Presence of protein-bound unconjugated bile acids in the cytoplasmic fraction of rat brain

Nariyasu Mano,† Takaaki Goto,*, Masashi Uchida,*, Koji Nishimura,*, Masayuki Ando,† Norihiro Kobayashi,§ and Junichi Goto*§,**

Graduate School of Pharmaceutical Sciences,† Tohoku University, Aobayama, Aoba-ku, Sendai 980-8578, Japan; Department of Analytical Chemistry,‡ Niigata College of Pharmacy, 5-13-2 Kamishinei-cho, Niigata 950-2081, Japan; Kobe Pharmaceutical University,§ 4-9-1 Motoyama-Kitamachi, Higashinada-ku, Kobe 658-8558, Japan; and Department of Pharmaceutical Sciences,** Tohoku University Hospital, 1-1 Seiryo-machi, Aoba-ku, Sendai 980-8574, Japan

Abstract

Using liquid chromatography/electrospray ionization mass spectrometry, we have found three unconjugated bile acids [cholic acid (CA), chenodeoxycholic acid (CDCA), and deoxycholic acid (DCA)] in the rat brain cytoplasmic fraction. CDCA was detected only upon extraction with high concentrations of guanidine, indicating that it is bound noncovalently to protein in the brain. The most abundant of the three, it was present at a concentration of 1.6 nmol/g wet weight (~15 mg of protein) of brain, corresponding to almost 30 times its serum concentration. CA and DCA were present at 1/30th the concentration of CDCA. Bile acids conjugated with amino acids, sulfuric acid, and glucuronic acid were not detected. These data clearly demonstrate that unconjugated CDCA and, to a lesser extent, CA and DCA, exists in the rat brain.—Mano, N., T. Goto, M. Uchida, K. Nishimura, M. Ando, N. Kobayashi, and J. Goto. Presence of protein-bound unconjugated bile acids in the cytoplasmic fraction of rat brain. J. Lipid Res. 2004. 45: 295–300.

Supplementary key words chenodeoxycholic acid • cholic acid • deoxycholic acid • liquid chromatography/mass spectrometry

Bile acids are synthesized in the liver from cholesterol by the action of hepatic enzymes and excreted into the small intestine via the bile duct. In the intestinal lumen, they assist lipolysis and the absorption of fats by forming mixed micelles and then return to the liver upon absorption in the ileum and proximal colon. Because of their efficient hepatic uptake, bile acids have low concentrations in the peripheral blood. Recent observations also indicate that the nuclear bile acid receptor, the farnesoid X receptor, regulates the bile acid pool by repressing the transcription of genes encoding hepatocyte transporters (1) as well as cholesterol 7α-hydroxylase (2, 3), which is the rate-limiting enzyme for bile acid biosynthesis. In the last few decades, much research has been focused on neurosteroids, which are synthesized in the central nervous system from cholesterol or steroidal precursors imported from peripheral sources (4). Steroids having a 3β-hydroxy-Δ5 structure, which include the sulfated and nonsulfated forms of pregnenolone and dehydroepiandrosterone, are known to act as allosteric modulators of neurotransmitter receptors such as γ-aminobutyric acid type A (GABA_A) (5–7) and N-methyl-d-aspartic acid receptors (8, 9) but not of classic receptors, which modulate adenylate cyclase activity in a steroid ligand-dependent manner. The neurosteroids 3α-hydroxy-5α-pregnan-20-one and 3α,21-dihydroxy-5α-pregnan-20-one, which are ring A-reduced progesterone derivatives of progesterone and deoxycorticosterone, respectively, were the first steroids to be characterized as potent positive allosteric modulators of the GABA_A receptor (5, 10). In contrast, virtually no similar studies have been performed on bile acids, which also possess a steroid nucleus. Weil (11) noted that taurocholic acid caused marked demyelination in vitro and proposed that bile acids of some type might act as a natural demyelinating agent, which might be formed biosynthetically within the brain or enter into the brain from the bloodstream (12). Strangely, a high dose of 14C-labeled cholic acid (CA) injected intracerebrally could not be detected in appreciable amounts in central nervous system tissue within 1 week of the injection (13). In a recent publication, systemically administered tauroursodeoxycholic acid led to a significant reduction in the striatal neuropathology of a transgenic animal model of Huntington’s disease, indicating that this bile acid may act as a neuroprotective

Abbreviations: CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; ESI, electrospray ionization; IS, internal standard; LC/MS, liquid chromatography/mass spectrometry; SIM, selected ion monitoring.

1 To whom correspondence should be addressed.

e-mail: n-mano@mail.pharm.tohoku.ac.jp
subject by reducing striatal atrophy or decreasing striatal apoptosis (14).

Akwa et al. (15) have demonstrated that conversion of two neurosteroids, dehydroepiandrosterone and pregnenolone, to their corresponding 7α-hydroxylated derivatives occurs in rat brain microsomes. In addition, 24-hydroxylated cholesterol has been detected at very high levels in brain (16). Previously, it has also been reported that 3β-hydroxy-5-cholestan-24-oic acid, whose biosynthetic pathway may pass through a 24-hydroxycholesterol intermediate, is a possible precursor for the alternative formation of chenodeoxycholic acid (CDCA) in the liver (17). Meanwhile, even more recent observations have demonstrated that a specific mechanism exists that allows the efflux of taurocholic acid from the bloodstream across the blood-brain barrier (18). In support of this, it was found that the organic anion-transporting polypeptide, which is known to function as an anion exchanger that facilitates the transport of anions such as bile acids from systemic portal blood into hepatocytes, is abundant in human brain (19).

To expand upon these previous findings, the present study examines the existence of bile acids in rat brain using HPLC combined with electrospray ionization mass spectrometry (LC/ESI-MS) and an immunological method using a monoclonal antibody.

METHODS

Chemicals

Unconjugated bile acids were purchased from Nacalai Tesque, Inc. (Kyoto, Japan), except for ursodeoxycholic acid, which was generously donated by Mitsubishi Pharma Co. (Tokyo, Japan). Bile acids labeled with stable isotopes and 12-oxo-lithocholic acid used as internal standards (ISs) and amino acid-conjugated bile acids were synthesized in our laboratory by previously reported methods (20, 21). A Sep-Pak C18 cartridge was purchased from Millipore (Milford, MA) and washed successively with ethanol (20 ml), water (10 ml), 5% BSA aqueous solution (5 ml), and again with water (10 ml) before use. Horseradish peroxidase (EC 1.11.1.7, grade LC, 263 U/mg) was obtained from Toyobo (Osaka, Japan), and horseradish peroxidase-labeled deoxycholic acid (DCA), an enzyme-labeled antigen, was synthesized in our laboratory (22). AffiniPure rabbit anti-mouse IgG + IgM antibody (the second antibody in ELISA) was purchased from Jackson ImmunoResearch (West Grove, PA). Ninety-six-well EIA/RIA plates (No. 3590) were purchased from Costar (Cambridge, MA). Water used was purified by a Millipore water filtration system (Milli QUV Plus). All glassware used was silanized with trimethylchlorosilane, and other chemicals and solvents were of analytical grade.

LC/MS analysis

Analysis by LC/MS was performed using a JMS-LCmate (JEOL, Tokyo, Japan) double-focusing magnetic mass spectrometer equipped with an ESI probe under the negative ion detection mode. The resolution of the mass spectrometer was set at 750 or 3000, and the voltages for the electrospray, orifice, and ring lens were −2.5 kV, −15 V, and −80 V, respectively. The temperatures of the orifice and desolvating plate were 150 and 250°C, respectively. Liquid chromatographic separation was performed on an Inertsil ODS-2 (5 μm, 2.1 mm inner diameter × 150 mm; GL Science, Tokyo, Japan) at a flow rate of 220 μl/min.

High-resolution selected ion monitoring (SIM) was performed using polyethylene glycol sulfate as a calibration standard added through a postcolumn additive mode. Samples were analyzed by low-resolution (m/z 391) and high-resolution (m/z 391.2848) SIM using a 20 mM ammonium acetate solution (pH 7.0) 12-oxo-lithocholic acid acetate solution (pH 4.0, 5.0, and 7.0)-acetonitrile at 53:47, 11:9, and 3:2 (v/v) for each analytical condition.

To compare the relative retention factors (Rks) of target compounds with those of standards, peaks on the chromatogram corresponding to the retention times of authentic bile acids were collected and mixed with 12-oxo-lithocholic acid as an IS, and their Rks relative to IS (tR – tIS / tR0 – tIS0) were determined under three different separation conditions [20 mM ammonium acetate solution (pH 4.0, 5.0, and 7.0)-acetonitrile at 53:47, 11:9, and 2:1 (v/v), respectively].

Sample preparation

Male or female Wistar rats (200–250 g) fed on a commercial pellet diet and water ad libitum were used. Venous blood was collected and, after decapitation, whole brains (about 1.9 g wet weight) were washed with saline and homogenized in 7 vol of ice-cold 100 mM Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose. The homogenate was centrifuged first at 9,000 g for 20 min and then at 105,000 g for 60 min, and the final supernatant fluid was stored below 4°C. All procedures were carried out at 0–4°C.

An aliquot of the 105,000 g supernatant fluid from the rat brain (200 μl, 30 mg wet weight) was added dropwise to 500 μl of 1.65 M Tris-HCl buffer (pH 8.6) containing 0.03 M EDTA and 7.3 M guanidine hydrochloride and mixed gently for 60 min. After the addition of 3,7-[18O]-3,7m, 2.1 mm inner diameter

Recovery of bile acids during the extraction procedure

The recovery of bile acids was investigated using 18O-labeled CA, CDCA, and DCA spiked into samples of 105,000 g supernatant fluid from the rat brain to avoid the effect of endogenous bile acids. To evaluate the intra-day variation, we prepared six samples each of rat brain cytoplasmic fraction spiked with three different amounts (0.3, 3.0, and 30 ng) of each labeled bile acid on the same day and analyzed them using the procedure de-
ELISA for DCA

To characterize DCA, the fraction having a chromatographic retention time corresponding to that of authentic DCA was collected and subjected to ELISA using a monoclonal antibody with high specificity to DCA and markedly low affinity to the other bile acids (23). AffiniPure rabbit anti-mouse IgG + IgM antibody diluted 1:400 with 50 mM sodium phosphate buffer (pH 7.3) (buffer I) was distributed in each well of the EIA/RIA plates, diluted 1:400 with 50 mM sodium phosphate buffer (pH 7.3) containing 0.9% sodium chloride (buffer II), the wells were blocked with a 5% skim milk solution in buffer II (200 µl) at 37°C for 1 h. The wells were washed three times with 50 mM sodium phosphate buffer (pH 7.3) containing 0.9% sodium chloride and 0.05% (v/v) Tween 20 (buffer III), and then anti-DCA monoclonal antibody diluted 1:5,000 with 100 µl of 50 mM sodium phosphate buffer (pH 7.3) containing 0.1% gelatin and 0.9% sodium chloride (buffer IV) was added. After incubation at room temperature for 1 h, the solutions were aspirated off and the wells were washed three times with buffer III. Horseradish peroxidase-labeled antigen (4 ng) dissolved in 100 µl of buffer II and either standard DCA solutions for calibration or the fractions collected for ELISA as described above were then added, and the samples were incubated at room temperature for 3 h. After washing, bound enzyme activity on the plate was measured colorimetrically using a substrate solution (100 µl) containing 0.04% o-phenylenediamine dihydrochloride and 0.018% H2O2. After incubation at room temperature for 60 min, the enzymatic reaction was terminated by adding 100 µl of 1 M H2SO4. The absorbance at 492 nm was measured using an MPR A4I microplate reader (Tosoh, Tokyo, Japan).

CA, DCA, and CDCA are present in the rat brain

After the addition of guanidine, commonly used to unfold proteins in solution (24), the rat brain cytoplasmic fraction was added dropwise to ethanol and the supernatant was subjected to LC/ESI-MS analysis. This extraction procedure, using a large quantity of guanidine, gave rise to remarkable results (Fig. 1A) in which a major peak (peak c) corresponding to the retention time of DCA was observed, along with minor peaks (peaks a and b) corresponding to the retention times of CA and DCA, respectively. In contrast, when a standard extraction procedure was used, either a solid-phase extraction cartridge coated with BSA for serum bile acids (25) or ethanol alone for neurosteroids (26), chromatograms of the resulting extracts displayed only peaks a or b, respectively (Fig. 1B, C). None of these three extraction procedures, however, yielded any other bile acids or their amino, sulfuric, or glucuronic acid conjugates.

Under various ionization conditions, the ESI mass spectrum and isotopic distribution of deprotonated molecules for the major peak c was identical to that of the CDCA standard (Fig. 2). Furthermore, the exact mass (C24H38O4) of peak c, as determined by high-resolution MS, matches the calculated mass of deprotonated CDCA. Bile acids have a characteristic chromatographic behavior attributable to the number and position of the hydroxyl group on the steroid nucleus, which is very useful for the identification of trace amounts of these compounds in biological fluids (27). Thus, the fractions corresponding to peaks a, b, and c were collected and subjected to LC/ESI-MS analysis using three mobile phases at different pH values. The Rf values of peaks a, b, and c relative to that of the IS, 12-oxo-lithocholic acid, were identical to those of CA, DCA, and CDCA, respectively (Table 1). Further confirmation of the identity of peak b was provided by ELISA, which demonstrated its high affinity to an anti-DCA monoclonal antibody (23). The amount of DCA in the sample, as measured by ELISA (0.06 nmol/g wet weight of brain) was comparable to the result obtained by LC/ESI-MS. These
results clearly identify the chromatographic peaks a, b, and c as CA, DCA, and CDCA, respectively.

**Determination of the identified bile acids in the brain**

Bile acids exist in the cerebral blood flow, and it is possible that simple diffusion from blood to brain is responsible for the existence of brain bile acids. Therefore, we compared the bile acid levels determined here in the brain to previously reported serum levels. As shown in Table 2, CDCA was the major bile acid present in the rat brain cytoplasmic fraction, with CA and DCA present at significantly lower levels. No other unconjugated or amino acid-conjugated bile acids were detected in this fluid. Intra- and inter-day assay validation was carried out using the rat brain cytoplasmic fraction spiked with 0.3, 3.0, or 30 ng of each of three unconjugated bile acids. All of the tested bile acids were recovered efficiently in a range from 98.7% to 102.4%. Although the absolute concentrations of brain bile acids varied from one animal to another, the amount of CDCA found in the rat brain tissue was up to 30-fold higher than that seen in 1 ml of serum.

**DISCUSSION**

The present study demonstrates that unconjugated CA, DCA, and CDCA exist in the rat brain. Of these, CDCA is the most abundant, being present at 10-fold the concentration of pregnenolone, which is known to be the most abundant neurosteroid (29). The brain levels of CA and DCA are 1/14th and 1/25th that of CDCA, respectively, but they are still higher than that of a typical neurosteroid, dehydroepiandrosterone. The factors contributing to the wide variation in brain bile acid levels between individual animals are not known. Although some neurosteroids are present in a sulfuric acid-conjugated form (5, 6, 8, 9), neither bile acid sulfates nor their glucuronides were detected. It is noteworthy that, in the brain, only unconjugated bile acids are observed, whereas bile acids exist in serum in both unconjugated and taurine-conjugated forms (28). Several bile acids, such as 6-hydroxylated α-muricholic, β-muricholic, and hyodeoxycholic acids, which are abundant in rat serum (28), are not found in the brain. These observations, taken together, suggest that bile acids are transported to the brain by a mechanism other than simple diffusion.

Coating of the solid-phase extraction cartridge with BSA increases the efficiency of bile acid extraction from

**TABLE 1. Relative retention factors of bile acids in the rat brain cytoplasmic fraction**

<table>
<thead>
<tr>
<th>Bile Acid or Peak</th>
<th>Relative Retention Factor Values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 4.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Peak a</td>
<td>0.37</td>
</tr>
<tr>
<td>CA</td>
<td>0.37</td>
</tr>
<tr>
<td>Peak b</td>
<td>1.68</td>
</tr>
<tr>
<td>DCA</td>
<td>1.68</td>
</tr>
<tr>
<td>Peak c</td>
<td>1.47</td>
</tr>
<tr>
<td>CDCA</td>
<td>1.47</td>
</tr>
</tbody>
</table>

CA, cholic acid; DCA, deoxycholic acid; CDCA, chenodeoxycholic acid.

<sup>a</sup> Twenty millimolar ammonium acetate buffer (pH 4.0)/acetonitrile (53:47).

<sup>b</sup> Twenty millimolar ammonium acetate buffer (pH 5.0)/acetonitrile (11:9).

<sup>c</sup> Twenty millimolar ammonium acetate buffer (pH 7.0)/acetonitrile (2:1).
serum attributable to the facilitated transfer of bile acids from serum albumin to BSA (25). However, CDCA was detected in the rat brain cytoplasmic extract only upon treatment with a high concentration of guanidine. Protein denaturation with ethanol often causes the coprecipitation of low molecular weight biological ligands, which bind noncovalently to the proteins. Guanidine is known to promote protein unfolding (24). Hence, CDCA may strongly bind to cytoplasmic proteins in the rat brain. Bile acids incorporated into hepatocytes are thought to be transferred to the bile duct in a cytoplasmic protein-bound form (30). In human liver, this high-affinity bile acid binding protein, a member of the 3α-hydroxysteroid dehydrogenase family, assists in the rapid intracellular transport of bile acids from the sinusoidal to the canicular pole of the cell (31). In addition, an ileal bile acid-binding protein binds bile acids recovered from the intestinal lumen and may play an important role not only in intracellular transport but also more generally in the enterohepatic circulation system (32). Thus, it is reasonable to believe that a protein with a high affinity to CDCA exists in the cytoplasm of cells in the rat brain. Previously, it was reported that unconjugated and conjugated bile acids inhibit brain respiration in vitro (33, 34). Because brain oxygen uptake is reduced in patients with hepatic coma, bile acids are believed to be involved in the pathogenesis of hepatic coma (35). Therefore, binding of CDCA with proteins in the brain might protect against CDCA's deleterious effects.

It is not at all clear whether these bile acids are transported from the bloodstream through the blood-brain barrier or biosynthesized in the brain. The physicochemical properties of dihydroxy bile acids determine their membrane permeability, which is especially sensitive to protonation of a carboxyl group at the C-24 position (36), which is almost completely ionized under physiological conditions. Hepatic bile acid uptake occurs predominately via a Na⁺/taurocholate cotransporter (37), with some minor activity provided by organic anion transporter family members (38). Hepatic bile acids are secreted by the liver through the canicular membrane, which provides a major driving force for the flow of biliary fluid into the canicular space (39). The overall process of bile acid transport allows the hepatocyte to maintain a concentration gradient for bile acids, with 100- to 1,000-fold higher levels in bile than in systemic portal blood. Recently, an examination of taurocholic acid elimination after microinjection into the cerebral cortex (18) elucidated the specific mechanism for its movement across the blood-brain barrier. Moreover, organic anion transporters have also been found to be abundant in human brain (19). These recent observations support the hypothesis that bile acids are transported from the peripheral bloodstream to the central nervous system through an organic anion transporter expressed in the blood-brain barrier. These bile acids may then be returned to the peripheral blood through another transporter whose identity is unknown. Because it binds strongly to a protein in the brain, only CDCA accumulates in the central nervous system. Previous studies showing that 14C-labeled cholic and lithocholic acids injected intracerebrally rapidly disappear from the brain (40) support this hypothesis.

It has been reported that hepatic enzymes convert 3β-hydroxy-5-cholenic acid, a compound present in human biological fluids, to CDCA via a 7α-hydroxylated intermediate (17). In addition, high levels of 24-hydroxycholesterol (16), which is assumed to be a possible intermediate in the biosynthesis of 3β-hydroxy-5-cholenic acid, and 7α-hydroxylated dehydroepiandrosterone, a compound that also features a 3β-hydroxy-Δ5 structure, have also been confirmed in the brain (15). Finally, a previous study has proposed a possible route for the synthesis of lithocholic acid from cholesterol in brain tissue (41). These observations suggest that bile acids may be synthesized directly from cholesterol in the central nervous system.

In conclusion, the existence of unconjugated CA, DCA, and CDCA in the rat brain was demonstrated. Among these compounds, CDCA was found to be present at high concentrations in a noncovalently protein-bound form. These observations suggest that bile acids may play a previously unrecognized but significant role in the central nervous system.

The authors thank Dr. Hiroshi Asaba and Dr. Tetsuya Terasaki of the Graduate School of Pharmaceutical Sciences of Tohoku University for their technical assistance in the preparation of rat brain tissue and for useful discussion. This work was sup-

---

**TABLE 2. Concentration of three unconjugated bile acids in the rat brain cytoplasmic fraction and the serum**

<table>
<thead>
<tr>
<th>Brain Sample No.</th>
<th>Sex</th>
<th>CA</th>
<th>CA*</th>
<th>DCA</th>
<th>DCA*</th>
<th>CDCA</th>
<th>CDCA*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol/g tissue</td>
<td>%</td>
<td>nmol/g tissue</td>
<td>%</td>
<td>nmol/g tissue</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Male</td>
<td>0.048</td>
<td>2.3</td>
<td>0.068</td>
<td>3.3</td>
<td>1.974</td>
<td>94.4</td>
</tr>
<tr>
<td>2</td>
<td>Male</td>
<td>0.049</td>
<td>2.3</td>
<td>0.065</td>
<td>3.1</td>
<td>1.997</td>
<td>94.6</td>
</tr>
<tr>
<td>3</td>
<td>Male</td>
<td>0.020</td>
<td>2.0</td>
<td>0.017</td>
<td>1.7</td>
<td>0.983</td>
<td>96.4</td>
</tr>
<tr>
<td>4</td>
<td>Male</td>
<td>0.534</td>
<td>18.6</td>
<td>0.165</td>
<td>5.8</td>
<td>2.166</td>
<td>75.6</td>
</tr>
<tr>
<td>5</td>
<td>Male</td>
<td>0.005</td>
<td>1.7</td>
<td>0.003</td>
<td>1.0</td>
<td>0.279</td>
<td>97.2</td>
</tr>
<tr>
<td>6</td>
<td>Female</td>
<td>0.117</td>
<td>5.5</td>
<td>0.070</td>
<td>3.3</td>
<td>1.937</td>
<td>91.2</td>
</tr>
<tr>
<td>7</td>
<td>Female</td>
<td>0.033</td>
<td>1.6</td>
<td>0.061</td>
<td>3.0</td>
<td>1.999</td>
<td>95.3</td>
</tr>
<tr>
<td>Average (mean ± SD)</td>
<td>0.115 ± 0.188</td>
<td>4.9 ± 6.2</td>
<td>0.064 ± 0.052</td>
<td>3.0 ± 1.5</td>
<td>1.606 ± 0.701</td>
<td>92.1 ± 7.5</td>
<td></td>
</tr>
<tr>
<td>Serum (nmol/mL)</td>
<td>Male</td>
<td>0.231</td>
<td>58.9</td>
<td>0.107</td>
<td>27.3</td>
<td>0.054</td>
<td>13.8</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>0.052</td>
<td>3.0</td>
<td>0.701</td>
<td>92.1</td>
<td>0.188</td>
<td>4.9</td>
</tr>
</tbody>
</table>

*Results are expressed as the percentage of three unconjugated bile acids.
Serum was prepared from the brain of rat number 1.*
ported in part by grants from the Ministry of Education, Culture, Sports, Science, and Technology.

REFERENCES


