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Determination of β -hydroxy- β -methylbutyrate concentration and enrichment in human plasma using chemical ionization gas chromatography tandem mass spectrometry

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Abstract

Our objective was to develop a quick and simplified method for the determination of β -Hydroxy- β -methylbutyrate (HMB) and α -ketoisocaproic acid (KIC) concentrations and enrichments by GC/MS/MS to determine the turnover rate of HMB in humans.

In experiment 1, we provided a pulse of L-[5,5,5- $^2\text{H}_3$]leucine to younger adults in the postabsorptive state then collected blood samples over a 4 h time period. In experiment 2, we provided a pulse of [3,4,methyl- $^{13}\text{C}_3$]HMB to older adults in the postabsorptive state then collected blood samples over a 3 h time period. Plasma concentrations of KIC and HMB and MPE of KIC and HMB were determined by GC/MS/MS. Plasma enrichment of leucine was determined by LC/MS/MS.

To determine plasma enrichment of [5,5,5- $^2\text{H}_3$]HMB and [3,4,methyl- $^{13}\text{C}_3$]HMB, samples were derivatized using pentafluorobenzyl bromide and analyzed using chemical ionization mode. The final methods used included multiple reaction monitoring of transitions 117.3 > 59.3 for M + 0 and 120.3 > 59.3 for M + 3. In experiment 1, peak MPE of Leu peaked at 9.76% generating a peak MPE of KIC at 2.67% and a peak HMB MPE of 0.3%. In experiment 2, the rate of appearance for HMB was 0.66 $\mu\text{mol/kg}$ ffm/h. We calculated that production of HMB in humans accounts for 0.66% of total leucine turnover.

Keywords

HMB; Chemical ionization; Gas chromatography; Tandem mass spectrometry; Turnover

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jchromb.2016.11.010>.

1. Introduction

β -Hydroxy- β -methylbutyrate (HMB), also referred to as β -hydroxyisovaleric acid, is a product of α -ketoisocaproic acid (KIC) oxidation, which is produced primarily in muscle, liver, and adipose tissue from the amino acid leucine [1,2]. Initially, leucine is deaminated to α -ketoisocaproic acid (KIC) by branched chain aminotransferase and subsequent HMB production occurs *via* the cytosolic enzyme KIC dioxygenase [3,4]. Additionally, KIC is converted to isovaleryl-CoA and, through a number of reactions, is converted to HMB [3,4]. In conditions such as type II diabetes [5], biotin deficiency [6], isovaleric acidemia [7], β -methylcrotonyl-CoA carboxylase deficiency [8], and ketoacidosis [9], HMB accumulates in the urine due to altered leucine metabolism and as such is used as a marker for these conditions.

HMB supplementation, on the other hand maintains muscle mass in situations conducive to muscle loss. We found that HMB supplementation during bed rest reduced muscle loss [10] or when added to a nutritional supplement could reduce mortality after hospital admission in older adults [11]. Others have found increases in muscle mass under various conditions [12–17]. HMB supplementation in general results in reduced muscle loss and increased muscle gain [18], enhanced anabolic signaling [19], and improved muscle strength [20].

The use of isotopes in human studies has provided a unique tool that enables investigations into metabolic research [21]. The isotope, being identical to the natural compound of interest in every aspect but molecular weight, is traced throughout the body and thus metabolic information is obtained that can be applied to the naturally occurring compound. Stable isotopes are preferred, albeit more expensive, over radioactive isotopes due to the radiation exposure associated with radioactive isotopes. We [22,23] and others [24,25] use stable isotopes in protein/amino acid metabolisms studies, while others have used them to investigate carbohydrate/glucose [26–28], and fat [29–31] metabolism. Although isotopes (some radioactive) have been used to study HMB metabolism in animals [32,33], no studies have used HMB isotopes to study HMB metabolism in humans.

The gold standard for HMB analysis is gas chromatography with mass spectrometry (GC–MS) using trimethylsilyl (TMS) derivatives [33–37]. Although this derivatization method has been used extensively, it does require large amounts of sample and laborious preparations prior to injections onto a GC. Using a LC tandem mass spectrometry (LC/MS/MS) system [38], improved upon the latter method in that their method utilized less sample and reduced the preparation time. Our objective was to develop a quick and simplified method for sample derivatization and analysis of HMB and KIC enrichments by GC/MS/MS, to capitalize on its improved selectivity over GC/MS. In combination with an existing LC/MS/MS method for measuring leucine enrichment, this method was then applied to a stable isotope human clinical study to determine leucine, KIC, and HMB MPE in plasma samples.

2. Materials and methods

2.1. Materials

All solvents were GC/MS and/or HPLC grade, purchased from Sigma-Aldrich. Pentafluorobenzyl bromide was purchased from Sigma-Aldrich. L-[5,5,5-²H₃]Leucine and [3,4,methyl-¹³C₃]HMB were purchased from Cambridge Isotopes.

2.2. Sample preparation

After sampling, blood was immediately transferred into lithium-heparin or EDTA containing blood tubes and centrifuged at $8,000 \times g$ for 5 min to yield plasma, an aliquot of which was transferred to a 1.5 mL eppendorf tube. Plasma can be used for immediate analysis or stored at -80°C for later analysis. For sample preparation for GC/MS/MS analysis, 100 μL of either plasma, a standard, or a blank was reacted with 200 μL of 100 μM pentafluorobenzyl bromide (diluted in acetone) for 1 h at 65°C as previously described [39] with slight modifications (described below). One mL of hexane was added prior to centrifugation at $300 \times g$ for 1 min. After gentle mixing, the upper phase was transferred to a threaded 2 mL borosilicate GC vial. The GC vial was closed with a septum cap of a type previously determined to form a gas-tight seal.

2.3. Gas chromatography conditions

Analyses were carried out on a Bruker GC/MS/MS system consisting of a Scion TQ triple quadrupole mass spectrometer, a 436-GC gas chromatograph with programmable temperature vaporization (PTV) inlet, and a CP-8400 autosampler. The GC was equipped with a $30 \text{ m} \times 0.25 \text{ mm}$, $0.20 \mu\text{m}$ SP-2330 column with a Poly (80% biscyanopropyl/20% cyanopropylphenyl siloxane) stationary phase (Supelco, Inc. Bellefonte, PA). Helium was used as the carrier gas at a rate of 1.8 mL min^{-1} . One μL was autosampled and injected into the PTV inlet fitted with a Sky $3.4 \text{ mm} \times 5.0 \text{ mm} \times 54 \text{ mm}$ split inlet liner with glass frit (Restek #23462.5). The inlet was set initially to 250°C for 2 min, ramped to 300°C at 50°C/min and held for 12.25 min, then at 15.25 min ramped down to 250°C at 200°C/min . Splitless mode was held for 2 min following injection, then set to 1:100 for 15 s, 1:10 at 2.15 min, 1:100 at 13 min, and 1:10 at 13.15 min. The oven temperature program consisted of an initial temperature of 100°C and held for 1 min, ramped to 194°C at 10°C/min , ramped to 220°C at 15°C/min , held at 220°C for 2.87 min, and ramped down to 100°C at 120°C/min .

2.4. Tandem mass spectrometry conditions and method development

To conduct the following methods, an electron capture negative ion chemical ionization method using methane as the ionization gas was used. The transfer line temperature was set at 250°C and the chemical ionization (CI) source of the Scion TQ at 200°C . The source voltage was -120 eV . The filament current was set to $45 \mu\text{A}$. To develop the method, a solution in hexane of the PFB derivative of HMB (formed as described below) was injected onto the GC with the spectrometer operated in full scan mode. A peak representing HMB was identified as m/z 117.3. A subsequent injection was made and the signal was acquired in product ion scan mode to identify potential fragments. The product ion scan was acquired

using a collision energy (CE) of 10 eV and a collision-induced dissociation (CID) argon gas pressure of 1.0 mTorr. A fragment was identified having a mass of 59.3. To further optimize conditions favoring the m/z 117.3 to 59.3 transition, injections were made and data were acquired in multiple reaction monitoring (MRM) mode while stepping the CID gas pressure (0.1, 0.4, 0.7, 1.0, 1.3, 1.6, and 1.9 mTorr) and the CID energy (0 to 50 eV). A collision energy of 14 eV was found to be optimal for HMB analysis. Details for chromatography and mass spectrometer for KIC and Leucine can be found in the supplemental material.

2.5. HMB concentration

Plasma samples collected prior to isotope administration at baseline were used to determine postabsorptive HMB concentrations. Prior to processing of plasma and standards, [3,4,methyl- $^{13}\text{C}_3$]HMB was added as an internal standard (IS) and samples were analyzed as described above. Concentrations were determined by using the M + 0 HMB standard to M + 3 HMB IS peak area ratio linear calibration curve constructed by serial dilution.

2.6. Human clinical experiment

All participants gave informed written consent before participation, which was approved by the Institutional Review Board of Texas A&M University. For experiment 1, younger adults ($n = 10$) 20–26 years of age were used in this study and are a subset of a larger study (Clinical Trials #NCT01787682). A background blood sample was collected prior to IV administration of a 9 mL pulse containing 9.66 mM L-[5,5,5- $^2\text{H}_3$]Leucine, a precursor for KIC and HMB production. As part of the larger study, subjects received other stable isotope *via* IV infusions. Blood samples were subsequently collected at 2, 5, 10, 15, 20, 30, 40, 50, 60, 90, 120, 150, 180, 210, and 240 min. Subjects started the study fasted and laid supine for the duration of the study. The enrichment and concentration of KIC and HMB was measured by GC/MS/MS method as described above and the enrichment of leucine was measured by LC/MS/MS as described above. The rate of appearance of Leucine was calculated as previously described [40]. The concentration of leucine during the pulse did not change and is presented in Fig. S4.

For experiment 2, older adults ($n = 4$) 39–71 years of age were used in this study and are a subset of a larger study (Clinical Trials #NCT02157844). A background blood sample was collected prior to IV administration of a 8.5 mL pulse containing 0.94 mM [3,4,methyl- $^{13}\text{C}_3$]HMB. As part of the larger study, subjects received other stable isotope *via* IV infusions. Blood samples were subsequently collected at 2, 5, 10, 15, 20, 30, 40, 50, 60, 90, 120, 150, and 180 min. Subjects started the study fasted and laid supine for the duration of the study. The enrichment of [3,4,methyl- $^{13}\text{C}_3$]HMB was measured by GC/MS/MS method as described above. The rate of appearance of HMB was calculated as previously described [40]. The concentration of HMB during the pulse did not change and is presented in Fig. S5.

3. Results and discussion

Our objective was to develop and implement a quick and simplified method for sample derivatization for analysis of HMB concentration and enrichment while maintaining

improved sensitivity and reduced signal-to-noise by using gas chromatography coupled with chemical ionization tandem mass spectrometry. Due to the increased research interest in using HMB as a therapy to reduce muscle mass loss in situation conducive to muscle wasting and the dysregulation of HMB production and excretion during various metabolic derangements (e.g., ketoacidosis, isovaleric acidemia, etc.), creating a simplified yet sensitive method utilizing a GC/MS/MS is warranted. Following development of this method, we set out to determine the enrichment of HMB in plasma samples collected from healthy individuals that were given an intravenous one time pulse dose of L-[5,5,5-²H₃]Leucine over a 4 h time period. Furthermore, enrichment of HMB was determined in blood samples collected from older adults following a bolus injection of [3,4,methyl-¹³C₃]HMB over a 3 h period.

3.1. Sample preparation and HMB derivatization

Samples were processed and derivatized as previously described with slight modifications [39]. In this protocol, the ratio of sample to pentafluorobenzyl bromide (PFBBBr) is 1:2 and uses 200 µL of sample. In our study and for determining isotopic enrichment, we reduced the sample volume to 100 µL, but maintained the 1:2 ratio by using 200 µL of PFBBBr instead. With conventional methods using GC/MS, the derivatizing agent of choice is TMS derivatives. However, we determined that using PFBBBr instead would provide an advantage over TMS derivatives. Furthermore, the PFB derivative is commonly used for fatty acid [41] and prostaglandin [42] analysis using GC/MS chemical ionization analysis. Using PFBBBr would allow the use of chemical ionization that provides a softer ionization as opposed to electron impact ionization, which can lead to excessive fragmentation in the source. Furthermore, during chemical ionization, the PFB derivative is lost following ionization in the source leaving a lower molecular weight carboxylate anion, which reduces the number of carbons entering the mass spectrometer and results in a lower background isotopic enrichment. The remaining keto and hydroxyl groups on HMB do not require further derivatization and due to the preponderance of carboxylate anions produced in the source, sensitivity and detectability are greatly improved [43].

3.2. HMB method development

Repeated injections of plasma samples and standards onto the GC maintained signal as the coefficient of variations was 0.27 and 1.72%, respectively. Potential carryover contamination between injections was eliminated by ramping the column temperature post-run to 220 °C and held for 2.87 min and ramping the injection port temperature to 300 °C and held for 12.75 while intermittently ramping split mode to 100:1 for 15 s. This was confirmed by injecting the method solvent, hexane, which resulted in the absences of detectable peaks over background noise (data not shown).

Injection of standard/plasma samples onto the GC yields one chromatographic peak on full-scan single-quadrupole mode (Fig. 1) that eluted at 9.00 min. Mass chromatograms showed a single peak substantially free of baseline noise. When the spectra was acquired, a peak (117 *m/z*) was identified representing HMB (Fig. 2a). After optimization of MS voltage and gas settings to maximize *m/z* 117 ion transmittance, using product ion scan mode, that ion was subjected to fragmentation in Q2 of the MS/MS. Fragments generated from

fragmentation in Q2 were 59.3 (Fig. 2b). An illustration of that fragmentation is shown in Fig. 3. Following identification of the fragment, subsequent analysis was conducted using a precursor ion scan with Q3 set to m/z 59.3 and Q1 scanning for the precursor mass, which was confirmed as m/z 117 (Fig. 2c). Subsequently, a MRM method was generated by Q1 scanning for m/z 117.3 and Q3 scanning for fragment m/z 59.3 as $M + 0$. Fragmentation of the 59.3 ion in Q2 was optimized by conducting MRM experiments while varying the collision energy (CE) and collision-induced dissociation (CID) gas pressure. Collision energy of 14 eV and a CID gas pressure of 1.0 mTorr were determined to be optimal in generating the m/z 59.3 fragment. For sample HMB $M + 3$ enrichment analysis, an additional channel was included such that Q1 scanned for m/z 120.3 and Q3 scanned for fragment m/z 59.3. Due to the location of the deuterium labels in HMB following conversion from [5,5,5- $^2\text{H}_3$]leucine in experiment 1 and the location of the labels in HMB following the bolus injection of [3,4,methyl- $^{13}\text{C}_3$]HMB in experiment 2, the aforementioned mass spectrometry conditions to detect each tracee/tracer was the same for both experiment 1 and experiment 2.

Identification of the HMB peak is shown in (Fig. 1). Limits of detection for HMB concentration was 75 nM and for MPE of [3,4,methyl- $^{13}\text{C}_3$]HMB was 0.0335. Limits of quantitation for HMB was 150 nM and for MPE of [3,4,methyl- $^{13}\text{C}_3$]HMB was 0.07. Variability at the limit of quantitation for concentration of KIC and HMB were 5.62 and 8.7%, and for MPE of [3,4,methyl- $^{13}\text{C}_3$]HMB was 4.73%. Intra-day variability for HMB concentration in plasma was 5.08% and for MPE of [3,4,methyl- $^{13}\text{C}_3$]HMB in plasma was 4.85%. Inter-day variability across a 30 day period for HMB concentrations in plasma was 7.68% and for MPE of [3,4,methyl- $^{13}\text{C}_3$]HMB in plasma was 6.53%, respectively. Data for KIC and Leucine are shown in the supplemental material.

3.3. Human clinical study

Following validation of the analytical methods, plasma samples from a human clinical study (experiment 1) were collected over a 4 h time period from subjects in the postabsorptive state that were given an intravenous one time pulse dose of L-[5,5,5- $^2\text{H}_3$]leucine and analyzed for [5,5,5- $^2\text{H}_3$]leucine, [5,5,5- $^2\text{H}_3$]KIC, and [5,5,5- $^2\text{H}_3$]HMB MPE. The time course of plasma MPE of [5,5,5- $^2\text{H}_3$]leucine, [5,5,5- $^2\text{H}_3$]KIC, and [5,5,5- $^2\text{H}_3$]HMB are shown in Fig. 4. Plasma MPE of [5,5,5- $^2\text{H}_3$]leucine is highest at the first sample point at 2 min (9.76), while for [5,5,5- $^2\text{H}_3$]KIC, it is at 5 min (2.67). [5,5,5- $^2\text{H}_3$]HMB highest MPE is 0.3 at 40 min following the bolus injection. By 4 h, the MPE of [5,5,5- $^2\text{H}_3$]leucine was 0.054, of [5,5,5- $^2\text{H}_3$]KIC was 0.163, and of [5,5,5- $^2\text{H}_3$]HMB was 0.14. Clearly, both the highest and lowest MPE were measured with sufficient precision. Provided that KIC and HMB stable isotopes were not administered in experiment 1, the conversion of isotopically labeled leucine to KIC and KIC to HMB is evident. Leucine is initially transaminated in a reaction with α -ketoglutarate to produce KIC and glutamate[44–46]. KIC can be further oxidized to its branched chain acyl-CoA or converted to HMB[47]. The average concentration of HMB at baseline was 3.04 μM and was determined using a linear calibration curve (slope = 3.015, intercept = 0.8105).

A second experiment was performed to determine 1) the ability to detect and use [3,4,methyl- $^{13}\text{C}_3$]HMB in a bolus injection in human clinical studies and 2) determine the turnover rate of HMB in older adults. Following the bolus injection, plasma samples were collected over a 3 h time period from subjects in the postabsorptive state. Linear calibration curve for [3,4,methyl- $^{13}\text{C}_3$]HMB MPE, in the 0 to 35 range, were acquired with a slope of 1.006 (Fig. 5). The observed MPE vs. the expected MPE was linear ($R^2 = 1.00$). Plasma MPE of [3,4,methyl- $^{13}\text{C}_3$]HMB (Fig. 6) was the highest at 5 min (61.2) following the bolus injection, while at 3 h, the MPE dropped to 7.1. The turnover rate was calculated from the [3,4,methyl- $^{13}\text{C}_3$]HMB MPE curve following the bolus injections as previously described [40] and was calculated to be $0.66 \mu\text{mol/kg ffm/h}$. Previously, we [48] reported the turnover rate of leucine in healthy older adults to be approximately $100 \mu\text{mol/kg ffm/h}$ in healthy older adults. Therefore, this would suggest that HMB production in older adults accounts for 0.66% of leucine turnover. This is in contrast to HMB production representing 2–10% of leucine turnover reported in sheep and pigs[33], but more studies are needed to support this notion.

4. Conclusions

In conclusion, we describe a method to determine high to very low enrichments of HMB in plasma by using GC/MS/MS. Considering this is the only report, that we are aware of, that has used GC/MS/MS, our developed analytical method is less laborious and time consuming, and very sensitive over a wide range of isotope enrichments. This approach was successfully applied to human clinical studies where a single pulse injection of stable isotopically labeled leucine in experiment 1 and of stable isotopically labeled HMB in experiment 2 was administered to young and older adults, respectively. This permitted the determination of plasma concentrations and turnover rate of HMB.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Fig. 1. Mass chromatogram acquired in MRM mode in plasma, a neat HMB standard (at a 2x plasma concentration), and plasma spiked with a HMB standard. A single peak is observed at 9.0 min post-injection. Both standard and plasma samples were normalized to an internal standard.

Fig. 2.

Spectrum generated from (A) full scan, (B) product ion scan, and (C) precursor ion scan of plasma and of a neat HMB standard. Using product scan mode, the precursor m/z 117.2 ion is fragmented in Q2, generating a m/z 59.2 product ion. Doing so will result in lower natural background in a $M + 1$ isotope channel ($118.2 > 60.2$) due to the presence of fewer carbon atoms in the product. Using precursor scan mode, specificity of the precursor m/z 117.2 is verified to generate the 59.2 fragment in Q2.

Fig. 3.

Proposed collisional breakage of m/z 117 HMB to yield a m/z 59 product ion and neutral radical with an unknown structure. A neutral loss method is used when detecting the isotope labels from [5,5,5- $^2H_3$]HMB and [3,4,methyl- $^{13}C_3$]HMB as the labels are lost in the neutral radical during fragmentation.

Fig. 4. MPE curve spanning a 4 h time period of [5,5,5- $^2\text{H}_3$]KIC and [5,5,5- $^2\text{H}_3$]HMB generated from L-[5,5,5- $^2\text{H}_3$]Leucine given in an IV pulse to healthy older human subjects.

Fig. 5. Standard curve of $^{13}\text{C}_3$ -enrichment of HMB. The inset shows the plot from 0.00035 to 0.007 MPE.

Fig. 6.
MPE curve of [3,4,methyl- $^{13}\text{C}_3$]HMB over a 3 h time period generated from a bolus injection of [3,4,methyl- $^{13}\text{C}_3$]HMB given to young adult subjects.