Analysis of Branched-Chain Keto Acids in Cell Extracts by HPLC-Fluorescence Detection

Ayuna HATTORI¹, Takahiro ITO¹, Makoto TSUNODA*²

¹Department of Biochemistry and Molecular Biology, The University of Georgia, 500 DW Brooks Drive, Athens, GA 30602, USA
²Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

Abstract
An analytical method for quantifying branched-chain keto acids (BCKAs) - namely α-keto-isovaleric acid (KIV), α-keto-isocaproic acid (KIC), and α-keto-β-methylvaleric acid (KMV) - in cell extracts was developed and applied to a human chronic myelogenous leukemia cell line. Reversed-phase liquid chromatography was conducted for separation of the BCKAs after derivatization into fluorescent quinoxalines by reaction with o-phenylenediamine. The calibration curve was linear over the range from 0.5 to 50 μM. The precision values in intra- and inter-day assays were less than 3.1% and 5.6% (n = 4), respectively. Intracellular concentrations of KIV, KIC, and KMV in K562 cells were 47.6 ± 10.2, 312.4 ± 40.6, and 282.4 ± 71.6 pmol/1 x 10⁶ cells, respectively (n = 5 for each BCKAs). Furthermore, this method was applied to study the effect of gabapentin - an inhibitor of branched-chain amino acid aminotransferase 1 - on intracellular BCKA levels.

Keywords: K562 cells; o-Phenylenediamine; Branched-chain amino acids; Cancer

1. Introduction
Branched-chain amino acids (BCAAs) are amino acids that have aliphatic side-chains with a branch. There are three BCAAs - leucine (Leu), isoleucine (Ile), and valine (Val) - among the essential amino acids in humans. BCAAs have been shown to modulate muscle protein synthesis, reduce protein catabolism, and affect the mammalian target of rapamycin (mTOR) pathway, which could increase the basic metabolic rate [1]. BCAAs are useful biomarkers of some diseases such as diabetes, liver disease, and cancer. Hence, there are many application of BCAAs present in biological fluids and cell extracts [2,3]. However, the analysis of BCAAs alone is not sufficient for gaining a better understanding of their physiological roles.

BCAAs are metabolized to branched-chain keto acids (BCKAs) by branched-chain amino acid aminotransferase (BCAT) [4]. BCAT catalyzes the reversible conversion of the α-amino group of a Leu, Ile, and Val to α-ketoglutaric acid, producing α-keto-isocaproic acid (KIC), α-keto-β-methylvaleric acid (KMV), and α-keto-isovaleric acid (KIV), respectively, as well as glutamic acid (Fig. 1). Since the reaction is reversible, BCAT can also synthesize BCAAs from BCKAs and glutamate. Therefore, a change in the equilibrium of the BCAT-catalyzed reaction has a significant impact on intracellular BCAA metabolism [5,6]. Alterations in BCAA metabolism are drivers of several diseases, such as insulin resistance and cancer development [7,8]. Recently, we reported that the aberrant activation of BCAT1 promotes transformation of hematopoietic progenitors and thereby drives cancer progression in myeloid leukemia [9].

Prior techniques for the quantification of BCKAs include high-performance liquid chromatography (HPLC)-fluorescence detection [10-13], liquid chromatography-mass spectrometry (LC-MS) [14-16], and gas chromatography-mass spectrometry (GC-MS) [17]. These methods have been applied to various biological fluids such as plasma and serum; but very few methods...
have been established for the reliable quantification of intracellular BCKAs from cell or tissue samples: Mühling et al. determined intracellular α-keto acid levels in neutrophils [12], and Olson et al. developed an ultra-fast LC–MS method for the quantification of BCKAs in mouse tissues [14]. In this study, we quantified intracellular BCKAs in K562 human myeloid leukemia cells by HPLC-fluorescence detection. We also analyzed the effect of gabapentin - a chemical inhibitor of BCAT1 activity - on intracellular BCKA levels.

2. Materials and methods

2.1. Reagents

α-Keto-isovaleric acid (KIV, sodium 3-methyl-2-oxobutyrate), α-keto-isocaproic acid (KIC, sodium 4-methyl-2-oxovalerate), α-keto-β-methylvaleric acid (KMV, 3-methyl-2-oxopentanoic acid sodium salt), and o-phenylenediamine (OPD) were purchased from Sigma-Aldrich (St. Louis, MO, USA). α-Ketovaleric acid (KV, 2-oxovaleric acid, internal standard) was obtained from Tokyo Chemical Industry (Tokyo, Japan). HPLC-grade methanol was from Merck (Darmstadt, Germany). The HPLC-grade water was purified by a Milli-Q water purification system (Merck Millipore, Darmstadt, Germany). All other reagents used in this study were of analytical grade.

2.2. Cell culture and BCAT inhibitor experiments

The human blast crisis myeloid leukemia cell line K562 was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and treated essentially as described previously [9]. In brief, K562 cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 IU/ml penicillin and 100 μg/ml streptomycin. Cell line authentication testing was performed by ATCC-standardized short tandem repeat analysis. For the BCAT1 inhibitor experiments, K562 cells were plated at 2 x 10^5 cells/mL. After 24 hr, either phosphate-buffered saline (PBS) or 20 mM gabapentin (Tokyo Chemical Industry) was added to the medium. At 24 or 48 hr post-treatment, cells were collected, washed with PBS and treated with 80% methanol. After removing the insoluble particles by centrifugation, the supernatants were collected and dried using a vacuum centrifuge for keto acids analysis.

2.3. Sample preparation and derivatization

Sample preparation and derivatization were performed according to previous studies [18]. Twenty-five microliter of the sample was added to 225 μL of 12.5 mM OPD in 2 M HCl, followed by incubation at 80 °C for 20 min. The reaction solution was transferred to a glass tube containing 40 mg of Na₂SO₄; 250 μL of ethyl acetate was added, and the tube was vortexed, then centrifuged at 1000 g for 8 min at 4 °C. The upper phase was transferred into a fresh glass tube with 40 mg of Na₂SO₄. Ethyl acetate (250 μL) was added to the original tube, which was vortexed, then centrifuged at 1000 g for 8 min at 4 °C. The upper phase was combined with the first extracted solution, dried using a vacuum centrifuge, and re-solubilized in 200 μL of methanol. Ten microliter of the sample was injected into an HPLC system.

2.4. HPLC analysis

The HPLC system consisted of a PU-2080 pump (Jasco, Tokyo, Japan), an AS-950 autosampler (Jasco), an Inertsil ODS-4V column (25 cm length, 3.0 mm I.D., 5 μm particle size, GL Sciences, Tokyo, Japan) in a CO-965 column oven (Jasco) and a FP-2020 fluorescence detector (Jasco). The mobile phase was water/methanol (50/50, v/v). The flow rate was maintained at 0.6 mL/min. The column temperature was set at 40 °C. The detection wavelength was set at 415 nm; the excitation wavelength was set at 360 nm.

2.5. Method validation

Samples containing 5, 25, 50, 250, or 500 μM of KIV, KIC, or KMV were injected into the HPLC system after the derivatization. The calibration curves for the relative sample peak area/the area of the internal standard (KV) versus the concentrations of these compounds were obtained. Least-squares regression was used for the calibration of the slope, intercept, and correlation coefficient. The limits of detection (LOD) and the limits of quantitation (LOQ) were defined as the sample concentrations that produced a signal-to-noise ratio of 3 (S/N = 3) and a signal-to-noise ratio of 10 (S/N = 10), respectively. Intra- and inter-day variations were calculated by repeated analysis (n = 4) of cell samples in a single.

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Fig. 1. Chemical structures of branched-chain keto acids (BCKAs). (a) α-Keto-isovaleric acid (KIV), (b) α-keto-isocaproic acid (KIC), (c) α-keto-β-methylvaleric acid (KMV), and (d) α-ketovaleric acid (KV, internal standard).
3. Results and discussion

While there are several analytical methods reported for keto acids, there are very few that can be applied to cell samples. In this study, we employed HPLC-fluorescence detection with o-phenylenediamine (OPD) as a fluorescent derivatization reagent, since most studies concerning the analysis of keto acids have utilized this compound. Derivatization of keto acids with OPD resulted in quinoxaline derivatives, with fluorescence in the 410 nm to 500 nm region [10].

3.1. Method optimization and analysis of cell extracts

On the basis of previous studies [10-12], chromatographic parameters were optimized for better resolution of OPD-derivatized BCKAs and the internal standard. Optimized LC conditions are described in the Methods section. A representative chromatogram of the standard solution consisting of the quinoxaline derivatives of the standard BCKAs is shown in Fig. 2(a). The method was applied to K562 cells, which are human chronic myelogenous leukemia cells.

A typical chromatogram of K562 cell samples is shown in Fig. 2(b). Intracellular concentrations of KIV, KIC, and KMV were estimated to be 47.6 ± 10.2, 312.4 ± 40.6, and 282.4 ± 71.6 pmol/1 x 10^6 cells, respectively (n=5 for each BCKA). The values were similar with neutrophils [12] and about 10 times higher than those of leukemia cells from the spleens of mice in the blast crisis phase [9]. Additionally, the concentration ratio of the three BCKAs varied between different cell types: in K562 cells, the concentrations of KIC and KMV were higher than that of KIV and vice versa in neutrophils. This is probably due to the different amino acid concentrations in different cell types.

3.2. Method validation

Under optimal experimental conditions, validation data was collected as shown in Table 1. The LODs for the BCKAs were 18-40 nM. These values were similar to those in the previous study [12] and better than other method using different fluorescence reagents [13]. The LOQs for the BCKAs were 60-134 nM. The method was linear over the concentration range of 0.5-50 μM per injection, with the correlation coefficients equal to or better than 0.99988, indicating excellent linearity between the peak height ratio of each keto acid to the internal standard and the sample concentration. The intra- and interday precision values were less than 3.1% and 5.6%, respectively. To test the accuracy, known concentrations of BCKAs were added to the cell samples, then measured as described; the obtained accuracy values were 84.4-95.6%. Accordingly, the method reported here is considered suitable for the precise determination of.

Table 1. Validation data of the developed method for BCKAs

|               | LOD (nM) | LOQ (nM) | Linearity (μM) | Intraday precision (%) | Interday precision (%) | Accuracy (%) |
|---------------|----------|----------|----------------|------------------------|------------------------|--------------|
| KIV           | 40.1     | 134      | 0.5-50         | 2.1                    | 3.4                    | 84.4         |
| KIC           | 18.0     | 60.0     | 0.5-50         | 2.0                    | 3.9                    | 95.6         |
| KMV           | 31.6     | 105      | 0.5-50         | 3.1                    | 5.6                    | 91.4         |

Fig. 2. Representative chromatograms of (a) standards and (b) K562 cell samples. Peaks: (1) α-ketovaleric acid (KV, internal standard), (2) α-keto-isovaleric acid (KIV), (3) α-keto-isocaproic acid (KIC), (4) α-keto-β-methylvaleric acid (KMV).
BCKA levels in cell samples.

3.3. Effect of gabapentin on intracellular BCKA levels

With this method, we investigated the effect of gabapentin on the intracellular BCKA levels. Gabapentin is a competitive inhibitor of the BCAT enzyme. We and others have studied the effect of gabapentin on intracellular BCAA levels, and found that 20 mM gabapentin significantly lowered the BCAA levels [9,19]; hence, 20 mM was chosen as the experimental concentration of gabapentin. In this study, the intracellular BCKA concentrations were measured after the collection of cells treated with 20 mM gabapentin or PBS as a control. As shown in Fig. 3, the BCKA levels remained unchanged or at least did not decrease after gabapentin treatment. When the breakdown of BCAAs is the predominant in BCAT reaction, BCKA levels should be decreased when the enzyme is inhibited. Since gabapentin decreases the intracellular concentration of BCAAs, these results provide further evidence that BCAT mediates BCAA production in leukemia cells [9].

4. Conclusion

In this study, we developed a robust and sensitive analytical method for intracellular BCKA quantitation through the derivatization of BCKAs with OPD. This method is effective and reliable, as it shows good linearity and high precision. The sensitivity is sufficient for the analysis of intracellular BCKA concentrations in 1 x 10^6 K562 cells. Although there are several studies that used OPD for fluorescence labeling of keto acids, this is the first report in which BCKAs were quantified in K562 cells.

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