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Unconjugated bile acids in rat brain: Analytical method based on LC/ESI-MS/MS with chemical derivatization and estimation of their origin by comparison to serum levels

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ABSTRACT

Although some studies have revealed the implication of bile acids (BAs) and neurological diseases, the levels and origin of the BAs in the brain are not fully understood. In this study, we first developed and validated a sensitive and specific method for the determination of three unconjugated BAs [cholic acid (CA), chenodeoxycholic acid (CDCA) and deoxycholic acid (DCA)] in the rat brain by liquid chromatography/electrospray ionization-tandem mass spectrometry combined with chemical derivatization. The measured brain concentrations (mean ± standard deviation, $n = 10$) of normal rats were 58.7 ± 48.8, 14.2 ± 11.7 and 13.2 ± 8.7 ng/g tissue for CA, CDCA and DCA, respectively. For their origin, we developed the hypothesis that they might be mostly derived from the periphery. To test this hypothesis, the brain BA levels were compared with the serum levels. The brain levels had high correlations with the serum levels, and were always lower than the serum levels for the three unconjugated BAs. Furthermore, the higher brain-to-serum concentration ratios were found for the BAs with higher log*D* values (higher lipophilicity). Moreover, the brains of the rats intraperitoneally administered with deuterium-labeled CA and CDCA were also analyzed; the deuterium-labeled BAs were detected in the brain of the rats administered with these compounds. Based on all the results, we concluded that the BAs found in the brain are mostly derived from the periphery and the major mechanism for the transportation of the unconjugated BAs to the brain is by passive diffusion.

1. Introduction

The naturally-occurring common bile acids (BAs) are saturated C_{24} steroid carboxylic acids and their conjugates with glycine or taurine. Cholic acid (CA) and chenodeoxycholic acid (CDCA) are the primary BAs synthesized from cholesterol by the action of hepatic enzymes and excreted into the small intestine via the bile duct. Before being excreted, the primary BAs are mostly conjugated with glycine or taurine at the C_{24} carboxy group. In the intestine, the primary BAs are deconjugated and converted into the secondary BAs, deoxycholic acid (DCA), ursodeoxycholic acid (UDCA) and lithocholic acid (LCA), by microbiota. Most BAs then return to the liver upon absorption in the ileum and proximal colon, conjugated by hepatocytes, and re-excreted into the small intestine to complete the enterohepatic circulation. The BAs assist lipolysis and the absorption of fats by forming mixed micelles. The BAs also act as the signaling molecules with broad paracrine and endocrine functions [1,2].

In the past decade or so, the cholesterol metabolites, such as the BAs and oxysterols, in the brain have been intensively studied with respect to neurological diseases [3–5]. Cerebrotendinous xanthomatosis (CTX) is caused by a 27-hydroxylase enzyme deficiency, leading to the CDCA deficiency. Progressive neurological dysfunctions in patients with CTX can be prevented by a daily supplementation with CDCA [6]. Although the physiological functions of the BAs in the brain are almost unknown, it has been reported that CDCA serves as a potent antagonist at the *N*-methyl-D-aspartic acid and γ-aminobutyric acid type A receptors [7]. It has also been reported that the taurine conjugate of UDCA has neuroprotective effects in several animal models of neurodegenerative diseases, such as Alzheimer's disease, Huntington's disease and Parkinson's disease [8]. In the meantime, the BAs could act as possible toxic compounds for the brain and blood–brain barrier (BBB), and participate in the pathogenesis of hepatic encephalopathy in patients with acute and

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chronic liver diseases [9]. Some studies have revealed the implication of the BAs and neurological diseases, but the occurrence of the BAs, apart from the intermediates of the BA biosynthetic pathways [3], in the brain is still inconclusive. As far as we know, the first experimental evidence for the presence of the common BAs in the brain was demonstrated by Mano et al. [10], in which the unconjugated CA, CDCA and DCA were identified in the rat brain by liquid chromatography (LC)/electrospray ionization (ESI)-mass spectrometry (MS), and the BAs conjugated with amino acids, sulfuric acid and glucuronic acid were not detected in the brain.

BAs are ionized by ESI operating in the negative-ion mode and produce their deprotonated molecules ($[M-H]^-$) [10,11]. However, no characteristic product ion is formed during the collision-induced dissociation of the deprotonated molecules of the unconjugated BAs [11,12], and in such a case, the selected reaction monitoring (SRM) produces a limited benefit in increasing the sensitivity (signal-to-noise ratio, S/N) and selectivity. The brain tissue is highly lipid-rich (sterol-rich), which makes it difficult to selectively detect the target steroidal compounds including the BAs in the brain. Furthermore, for the BAs, adequate reversed-phase chromatographic retention and resolution are achieved when using an acidic mobile phase, which, however, suppresses the deprotonation of the carboxy groups during the ESI, leading to a decreased sensitivity. To enhance the detection responses and selectivity of the BAs in the SRM mode, chemical derivatization is an effective technique [13,14], and we previously developed a reagent, 1-[(4-dimethylaminophenyl)carbonyl]piperazine (DAPPZ, Fig. 1), suitable for the quantification of the carboxylic acids by the positive-SRM mode [15]. Given this background, in this study, we first developed and validated a method for the determination of three unconjugated BAs (CA, CDCA and DCA) by LC/ESI-tandem MS (MS/MS) combined with the DAPPZ-derivatization, which made the occurrence of the BAs in the brain more definitive, and could provide more reliable quantitative values.

The origin of the BAs present in the brain is one of the major concerns; it is unclear whether they are derived from the bloodstream through the BBB or locally synthesized in the brain. It has been demonstrated that rat brain contains enzymes capable of converting 3β-hydroxy-5-cholenoic acid to CDCA through the intermediates, 3β,7α-dihydroxy-5-cholenoic acid and 7α-hydroxy-3-oxo-4-cholenoic acid [16]. However, the enzymic activity for conversion of 7α-hydroxy-3-oxo-4-cholenoic acid, which was found in human cerebrospinal fluid [3], to CDCA was very low [16]. Based on these observations, we developed the hypothesis that the BAs found in the brain might be mostly derived from peripheral sources. The comparison of the brain BA levels to the serum levels will be contributory to estimate the origin of the BAs present in the brain. To test the hypothesis, the brain and serum BA levels were measured and compared in the normal and thyroidectomized (Tx) rats, in which the serum CA and CDCA levels are significantly elevated [17]. The brains of the rats intraperitoneally administered with deuterium-labeled CA and CDCA were also analyzed to examine the transportation of the unconjugated BAs to the brain from the periphery.

2. Experimental

2.1. Materials and chemicals

BAs including CA, CDCA and DCA were purchased from Tokyo Chemical Industry (Tokyo, Japan) or Nacalai Tesque (Kyoto, Japan). Stock solutions of the BAs were prepared as 100 μg/mL solutions in methanol. Subsequent dilutions were carried out with methanol to prepare 2.0, 5.0, 10, 20, 50, 100, 200, 500, 1000, 2000, 5000 and 10000 ng/mL solutions. [2,2,4,4-$^2H_4$]-CA (D_4-CA) and [2,2,4,4,-$^2H_4$]-CDCA (D_4-CDCA) were obtained from CDN Isotopes (Quebec, Canada) and used as the internal standards (ISs). The ISs were dissolved in and diluted with methanol to prepare 10, 1000 or 5000 ng/mL solutions. 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) was from Kanto Chemicals (Tokyo). DAPPZ was synthesized in our laboratories [15]. Strata-X™ cartridges (60 mg adsorbent; Phenomenex, Torrance, CA) were successively washed with methanol (2 mL) and water (2 mL) prior to use. All other reagents and solvents were of analytical grade or LC/MS grade.

2.2. LC/ESI-MS/MS

LC/ESI-MS/MS was performed using a Shimadzu LCMS-8030 triple quadrupole mass spectrometer connected to a Shimadzu LC-20AD chromatograph (Kyoto, Japan). An Ascentis Express C18 column (5 μm, 150 × 2.1 mm i.d., Sigma-Aldrich Japan, Tokyo) was used at the flow rate of 0.2 mL/min and at 40 °C. A gradient elution program with mobile phase A [methanol-10 mM ammonium formate (8:3, v/v)] and mobile phase B (methanol) was performed; B = 0% maintained (0–14 min), 67% linearly increased (14–16 min) and maintained (16–21.5 min), and 0% maintained (21.5–31.5 min). The derivatized BAs were analyzed in the positive-ion mode. The MS conditions were as follows: interface voltage, 4.5 kV; Q1 pre-rod bias voltage, −20 V (derivatized CA and D_4-CA) or −24 V (derivatized CDCA, CDA and D_4-CDCA); Q3 pre-rod bias voltage, −18 V; collision energy, 38 eV (derivatized CA and D_4-CA) or 41 eV (derivatized CDCA, CDA and D_4-CDCA); nebulizer gas flow rate, 3 L/min; drying gas flow rate, 15 L/min; desolvation line temperature, 250 °C; heat block temperature, 400 °C and collision gas, 230 kPa. The SRM transitions (precursor and product ions) were *m/z* 624.4 → 148.1 (CA-DAPPZ), 628.4 → 148.1 (D_4-CA-DAPPZ), 608.4 → 148.1 (CDCA-DAPPZ and DCA-DAPPZ) and 612.4 → 148.1 (D_4-CDCA-DAPPZ). The LC eluent entered the mass spectrometer from 5 to 20 min after injection through a diversion valve.

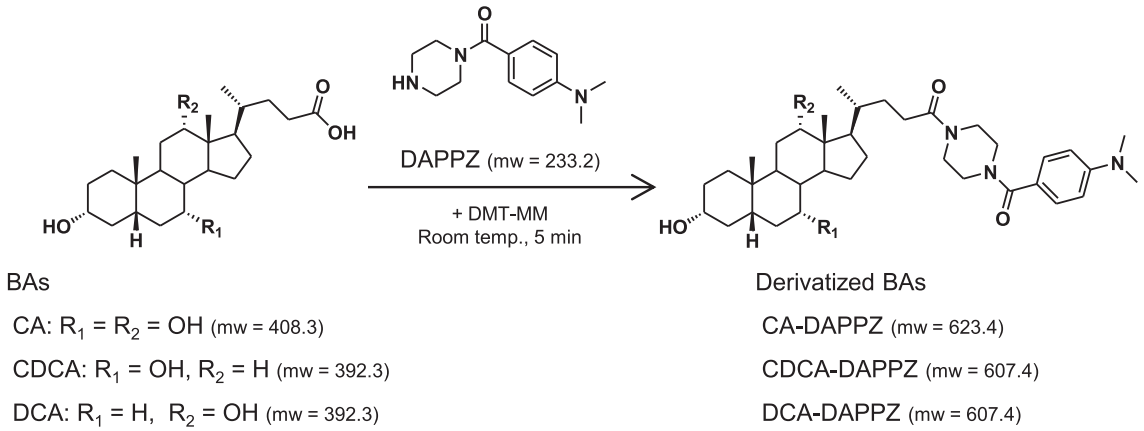

Fig. 1. Chemical structures of unconjugated BAs examined in this study and derivatization scheme of the BAs with DAPPZ.