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Journal of Chromatography A, 724 (1996) 354–357

JOURNAL OF CHROMATOGRAPHY A

Short communication

Postcolumn co-immobilized leucine dehydrogenase–NADH oxidase reactor for the determination of branched-chain amino acids by high-performance liquid chromatography with chemiluminescence detection

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Received 4 July 1995; accepted 9 September 1995

Abstract

A liquid chromatographic system with a co-immobilized leucine dehydrogenase–NADH oxidase reactor is described for the determination of branched-chain amino acids such as D-leucine, D-isoleucine and D-valine. The enzymes were simultaneously immobilized on tresylate-containing poly(vinyl alcohol) beads. The separation was achieved by means of an ODS column with elution with phosphate buffer (pH 7.5). The hydrogen peroxide produced was detected chemiluminometrically via a luminol–hexacyanoferrate(III) reaction. The system gave a linear response from 0.3 to 300 μM for each amino acid and the detection limit was 0.1 μM.

Keywords: Post-column reactors; Amino acids, branched-chain; Leucine; Isoleucine; Valine

1. Introduction

The measurement of branched-chain amino acids (BCAAs) [L-leucine (Leu), L-isoleucine (Ile) and L-valine (Val)], is of clinical importance in the diagnosis of inborn errors of metabolism [1]. Enzymatic methods have been developed for the selective determination of total amounts of BCAAs in neonatal blood samples [2–5]. The methods are unsuitable for monitoring patients under therapy and for the diagnosis of several disorders of metabolism such as maple-syrup urine disease, hypervalinaemia and hyper-leucine–isoleucinaemia, because the individual BCAAs cannot be measured separately. In routine analysis the amino acids are separated and determined by amino acid analysers, which employ high-performance liquid chromatography (HPLC) on the basis of post-column derivatization with ninhydrin or *o*-phthalaldehyde [6,7]. They produce an excessive amount of information that is usually not required for practical use. For the selective determination of individual BCAAs an HPLC system with fluorescence detection using a reactor containing immobilized leucine dehydrogenase (EC 1.4.1.9), (LeuDH) have been reported [8]. The method is moderately sensitive and requires a tedious deproteinization procedure.

This paper describes an HPLC system with

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0021-9673/96/$15.00
SSDI 0021-9673(95)01041-6

co-immobilized LeuDH–NADH oxidase (NAOD) in a postcolumn reactor for the sensitive detection of BCAAs in plasma. BCAAs were separated on a reversed-phase ODS column. In the reactor, the LeuDH catalyses the deamination of the BCAAs in the presence of nicotinamide adenine dinucleotide (NAD^+) and the resulting reduced form NAD^+ (NADH) formed is removed by the NADH oxidase with concomitant formation of hydrogen peroxide. The hydrogen peroxide produced in the reactor was detected by measuring the chemiluninescence emitted when mixed with luminol and potassium hexacyanoferrate(III). This method was applied to the determination of BCAAs in plasma.

2. Experimental

2.1. Reagents

LeuDH (from *Bacillus stearothermophilus*, 65 U mg^{-1}) and NADH oxidase (EC number not assigned, from *Bacillus megaterium*, 56 U mg^{-1}) were obtained from Unitika (Osaka, Japan) and Asahi Kasei (Tokyo, Japan), respectively. NAD^+ (free acid, 95%) was purchased from Kohjin (Tokyo, Japan). Poly(vinyl alcohol) beads (GS-520, 13 μm diameter) were purchased from Showa Deno (Tokyo, Japan). All other chemicals were of analytical-reagent grade.

A stock solution (1 m*M*) of each BCAA was prepared by dissolving in 0.02 *M* phosphate buffer (pH 8.0). A potassium hexacyanoferrate(III) stock solution (200 m*M*) was prepared and diluted tenfold with water before use. Luminol solution (5 m*M* luminol in carbonate buffer (pH 10.5) consisting of 0.4 *M* sodium carbonate–0.4 *M* sodium hydrogencarbonate) was prepared and stored for 3 days in a refrigerator to stabilize it. NAD^+ solution [5 m*M* in 0.02 *M* phosphate buffer (pH 7.0)] was prepared daily.

2.2. Immobilization of enzymes

The method for activation of the beads with 2,2,2-trifluoroethanesulfonyl chloride (tresyl chloride) was similar to that described by Nilsson and Mosbach [9]. The beads (1 g) were washed with dry acetone (50 ml). The beads were suspended in 20 ml of dry acetone–pyridine (1:1, v/v). With vigorous magnetic stirring, 1 ml of tresyl chloride was added dropwise to the suspension over 2 min and the reaction was continued for 10 min. The beads were washed with acetone (10 ml) and 1 m*M* HCl (20 ml). The activated beads was stable for 3 days when stored in 1 m*M* HCl in a refrigerator. The time taken to prepare the tresylate-containing beads was about 20 min. The beads were packed into a stainless-steel column (5 cm × 4 mm I.D.) by the slurry-packing method. Enzyme solution [LeuDH 5 mg (325 U) and NAOD 5 mg (280 U) in 10 ml of 0.1 *M* phosphate buffer (pH 7.0)] was circulated through the column at 0.2 ml min^{-1} for 4 h. The time course of the immobilization process for NAOD was monitored by a spectrophotometer (Jasco Uvidec-100-VI) with a flow-through cell at 380 nm, which is one of the absorption maxima of NAOD [10]. The immobilization yield was evaluated by the decrease in absorbance. NAOD was immobilized with a 98% yield.

2.3. HPLC

Chromatography was carried out with Hitachi L-6000 pumps, a Sanuki SVI-6M2 injector with a 20-μl loop, a separation column (15 cm × 4 mm I.D.) which was filled with Capcell C_{18} SG120 (5 μm), a co-immobilized enzyme column reactor and a Soma S-3400 luminometer with a 100-μl flow-through cell, connected to a SIC SC77 signal cleaner and a System Instruments Chromatocorder II data processor. The column and the reactor were held constant at 50°C in the column oven. The samples were injected into the separation column at a flow-rate of 0.4 ml min^{-1} in 0.02 *M* phosphate buffer (pH 7.5). The eluate from the column was combined with the NAD^+ solution and the luminol solution at a four-way valve prior to elution through the reactor. The solutions were each pumped at a flow-rate of 0.15 ml min^{-1} and the total flow-rate through the reactor was 0.7 ml min^{-1}. The eluate from the reactor was combined with the potassium hexa-

cyanoferrate(III) solution at a three-way valve and then mixed with a mixing tube (90 cm × 0.5 mm I.D.) prior to the detector cell. The total flow-rate through the detector cell was 1.1 ml min^{-1}. Peak areas were measured and the responses were calibrated with standards. When not in use, the reactor was stored in the NAD^+ solution at ca. 5°C.

2.4. Procedure

Venous blood was collected in plastic tubes containing lithium heparin, mixed by inversion and centrifuged (2000 *g*) without delay for 15 min at room temperature. Subsequently, plasma was stored in a glass tube at −20°C until used. Plasma (5 μl) was added to 200 μl of 0.02 *M* phosphate buffer (pH 8.0) and filtered through an ultrafiltration membrane (Advantec Q0100, nominal molecular mass cut-off 10 000). The filtrate (20 μl) was injected via the sample injector.

The results obtained by the present method were compared with those obtained with an amino acid analyser (Kyowa Seimitsu K-201; column, TSKgel Aminopak; eluent, citrate buffers; postcolumn derivatization with *o*-phthalaldehyde).

3. Results and discussion

3.1. Reactor performance

The properties of the reactor were evaluated by using the system without using the separation column. The influence of pH on the peak height was studied over the pH range 9.5–11.5 by injecting 10 μ*M* Leu (20 μl). The optimum pH for the enzymatic reaction was about 10.5. The reactor was placed in a water-bath and the temperature was varied between 35 and 60°C. The optimum temperature for the reactions was 50–55°C. The effect of the concentration of the NAD^+ solution was examined in the range 0.5–20 m*M*. The optimum concentration of NAD^+ was 5 m*M*; for NAOD reaction lower NAD^+ concentrations are desirable for the production of H_2O_2 and, on the other hand, for LeuDH reaction lower concentrations are undesirable for the formation of NADH. The 5 m*M* NAD^+ solution was used in this system; at this concentration, the NAD^+ concentration in the reactor was 1 m*M*. The influence of the concentration of the luminol solution on the peak height was studied from 2 to 30 m*M*. The reactor exhibited maximum response at 25 m*M*, but the inactivation of the reactor commenced at concentrations above 20 m*M*. A 5 m*M* luminol solution in 0.4 *M* carbonate buffer (pH 10.5) was used; the luminol concentration in the reactor was 1 m*M*. The reactor was used for repeated injections of Leu solution (10 μ*M*) at a sample rate of 12 h^{-1} for 8 h per day (96 injections) and then thoroughly washed with the NAD^+ solution, and stored at 5°C when not in use. The activity decreased slowly to 91% of the initial value after 3 weeks.

Under the conditions of carbonate buffer (pH 10.5) containing 1 m*M* NAD^+ and 1 m*M* luminol at a flow-rate of 0.7 ml min^{-1} at 50°C, each standard BCAAs solution (10 μ*M*) was injected into the system and the peak heights were compared with the peak height for H_2O_2. The conversion efficiencies of the reactor for Leu, Ile and Val to H_2O_2 were 66.5, 60.1 and 57.0%, respectively; the relative peak heights for Leu, Ile and Val were 100:90.4:86.0.

3.2. Separation of the BCAAs

The separation of BCAAs was effected on a Capcell C_{18} SG120 (5 μm) column (15 cm × 4 mm I.D.) with 0.02 *M* phosphate buffer (pH 8.0) as mobile phase. The peak-area ratio for Leu, Ile and Val was 100:90:87. The peak area was plotted against the concentration of the compounds. The concentration range of linear response was from 0.3 to 300 μ*M* for each BCAA. The detection limit (signal-to-noise ratio = 3) for each BCAA was 0.1 μ*M*.

3.3. Application

This system was used to determine the amount of Leu, Ile and Val in plasma from normal adult subjects. A typical chromatogram for a plasma sample is shown in Fig. 1.

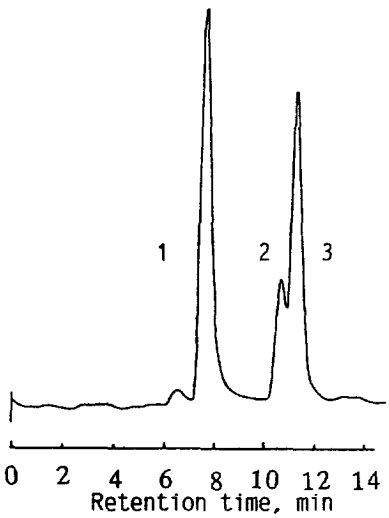

Fig. 1. Chromatogram of a plasma sample from a normal adult subject. Plasma (5 μl) was diluted with 200 μl of 0.02 *M* phosphate buffer (pH 7.5) and filtered with a membrane filter. Peaks: 1 = L-valine; 2 = L-isoleucine; 3- = L-leucine.

3.4. Precision and reproducibility

Plasma was repeatedly analysed during 3 weeks. The reactor was used for analyses of 40 samples per day and standards were measured at 20-sample intervals, in order to check the variation of the conversion efficiencies. The reactor was renewed every 10 days. The system gave satisfactorily precise and reproducible results; for plasma containing 161 μM Leu, 67.3 μM Ile and 235 μM Val, the within-day relative standard deviations (R.S.D.s) for Leu, Ile and Val were 2.5%, 3.0% and 2.3%, respectively, and the day-to-day R.S.D.s for Leu, Ile and Val were 3.1%, 3.8% and 2.6%, respectively.

3.5. Comparison

The results for plasma BCAAs obtained using this system compared well with the results obtained with the amino acid analyser. Plasma samples ($n = 20$) were obtained from 15-h fasted normal subjects (18–60 years old, 11 men). The calculated linear regressions and correlation coefficients for Leu (117–176 μM), Ile (60–94 μM) and Val (211–255 μM) were $y = 1.001x - 0.016$ and $r = 0.998$, $y = 0.999x - 0.057$ and $r = 0.995$ and $y = 1.001x - 0.022$ and $r = 0.999$, respectively, where y = peak height (cm) and x = concentration (μM). This method was simpler than using the amino acid analyser (HPLC with *o*-phthalaldehyde) for the determination of BCAAs, since only the separation of the three amino acids from each other is required for the assay. The sample throughputs for this method and for the amino acid analyser were 5 and 1 h^{-1}, respectively.

4. Conclusion

It was demonstrated that the co-immobilized LeuDH–NAOD reactor is useful for the sensitive detection of individual BCAAs using reversed-phase HPLC and chemiluminescence detection. The reactor in a postcolumn system was stable enough to permit the measurement of more 400 samples in 10 days. Compared with the fluorimetric HPLC method with an immobilized LeuDH reactor [8], the sensitivity for each amino acid is five times higher. The method can easily be used routinely for the determination of BCAAs in plasma with a simpler deproteinization procedure.

References

[1] C. Scriver and L. Rosemberg (Editors), Major Problems in Clinical Pediatrics, Vol. 10, Saunders, London, 1973, p. 256.
[2] G. Livesey and P. Lund, Biochem. J., 188 (1980) 705.
[3] H.-J. Rudolph, K.-D. Gerbitz, G. Michal and O.H. Wieland, Clin. Chem., 27 (1981) 431.
[4] J.M. Burrin, J.L. Paterson and G.M. Hall, Clin. Chim. Acta, 153 (1985) 37.
[5] S. Girotti, A. Roda, and M.A. Angellotti, S. Ghini, G. Carrea, R. Bovara, S. Piazzi and R. Merighi, Anal. Chim. Acta, 205 (1988) 229.
[6] D.G. Drescher and K.S. Lee, Anal. Biochem., 84 (1978) 559.
[7] M.K. Radjai and R.T. Hatch, J. Chromatogr., 196 (1980) 319.
[8] N. Kiba, S. Hori and M. Furusawa, J. Chromatogr., 463 (1989) 177.
[9] K. Nilsson and K. Mosbach, Biochem. Biophys. Res. Commun., 102 (1981) 449.
[10] Y. Saeki, M. Nozaki and K. Matsumoto, J. Biochem., 98 (1985) 1433.