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Note

Determination of α-keto acids in rat hindquarter muscle by high-performance liquid chromatography with fluorescence detection

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In recent years, considerable attention has been focused on the branched-chain amino acids (BCAA) and their corresponding α-keto acids (BCKA). Both starvation [1–4] and diabetes mellitus [5] can result in significant alteration in concentrations of BCKA and BCAA. Furthermore, nitrogen-sparing effects of BCKA have been studied for some time [6,7]. For these reasons there has been an increased demand for sensitive methods for the determination of BCKA. Several methods have been reported for the determination of BCKA in biological samples. The derivatization of BCKA with 2,4-dinitrophenylhydrazine has been followed by paper chromatography [8] and high-performance liquid chromatography (HPLC) [9,10]. However, such chromatographic separations of the derivatives are complicated and unsatisfactory. BCKA react with *o*-phenylenediamine in acidic solution to form stable quinoxalinol derivatives [11]. Recently, these fluorescent quinoxalinol derivatives of BCKA have been analysed by gas chromatography (GC) [12–16] and HPLC [17–19].

In this report, we describe the HPLC analysis of BCKA in rat hindquarter muscle using fluorescent quinoxalinol derivatives and discuss a new method of prepurification.

EXPERIMENTAL

Chemicals

Sodium α-ketoisocaproate (KIC), sodium α-keto-β-methylvalerate (KMV), sodium α-ketoisovalerate (KIV) and sodium α-ketocaproate (KC) were pur-

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chased from Sigma (St. Louis, MO, U.S.A.). *o*-Phenylenediamine and all other reagents were purchased from Wako (Osaka, Japan).

A 0.13% *o*-phenylenediamine solution was prepared daily by dissolving 133 mg in 100 ml of 3 *M* hydrochloric acid. KC was chosen as the internal standard (I.S.) since it is not found in mammalian tissues and is completely separated from other BCKA. Absolute methanol containing 4 μmol/l KC was prepared as the I.S. Sample collection racks and disposable Bond Elut SAX (quaternary amine) 3-ml anion-exchange columns (Analytichem International, Harbor City, CA, U.S.A.) were purchased from Wako.

Apparatus

A Shimadzu Model LC-4A high-performance liquid chromatograph equipped with a Model SIL-2AS loop injector, a Model RF-530 fluorescence spectromonitor, a Model CTO-2AS column oven and a Model CR-2AX reporting integrator (Shimadzu, Kyoto, Japan) was used in these studies.

Chromatographic separations were carried out with 250×4.0 mm I.D. LiChrosorb RP-18 (5 μm particle size) analytical column (Wako) and a 45.0×4.0 mm I.D. Zorbax-ODS (45 μm particle size) guard column (Wako).

Sample preparation

Wistar strain male rats weighing 180–220 g were used. The fasted group was starved for 24 h before use. The fed group was fed on standard diet. Immediately after decapitation of the animal, the hindquarter muscle was removed and clamped between plates of aluminium tongs, which had been cooled in liquid nitrogen. These procedures, from removal of the muscle to freezing, were done as quickly as possible. The clamped frozen tissues were pulverized in a motor pestle which was maintained at the temperature of liquid nitrogen. The powdered tissue was stored at below −70 °C until use. Frozen powder (0.3 g) was weighed in a Cornex test-tube (110×13 mm), and ten volumes of cold I.S. were added. The mixture was homogenized with a Polytron homogenizer for three 10-s periods with cooling and centrifuged for 10 min at 30 000 *g* at 4 °C. The supernatant was used for analysis, as described later.

Prepurification of tissue extract

The Bond Elut anion-exchange columns were conditioned by washing them once with one column volume of methanol, followed by one column volume of distilled water; during this procedure it is important to keep the sorbent wet [20]. Then 2 ml of tissue extract were passed through the column, which was subsequently washed twice with one column volume of distilled water. A 0.5-ml volume of 0.5 *M* sulphuric acid was then added to the column twice, and 1 ml of the total effluent containing the BCKA was collected in a glass test-tube.

Preparation of quinoxalinol derivatives

Fluorescent labelling of BCKA was done according to the method described by Koike and Koike [19]. Exactly 0.5 ml of the Bond Elut SAX column effluent was placed in a 15-ml screw-capped tube. To this, 1.5 ml of 0.13% *o*-phenylenediamine

solution (freshly prepared) and distilled water were added to a final volume of 3 ml. The mixture was then placed in a hot block at 100°C for 30 min and allowed to cool to room temperature. Then, 0.5 g of anhydrous sodium sulphate was added, to ensure good extraction of the quinoxalinol derivatives of BCKA [17]. The quinoxalinol derivatives of BCKA in the reaction mixture were extracted twice with 3 ml of chloroform. Substitution of methylene chloride for chloroform in the extraction procedure produced identical results. The double extracts were combined and evaporated to dryness under reduced pressure at 40°C using a Yamato Model RD-31 centrifugal evaporator (Tokyo, Japan). The dried quinoxalinol residue was dissolved in 0.4 ml of methanol and filtered through a Millipore HV4L column filter. A portion of this filtrate (10–40 μl) was directly injected into the chromatograph.

Chromatographic conditions

Chromatography was performed at 50°C by a linear gradient at a flow-rate 0.7 ml/min. Solvent A contained 39% (v/v) aqueous acetonitrile and solvent B was 100% acetonitrile. The mobile phase composition was 100% solvent A at 0–5 min, 79% solvent A–21% solvent B at 19 min and 100% solvent B at 19–25 min. The original conditions were reestablished by a reverse gradient to 100% solvent A from 25 to 26 min. This composition was maintained for 29 min from 26 to 55 min, at which time the column was ready for injection of the next sample. The quinoxalinol derivatives of BCKA were detected by monitoring the fluorescence emission at 410 nm (with excitation at 350 nm).

RESULTS AND DISCUSSION

Identification of BCKA

A representative elution profile of the quinoxalinol derivatives of the standard BCKA is presented in Fig. 1. Capacity factors (k') and retention times (t_R) of several BCKA are shown in Table I. For injected samples in the range from 40 pmol to 4 nmol, the detector response was linear ($r^2 \geqq 0.98$) for each BCKA.

Recoveries of BCKA from Bond Elut SAX column

To examine losses of BCKA from the Bond Elut SAX column, we examined the recovery of BCKA using known amounts of standard solution.

When measured without (a) and with (b) the Bond Elut SAX column, the following ratios (a/b) were obtained: 103.6 ± 10.4 for KIV; 105.7 ± 14.6 for KIC; 93.9 ± 4.6 for KC; 98.7 ± 11.1 for KMV.

Comparison of chromatograms with and without Bond Elut

Fig. 2 represents the chromatogram of the quinoxalinol derivatives of BCKA in hindquarter muscle of the fasted rat. Fig. 2a shows that when the Bond Elut SAX was used for prepurification, a good separation was obtained for KIV, KIC, KC (I.S.) and KMV. Fig. 2b shows a chromatogram obtained without using the Bond Elut SAX. Many impurities were present and the identification of BCKA was difficult.

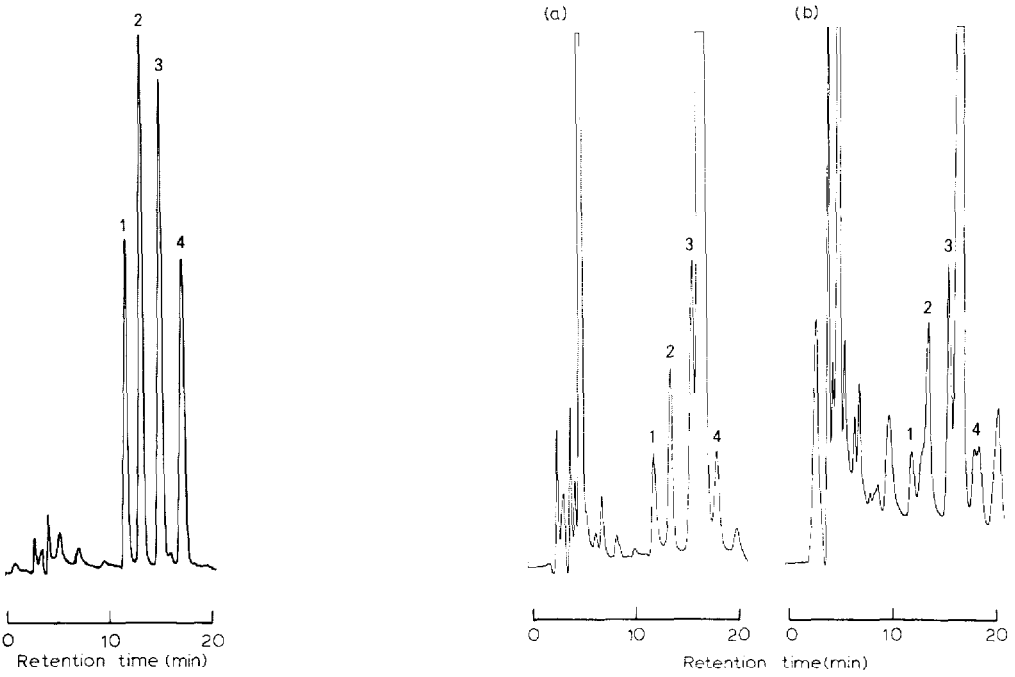

Fig. 1. HPLC profile of the quinoxalinol derivatives of the standard α-keto acids; 20 μl of the final extract containing 200 pmol of each of the derivatives were injected. Peaks: 1 = KIV; 2 = KIC; 3 = KC (I.S.); 4 = KMV. The chart speed was 0.25 cm/min.

Fig. 2. HPLC profiles of the quinoxalinol derivatives of α-keto acids in hindquarter muscle of the fasted rat. (a) The Bond Elut SAX anion-exchange column was used before quinoxalinol labelling. All procedures were performed as described in the text, and 20 μl of the final extract (400 μl) were injected. (b) The Bond Elut Sax anion-exchange column was not used. The tissue extract was labelled with a fluorescent tap directly, and a 20-μl sample of the final extract (400 μl) was analysed.

TABLE I

CAPACITY AND SELECTIVITY FACTORS FOR QUINOXALINOL α-KETO ACID DERIVATIVES

α-Keto acid	Capacity factor* (k')	Retention time (t_R) (min)	Selectivity factor** (α)
KIV	4.69	11.56	
KIC	5.33	12.84	1.14
KC	6.14	14.50	1.15
KMV	7.11	16.47	1.16

*$k' = \frac{t_R - t_0}{t_0}$, where t_0 (2.03 min) is the retention time of the solvent front.

**$\alpha = \frac{k'_2}{k'_1}$, where k'_2, are the column capacity factors of later and earlier eluting plots, respectively.

TABLE II

CONTENTS AND RECOVERIES OF α-KETO ACIDS FROM FREEZE-CLAMPED RAT HINDQUARTER MUSCLE

α-Keto acid	Content (mean ± S.D., $n=9$) (nmol/g wet weight)		Recovery (mean ± S.D., $n=18$) (%)
	Starved	Fed	
KIV	15.64 ± 1.29	7.48 ± 0.59	105.1 ± 3.4
KIC	12.70 ± 1.08	10.55 ± 0.96	99.8 ± 5.5
KMV	8.35 ± 0.72	6.11 ± 0.43	105.9 ± 2.4

Contents and recoveries of BCKA from rat hindquarter muscle

Known amounts of BCKA standard were added to powdered hindquarter muscle, which was then subjected to the full procedure described in Experimental. The percentage recoveries are shown in Table II. BCKA in normal rat hindquarter muscle was determined by this method (Table II). These concentrations of BCKA were in good agreement with the average values of Hutson and coworkers [1,2] who found that KIV, KIC and KMV concentrations from the fed group were 7.4 ± 0.5, 7.7 ± 0.5 and 4.9 ± 1.0 nmol/g of tissue, and that the same concentrations after three days of starvation were 18.5 ± 1.8, 12.1 ± 1.5 and 5.8 ± 0.7 nmol/g of tissue, respectively, using the GC method of Cree et al. [15].

Our method is simple, rapid and reproducible. The prepurification procedure using the disposable anion-exchange column permits the enrichment of BCKA from crude samples. It enables the determination of BCKA in muscle fibre and eliminates previous problems caused by impurities and difficulties in measurement.

The use of a reversed-phase LiChrosorb RP-18 column permits the direct injection of quinoxalinol derivatives of BCKA without trimethylsilylation, which is a necessary step in the GC procedure.

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