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Subchronic toxicity study of β-hydroxy-β-methylbutyric free acid in Sprague–Dawley rats

John C. Fuller Jr. [a,*], Lawrence H. Arp [b], Lisa M. Diehl [c], Kelly L. Landin [c], Shawn M. Baier [a], John A. Rathmacher [a,d]

[a] Metabolic Technologies Inc., 2711 S. Loop Dr., Suite 4400, Ames, IA 50010, USA
[b] Charles River Laboratories, Pathology Associates-Maryland, 15 Worman's Mill Ct., Suite I, Frederick, MD 21701, USA
[c] Charles River Laboratories, Preclinical Services, 640 N. Elizabeth St., Spencerville, OH 45887, USA
[d] Department of Animal Science, Iowa State University, 313 Kildee Hall, Ames, IA 50010, USA

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ABSTRACT

Calcium β-hydroxy-β-methylbutyrate-monohydrate (CaHMB) is a dietary supplement used as an ergogenic aid and in functional and medical foods. A new delivery form has been developed, β-hydroxy-β-methylbutyric free acid (HMBFA), which has improved bioavailability. While the safety of CaHMB is well documented, there are few published studies demonstrating the safety of HMBFA. Because HMBFA results in greater serum levels of β-hydroxy-β-methylbutyrate (HMB) and greater clearance rates, a 91-day subchronic toxicity study was conducted in male and female Sprague–Dawley Crl:CD rats assigned to HMBFA treatments at either 0%, 0.8%, 1.6%, or 4% of the diet by weight. No deaths or untoward clinical observations, and no negative clinical chemistry or hematology were attributed to the administration of HMBFA. Gross pathology and histopathology results showed no tissue abnormalities due to HMBFA and all measures were within a normal physiological range for the animals or were expected in the population studied. In conclusion, the no-observed-adverse-event-level (NOAEL) for HMBFA was the highest level administered, 4% of the diet, which corresponded to an intake of 2.48 and 2.83 g/kg BW d^{-1} in the males and females, respectively. The equivalent human dosage using body surface area conversion would be 402 and 459 mg/kg BW d^{-1} for men and women, respectively.

1. Introduction

The dietary supplement calcium β-hydroxy-β-methylbutyrate-monohydrate (CaHMB), the calcium salt of HMB, a leucine metabolite, is widely used as an ergogenic aid to increase muscular strength and lean body mass gains with resistance training and improve recovery after exercise (Gallagher et al., 2000a; Jówko et al., 2001; Nissen et al., 1996; Nissen and Sharp, 2003; Panton et al., 2000; Wilson et al., 2008, 2013a). Recently a new delivery form, HMB free acid (HMBFA), has been developed which achieves greater (+185%) peak serum concentrations in about one fourth the time and has a 25% improved clearance by tissues when compared with CaHMB administration in humans (Fuller et al., 2011); further, urinary losses were not different between CaHMB and HMBFA administration. The International Society of Sports Nutritionists has concluded that chronic consumption of HMB is safe in both young and old populations (Wilson et al., 2013a); however, the current study is the first to report results of toxicology studies of this new delivery form, HMBFA.

The calcium form of HMB, CaHMB, has been extensively studied in humans and has been shown to improve strength and lean gains with exercise (Nissen and Sharp, 2003; Panton et al., 2000), increase aerobic metabolism (Lamboley et al., 2007; Vukovich and Dreifort, 2001), and decrease muscle damage and protein breakdown in response to strenuous exercise (Jówko et al., 2001; Nissen et al., 1996). Recent studies are similarly showing the HMBFA form of HMB is also effective in improving strength gains (Dunsmore

Abbreviations: A/G ratio, albumin to globulin ratio; ALT, alanine aminotransferase; ANOVA, analysis of variance; AST, aspartate aminotransferase; CaHMB, calcium β-hydroxy-β-methylbutyrate-monohydrate; FDA, Food and Drug Administration; GGT, gamma-glutamyltransferase; GLP, Good Laboratory Practice; HMB, β-hydroxy-β-methylbutyrate; HMBFA, β-hydroxy-β-methylbutyric free acid; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; mTOR, mammalian target of rapamycin; NOAEL, no-observed-adverse-effect-level; RDW, red cell distribution width.

* Corresponding author. Tel.: +1 515 296 8065; fax: +1 515 296 0908.
E-mail addresses: Fuller@mti-hmb.com (J.C. Fuller Jr.), Lawrence.Arp@crl.com (L.H. Arp), Lisa.Diehl@crl.com (L.M. Diehl), Kelly.Landin@crl.com (K.L. Landin), Baier@mti-hmb.com (S.M. Baier), Rathmacher@mti-hmb.com (J.A. Rathmacher).

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et al., 2012) and in decreasing muscle damage and improving recovery after resistance training (Wilson et al., 2013b).

Studies evaluating the mechanisms of action of HMB have demonstrated its ability to stimulate protein synthesis and attenuate protein degradation in muscle. HMB decreases muscle protein breakdown by down regulating the ubiquitin–proteasome proteases, particularly caspase-3 and -8 (Eley et al., 2008b; Smith et al., 2005). Its stimulatory effects on muscle protein synthesis are mediated through the mammalian target of rapamycin (mTOR) pathway (Eley et al., 2007, 2008a). Recent human observations have confirmed the anabolic effects of HMBFA and demonstrated its ability to enhance muscle protein synthesis by 70%, via the mTOR signaling pathway, and decrease muscle protein breakdown by 57% in an insulin-independent manner (Wilkinson et al., 2013). More detailed mechanistic information is available in review papers summarizing efficacy and mechanistic data on HMB (Fitschen et al., 2013; Wilson et al., 2008, 2013a; Zanchi et al., 2011).

The safety of CaHMB has been well established through clinical studies (Gallagher et al., 2000b; Nissen et al., 2000; Rathmacher et al., 2004) and through rodent toxicity data establishing a no-observed-adverse-effect-level (NOAEL) of 3.49 and 4.16 g/kg BW d^{-1} in male and female rats, respectively (Baxter et al., 2005). Additionally, Nissen and colleagues had examined the acute effects of CaHMB in young pigs (Nissen and Abumrad, 1997). Briefly, three approximately 20 kg pigs were fed 100 g of CaHMB per day for 4 days. Blood chemistry and hematology and organ pathology and histology were compared with that of 2 control fed pigs. There were no untoward effects of CaHMB found on any of the parameters measured. CaHMB has been safely consumed by humans in a variety of clinical conditions; by young and old, males and females, with and without exercise (Nissen et al., 2000); in AIDS (Clark et al., 2000), cancer (Eubanks May et al., 2002), and trauma patients (Kuhls et al., 2007). Data on the safety of HMBFA consumption are lacking, and relatively few human studies have been conducted thus far with HMBFA (Fuller et al., 2011; Wilkinson et al., 2013; Wilson et al., 2013b). In a recent study HMBFA was supplemented for 12-weeks in males undergoing a rigorous resistance-training protocol with and overreach cycle of training (Davis et al., 2012; Dunsmore et al., 2012). No untoward study-related events have been reported for over 18 months following the HMBFA supplementation.

Given that HMBFA has improved efficacy through quicker and greater tissue utilization, the establishment of the NOAEL level for HMBFA is an important factor in designing human clinical studies to further assess the efficacy of HMBFA on muscle protein metabolism. The results from the current 91-day subchronic toxicity study in rats establish the NOAEL level and document detailed toxicological data for HMBFA following Good Laboratory Practice (GLP, 21 CFR 58) and FDA Redbook protocol for 91-day oral toxicity studies (FDA Office of Food Additive Safety, 2003).

2. Materials and methods

The study was conducted following an approved animal use protocol at the Charles River Laboratories, Spencerville, OH research facility. The Good Laboratory Practice (GLP, 21 CFR 58) study was conducted following FDA Redbook protocol for 91-day oral toxicity studies (FDA, 2003). Animals were housed and animal care was given as specified in the USDA Animal Welfare Act (9 CFR, Parts 1, 2, and 3) and as described in the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996).

2.1. Animals and feed

Eighty male and 80 female Sprague–Dawley Crl:CD(SD) rats (Charles River Laboratories, Portage, MI) were randomly allocated to one of four dietary treatments consisting of 0 (control), 0.8%, 1.6%, and 4.0% HMB free acid (HMBFA) by weight mixed into the basal diet, with 20 males and 20 females assigned to each dietary treatment. Group size and the number of groups were based upon guidelines of the FDA Redbook (FDA Office of Food Additive Safety, 2003). At the initiation of dosing, the animals were approximately 8 weeks of age and body weights ranged from 221 to 257 g for the males and 159–200 g for the females. For identification purposes the animals were marked with a tail tattoo. The animals were individually caged in a controlled temperature (18–26 °C) and humidity (50 ± 20%) animal room with an air flow of 10 plus air changes per hour with fresh air. The lighting cycle was 12 h on and 12 h off unless otherwise called for to conduct study procedures, and feed and water were provided *ad libitum*. Animal enrichment such as a chewing object was provided and veterinary care was available throughout the study if needed.

The basal diet was PMI Nutrition International Certified Rodent Chow No. 5CR4 (14% protein, Richmond, IN). Feed was analyzed for nutrient content and environmental contaminants prior to use and was determined to be within nutritional specifications and did not contain any environmental contaminants that would interfere with the objectives of the study. The test substance, HMBFA, was obtained from TSI (USA), Inc. (Missoula, MT) and was from one commercial production lot. Purity of the HMBFA was 99.2%. The HMBFA, adjusted for purity, was mixed into the test rations weekly at either 0.8%, 1.6%, or 4% of the ration by weight with the control group (0%) receiving the basal ration alone. The HMBFA was added in free acid form with no neutralization and no flavorings to mask the taste. Test article concentration, homogeneity, and stability in the feed under the conditions of use were determined. Levels of HMBFA in the prepared rations were determined for concentration and homogeneity and were found to be within the protocol limits of ±15% weight percent for concentration and ±10% weight percent across top middle and bottom samples of a batch. The HMBFA was also determined to be stable in the feed rations at the low and high dosages formulated during the conditions of mixing and use.

2.2. Clinical observations and measurements

The animals were observed throughout the study for general health/mortality and moribundity twice daily, once in the morning and afternoon. Cage side observations were performed once daily on days 1–92, except on the days of detailed clinical observations. Each animal was removed from the cage and observed in detail weekly, beginning with week-1. Body weight of each animal was measured on the day of randomization (day-4) and on days 1, 8, 15, 22, 29, 36, 43, 50, 57, 64, 71, 78, 85, and 91 of the treatment period. A final fasted body weight was recorded for each animal on the day of scheduled euthanasia for calculation of organ to body weight ratios (days 92 and 93). Food consumption was quantitatively measured for each animal weekly beginning with week-1 (day-4) and continuing throughout the dosing phase.

2.3. Ophthalmological examinations

Ophthalmological examinations were performed by a board-certified veterinary ophthalmologist prior to in-life initiation (day-4) and during the last week of dosing (day 86). The ocular examinations were conducted using a hand-held slit lamp and indirect ophthalmoscope. A short acting mydriatic solution was used to dilate the eyes and facilitate the indirect ocular examinations.

2.4. Hematology, blood chemistry, urinalysis

Blood was collected from the jugular vein (under isoflurane anesthesia) during week 6 and from the vena cava (under isoflurane anesthesia at gross necropsy) at the end of the study (week 13). The animals were fasted overnight before scheduled clinical pathology sample collections, but had access to water *ad libitum*. Clinical hematology included red blood cell count, hemoglobin concentration, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular hemoglobin (MCH), reticulocyte count (absolute), platelet count; red cell distribution width (RDW), white blood cell count; neutrophil count (absolute), lymphocyte count (absolute), monocyte count (absolute), eosinophil count (absolute), basophil count (absolute), large unstained cells, and other cells (as appropriate). Clinical chemistry measures included aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase; gamma-glutamyltransferase (GGT), total bilirubin, cholesterol, triglycerides, glucose, total protein, albumin, globulin, urea nitrogen, creatinine, calcium, phosphorus, sodium, potassium, and chloride.

Urine was collected overnight using urine collection cages containing water bottles with ball-bearing sipper tubes. After collection, samples were transferred to the clinical pathology laboratory for processing and color, clarity, specific gravity, microscopic evaluation of urine sediment, total volume, pH, protein, glucose, bilirubin, ketones, and blood were measured.

2.5. Necropsy and pathology

The study animals were subjected to a complete necropsy examination, which included evaluation of: the carcass and musculoskeletal system; all external surfaces and orifices; the cranial cavity and external surfaces of the brain; and the thoracic, abdominal, and pelvic cavities with their associated organs and tissues.