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Development of analytical method for simultaneous determination of five rodent unique bile acids in rat plasma using ultra-performance liquid chromatography coupled with time-of-flight mass spectrometry

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ABSTRACT

Bile acids (BAs) are crucial for the diagnosis, follow-up, and prognostics of liver injuries and other BA metabolism related diseases. In particular, rodent unique BAs, α-muricholic acid (α-MCA), β-MCA, ω-MCA, tauro-α-MCA (α-TMCA), and β-TMCA, are valuable biomarkers for preclinical drug development. To the best of our knowledge, however, a simple, selective, sensitive, and robust analytical method for ω-MCA and taurine-conjugated MCAs has never been reported. We have developed a simple, selective, and sensitive analytical method for measurement of 16 BAs including the five rodent unique BAs in rat plasma using an ultra-performance liquid chromatography time-of-flight mass spectrometry (UPLC-TOF-MS) method. Activated charcoal was utilized to prepare BA-free plasma, which served as the surrogate matrix for the preparation of calibration standards and quality control (QC) samples. Results of matrix effects evaluation suggested that the BA-free plasma could be adequate as a surrogate matrix for BAs determination. Three stable isotope labelled internal standards were separated by reverse phase UPLC using gradient elution and were detected by TOF-MS in negative ion mode. The calibration curve was linear for all BAs over a range of 10–25 ng/mL to 1000–10,000 ng/mL, with overall imprecision below 15% and 20% at lower limit of quantification (LLOQ), respectively. This analytical method was used to determine BA concentrations in more than 300 plasma samples from rats with liver injuries induced using α-naphthylisocyanate, carbon tetrachloride, or flutamide. The alteration of BA concentrations was most evident for necrosis, and cholestasis hepatotoxins, with more subtle effects by steatosis and idiosyncratic hepatotoxins. In conclusion, we have developed a simple, selective, and sensitive analytical method to measure plasma 16 BAs including 5 rodent unique BAs, α-MCA, β-MCA, ω-MCA, α-TMCA, and β-TMCA. Our data suggested that α-TMCA and β-TMCA could be useful for identification or prediction of liver injuries, a currently unmet need in preclinical toxicity. Our method using TOF-MS is useful to determine BAs in rat plasma and of use in structural analyses of metabolites in early stage of drug development.

1. Introduction

Bile acids (BAs) are steroidal carboxylic acids derived from cholesterol metabolism in hepatocytes. BA synthesis produces primary cholic acid (CA) and chenodeoxycholic acid (CDCA) in human and α- and β-muricholic acid in rodents [1,2]. Before being excreted into bile canaliculi, primary BAs are mainly conjugated with taurine or glycine through the terminal side-chain carboxylic group. In the intestine, primary BAs are deconjugated and converted into secondary BAs by microbiota. Then, most BAs are re-absorbed back to the liver, conjugated by hepatocytes, and re-excreted into bile to complete enterohepatic circulation [3]. The BAs that

Abbreviations: CA, cholic acid; TCA, taurocholic acid; α-MCA, α-muricholic acid; β-MCA, β-muricholic acid; ω-MCA, ω-muricholic acid; α-TMCA, taurine-α-muricholic acid; β-TMCA, taurine-β-muricholic acidmuricholic acid; GCA, glycocholic acid; TLCA, taurolithocholic acid; TDCA, taurodeoxycholic acid; THCA, taurohyocholic acid; CDCA, ahenodeoxycholic acid; THDCA, taurohyodeoxycholic acid; TUDCA, tauroursodeoxycholic acid; TCDCA, taurochenodeoxycholic acid; GCDCA, glycochenodeoxycholic acid; ANIT, α-Naphthylisothiocyanate.

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escape the enterohepatic circulation pass into the colon where they undergo further bacterial metabolism such as deconjugation, oxidation-reduction, epimerization, 7-dehydroxylation, and esterification [4]. BAs have many important physiologic functions including regulation of their own synthesis, endocrine, and energy expenditure [5,6]. BAs act as the signaling molecules in controlling the metabolism of glucose and lipids in the enterohepatic system. Since BAs have diverse functions and roles, a more comprehensive determination is important for the diagnostic, therapeutic, and prognostic management of the associated diseases. In hepatic disease, the synthesis and clearance of BAs in the liver and their intestinal absorption are disturbed, manifest as elevated levels of total BAs and their conjugates in plasma [7]. Usually, obvious changes in the concentration of individual BAs and their metabolic profiles in plasma, urine, and feces can be observed and have been considered important biochemical markers for diagnosis of liver disorders and other diseases affecting BA metabolism. Because of structural similarities, especially isomers, and lower concentrations of BAs in biological samples such as plasma, highly selective and sensitive analytical methods are needed for simultaneous monitoring of individual BAs. The concentrations of BAs in humans have been well documented; however, only a few studies have determined BA concentrations in experimental animals, despite the common use of rats in preclinical studies. Muricholic acids (MCAs) are one of the main groups of BAs found in mice and rats, and at low concentrations in other species [1,8]. The three major BAs in germ-free mice are CA, α-MCA, and β-MCA [9]. In intestine, bacterial transformation of β-MCA occurs to synthesize ω-MCA. It was suggested that the gut microbiota might play a role in the pathogenesis or progression of certain liver disease [10–13]. Conjugation of BAs with taurine or glycine takes place in the liver before secretion. Taurine-conjugated serum BAs showed a higher correlation than alanine aminotransferase (ALT) with histopathological necrosis severity scores [14]. To the best of our knowledge, however, a simple, selective, sensitive, and robust analytical method for ω-MCA and taurine-conjugated MCAs has never been reported. In preclinical studies, highly selective, sensitive, and reliable analytical methods are needed for simultaneous monitoring of individual BAs including MCAs in biological samples.

Liquid chromatography–mass spectrometry/mass spectrometry (LC–MS/MS) is capable of simultaneously and accurately detecting specific compounds in various specimens. A recent analytical development is ultra-performance liquid chromatography (UPLC), which greatly improves the chromatographic separation of complex biofluid samples, and hence, reduces ion suppression problems [15–17]. The hyphenated analytical methods have proven to be a very powerful tool for separation, characterization, and structural elucidation of metabolic components in biological fluids. Mass spectrometric analysis of plasma samples from *in vivo* studies provides accurate and precise measurement of metabolites, and such technology has contributed to a better understanding of liver disorders and other diseases affecting BA metabolism in rodents.

The aim of the present study was to establish highly simple, selective, sensitive, and reliable analytical methods for simultaneous monitoring of individual BAs including α-MCA, β-MCA, ω-MCA, α-TMCA, and β-TMCA in rat plasma samples.

2. Materials and methods

2.1. Chemicals

A total of 16 BAs (Fig. 1) and 3 internal standards (ISs) were included in this study: α-MCA, β-MCA, ω-MCA, taurine-α-MCA (α-TMCA), taurine-β-MCA (β-TMCA), glycochenodeoxycholic acid (GCDCA), taurolithocholic acid (TLCA), taurohyodeoxycholic acid (THDCA), taurohyocholic acid (THCA), and taurocholic acid (TCA) were purchased from Steraloids UK Ltd. (London, England). Glycocholic acid (GCA), tauroursodeoxycholic acid (TUDCA), taurochenodeoxycholic acid (TCDCA), and taurodeoxycholic acid (TDCA) were purchased from Merck (Darmstadt, Germany). CA and chenodeoxycholic acid (CDCA) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The ISs cholic acid-2,2,3,4,4-d_5 (CA-d_5) and taurocholic acid-2,2,4,4-d_4 (TCA-d_4) were purchased from Toronto Research Chemicals Inc. (Toronto, Canada), and glycocholic acid-2,2,4,4-d_4 (GCA-d_4) was obtained from C/D/N isotopes Inc. (Quebec, Canada). α-Naphthylisothiocyanate (ANIT), carbon tetrachloride (CCl_4), and flutamide were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Deionized water used for the UPLC mobile phase was prepared with a Milli-Q advantage A10 water purification system (Millipore, Bedford, MA, USA). HPLC grade methanol and acetonitrile were obtained from Kanto Chemical Inc. (Tokyo, Japan). Formic acid, ammonium acetate, and activated charcoal were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2.2. Equipment

The chromatographic system consisted of an ACQUITY UPLC (Waters, Milford, MA, USA). Chromatographic separation was carried out on an XBridgeBEH C18 column (100 × 3 mm, i.d., 2.5 μm; Waters, Milford, MA, USA) at 40 °C. The UPLC system was coupled online to a Xevo Q-TOF mass spectrometer (Waters, Milford, MA, USA) in negative electrospray mode with a scan range of *m/z* 50–1000, a capillary voltage of 2.3 kV, a sample cone of 70 V, a desolvation temperature of 500 °C, a source temperature of 150 °C, and a desolvation gas flow of 1000 L/h fitted to the electrospray ionization (ESI) source. Leucine enkephalin (*m/z* 554.262, 236.104) was used as the lockmass standard; a solution of 200 pg/μL [acetonitrile-0.1% formic acid (1:1)] was infused into the system at 20 μL/min *via* an auxiliary sprayer. Data were collected in centroid mode with a scan range of *m/z* 50–1000, and lockmass scans were collected every 20 s and averaged over 3 scans to perform mass correction.

2.3. BA-free plasma preparation

Pooled drug-free plasma was purified using activated charcoal (Norit SX PLUS, Cabot Corporation, Boston, MA) to remove endogenous BAs. The BA-free plasma was used as a matrix for calibration standards and quality control (QC) samples. Specifically, 50 mL of plasma was mixed with 5 g of activated charcoal, and the mixture was shaken moderately on an orbital shaker overnight (about 17 h) at room temperature. After centrifugation at 1600 × *g* for 10 min at 4 °C, the supernatant of the purified plasma was transferred to clean tubes. Then, the supernatant was centrifuged at 27,000 × *g* for 30 min at 4 °C, and the obtained supernatant was applied to a 0.45 μm filter. Furthermore, the obtained filtrate was centrifuged at *ca* 40,000 × *g* for 90 min at 4 °C, and then the obtained supernatant was again centrifuged at 40,000 × *g* for 90 min at 4 °C. This final supernatant was used as BA-free plasma.

2.4. Standard solutions

BA stock solutions were prepared in methanol to achieve a concentration of 10 mg/mL. The 16 BA stock solutions were mixed in equal amounts and were diluted with methanol to achieve a concentration of 500 μg/mL. Three ISs, CA-d_5, TCA-d_4, and GCA-d_4, were mixed in equal amounts and were diluted with methanol to achieve a concentration of 2000 ng/mL.