method has many advantages such as rapidity, simplicity, accuracy, and sensitivity, which can be used for determining amino acids in both clinical practice and research.

Since most amino acids cannot exhibit a useful response with UV absorption or fluorescence detection, chemical derivatization of amino acids with chromophores is needed to improve detectability. There are many kinds of derivative reagents that have been introduced. Among them, UV [44–46] and fluorescence [47–49] reagents are often used. The former includes DNFB, dabsyl chloride and phenylisothiocyanate, and the latter includes *o*-phthalaldehyde, dansyl chloride, fluorescamine, 9-fluorenylmenthyl chloride, naphthalene dicarboxaldehyde and fluorescein isothiocyanate. The DNFB reagent was adopted since it showed a good sensitivity and stability in this study. The improved

Table 2
Recovery of amino acids from spiked human serum

Amino acid	Level 1 (%)[a]	Level 2 (%)[b]	Mean (%)
Arg	95.3	96.8	96.1
Tyr	105.8	97.2	101.5
Lys	103.5	101.7	102.6
Trp	96.4	104.5	100.4
His	85.2	87.4	86.3
Phe	96.1	103.5	99.8
Ile	105.6	103.8	104.7
Leu	94.0	96.3	95.2
Cys	104.3	97.5	100.9
Met	96.2	86.7	91.4
Val	91.4	101.6	96.5
Ser	104.7	90.9	97.8
Thr	92.9	93.5	93.2
Pro	102.8	112.0	107.4
Ala	101.9	92.8	97.4
Gly	93.5	90.7	92.1

[a] A 100-μl aliquot of 500 μ*M* standard amino acids was added to 1000 μl of human serum.
[b] A 100-μl aliquot of 2000 μ*M* of standard amino acids was added to 1000 μl of human serum.

Table 3
The concentration of amino acids in serum of 40 healthy subjects and 32 patients with renal failure

Amino acid	Healthy subjects (μ*M*)	Patients with renal failure (μ*M*)
Arg	108.3±30.4	104.7±28.3
Tyr	65.8±12.7	54.2±10.6
Lys	209.1±38.5	197.5±43.2
Trp	35.2±7.6	94.8±26.7[a]
His	81.4±11.3	86.5±29.1
Phe	61.5±8.2	97.8±23.7[a]
Ile	94.8±43.6	41.3±18.5[a]
Leu	124.7±30.5	115.2±27.6
Cys	21.3±8.4	274.3±76.4[a]
Met	31.3±7.4	35.2±10.5
Val	249.8±41.5	181.9±78.6[a]
Ser	127.4±35.8	48.3±20.5[a]
Thr	119.2±28.0	121.4±30.7
Pro	205.3±58.4	230.1±63.5
Ala	406.7±81.3	382.4±84.7
Gly	238.0±51.4	259.6±62.3

[a] Significantly different from healthy subjects ($P<0.01$).

detection and derivatization of amino acids in capillary electrophoresis has been thoroughly reviewed by Szulc and Krull [50].

In order to obtain optimal separation conditions five experimental parameters, i.e. pH, detergents, organic modifiers, ionic strength, and temperature were optimized. The pH of running buffer was the most important parameter for changing the selectivity of capillary electrophoresis. Separation by electrophoresis depended upon differing mobilities of amino acid derivatives, which were directly related to their size and net charge. Increase in salt concentration increased the polarity of the running buffer, and should therefore increase both electroosmotic flow and the electrophoretic mobility. Increasing the ionic strength could improve efficiency, resolution and sensitivity of the separation, because amino acid derivatives spent more time in the capillary column. Detergents and organic modifiers added to the running buffer altered the polarity and the viscosity of electrolyte, thus affecting both electro-osmotic flow and the electrophoretic mobility of the analytes. Temperature also affected the separation resolution because amino acid derivatives spent more time in the capillary column at low temperature.

Acknowledgements

This study was supported by a grant from the Anhui Province Natural Sciences Foundation (no. 00044517).

References

[1] J.M. Ratten, in: 1st ed, Amino Acid Analysis, Ellis Horwood, Chicester, UK, 1981, p. 257.
[2] A.J. Barbul, Parenter. Enteral. Nutr. 10 (1986) 227.
[3] S. Moore, D.H. Spackman, W.H. Stein, Anal. Biochem. 30 (1958) 1185.
[4] L. Cynober, C. Coudray-Lucas, F. Zeigler, J. Automat. Chem. 7 (1985) 201.
[5] L.Y. Lee, R.H. Slocum, Clin. Chem. 34 (1988) 719.
[6] D.H. Spackman, W.H. Stein, S. Moore, Anal. Chem. 30 (1958) 1190.
[7] J.V. Benson, M.J. Gordon, J.A. Patterson, Anal. Biochem. 18 (1967) 228.
[8] R.F. Goldsmith, J.W. Earl, A.M. Cunningham, Clin. Chem. 33 (1987) 1736.
[9] T.N. Ferraro, T.A. Hare, Anal. Biochem. 143 (1984) 82.
[10] T.A. Graser, H.G. Godel, S. Albers, Anal. Biochem. 151 (1985) 142.
[11] S. Oguri, C. Uchida, M. Mishina, J. Chromatogr. A 724 (1996) 169.
[12] S.A. Cohen, K.M.D. Antonis, J. Chromatogr. A 661 (1994) 25.
[13] C. Saller, M.J. Czupryna, J. Chromatogr. 487 (1989) 167.
[14] K.L. Woo, S.H. Lee, J. Chromatogr. A 667 (1994) 105.
[15] P.A. Haynes, D. Sheumack, J. Kibby, J. Chromatogr. 540 (1991) 177.
[16] D.T. Blankenship, M.A. Krivanek, B.L. Ackermann, Anal. Biochem. 178 (1989) 227.
[17] M.A. Desiderio, P. Davalli, A. Perin, J. Chromatogr. 419 (1987) 285.
[18] G. Buzzigoli, L. Lanzone, D. Ciociaro, J. Chromatogr. 507 (1990) 85.
[19] T. Teerlink, P.A.M. van Leeuwen, A. Houdijk, Clin. Chem. 40 (1994) 245.
[20] S. Einarsson, B. Josefsson, S. Lagerkvist, J. Chromatogr. 282 (1983) 609.
[21] B. Gustavsson, I. Betner, J. Chromatogr. 507 (1990) 67.
[22] R.C. Dorresteijn, L.G. Berwald, G. Zomer, J. Chromatogr. A 724 (1996) 159.
[23] J.T. Smith, Electrophoresis 18 (1997) 2377.
[24] C.P. Ong, C.L. Ng, H.K. Lee, F.Y. Li, J. Chromatogr. 559 (1991) 537.
[25] H. Wan, M. Ohman, L.G. Bloberg, J. Chromatogr. A 916 (2001) 255.
[26] M. Qi, X.F. Li, C. Stathakis, N.J. Dovichi, J. Chromatogr. A 853 (1999) 131.
[27] G. Choudhary, C. Horvath, J.F. Banks, J. Chromatogr. A 828 (1998) 469.
[28] W. Lu, G. Yang, R.B. Cole, Electrophoresis 16 (1995) 487.
[29] A. Martin-Girardeau, M.F. Renou-Gonnord, J. Chromatogr. B 742 (2000) 163.
[30] T. Soga, D.N. Heiger, Anal. Chem. 72 (2000) 1236.
[31] C.W. Klampfl, W. Ahrer, Electrophoresis 22 (2001) 1579.
[32] T. Higashijima, T. Fuchigamin, T. Imasaka, N. Ishibashi, Anal. Chem. 64 (1992) 711.
[33] M. Chen, K.C. Waldron, Y. Zhao, N.J. Dovichi, Electrophoresis 15 (1994) 1290.
[34] J. Zhou, S.M. Lunte, Electrophoresis 16 (1995) 498.
[35] E. Zahou, H. Jornvall, T. Bergman, Anal. Biochem. 281 (2000) 115.
[36] Y.F. Cheng, N.J. Dovichi, Science 242 (1988) 562.
[37] J.Y. Zhao, D.Y. Che, N.J. Dovichi, J. Chromatogr. 608 (1992) 117.
[38] K.C. Chan, G.M. Janini, G.M. Muschik, H.J. Issaq, J. Chromatogr. A 653 (1993) 93.
[39] F. Robert, L. Bert, L. Lambas-Senas, L. Denoroy, B. Renaud, J. Neurosci. Methods 70 (1996) 153.

[40] J. Bergquist, M.J. Vona, C.O. Stiller, W.T. O'Connor, T. Falkenberg, R. Ekman, J. Neurosci. Methods 65 (1996) 33.
[41] L.A. Dawson, J.M. Stow, A.M. Palmer, J. Chromatogr. B 694 (1997) 455.
[42] Z. Chen, J. Wu, G.B. Baker, M. Parent, N.J. Dovichi, J. Chromatogr. A 914 (2001) 293.
[43] O. Boulat, D.G. McLaren, E.A. Arriaga, D.D. Chen, J. Chromatogr. B 754 (2001) 217.
[44] R.C. Morton, G.E. Gerber, Anal. Biochem. 170 (1988) 220.
[45] J.Y. Chang, R. Knecht, D.G. Braun, Biochem. J. 199 (1988) 547.
[46] A. Buzzigoli, L. Lanzone, D. Ciociaro, J. Chromatogr. 507 (1990) 85.
[47] E. Okuma, H. Abe, J. Chromatogr. 660 (1994) 243.
[48] S. Terabe, Y. Miyashita, Y. Ishihama, O. Shibata, J. Chromatogr. 636 (1993) 47.
[49] T. Ueda, R. Mitchell, F. Kitamura, T. Metcalf, T. Kuwana, A. Nakamoto, J. Chromatogr. 593 (1992) 265.
[50] M.E. Szulc, I.S. Krull, J. Chromatogr. A 659 (1994) 231.