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Note

High-performance liquid chromatographic determination of branched-chain α-keto acids in serum using immobilized leucine dehydrogenase as post-column reactor

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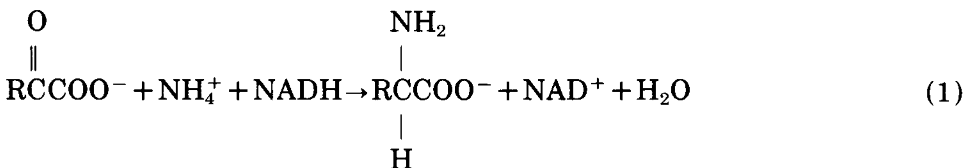
Branched-chain α-keto acids (BKAs) [α-ketoisovaleric acid (KIV), α-ketoisocaproic acid (KIC) and α-keto-β-methylvaleric acid (KMV)] are metabolites of branched-chain amino acids (valine, leucine and isoleucine). Therefore, elevated levels of serum BKAs have been associated with the occurrence of metabolic disorders. In recent years, high-performance liquid chromatography (HPLC) has been widely used for the determination of BKAs in physiological samples. Many fluorescent labelling reagents have been used for the pre-column derivatization of BKAs [1–6]. The methods are sensitive but require a long time for the derivatization. Other HPLC methods for the determination of BKAs have been developed by Nissen et al. [7] and Walser et al. [8]. BKAs were separated by reversed-phase HPLC and detected by UV absorbance at 214 nm. However, these methods require clean-up steps prior to the analysis to remove highly UV-absorbing substances that may coelute with BKAs.

Leucine dehydrogenase (L-leucine:NAD$^+$ oxidoreductase, deaminating, EC 1.4.1.9) (Leu-DH) catalyses the amination of α-keto acids in the presence of reduced β-nicotinamide adenine dinucleotide (NADH) according to the following reaction:



$$RC(=O)COO^- + NH_4^+ + NADH \rightarrow RCH(NH_2)COO^- + NAD^+ + H_2O \qquad (1)$$

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Leu-DH has been used as reagent for the determination of the total amount of BKAs [9,10].

This study aims to establish a specific HPLC method with fluorescence detection for the determination of BKAs in serum using immobilized Leu-DH as post-column reactor. Serum sample is deproteinized by ultrafiltration. BKAs are separated on an ODS column with a mobile phase containing ammonium salt buffer and NADH. The magnitude of decrease in intensity of fluorescence based on the NADH is dependent on the amount of BKA in the eluate.

EXPERIMENTAL

Materials

KIV, KIC, KMV, α-keto-n-butyric acid (KB), α-keto-n-valeric acid (KV) and α-keto-n-caproic acid (KC) were purchased from Sigma (St. Louis, MO, U.S.A.). NADH (grade II) was obtained from Boehringer Mannheim (Mannheim, F.R.G.). Leucine dehydrogenase (40 U/mg of solid) was purchased from Toyobo (Osaka, Japan) and purified by ultrafiltration through a membrane filter (cut-off at relative molecular mass 30 000) before use. The support used for enzyme immobilization was similar to that described previously [11]. All other chemicals were analytical-reagent grade.

Apparatus

A flow diagram of the HPLC system is shown in Fig. 1. A Model 655 HPLC pump (Hitachi, Tokyo, Japan) was connected to the Rheodyne Model 7125 injector (Cotati, CA, U.S.A.) with a 30-μl loop, a guard column (10 mm$\times$4 mm I.D. stainless-steel column), an analytical column (150 mm$\times$4 mm I.D. stainless-steel column) and an immobilized enzyme reactor (50 mm$\times$4 mm I.D. stainless-steel column). The system was equipped with a Model 650 fluorescence spectrophotometer (Hitachi) with a 18-μl flow-through cell and a data processor (Chromatocorder II, System Instrument, Tokyo, Japan). The mobile phase consisted of 0.5 M NH_4Cl–NH_4OH buffer (pH 8.5) and 5 μM NADH, at a flow-rate of 0.8 ml/min. The chromatographic columns were filled

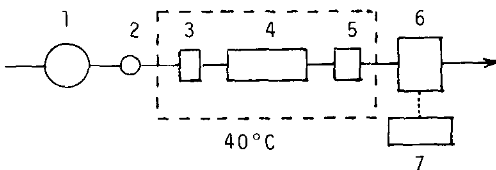

Fig. 1. Flow diagram of HPLC system for the determination of branched-chain α-keto acids. 1 = Pump; 2 = injector; 3 = guard column; 4 = analytical column; 5 = immobilized enzyme column reactor; 6 = fluorescence detector; 7 = data processor.

with Capecell 120 C_{18}, 5 μm (Shiseido, Tokyo, Japan). The columns and immobilized enzyme reactor were maintained at 40°C with a water-bath. The preparation of the immobilized enzyme reactor was described in detail in the previous paper [11].

Procedure

A portion of serum (200 μl) was mixed with 20 μl of 300 μM KC as an internal standard. The mixture was filtered through a membrane filter (cut-off at relative molecular mass 30 000). An aliquot of the filtrate (30 μl) was injected into the chromatograph.

The calibration graph was prepared by using the serum. A portion of serum (200 μl) was mixed with 20 μl of standard mixture containing 50–600 μM of each BKA and 300 μM of KC. The net peak-height ratio of the each BKA and KC was plotted against the concentration of each BKA.

RESULTS AND DISCUSSION

Reactor properties

To evaluate the immobilized enzyme reactor, KMV was selected as model compound.

The effect of pH on the enzyme activity was studied between pH 7.0 and 9.5. As shown in Fig. 2, maximum activity is observed at pH 8.5. Compared with the free enzyme (optimum pH 9.4), the pH maximum for the immobilized Leu-

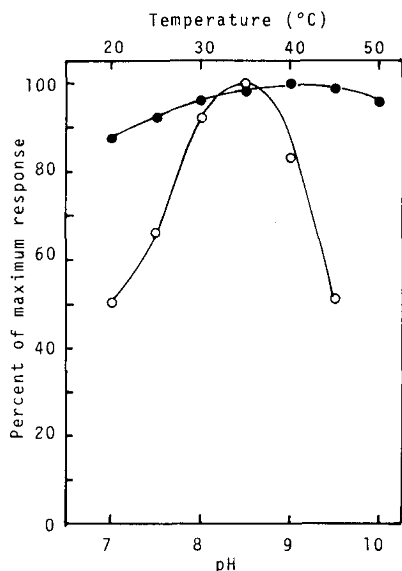

Fig. 2. Effects of pH (○) and temperature (●) on the activity of the immobilized enzyme.

DH is ca. 1 pH unit more acidic. The activity of the immobilized enzyme at different temperatures was examined. The reactor exhibits maximum activity at 40°C, as shown in Fig. 2.

The optimum concentration of ammonium ion in the buffer was also investigated. The response increased linearly with increasing concentration of the ion and became constant at above 0.4 *M*. The response decreased linearly as the flow-rate increased from 0.5 to 1.0 ml/min. The lower flow-rates were preferable for analytical sensitivity, but the peak broadening at lower rates was undesirable for the chromatographic system. The operational stability of the immobilized Leu-DH was evaluated for twelve weeks. The reactor was used for 8 h in a day and stored at 4°C in 0.1 *M* phosphate buffer (pH 7.0) when not in use. The activity remained at ca. 85% of the initial value for two weeks, and fell to 70% of the initial value after twelve weeks. Storage stability was examined for eight months. The enzyme was lyophilized and stored at −15°C in the presence of L-cysteine (1 g/g of the enzyme). The activity was 80% of the initial value after the period.

Under the conditions of 0.5 *M* NH_4Cl–NH_4OH (pH 8.5) containing 5 μM NADH at a flow-rate of 0.8 ml/min at 40°C, the relative activities of the immobilized enzyme for KMV, KIV, KIC, KB, KV and KC were 118, 106, 99, 85, 90 and 100, respectively. Other α-keto acids, such as pyruvic acid, α-ketoglutaric acid and phenylpyruvic acid, did not give any response. Under the same conditions, NADH in the presence of 30 μM KMV was converted into NAD in a 55% yield.

Separation of α-keto acids by HPLC

Fig. 3 shows a typical chromatogram obtained with a standard mixture of five α-keto acids. Separation of KMV and KIC is incomplete. The ratio of peak heights for KB, KIV, KMV, KIC and KC was 191:274:222:197:100. Since the baseline was affected by changes in column temperature, the temperature was kept constant at 40°C. Separation parameters of the α-keto acids are given in Table I.

Recovery, precision and comparison

Fig. 4 shows a chromatogram obtained from the serum sample to which KC (internal standard) was not added. Since the serum sample contained KB, KC was selected as internal standard. Linear relationships were observed between the peak-height ratios of BKAs to that of KC and the concentrations of BKAs in the range of 4–55 μM. The limit of detection for the BKAs was 1 μM.

To examine losses of α-keto acids from the deproteinization process (ultrafiltration), the recoveries of α-keto acids added to pooled normal serum were determined. The recoveries (mean ± S.D., each $n = 10$) of α-keto acids (each 27.3 μM) were 53.5 ± 1.3% (KIV), 56.5 ± 1.4% (KMV), 55.7 ± 1.3% (KIC) and 54.5 ± 1.2% (KC). The precision of this method for the determination of BKAs

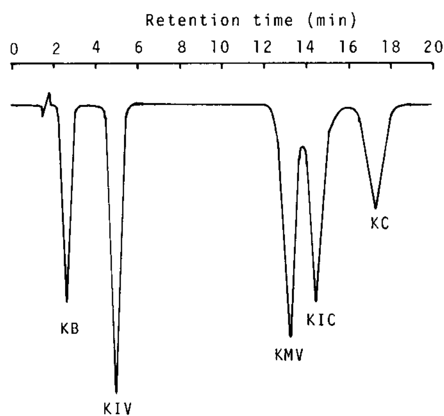

Fig. 3. Chromatogram of standard mixture of five α-keto acids (each 10 μM). For abbreviations see Table I.

TABLE I

SEPARATION PARAMETERS OF α-KETO ACIDS

α-Keto acid	Abbreviation	Capacity factor	Resolution	Separation factor
α-Ketobutyric acid	KB	0.64		
α-Ketoisovaleric acid	KIV	2.08	3.83	3.25
α-Keto-β-methylvaleric acid	KMV	7.23	11.8	3.48
α-Ketoisocaproic acid	KIC	7.98	1.33	1.10
α-Ketocaproic acid	KC	9.73	2.15	1.22

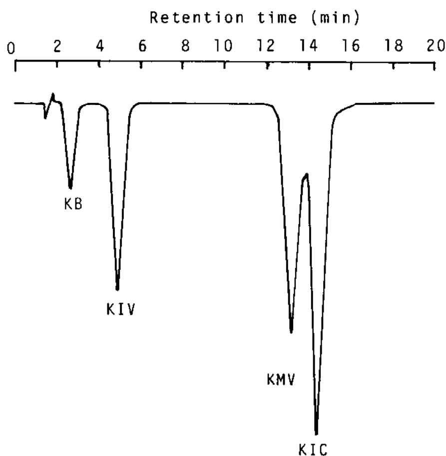

Fig. 4. Chromatogram of serum sample from a normal subject. For abbreviations see Table I.

TABLE II

COMPARISON STUDY OF BKAs IN SERUM

Values are means (μM) with C.V. (%) in parentheses.

Sample No.	Post-column method ($n=5$)			Direct method ($n=5$)		
	KIV	KMV	KIC	KIV	KMV	KIC
1	15.2 (2.3)	22.2 (2.0)	35.2 (2.8)	15.1 (2.8)	21.9 (3.4)	35.0 (3.5)
2	18.6 (2.0)	24.7 (1.9)	49.4 (2.5)	18.3 (2.7)	24.5 (3.2)	47.8 (3.0)
3	15.8 (2.3)	22.3 (1.9)	44.3 (2.6)	15.5 (3.0)	22.6 (3.3)	44.1 (3.1)
4	14.6 (2.4)	21.5 (2.1)	39.4 (2.8)	15.1 (3.0)	21.5 (3.3)	40.3 (3.1)
5	16.5 (2.1)	22.7 (2.1)	29.7 (2.8)	16.3 (2.9)	22.4 (3.3)	29.3 (4.1)
6	14.7 (2.4)	19.0 (2.2)	41.1 (2.6)	14.7 (3.2)	19.0 (3.8)	41.2 (3.6)
7	19.3 (2.0)	26.7 (1.9)	49.6 (2.5)	19.4 (2.8)	26.5 (3.2)	48.8 (3.5)
8	14.5 (2.5)	19.4 (2.2)	42.7 (2.5)	14.3 (3.0)	19.2 (3.9)	41.0 (3.2)
9	15.4 (2.3)	22.6 (1.8)	32.9 (2.9)	15.0 (2.6)	22.1 (3.6)	31.2 (3.3)
10	17.4 (2.2)	23.6 (1.7)	35.8 (2.7)	17.6 (2.7)	23.3 (3.6)	35.1 (3.6)

was examined by carrying out ten replicate analyses of pooled normal serum. The coefficients of variation (C.V.) for KIV (mean; 14.5 μM), KMV (23.0 μM) and KIC (44.3 μM) were 2.1, 1.7 and 2.4%, respectively.

The present method was compared with the direct HPLC method of Walser et al. [8] by analysing ten different pooled sera. The results are shown in Table II. The correlation coefficients, calculated by the least-squares method, were 0.986 for KIV, 0.994 for KMV and 0.993 for KIC. The Student's t-test (assuming similar S.D.s for the two methods) gave values of $t=1.00$ for KIV, 1.84 for KMV and 2.18 for KIC, which indicate no significant difference between the two methods at the 95% confidence level.

This study has provided an HPLC method that permits the determination of BKAs in normal sera. The method does not require any clean-up steps and/ or pre-column derivatization procedures. Thus, it is simple to carry out and it is available for routine use.

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