Journal of Chromatography B, 980 (2015) 72–78

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

An UPLC–MS/MS method for quantitative profiling of bile acids in sea lamprey plasma and tissues

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ARTICLE INFO

Article history:
Received 3 October 2014
Accepted 17 December 2014
Available online 25 December 2014

Keywords:
Bile acids
Sea lamprey
UPLC–MS/MS
Protein precipitation
Plasma
Tissue

ABSTRACT

Bile acids (BAs) have recently gained more attention because of their diverse roles from digestion to signaling. Simultaneous analyses of various BAs in biological samples are challenging due to their structural similarity, relatively low concentrations, and the presence of isomeric forms. In this study, we report a simple and sensitive UPLC–MS/MS method for simultaneous quantifications of 13 BAs including four unique sea lamprey BAs in sea lamprey plasma, liver, intestine, and gills. A straightforward protein precipitation (PPT) method was used to extract BAs from the biological samples. Separation of all target analytes was achieved on a reverse-phase UPLC column in 15 min, and detection was carried out on MS/MS with ESI in the negative ionization mode. This method was validated regarding its linearity, limits of detection (LOD), recovery, matrix effect, reproducibility, accuracy and precision. Significant improvements compared to previous LC–MS/MS methods were observed as a result of the application of UPLC and extensive optimization of experimental conditions. The method showed excellent linearity with high regression coefficients (>0.99) over a range of 0.5–1000 ng/mL and LODs ranged from 0.009 to 0.11 ng/mL. The applications of the developed method demonstrated that it simultaneously determined all target BAs in different biological sample matrices with excellent sensitivity, selectivity and reproducibility.

1. Introduction

Bile acids (BAs) are cholesterol derivatives with a carboxyl group at C-17 side chain and represent the characteristic constituents of human and animal bile [1,2]. Typically, primary BAs are synthesized in the liver and conjugated with taurine or glycine amino acids through the terminal side-chain carboxylic group in animals [3,4]. These BAs are deconjugated and converted to secondary BAs in the intestine, and reabsorbed and circulated back to the liver. BAs are involved in a number of metabolic processes, and play critical roles from digestion to signaling [5–8]. BAs are potent odorants that appear to attract adult migrating Arctic char (*Salvelinus alpines*) to spawning grounds [6]. The pheromone functions of BAs are well established in the sea lamprey [7]. Different BAs, even with slight difference in hydroxylation patterns, may exert drastically different biological and behavioral responses [8]. Reliable analytical methods are required for studies of the diverse functions and physiological importance of various BAs present in living organisms.

Various chromatographic methods are currently used to quantify BAs in biological matrix, such as the high performance liquid chromatography with UV detector (HPLC–UV) [9–11], HPLC–fluorescence detector (HPLC–FL) [12–14], and gas chromatography coupled with mass spectrometry (GC–MS) [15–19]. HPLC–UV/FL requires a long running time to separate the target analyst, and lacks the sensitivity and selectivity for quantitation of trace amounts of BAs. GC–MS is sensitive and specific but is time-consuming because of the multiple steps required for sample processing. On the contrary, liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) allows a single-step-measurement for both non-conjugated and conjugated BAs [20–25]. Moreover, LC/MS/MS could provide high sensitivity and wide dynamic range for quantitative analysis when operated in the multiple reactive monitoring (MRM) mode on a triple quadruple mass spectrometer [26–41]. For routine quantification of samples, LC–MS/MS is the most suitable method to determine BAs without tedious prior fractionation of conjugates [26,27]. However, detection of low-abundance and unusual BAs, such as the unique BAs in sea lamprey, remains a challenge for current analytical methods due to the complex biological matrix, differences in their physicochemical properties, wide range of concentrations, and existence of multiple isomeric forms.

In the present study, we report a simple and sensitive UPLC–MS/MS method for simultaneous quantifications of 13 BAs, including four unique sea lamprey BAs, in plasma and three

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http://dx.doi.org/10.1016/j.jchromb.2014.12.018

other tissue types relevant to BA biosynthesis (liver and intestine) and release (gill). The method was fully validated and significant improvements of the analytical performance parameters were obtained compared to previous LC–MS/MS methods as a result of the application of ultra-high performance LC and careful optimization of experimental conditions. This UPLC–MS/MS based method determined BAs in sea lamprey samples with excellent sensitivity, selectivity and reproducibility. The application of this method to quantitative profile of BAs in sea lamprey has resulted in the first discovery of intestinal biosynthesis of BA [42].

2. Experimental

2.1. Chemicals and reagents

Acetonitrile (ACN), methanol, formic acid (Mallinckrodt Baker, NJ, USA) and ammonium acetate (Sigma-Aldrich, MO, USA) were HPLC grade. De-ionized water was prepared by a Milli-Q system (Millipore,MA, USA). High-purity nitrogen (99.9%) and helium (99.99%) were from AGA gas Inc. (Lansing, MI). Cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), taurocholic acid (TC), taurochenodeoxycholic acid (TCDC), taurodeoxycholic acid (TDC), taurolithocholic acid (TLC), glycochenodeoxycholic acid (GCDC), and glycodeoxycholic acid (GDC) were purchased from Sigma-Aldrich (MO, USA). Deuterated taurocholic acid ([$^2H_4$]CA) was purchased from Steraloids Inc. (RI, USA). Petromyzonol sulfate (PZS), 3keto-petromyzonol sulfate (3kPZS), petromyzonamine disulfate (PADS), petromyzonol disulfate (PSDS) and deuterated 3keto-petromyzonol sulfate ([$^2H_5$]-3kPZS) were obtained from Bridge Organic Inc. (Vicksburg, MI, USA). The purity of the afore-mentioned compounds was above 95%.

2.2. Sea lamprey plasma sampling

Sea lampreys were collected by the U.S. Fish and Wildlife Service (Marquette, MI) and Canada Department of Fisheries and Ocean Sea Lamprey Control Centre, and transported to Michigan State University and kept at the University Research Containment Facility, where plasma samples were collected. For each set of experiments, all test subjects were captured from the same stream on the same day to reduce variation in levels of maturity. Standard operating procedures for transporting, maintaining, handling, anesthetizing, and euthanizing sea lampreys were approved by the Institutional Committee on Animal Use and Care of Michigan State University (AUF number 02/13-040-00). All animals were anesthetized with 0.05% MS-222 (Sigma-Aldrich) before handling or tissue sampling. Blood samples in the study were collected during daytime and extracted *via* cardiac puncture after the animals were euthanized with an overdose of MS-222 and immediately placed on ice until processing. Blood was centrifuged at $1000 \times g$ for 20 min at 4 °C. Plasma samples were collected and stored at −80 °C until use. Liver, intestine, and gills were collected, snap-frozen in liquid nitrogen, and stored at −80 °C until extraction.

2.3. Sample preparation

For plasma samples, 1 mL 75% ethanol/water solution and 20 μL of internal standard (IS) solution (4D-TC and 5D-3kPZS, 500 ng/mL) was added to 500 μL of plasma. The mixed solution was vortexed and centrifuged at $15{,}000 \times g$ for 10 min. The supernatant was transferred to a new tube, added 1 mL of ice cold ACN, vortexed and centrifuged at $15{,}000 \times g$ for 10 min. Supernatant was evaporated using a CentriVap Cold Trap with CentriVap Concentrator (Labconco Co., Kansas, MO, USA) and reconstituted in 100 μL of 50% ACN/water (v/v). For the liver, intestine and gill samples, tissues (20–50 mg) were mixed with 20 μL IS solution and homogenized in 1 mL 75% ethanol/water solution. The generated solutions were processed with similar procedure as plasma samples, and reconstituted in 100 μL of 50% ACN/water (v/v).

2.4. Standard solution preparation

Stock solutions of BAs and IS were prepared by dissolving the respective compounds in 50% methanol/water (v/v) to obtain concentration of 1 mg/mL. All stock solutions were stored at −20 °C until use. For each sample set, working solutions were prepared daily from the stock solution, and all labeled standards were analyzed by full scan MS to assure that there was no detectable contamination by unlabeled analytes.

To prepare calibration solutions and quality control (QC) samples, plasma and tissues (liver, intestine, and gill) were collected and each was pooled from ten sea lampreys. Tissue samples were homogenized according to the procedures described in Section 2.3. Plasma and each homogenate were incubated with 100 mg/mL activated charcoal for 1 h to strip the biological matrices of endogenous BAs. The charcoal was removed by centrifugation and filtration. The charcoal treated solutions were spiked with appropriate amount of standards to make calibration working solutions at nine levels from 0.5 ng/mL to 1000 ng/mL, and calibration curves were constructed by determining each analyte in each sample matrix. QCs at three levels (low, LQC: 1 ng/mL; middle, MQC: 10 ng/mL; high, HQC: 100 ng/mL) were prepared in charcoal treated plasma and tissue homogenates by spiking 20 μL standard solutions with appropriate amounts of BA standards and ISs.

2.5. LC/MS/MS

A Waters (Milford, MA) Xevo TQ-S mass spectrometer coupled to a Waters ACQUITY H-Class UPLC system was used. A Waters BEH C18 column (1.0 × 50 mm, 1.7 μm particle size) was used with column oven temperature at 30 °C. Mobile phase A was 10 mM ammonium acetate in water, and mobile phase B was ACN. Separation was achieved using the following gradient program at a flow rate of 200 μL/min for 15 min: 3% B for 0.5 min; increased to 40% B from 0.5 to 10 min; increased to 95% B from 10 min to 12 min, and then maintained at 95% B from 12 to 13 min; returned to 3% B from 13 to 15 min. The injection volume was 10 μL. Mass spectra were acquired using electrospray ionization in negative ion mode and MRM. The capillary voltage, extractor voltage, and rf lens settings were 3.17 kV, 4 V, and 0.3, respectively. The flow rates of cone gas and desolvation gas were 20 and 400 L/h, respectively. The source temperature and desolvation temperature were 110 and 350 °C, respectively. Collision-induced dissociation employed argon as collision gas at a manifold pressure of 2×10^{-3} mbar, and collision energies and source cone potentials were optimized for each transition using Waters QuanOptimize software. Data were acquired with MassLynx 4.0 and calibrated and quantified by QuanLynx software.

2.6. Method validation

2.6.1. Linearity, LOD and LOQ

Calibration linearity was acquired using internal standard spiked calibration solutions at nine concentrations, ranging from 0.5 ng/mL to 1000 ng/mL. Each point was the average of three injections with the injection volume of 10 μL. Integrated peak areas of the selected quantification MRM transitions were used to build the standard curves. The standard curves were fitted by a weighted ($1/\chi$) least squares regression analysis using the Quanlynx