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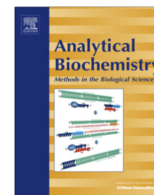

Quantification of branched-chain keto acids in tissue by ultra fast liquid chromatography–mass spectrometry

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ABSTRACT

Branched-chain keto acids (BCKAs) are associated with increased susceptibility to several degenerative diseases. However, BCKA concentrations in tissues or the amounts of tissue available are frequently at the limit of detection for standard plasma methods. To accurately and quickly determine tissue BCKAs, we have developed a sensitive ultra fast liquid chromatography–mass spectrometry (UFLC–MS) method. BCKAs from deproteinized tissue extractions were *o*-phenylenediamine (OPD) derivatized, ethyl acetate extracted, lyophilized in a vacuum centrifuge, and reconstituted in 200 mM ammonium acetate. Samples were injected onto a Shimadzu UFLC system coupled to an AB-Sciex 5600 Triple TOF mass spectrometer instrument that detected masses of the OPD BCKA products using a multiple reaction monitoring method. An OPD-derivatized ^{13}C-labeled keto acid was used as an internal standard. Application of the method for C57BL/6J (wild-type) and PP2Cm knockout mouse tissues, including kidney, adipose tissue, liver, gastrocnemius, and hypothalamus, is shown. The lowest tissue concentration measured by this method was 20 nM, with the standard curve covering a wide range (7.8–32,000 nM). Liquid chromatography–mass spectrometry run times for this assay were less than 5 min, facilitating high throughput, and the OPD derivatives were found to be stable over several days.

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Branched-chain keto acids (BCKAs)[1] are derived from the first step in the metabolism of branched-chain amino acids (BCAAs: leucine, isoleucine, and valine), a reversible transamination catalyzed by mitochondrial (BCAT2 or BCATm) and cytosolic (BCAT1 or BCATc) forms of branched-chain amino acid transaminase. BCAAs are essential amino acids needed to survive, but they also act as nutrient signals that regulate a number of processes either directly or during the process of metabolism. These actions include, but are not limited to, direct effects on glucose transport, insulin secretion in islet beta cells, satiety, and regulation of hepatic glucose output in the hypothalamus along with effects mediated by the mammalian target of rapamycin (mTOR) signaling pathway found in all mammalian tissues [1–9]. BCAAs and their keto acids are elevated in patients with insulin resistance, catabolic diseases, and obesity [10–12] as well as maple syrup urine disease (MSUD), an inborn error of metabolism. In MSUD, the branched-chain keto acid dehydrogenase (BCKDH) complex responsible for BCKA oxidation is mutated and defective [13,14].

Accumulation of BCKAs in MSUD has been linked to apoptosis and neuronal dysfunction [15,16]. Islet beta cells share many similarities with hypothalamic neurons. It is noteworthy, therefore, that recent studies suggest that plasma BCAA elevations in obesity may prognosticate the conversion to diabetes [17,18], which is associated with apoptosis of islet beta cells. The BCKDH phosphatase (*PPm1K,* PP2Cm), which elevates BCKAs when mutated [19,20], is a primary candidate gene for type II diabetes in human islets [21]. Furthermore, the E1-α subunit of BCKDH (*BCKDHA*) that PP2Cm dephosphorylates is a primary candidate gene for both obesity and diabetes [22]. Normal catabolism of the BCKAs also appears to be important for maintaining normal cardiac function and has been linked to cardiovascular disease [23–25]. Clearly, understanding the tissue-specific basis for the relationship of BCKAs with disease is important. Because BCKAs are formed from reversible intracellular transamination, it has been argued that BCKA concentrations provide a better reflection of the intracellular BCAA pools [26]. Changes in BCKA concentrations in tissues may also suggest altered oxidative capacity. Thus, given the emerging importance

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[1] *Abbreviations used:* BCKA, branched-chain keto acid; BCAA, branched-chain amino acid; MSUD, maple syrup urine disease; BCKDH, branched-chain keto acid dehydrogenase; HPLC, high-performance liquid chromatography; OPD, *o*-phenylenediamine; UFLC–MS, ultra fast liquid chromatography–mass spectrometry; WT, wild-type; KO, knockout; KIV, sodium α-keto isovalerate; KIC, sodium α-keto isocaproate; KMV, sodium α-keto β-methylvalerate; IS, internal standard; RT, room temperature; LC–MS, liquid chromatography–mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; XIC, extracted ion chromatogram.

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of tissue-specific BCKA metabolism in health and disease, it is important to have assays that can robustly measure their concentrations in tissues.

Previous methods have been developed to measure BCKAs in biological fluids, including reversed-phase high-performance liquid chromatography (HPLC) with fluorescence detection [27–33], gas chromatography–mass spectrometry [34], and gas–liquid chromatography [35,36]. These methods either are time-consuming or lack sensitivity for tissue as opposed to plasma BCKA concentrations. In addition, prior studies have not explored how to measure BCKAs from small tissue sizes, as would be available from mice or needle biopsies. For example, a previous HPLC method [35] modified by Hutson and Harper [37] was unable to detect certain BCKAs in some tissues/nutritional states due to low abundance [37].

Here we describe a rapid and sensitive assay for measuring BCKAs in tissue. It relies on the familiar derivatization of keto acids with *o*-phenylenediamine (OPD), resulting in stable cyclical 3-alkyl-2-quinoxalinol products with unique masses that could be detected by ultra fast liquid chromatography–mass spectrometry (UFLC–MS). It is quite sensitive, specifically measuring picomole quantities of BCKA per gram tissue, and has an instrument run time of less than 5 min—up to 8 times faster than the traditional HPLC method. We also show that it is widely linear, with reproducibility over a broad range of concentrations. We used this method to measure BCKAs in five tissues from C57BL/6J wild-type (WT) mice and PP2Cm knockout (KO) mice; these mice lack the BCKDH phosphatase and are a model of mild/intermittent MSUD [19]. Finally, we examined the long-term stability of the OPD adducts, which has previously been unclear. Stabilities ranging from hours [38] to weeks [39] have been reported. This new method should be quite useful for determining the tissue-specific role of BCKAs in health and disease.

Materials and methods

Mice

All procedures were conducted after review and approval by the Penn State Hershey institutional animal care and use committee. The Animal Resource Program, operated by the Department of Comparative Medicine, is accredited by Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International.

All animal living conditions are consistent with standards laid forth in the Guide for the Care and Use of Laboratory Animals, 8th edition, published by the National Research Council. C57BL/6J mice were obtained from Jackson Laboratories (http://www.jax-mice.jax.org). The metabolic phenotype and genotyping strategy of the second mouse strain used, PP2Cm KO (a generous gift from Yibin Wang, University of California, Los Angeles), was described previously [19].

Mice were maintained in open-top, solid-bottom polycarbonate cages with wire bar lids and bedding formed from pieces of dried corncob. Lighting was controlled with a 12:12-h light/dark cycle with lights on at 0700 h and lights off at 1900 h. Water and food (Teklad 2018 Global 18% Protein Rodent Diet, Harlan, Frederick, MD, USA) were available ad libitum. Freely fed mice were anesthetized with isoflurane and euthanized via cervical dislocation in order to obtain tissues for the BCKA assay. To compare this method with previous plasma determination methods, blood was obtained from the cheek vein of WT C57BL/6J mice using Medipoint mouse lancets and pediatric green top (hematocrit) BD Microtainer blood collection tubes. Plasma was prepared according to the manufacturer's directions.

Reagents

The BCKA standards, sodium α-keto isovalerate (KIV, CAS no. 3715-29-5), sodium α-keto isocaproate (KIC, CAS no. 4502-00-5), and sodium α-keto ß-methylvalerate (KMV, CAS no. 3715-31-9, a.k.a. (±)-3-methyl-2-oxovaleric acid sodium salt) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). [U-^{13}C]KIV uniformly labeled with the stable isotope of carbon, ^{13}C, at all five carbons was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Ethyl acetate was purchased from VWR (Radnor, PA, USA). OPD was purchased from Sigma (St. Louis, MO, USA). The Waters Acquity UPLC BEH C18 column (2.1 × 50 mm) was purchased from Waters (Milford, MA, USA). Na_2SO_4 and 70% (11.6 M) perchloric acid solution were purchased from Fisher Scientific (Pittsburgh, PA, USA).

Solutions for standard curve

The underivatized BCKA standard stock solutions (KIV, KIC, and KMV) were prepared at a stock concentration of 2 mM in water. To a fresh tube, 16 μl of each keto acid stock was added. Water was then added to that tube in order to obtain a final concentration of 32 μM (32,000 nM) in a 1-ml final volume. The diluted keto acid stock solution was then diluted in a series of 1:1 dilutions in separate tubes in order to achieve a set of standards with concentrations ranging from 7.8 to 32,000 nM. The [^{13}C]KIV internal standard (IS) was dissolved at 10 mg/ml (69.9 mM) in water and then diluted in a series of 1:10 dilutions to achieve 1 μg/ml (6.99 μM). A 2.5-μl aliquot of this IS was added to each sample and standard, giving a final amount of 2.5 ng/tube (87 nM). All keto acid stock solutions were aliquoted and stored at −80 °C until they were used once and not refrozen.

Procedure for processing tissues

Adipose tissue, gastrocnemius, liver, kidney, and hypothalamus tissues were harvested from each euthanized animal, freeze-clamped at the temperature of liquid nitrogen, wrapped in aluminum foil, and stored at −80 °C until used. The tissues were retrieved from −80 °C, maintained in liquid nitrogen on the bench, and then crushed one at a time using a metal mortar and pestle (cooled to the temperature of liquid nitrogen). The resulting powdered tissue was transferred to a tared microfuge tube (precooled in liquid nitrogen). The weight of the powdered tissue was recorded and was extracted with 3 M perchloric acid using the ratio of 300 μl of perchloric acid per 100 mg of tissue. The amount of tissue ranged from 70 to 400 mg. The tissue and acid mixture was sonicated twice for 10 s, cooled on ice, and then centrifuged at 25,000*g* for 15 min at 4 °C. After centrifugation, the supernatant was removed, aliquoted, and stored as tissue extractions at −80 °C until assay.

As part of a test of method accuracy, we analyzed some mouse plasma samples in order to compare our results with previous historic values obtained for human plasma [32] and rat plasma [37]. For this assay, 10 μl of plasma was precipitated with perchloric acid and centrifuged. Next, 25 μl of that supernatant was added to a new tube with 0.5 ml of 12.5 mM OPD. The following steps matched those for the tissue samples above.

BCKA derivatization and extraction

The above tissue extractions were thawed, and 50 μl of each was added to an Eppendorf tube with 2.5 ng of IS ([^{13}C]KIV—2.5 μl of 1000 ng/ml). A serial dilution of the unlabeled BCKAs that spanned from 7.8 nM to 32 μM generated the standard curve. Each standard had 2.5 ng of IS added. To each tube containing standard

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