Simultaneous Determination of L-Phenylalanine and Branched-chain L-Amino Acids in Plasma by Liquid Chromatographic System with a Coimmobilized Enzyme Reactor and Fluorescence Detection

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The determination of L-phenylalanine (Phe) and branched-chain L-amino acids (L-leucine (Leu), L-isoleucine (Ile) and L-valine (Val), BCAA) is important in the diagnosis of inborn metabolism disorders such as phenylketonuria and maple syrup urine disease. In routine work, a microplate fluorometric system has been used for multiple screening for the diseases.^{1,2} Such system is subject to interference from some compounds. Amino acid analyzers, which employ high-performance liquid chromatography on the basis of postcolumn derivatization with ninhydrin or *o*-phthalaldehyde, are used for the determination of the amino acids.^{3,4} The methods are reliable, but they produce an excessive amount of information that is not required for the purpose and result in high cost per analysis. Enzymatic methods have been developed for the selective determination of the amino acids in neonatal blood samples, using phenylalanine dehydrogenase (EC 1.4.1.1, PheDH)⁵⁻¹³ or leucine dehydrogenase (EC 1.4.1.9, LeuDH),¹⁴⁻¹⁷ which catalyzes the oxidative deamination of the amino acids with concomitant reduction of nicotinamide adenine dinucleotide (NAD⁺) to reduced nicotinamide adenine dinucleotide (NADH). Multi-channel flow-injection system with immobilized enzyme reactors have been developed for the simultaneous determination of Phe and BCAA.²⁰ PheDH has been immobilized and used in flow-injection (FI) systems for the determination of Phe in serum.¹⁸⁻²⁰ One of the major problems for practical use of the systems was the lack of the stability of the immobilized PheDH. It was reported that PheDH from *Bacillus badius* which was immobilized on a nylon coil and used in a bioluminescence continuous-flow system is relatively stable.²¹ But the system is unsuitable for use in routine work, because bioluminescence enzymes are not stable and the PheDH exhibits activities toward other amino acids such as Leu and Val.²²

To overcome these limitations, we attempted to develop a liquid chromatographic (LC) system with an enzyme reactor as postcolumn reactor for the simultaneous determination of Phe and BCAA. In this work, PheDH from *Bacillus badius* and LeuDH from *Bacillus stearothermophilus* were attached covalently to tresylate-poly(vinyl alcohol) beads to obtain coimmobilized enzyme reactor with high activity and stability.

The amino acids in the sample were separated with an ODS column (20 × 4 mm i.d.). The NADH produced in the reactor was monitored fluorometrically. The LC method was applied to the determination of Phe and BCAA in plasma.

Experimental

Materials and reagents

PheDH was prepared from *Escherichia coli* transformant in a similar manner to that described previously.²² The specific activity of the enzyme was 10 U mg-protein⁻¹. LeuDH (60 U mg⁻¹), NAD⁺ (free acid, 96%) and NADH (disodium salt, 99.5%) were purchased from Unichika (Osaka, Japan), Kohjin (Tokyo, Japan) and Boehringer Mannheim (Mannheim, Germany), respectively. Poly(vinyl alcohol) beads (GS-520, 13 µm diameter) and ODS column (20 × 4 mm i.d., Develosil ODS-UG-5 (5 µm)) were obtained from Showa Denko (Tokyo, Japan) and Nomura Chemical (Aichi, Japan), respectively. All other chemicals, which were purchased from Nacalai Tesque (Kyoto, Japan), were of analytical-reagent grade.

NAD⁺ solution (5 mM NAD⁺ in 0.02 M phosphate buffer (pH 6.8)) was prepared fresh daily. A carbonate buffer (pH 9.5) consisting of 0.2 M sodium carbonate-0.2 M sodium hydrogencarbonate was used.

PheDH and LeuDH were coimmobilized on the tresylate-poly(vinyl alcohol) beads. The methods for the preparation of the tresylate-beads was similar to that described previously.²³ The beads were packed into the stainless-steel column (40 × 4 mm i.d.). Enzyme solution [5 mg of PheDH and 1 mg of LeuDH in 5 ml of 0.05 M phosphate buffer (pH 6.8)] was circulated through the column at 0.2 ml min⁻¹ for 4 h at room temperature. After the coupling the reactor was washed with 10 mM glycine. The immobilization yield for PheDH was 98%.

Apparatus

The LC system used in this work is outlined in Fig. 1. The system consisted of two piston pumps (Hitachi L-6000), an injection valve (Sanuki SVI-6M2) fitted with a 30 µl loop, an ODS separation column, a reactor, connecting tubing (0.5 mm i.d.) and fittings. The fluorescence was measured with a Jasco FP-920 with a flow-through cell (15 µl) at 465 nm (excitation

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Fig. 1 Schematic representations of LC system employed for L-phenylalanine and branched-chain L-amino acids determination. A: 5 mM NAD⁺ solution; B: 0.2 M carbonate buffer (pH 9.5); S: injector with a 30 μ l loop; C: ODS column (20 $\times$ 4 mm i.d.); WB: water-bath thermostated at 35°C; CR: enzyme column reactor (4 cm $\times$ 4 mm i.d.); F: spectrofluorometer with a flow through cell (15 μ l); SC: signal cleaner; R: recorder; W: waste. All connecting tubing (0.5 mm i.d.) was made of PTFE.

wavelength: 340 nm). The detector output was recorded on a TOA FBR-251A recorder with a signal cleaner (SIC SC77). The analytical column and the reactor were maintained at 35°C.

Procedure

Venous blood was collected into plastic tubes containing lithium heparin, mixed by inversion and centrifuged (2000g) without delay for 15 min at room temperature. Subsequently, plasma was stored at -20°C until use. Plasma (100 μ l) was added to 500 μ l of the carbonate buffer (pH 9.5) and filtered through an ultrafiltration membrane (Advantec ULTRA FILTER 10000). The filtrate (30 μ l) was injected into the system. When not in use, the reactor was washed with the NAD⁺ solution and stored in a refrigerator.

The present method was compared with high-performance liquid chromatography [amino acid analyzer: Kyowa Seimitsu K-201; column: TSKgel SCX (150 $\times$ 6.0 mm i.d.); mobile phase: citrate buffer (solvent gradient); postcolumn derivatization with ninhydrin].

Results and Discussion

Reactor performance

The effects of temperature, pH and concentration of NAD⁺ on the activity of the reactor were evaluated by injecting 10 μ M Phe or Ile using the system shown in Fig. 1 without using the separation column. The temperature dependence was investigated over the range 20–40°C. The optimum temperature for both enzyme reactions was found to be 35°C. The effect of pH was studied in the range 9.0–11.0 by using carbonate buffer, glycine buffer and borate buffer. The carbonate buffer (0.2 M, pH 9.5) gave the best results for both amino acids. The effect of NAD⁺ solution was studied over the range 1–10 mM. The peak heights increased with increasing concentration, first rapidly and then gradually. Above 5 mM, the peak heights for both amino acids were almost constant; in the reactor the NAD⁺ concentration was one-half that of the solution.

Under the conditions of 2.5 mM NAD⁺ at pH 9.5 and 35°C, the peak height was measured by changing the flow rates of the buffer and the NAD⁺ solution, keeping the flow rate ratio of these solutions at 1:1. The peak height for Phe was almost constant from the total flow rate of 0.2 (0.1 ml min⁻¹ each) to 0.4 ml min⁻¹, and it decreased linearly above 0.7 ml min⁻¹. The peak height for Ile was constant from 0.2 to 0.5 ml min⁻¹.

Fig. 2 Stability tests for ODS column. Column: Develosil ODS-UG-5 (20 $\times$ 4 mm i.d.); mobile phase: 2.5 mM NAD⁺ in 0.1 M carbonate buffer (pH 9.5); flow rate: 0.4 ml min⁻¹; column temperature: 35°C; sample: 30 μ l containing Phe (10 μ M, A) and Ile (70 μ M, B).

The operational stability of the reactor was evaluated over 30 days. The reactor was used for analyses of 15 samples each for 2 h per day and stored in a refrigerator when not in use. The conversion efficiencies of the reactor for Phe and Ile were 100% immediately just after the preparation of the reactor and then peak height decreased gradually; the efficiencies were measured by using NADH. The half-life periods of the immobilized PheDH and LeuDH were 35 and 66 days, respectively. Compared with the immobilized PheDH from *Rhodococcus* sp. M4,¹⁸ the lifetime is about twice as long.

The reactor had activities against Phe, Leu, Ile, Val, L-methionine (Met) and L-tyrosine (Tyr), but other amino acids normally found in proteins did not give any response. The responses to Phe, Leu, Ile, Val, Met and Tyr were 100, 100, 100, 98, 5 and 2, respectively. No interferences from 1 mM L-ascorbic acid, 2 mM uric acid and 50 mM glucose were observed.

Separation of Phe and BCAA

Separation of Phe and BCAA was effected by a Develosil ODS-UG-5 column (20 $\times$ 4 mm i.d.) with 2.5 mM NAD⁺ in 0.1 M carbonate buffer (pH 9.5) as eluent at a flow rate of 0.4 ml min⁻¹ at 35°C. The stationary phase was chosen for the stability under basic pH conditions.^{24,25} The retention times (min) for Leu, Ile, Val, Met, Phe and Tyr were 1.4, 1.4, 1.3, 1.4, 2.4 and 2.5, respectively. The durability of the column was evaluated over 30 days. The column was used for 75 samples (mixture of Phe 10 and Ile 70 μ M) for 5 h per day and then washed with a water-methanol mixture (1:1 v/v). The capacity factors (*k*) for Phe and Leu were almost constant for 23 days, as shown in Fig. 2.

Calibration

Mixtures of Phe and Ile were used as a standard. Under the conditions shown in Fig. 1, the plot of peak height against the concentration was linear from 1 to 700 μ M for Phe with a correlation coefficient of 0.998 (10 data points) and from 0.5 to 600 μ M for Ile with 0.999.

Fig. 3 Chromatograms for five repeated injections of plasma sample. First peak: BCAA; second peak: Phe. Plasma was diluted 6-fold with 0.2 M carbonate buffer (pH 9.5) and filtered through an ultrafiltration membrane. The filtrate (30 μ l) was injected into the system.

For ten successive injections of a mixture (10 μ M Phe and 70 μ M Phe; the concentration of BCAA in plasma for healthy subjects is about 7 times that of Phe²⁶), the measured peak heights had a relative standard deviation (RSD) of 0.53% for Ile and of 0.69% for Phe. The detection limits (signal-to-noise ratio = 3) were 0.3 μ M for Ile and 0.8 for Phe.

Application

The LC system was used to determine Phe and BCAA concentrations in plasma. Plasma was repeatedly analyzed for 15 days with the reactor at a sampling rate of 15 h⁻¹. Figure 3 shows chromatograms obtained from the plasma sample; the concentrations of Phe, Leu, Ile, Val, Met and Tyr in the plasma, which were obtained by the amino acid analyzer, were 61, 139, 66, 233, 30 and 72 μ M, respectively. The reactor was used for analyses of 50 samples in a day and standards were measured every day, in order to correct the variation of the conversion efficiencies. The separation column was renewed every 15 days, since the resolution decreased gradually because of the accumulation of large molecules. The method gave precise and reproducible results; for plasma containing 61 μ M Phe and 438 μ M BCAA, the within-day RSDs for Phe and BCAA were 0.78% and 0.70, and the day-to-day RSDs were 1.4% and 1.2%.

Plasma of known Phe (62 μ M) and BCAA (427 μ M) concentrations was supplemented with Phe or Ile to give a final concentration of 0.562–2.06 mM for Phe or 0.927–3.43 mM for BCAA. The results are summarized in Table 1. The recoveries were in the range 99–101%.

For comparison, determinations were carried out by amino acid analyzer; $n=25$, ranges 48–91 μ M Phe and 388–502 μ M BCAA. The calculated linear regressions and correlation coefficients were $y=1.03x+0.04$ and 0.997 for Phe and $y=1.01x+0.02$ and 0.999 for BCAA, respectively. The present method gave slightly bigger values for Phe, because the reactor exhibited slight activities to Try in plasma; the mean concentration of Try in the plasma samples ($n=25$) was 79 μ M, with the amino acid analyzer.

Table 1 Recoveries of Phe and Ile added to plasma^a

Added/ μ M	Recovered ^b / μ M	Recovery, %
Phe		
500	505	101
1000	998	100
2000	2290	100
Leu		
500	502	100
1000	1010	101
2000	1990	100
3000	2980	99

a. Values corrected for Phe (62 μ M) or BCAA (427 μ M) already present in plasma.

b. All values are means ($n=5$).

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References

- U. Wendel, M. Koppelkamm, W. Hummel, J. Sander, and U. Langenbeck, *Clin. Chim. Acta*, **1990**, *192*, 165.
- N. Lubenow, F. Diepenbrock, H. Schickling, D. Bock, R. Heckler, and J. Sander, *Eur. J. Clin. Chem. Clin. Biochem.*, **1994**, *32*, 525.
- D. G. Drescher and K. S. Lee, *Anal. Biochem.*, **1978**, *84*, 559.
- M. K. Radjai and R. T. Hatch, *J. Chromatogr.*, **1980**, *196*, 319.
- T. Ohshima, H. Sugimoto, and K. Soda, *Anal. Lett.*, **1988**, *21*, 2205.
- W. Hummel, H. Scutte, and M.-R. Kula, *Anal. Biochem.*, **1988**, *170*, 397.
- U. Wendel, W. Hummel, and U. Langenbeck, *Anal. Biochem.*, **1989**, *180*, 91.
- U. Wendel, M. Koppelkamm, W. Hummel, J. Sander, and U. Langenbeck, *Clin. Chim. Acta*, **1990**, *192*, 165.
- H. Naruse, Y. Y. Ohshi, A. Tsuji, M. Maeda, K. Nakamura, T. Fujii, A. Yamaguchi, M. Matsumoto, and M. Shibata, *Screening*, **1992**, *1*, 63.
- K. C. Dooley, *Clin. Biochem.*, **1992**, *25*, 271.
- M. A. Vilaseca, C. Farre, and F. Ramon, *Clin. Chem.*, **1993**, *39*, 129.
- R. Taylor, I. C. Smith, and S. J. Standing, *Clin. Chim. Acta*, **1993**, *218*, 207.
- N. Lubenow, F. Diepenbrock, H. Schickling, D. Bock, R. Heckler, and J. Sander, *Eur. J. Clin. Chem. Clin. Biochem.*, **1994**, *32*, 525.
- T. Ohshima, H. Misono, and K. Soda, *Agric. Biol. Chem.*, **1978**, *42*, 1919.
- G. Livesey and P. Lund, *Biochem. J.*, **1980**, *188*, 705.
- J. M. Burrin, J. L. Paterson, and G. M. Hall, *Clin. Chim. Acta*, **1985**, *153*, 37.
- U. Wendel, J. Gonzales, and W. Hummel, *Clin. Chim. Acta*, **1993**, *219*, 105.
- N. Kiba, A. Itagaki, and M. Furusawa, *Talanta*, **1997**, *44*, 131.
- W. Hummel, S. Taushensky, U. Spohn, U. Wendel, and U.

- Langenbeck, "Biosensors: Application in Medicine, Environmental Protection and Process Control", ed. R. D. Schmid and F. Scheller, **1989**, Vol.13, VCH, Weinheim, 313.
20. W. Hummel and U. Wendel, "Flow Injection Analysis (FIA) Based on Enzymes or Antibodies", ed. R. D. Schmid, **1991**, Vol.14, VCH, Weinheim, 135.
21. S. Girotti, E. Ferri, S. Ghini, R. Budini, G. Carrea, R. Bovera, S. Piazza, R. Merighi, and A. Roda, *Talanta*, **1993**, 40, 425.
22. Y. Asano, A. Nakazawa, K. Endo, Y. Hibino, M. Ohmori, N. Numao, and K. Kondo, *Eur. J. Biochem.*, **1987**, 168, 153.
23. N. Kiba, M. Tachibana, K. Tani, and T. Miwa, *Anal. Chim. Acta*, **1998**, 375, 65.
24. N. Nagae, *Chromatography*, **1994**, 15, 175.
25. N. Nagae and D. Ishii, *Am. Lab.*, **1995**, 27, 20.
26. M. D. Armstrong and U. Stave, *Metabolism*, **1973**, 22, 561.
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