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Semi-quantitative profiling of bile acids in serum and liver reveals the dosage-related effects of dexamethasone on bile acid metabolism in mice

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ABSTRACT

Nuclear receptors, such as the pregnane X receptor (PXR) and glucocorticoid receptor (GR), play an important role in regulating the homeostasis of bile acids (BAs). In previous studies, two-week treatment of 1 mg/kg of dexamethasone (DEX) was used to activate GR in mice, whereas 4-day treatment of 75 mg/kg of DEX was chosen to activate PXR. However, little is known about the effect of DEX on circulating and hepatic BA profiles. In the present study, we reported a simple and rapid LC-MS method for semi-quantitative profiling of 39 BA species in mouse serum and liver. This method was applied to investigate the BA profiles in mice treated with either 1 mg/kg DEX for two weeks or 75 mg/kg DEX for 4 days. The gene expression, microsomal induction and liver enlargement in mice confirmed that PXR was activated by 4-day treatment of 75 mg/kg DEX, but not by two-week treatment of 1 mg/kg DEX. Two-week treatment of 1 mg/kg DEX markedly increased the circulating BAs, in particular conjugated primary BAs, suggesting a pro-cholestatic effect of DEX at low doses. In contrast, 4-day treatment of 75 mg/kg DEX increased BA hydroxylation and decreased hepatic BAs, in particular unconjugated secondary BAs, suggesting a BA-lowering and bacteria-suppressive effect of DEX at high doses. To conclude, a semi-quantitative LC-MS method was developed and applied to elucidate the dosage-related effects of DEX on serum and hepatic BA profiles in mice.

1. Introduction

Bile acids (BAs) are synthesized from cholesterol in the liver via two major pathways, namely the classic and alternative pathways [1]. Cholesterol is converted to cholic acid (CA) and chenodeoxycholic acid (CDCA), two primary BAs in human liver. In rodents, CDCA could be further metabolized to form α/β-muricholic acids (α/β-MCAs). The majority of BAs in the liver are conjugated with taurine and/or glycine, secreted into the bile, and stored in the gallbladder. After a meal, BAs are excreted into the intestine, where they are converted to secondary BAs by bacterial enzymes. Deoxycholic acid (DCA) and lithocholic acid (LCA) are two major secondary BAs in humans.

Individual BAs have distinct physiological and pathophysiological activities, and thus the BA profile determines their hydrophobicity, fat-solubility, and toxicity [2]. The BA profile is important for BA signaling pathways, because individual BAs have distinct potency to activate BA receptors, such as farnesoid X receptor (FXR) and the plasma membrane-bound G-protein-coupled BA receptor 1(TGR5) [3]. In addition, the BA profile could be a reliable hallmark for certain diseases, such as intrahepatic cholestasis of pregnancy, fatty liver disease, and bacterial infection [4]. Consequently, there is a growing interest in the development of methods to quantify and profile individual BAs.

Nuclear receptors are key regulators of various physiological processes, including BA synthesis, transport and metabolism [5]. Activation of pregnane X receptor (PXR) results in an induction of CYP3A, which is sufficient for the hydroxylation and detoxification of BAs in the liver [6]. Glucocorticoid receptor (GR) has been reported to regulate genes involved in BA synthesis and transport [7, 8]. Dexamethasone (DEX), a synthetic glucocorticoid (GC), has been used as an agonist for both PXR and GR in previous studies [9, 10]. DEX at higher doses (> 20 mg/kg) was able to activate pregnane X receptor (PXR) in mice [11], whereas lower doses (< 6 mg/kg) mainly activate GR signaling [12]. Furthermore, DEX has been used to treat the intrahepatic cholestasis of pregnancy, suggesting an important role of DEX in decreasing

Abbreviations: 1°BAs, primary bile acids; 2°BAs, secondary bile acids; BA, bile acid; CDCA, chenodeoxycholic acid; CA, cholic acid; DEX, dexamethasone; GR, glucocorticoid receptor; MCA, muricholic acid; PXR, pregnane-X-receptor; ΣBAs, total bile acids; T-BAs, taurine-conjugated bile acids; U-BAs, unconjugated bile acids; UPLC, ultra-performance liquid chromatography

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BAs [13]. Nevertheless, to our knowledge, it remains largely unknown about the dosage-related effect of DEX on BA homeostasis.

Numerous LC-MS methods have been developed for BA quantification in humans and rodents. Some methods had relatively complicated sample processing and limited BA species coverage (from 11 to 32 BAs quantified) [14, 15]. For instance, Hagio et al. developed a LC-MS/MS method to quantify 22 BAs in liver and intestine, and the extraction procedure included grinding, sonicating, heating, centrifuging, evaporating, and filtering [16]. Wegner et al. developed a rapid LC-MS/MS method to quantify 32 BAs in fermentation broth, which was limited by a strong matrix effect [17]. Some methods were powerful, but had high requirement for the instrumentation. For instance, Sarafian et al. reported a LC-MS method quantifying 146 BA species [18]. However, this method requires two different mass spectrometers (Q-TOF for profiling and TQ-S for targeted detection), which are unaffordable for most laboratories. Additionally, this method included 88 sulfated BAs, which are not commercially available, and are almost undetectable in rodents [19]. Therefore, there is still a need for a simple and rapid method, which should not be limited to certain instruments and could be used as a routine tool for the laboratory. In our previous studies, we have performed BA quantification by using a Waters ACQUITY ultra performance LC system (Waters, Milford, MA) coupled with a Waters Quattro Premier XE triple quadrupole mass spectrometer [15, 20, 21]. In the present study, we developed a simple and semi-quantitative BA profiling method by using an Agilent 1290 UPLC system (Agilent Technologies, USA) coupled to a quadrupole time-of-flight mass spectrometer (Bruker micrOTOF-QII, Germany), which was the only LC-MS system available in the laboratory. With this BA profiling method, we investigated the BA concentrations in serum and livers of mice treated with two dosages of DEX and revealed dosage-related effects of DEX on BA profile.

2. Experimental

2.1. Chemicals and reagents

Sulfated BAs were kindly provided by Dr. Curtis D. Klaassen at the University of Washington (Seattle, Washington). Other BA standards were purchased from either Sigma-Aldrich (St. Louis, MO) or Steraloids, Inc. (Newport, Rhode Island). Total 39 BAs were quantified in the present study, with their structures and abbreviations shown in Fig. 1. Deuterated internal standard (IS) cholic acid-2,2,4,4-d_4 was purchased from C/D/N Isotopes, Inc. (Pointe-Claire, Quebec, Canada). Dexamethasone was purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol (HPLC Grade) was purchased from Concord Technology Co., Ltd. (Tianjin, China). Acetonitrile (HPLC Grade) was purchased from Fisher Scientific (USA). Ultra-pure water was generated by Q-Gard® 1 Water Purification System (Merck Millipore, USA). Ammonium acetate (GR) was purchased from Tianjin Kemiou Chemical Reagent Co., Ltd. (Tianjin, China). Glacial acetic acid (HP) and ammonium hydroxide (25%) were bought from Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China). All other reagents were purchased from commercial vendors and were of the highest purity grade available.

2.2. Animals and treatments

Eight-week-old adult male C57Bl/6 mice were purchased from Vital River Laboratory Animal Technology (Partner of Charles River Laboratories, Beijing, China), and housed in the certified animal facility at the Institute of Radiation Medicine of the Chinese Academy of Medical Sciences (CAMS, Tianjin, China). Mice were acclimated for three days before starting the treatment. According to previous literature, two-week treatment of 1 mg/kg of DEX was chosen to activate GR in mice, whereas 4-day treatment of 75 mg/kg of DEX was used to activate PXR in mice [22]. Mice (N = 5–6/group) were thus divided into 4 groups: Cont-1 (saline, 10 mL/kg, i.p.), DEX-1 (1 mg/kg, i.p. in saline), Cont-75 (saline with 2% carboxymethyl cellulose, 10 mL/kg, i.p.), and DEX-75 (75 mg/kg, i.p. in saline with 2% carboxymethyl cellulose). The experimental protocols were approved by the Animal Use Committee at the CAMS (No. 1203). At the end of treatment, mice were anesthetized with 50 mg/kg pentobarbital. The blood was collected from the retro-orbital sinus of mice, and serum was obtained by centrifuging blood at 6000 g for 15 min. Livers, with gallbladders removed, were harvested from the same animals, washed, frozen in liquid nitrogen, and stored at −80 °C.

2.3. Histopathology

Liver samples were fixed in 10% formalin prior to routine processing and paraffin embedding. The paraffin livers were cut into slices (5 μm). Liver sections were stained with hematoxylin and eosin for the evaluation of hepatocellular pathology.

2.4. Sample preparation

The BA extraction process was consisted of four steps: protein precipitation, extraction, dry and reconstitution. For serum samples, IS was added to 100 μL of serum sample and mixed thoroughly. The mixture was added with 1 mL of ice-cold acetonitrile and then vortexed vigorously. The final mixture was centrifuged at 12,000 × g for 10 min. The supernatant was evaporated under vacuum and reconstituted in 50 μL of 50% methanol. The suspension was centrifuged at 20,000 × g for 10 min, and the supernatant was then ready for injection. For liver samples, approximately 120 mg of liver was homogenized in 5 volumes of ddH_2O. An amount of 600 μL of liver homogenate was spiked with IS (d_4-CA), mixed thoroughly, and equilibrated on ice for 10 min. The homogenate was then mixed with 3 mL of ice-cold acetonitrile (3% ammonia), and shaken for 30 min at room temperature. Afterward, the mixture was centrifuged at 12,000 × g for 10 min, and the precipitate was re-mixed with 1 mL of ice-cold acetonitrile and centrifuged at 12,000 × g for 10 min. The two supernatants were combined, evaporated under vacuum, and reconstituted in 100 μL of 50% methanol. The suspension was centrifuged at 20,000 × g for 10 min, and the supernatant was then ready for injection.

2.5. Standard solutions and quality controls

The stock solutions of BAs and IS were prepared in 50% methanol to achieve a concentration of 500 μg/mL. In the present study, BA-free matrices were prepared for both serum and liver tissue. Liver homogenate was obtained by homogenizing liver tissue in water (1:2 w/v). The serum and liver homogenate were then treated with 100 mg/mL activated charcoal for 2 h to strip the endogenous BAs. The mixtures were centrifuged at 12,000 g for 10 min to get the supernatant, which were filtered. The filtrates were used as the stripped matrices to construct the calibration curve and quality controls (QCs). Seven standard solutions (0.01, 0.1, 1, 2.5, 5, 7.5, 10 μg/mL) containing internal standard (d_4-CA, 5 μg/mL) were prepared by spiking the stripped matrices with appropriate amounts of stock solutions of BAs and IS. The QCs at three concentrations 2.5, 5 and 7.5 μg/mL were prepared in 50% methanol. It should be noted that blank biological matrices do not exist for endogenous compounds, such as BAs. In the literature, both BA-free matrices and “neat” solvents have been used to prepare BA standard solutions and quality controls (QC) [19, 23, 24]. These “surrogate” matrices showed similar recovery ratios as original matrices for all analytes and QCs.

2.6. Instrumentation

The study was performed on an Agilent 1290 UPLC system (Agilent Technologies, USA) coupled to a quadrupole time-of-flight mass spectrometer (Bruker micrOTOF-QII, Germany) equipped with an ESI