The Elevation of L-3-Hydroxybutyrate Concentrations in the Serum of Aristolochic Acid-Treated Mice

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Abstract

The concentrations of L-3-hydroxybutyrate (L-3HB) in the serum of aristolochic acid (AA)-treated mice were determined by column-switching high-performance liquid chromatography (HPLC). The samples were derivatized with 4-nitro-7-piperazino-2,1,3-benzoxadiazole (NBD-PZ) and determined fluorimetrically. Total 3-HB derivatives in serum were separated by an ODS column and then introduced to a sample loop for chiral separation by two tandem CHIRALCEL OD-RH columns. The results showed that the concentration of L-3HB in the serum of normal mice was 0.81 ± 0.15 μM compared to that of AA-treated mice with 4.33 ± 2.81 μM after 3 days and 8.65 ± 2.60 μM after 5 days of AA administration ($p < 0.05$), suggesting that serum L-3HB may be used as an indicator to determine the level of renal damage.

Keywords: *L-3-hydroxybutyrate; renal damage; column-switching HPLC*

1. Introduction

The 3-HB has a chiral center at the third carbon, and thus exists as two enantiomers. It has long been believed that the enantiomer of D-3HB, L-3HB, is absent in mammalian tissues or body fluids under physiological conditions. However, recent reports have demonstrated free L-3HB in a variety of rat tissues, such as serum, liver, heart, and kidney [1, 2]. It was reported that L-3HB can also be used the same as D-3HB in the biosynthesis of hepatic lipids, brain proteins, and amino acids in neonatal rats [3, 4]. Furthermore, L-3HB was shown to be a more-favorable substance than D-3HB, acetoacetate or acetone for sterol and fatty acid synthesis in the brain, spinal cord, and kidney [5]. Rho et al. reported that neither D-3HB nor (D+L)-3HB, but only L-3HB showed anticonvulsant effect in the animal studies [6]. The effect of L-3HB may be via the block of NMDA receptors [7]. In addition, in patients with β-ketothiolase deficiency or medium-chain acyl-CoA dehydrogenase deficiency, L-3HB was found to be comprised of a minor amount of about 3–5% of the total (D+L)-3HB by gas chromatography-mass spectrometry [8]. The results suggest that the differences in the enantiomeric ratio of 3HB may originate from the enantioselectivity of different

enzyme systems [8]. Therefore, the purpose of this study was to determine L-3HB concentrations in the serum of normal and AA-induced mice in order to elucidate whether L-3HB is an adequate indicator of renal damage.

2. Materials and methods

2.1 Chemicals

NBD-PZ, triphenylphosphine (TPP), and 2,2'-dipyridyl disulfide (DPDS) were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Sodium D- and L-3HB were obtained from Wako Pure Chemicals (Osaka, Japan). Trifluoroacetic acid (TFA) was from Riedel-de Haën (Seelze, Germany), and propionic acid was from Nacalai Tesque (Kyoto, Japan). HPLC-grade acetonitrile (MeCN), ethanol (EtOH), and methanol (MeOH) were the products of the Merck Co. (Darmstadt, Germany). TSKgel ODS-80Ts (150 × 4.6 mm i.d.) and CHIRALCEL OD-RH (OD-RH) (150 × 4.6 mm i.d.) were from the Tosoh Co. (Tokyo, Japan) and Daicel Co. (Osaka, Japan), respectively.

2.2 Derivatization of D- and L-3HB with NBD-PZ

The dericatization of D- and L-3HB with NBD-PZ were carried out according to our previous method [1]. Briefly, One hundred microliters of the sample solution was added to 100 μL of 2 mM NBD-PZ dissolved in MeCN, and then 50 μL each of 280 mM TPP and DPDS in MeCN was added and mixed. The derivatization was allowed to stand for 3 h at 30°C; followed by addition of 250 μL of 0.1% TFA in H_2O to terminate the reaction. An Empore^{TM} SDB-RPS cartridge (3M, St. Paul, MN, USA) was preconditioned with 100 μL of an eluting solution composed of EtOH/MeCN/H_2O (20/30/50, v/v/v), and the eluent was discarded. We than loaded 100 μL of the resultant solution onto the cartridge and collected the eluent. Another elution was performed by loading 100 μL of the eluting solution to thoroughly elute D- and L-3HB. Two portions of the eluent were combined and filtrated through 0.20-μm filters (Sartorius AG, Göttingen, Germany), and 20 μL of the filtrate was injected into the HPLC.

2.3 Preparation of mouse serum

Animal experiments were approved by the Laboratory Animal Research Committee of Taipei Medical University. Serum used for L-3HB analysis was from 6-week-old C3H/He mice (National Laboratory Animal Breeding and Research Center, Taipei, Taiwan), which were reared in cages with food and tap water provided ad libitum. To induce renal damage, aristolochic acid sodium salt dissolved in distilled water was administered orally at a dose of 3.0 μg/mL (0.5 mg/Kg/day). After 3 and 5 days, the blood of experimental animals from normal and AA-treated mice was drawn from the tail vein and centrifuged with Mikro 22R Centrifuge (Hettich Zentrifugen, Tuttlingen, Germany) at 700 g for 15 min at 4°C immediately. Ten microliters of 1.0 mM propionic acid in H_2O as an internal standard (I.S.) was added to 50 μL of serum, and was then brought to 200 μL with EtOH for deproteinization. The solution was vigorously mixed using a vortex mixer and centrifuged at

700 g for 5 min. One hundred microliters of the supernatant was supplemented with fluorogenic reagents and TPP and DPDS to perform the derivatization as described above.

2.4 HPLC conditions

A column-switching HPLC system, as described in our previous publications [1, 2, 9], was used in the present work. As illustrated in Fig. 1, the HPLC system was equipped with a AS-950 intelligent sampler (Jasco, Tokyo, Japan) with a 20-μL loop, two pumps (L-7100; Hitachi, Tokyo, Japan), an L-2480 and an L-2485 fluorescence detector (Hitachi), two D-2500 chromatointegrators (Hitachi), and a Rheodyne Model 7000 switching valve with a 100-μL loop as a sample trap. A TSKgel ODS-80Ts column was used for separation and quantification of the total 3HB in mouse serum; the mobile phase was MeOH/H_2O (33/67, v/v) at a flow rate of 0.7 mL/min. Two OD-RHs connected in tandem (tandem OD-RHs) were used for the enantiomeric separation of D- and L-3HB eluted with MeCN/H_2O (40/60, v/v); the flow rate was 0.3 mL/min. Fluorescence detection (detectors 1 and 2) was performed at 547 nm with a 491 nm excitation wavelength.

3. Results and discussion

By using the proposed column-switching HPLC method, D- and L-3HB concentrations in the serum of normal and AA-treated mice were investigated. The derivatized total 3HB could be separated from other carboxylate components in mouse serum using a TSKgel ODS column with isocratic elution of MeOH/H_2O (33/67) at a flow rate of 0.7 mL/min. The peak of total 3HB on the chromatogram emerged at about 23 min, and the retention time of the internal standard was about 38 min (Fig. 2a, 2c). Neither the total 3HB nor the internal standard peaks were found to overlap with any other interfering peaks, and quantification of the total 3HB concentration was accomplished through the calibration curve.

For the determination of L-3HB in mouse serum, an octylsilica column (TSKgel ODS-80Ts) was selected as the first non-chiral column, because it gave good separation of 3HB from the other endogenous compounds in mouse serum (Fig. 2a and c). After the separation on the octylsilica column, a portion of 3HB fraction was introduced into the cellulose-based chiral column through a six-port valve. As a result, both peaks of D-3HB and L-3HB were clearly observed in the chromatogram (Fig. 2b and d). A significant increase of total 3HB was observed in the serum of AA-treated mice. The results are shown in Fig. 3. In normal mice, the concentrations of L-3HB in serum was 0.81 ± 0.15 μM (mean ± SD; 3days) and 0.86 ± 0.81 μM (5 days), while those in serum of AA-treated mice were 4.33 ± 2.81 μM (3 days; $p<0.01$) and 8.65 ± 2.60 μM (5 days; $p<0.05$), respectively. Not only L-3HB, but also D-3HB in serum, was significantly increased in the AA-treated mice (data not shown). The average L-3HB percentage was about 2.88% of the total 3HB in normal mouse serum, and that was about 2.03% in AA-treated ones. To our knowledge, the present work is the first study of the identification and quantification of L-3HB under disease conditions.

In conclusion, employing the column-switching HPLC system, we were able to detect and identify L-3HB in normal and AA-treated mouse serum. A significant increase of L-3HB was observed in the serum of AA-treated mice. It suggests that serum L-3HB may be used as an indicator to determine the level of renal damage.

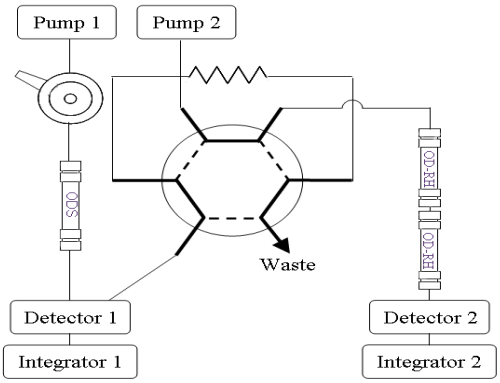

Figure 1. A schematic diagram for the column-switching HPLC system used in this study. The solid and dotted lines indicate six-port valve position A and B, respectively

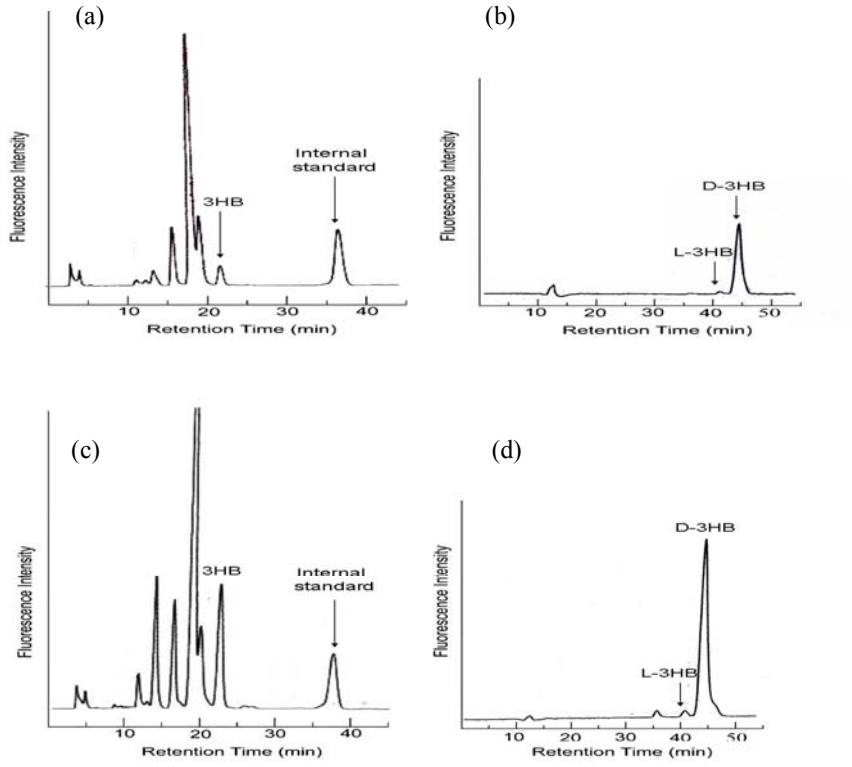

Figure 2a-d. Representative chromatograms obtained by proposed column-switching HPLC system: (a) Analysis of L-3HB in normal mouse serum. The retention times of the total 3HB derivative and the I.S. were about 23 and 38 min, respectively. (b) Enantiomeric separation of D- and L-3HB derivatives isolated from mouse serum. (c) (A) Analysis of L-3HB in AA-treated mouse serum. (B) Enantiomeric separation of D- and L-3HB derivatives isolated from AA-treated mouse serum.

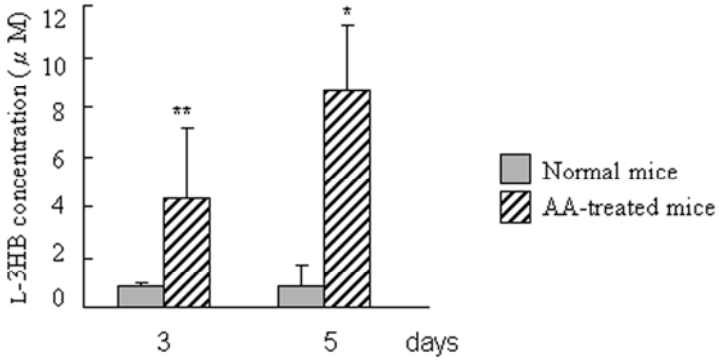

Figure 3. Concentrations of L-3HB in the serum of normal and AA-treated mice after 3 and 5 days of AA administration. (n = 5). *$p < 0.05$, **$p < 0.01$ vs normal rats

Acknowledgments

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