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Quantification of β-hydroxymethylbutyrate and leucine by ultrahigh performance liquid chromatography tandem mass spectrometry at different situations and stages of a rodent life

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ABSTRACT

The main objective of this work was to develop a method to measure Leucine (Leu) and β-hydroxymethylbutyrate (HMB) at basal levels in serum, urine, milk and brain microdialysates in rats. Ultrahigh performance liquid chromatography–electrospray-tandem mass spectrometry (UHPLC-ESI-MS/MS) was used as analytical technique. The sample treatment was simple and consisted of dilution with methanol and centrifugation for serum and urine, dilution with water and filtration with an Amicon filter for milk, and treatment with formic acid with no further dilution for microdialyzates. The procedures for sampling and the UHPLC-MS/MS parameters were accurately optimized to achieve the highest recoveries and to enhance the analytical characteristics of the method. For chromatographic separation, an Acquity UPLC BEH Amide column using acetonitrile-water gradient with formic acid as additive was used. The total run time was 4 min. The analytical characteristics (accuracy, selectivity and sensitivity) of the proposed method were evaluated. The limits of detection (LODs) obtained ranged from 0.4 to 7 ng mL^{-1} and the limits of quantification (LOQs) from 1 to 22 ng mL^{-1}. Precision, expressed as relative standard deviation (% RSD), was lower than 15% in all cases, and the determination coefficient (R^2) was equal or higher than 99.0% with a residual deviation for each calibration point lower than ±25%. Mean recoveries were between 85 and 115%. The method was successfully applied to these matrices being able to detect significant differences between physiological situations, strains and stages of life.

1. Introduction

β-hydroxy-β-methylbutyrate (HMB) is a metabolite of the amino acid leucine (Leu). The first step in HMB metabolism is the reversible transamination of Leu to α-ketoisocaproate (α-KIC) that occurs mainly extrahepatically. Following this enzymatic reaction, the majority of α-KIC is irreversibly oxidized to isovaleryl-CoA via the enzyme branched chain keto acid dehydrogenase (BCKAD). In the minority alternative pathway (approximately 5% of metabolized Leu), HMB is produced from α-KIC by the cytosolic enzyme KIC dioxygenase [1,2] (See Fig. 1). HMB functions are well known. It is an anti-catabolic agent associated with protein synthesis and attenuation of protein degradation [3,4], which makes it suitable for increasing strength and muscle mass in sport nutrition [5–9], as well as for clinical situations of increased protein degradation (cachexia), decreased rate of muscle protein synthesis (inactivity), or alteration of both (sarcopenia) [10–12]. In addition, it is a substrate for the synthesis of cholesterol needed to form and stabilize sarcolemmas in muscle [5], it has immune modulator properties [13,14], it downregulates apoptosis during immobilization and recovery [15] and also it produces improvement in oxidative metabolism [16].

Due to the catabolism of Leu people have a daily endogenous synthesis of HMB between 0.3 and 0.4 g, but plasma HMB levels can be increased by five- to ten-fold after feeding Leu (60 g) or HMB (3 g). Kinetic studies in animals have reported that the half-life of HMB in plasma is about 2 h and that about 34% of HMB is excreted in urine. In humans, the timecourse kinetics of HMB in plasma and urine showed that the half-life of HMB is also about 2 h while only 14–29% of the HMB is excreted in urine, depending on the dose. Those data suggest that 70–85% of the ingested oral HMB is retained in the body for further metabolism [1,2,17]. There may be a number

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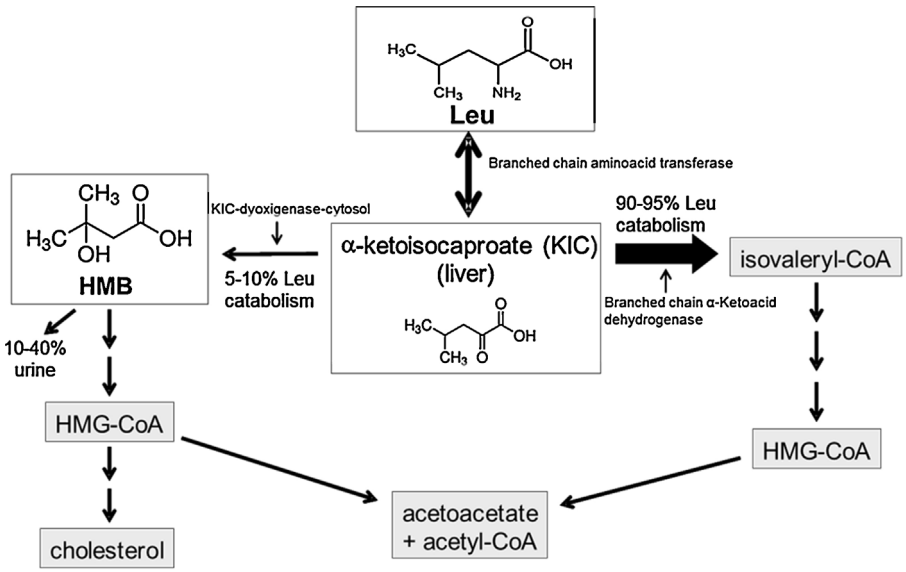

Metabolic route of Leu with chemical structures

Fig. 1. Chemical structures of HMB and Leu and the metabolic route.

of factors such as gender, race, age or pathologies that could affect the basal levels and excretion of HMB and/or Leu. It is important to explore these levels of HMB and Leu in different biological fluids to have knowledge about how they vary naturally throughout the life of an animal under different conditions. This information could be relevant for the use of these compounds as dietary supplements or nutritional products with specific health outcomes.

Accurate and sensitive analytical methods are necessary for the analysis of low levels of HMB and Leu present in specific biological fluids. Classically, several methods have been published for determining amino acids such as Leu [18], with techniques such as HPLC [19–24] or gas chromatography [25–27]. So far, these methods had some shortcomings such as low specificity for HMB or the sample treatment complexity. Since HMB contains an α-hydroxy in place of the α-amino group, the techniques involving derivatization steps are not recommended. Nissen et al. [28] published a method to determine HMB in plasma by gas chromatography coupled with mass spectrometry (GC–MS) but this method requires high sample volumes and laborious protocols including derivatization steps. Recently, two analytical methods have been published to improve these inconveniences. First, in 2013 Deshpande et al. published a study of the bioavailability of HMB in plasma by LC–MS [29] and second, our research group has recently published a method to measure HMB and Leu by UHPLC-ESI-MS/MS in relatively clean biological fluids, namely cell cultures and brain microdialysates [30].

The present work aims to extend the use of this method to other biological fluids such as serum, urine and milk. Brain microdialysates were also revisited to improve sensibility. Sample treatments were optimized for the fluids mentioned above and the method was validated according to the US Food and Drugs Administration (FDA) guideline for bioanalytical assays [31]. Subsequently, basal levels HMB in these sample matrices and on different biological conditions and stages of life were measured.

2. Materials and methods

2.1. Chemicals and reagents

All reagents were analytical grade unless specified otherwise. Water (18.2 MΩ cm) was purified and filtered by a specific LC–MS filter using a Milli-Q system from Millipore (Bedford, MA, USA). β-Hydroxymethylbutyrate (HMB) and Leu were supplied by Sigma-Aldrich (Madrid, Spain). LC-MS grade methanol (MeOH), acetonitrile (MeCN), ethanol (EtOH) and formic acid (FA) were purchased from Scharlab (Barcelona, Spain). Artificial cerebrospinal fluid (aCSF) was purchased from Harvard Apparatus (Holliston, Massachusetts, USA).

A stock solution of compounds was prepared by weighing 0.04 g HMB and 0.01 g Leu into a 10 mL flask and dilution with water. The solution remained stable for at least one month at 4 °C. Five work standard solutions for calibration purposes were prepared specifically depending on the studied matrix. For serum and urine, an intermediate solution (No. 1) was obtained by dilution of 50 μL of the stock solution to a final volume of 10 mL with MeOH. A second intermediate solution (No. 2) was prepared by diluting 200 μL of solution No. 1 to 1 mL with MeOH. Work standards for calibration purposes, named WS1, WS2, WS3 and WS4 were prepared by diluting 10, 50, 200 and 500 μL of the intermediate solution N° 2 to a final volume of 1 mL with MeOH in a maximum recovery vial. The standard WS5 was the solution No. 2. The same process was followed for other matrices, but in that case the solvent used for dilutions was different. Specifically, to measure HMB and Leu in milk the solvent was LC–MS water.

For microdialysates from rat brain a modification of the previously published article was done. The use of an improved chromatographic column by the manufacturer caused higher sensitivity than in the previous work due to narrower and higher peaks were obtained compared with those obtained in previous experiments by HILIC column. So with this improvement basal levels could be detected. Furthermore, as these levels were close to the limit of quantification, it was decided avoid dilution to improve the signal and work within the linear dynamic range of the method. For both calibration and samples processing, and in order to concentrate the basal level to the optimal value, no dilution of samples was made.

The calibration standards were injected at the beginning and end of each sample series. A quality control standard (WS3) was injected after every twenty injections. Calibration standards were freshly prepared from the original stock solution in each experiment.