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Profile of bile acids in fetal gallbladder and meconium using liquid chromatography-tandem mass spectrometry

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ABSTRACT

Background: The primary bile acids found in meconium vary with the gestational age of the fetus and the intestinal location of the meconium. We determined the composition of bile acids in samples that were collected from the gallbladder and intestine.
Methods: The bile-acid profiles of intestinal contents and the gallbladder were obtained from nine fetuses who died from abortion or respiratory failure within 72 h after birth. Intestinal content samples were collected from seven intestinal locations. The bile-acid profiles of meconium were also obtained from seven full-term live births for comparison. The profiles were analyzed using liquid chromatography-tandem mass spectrometry.
Results: The bile acids in meconium collected from stillborn and live births were mainly chenodeoxycholic acid and cholic acid, conjugated with taurine, glycine, and sulfate. The same bile acids were found in the gallbladder, except that sulfate was not found.
Conclusions: Sulfate-conjugated bile acid is found in urine, but rarely in stool. In this study, the gallbladder bile acid contained no sulfate conjugates, but these were present in intestinal contents and meconium. These results indicate that sulfate-conjugated bile acids are not excreted into the intestine through the biliary tract but originate from swallowed amniotic fluid that contains fetal urine.

1. Introduction

Meconium is composed of 80% water and digestive juice, intestinal epithelial cells, hair, bile, and swallowed amniotic fluid. The development of bile-acid metabolism during the growth of the fetus may be reflected in the meconium, so there have been many studies into its composition [1–3]. Cholic acid (CA) and chenodeoxycholic acid (CDCA) are produced from hepatic cells, and these are conjugated with amino acids such as glycine and taurine [4,5]. Fetal bile acids are mainly conjugated with taurine, in contrast to the glycine conjugates mainly seen in adults [4,6,7]. With the exception of certain bile acids such as sulfate conjugates and deoxycholic acids (DCA), the bile, which includes bile acids, is secreted into the duodenum through the bile duct [8].

The intestinal tract, which is sterile at birth, is rapidly colonized by microflora, increasing the proportions of bacterially derived secondary bile acids such as DCA and lithocholic acid (LCA). The bile-acid profiles of gallbladder bile and meconium of newborns have been analyzed by high performance liquid chromatography (HPLC) and gas chromatography (GC/MS) [9,10]. The bile acid is mainly composed of CA, CDCA, and hyocholic acid (HCA), with the composition varying between reports [1,2,9,11]. Furthermore, conjugates of the bile acids have not been examined because analysis by HPLC or GC/MS requires the bile acids to be hydrolyzed and separated from conjugates [12,13]. In contrast, liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) enables analysis of bile acids and conjugates, reflecting physiological conditions. There have been no reports to date of the analysis of bile-acid profiles of intestinal content using LC-ESI-MS/MS: our purpose here is to elucidate the developmental process of fetal bile-acid metabolism by analyzing the bile acids of preterm or term infants.

2. Patients and methods

2.1. Patients

The infants had no hepatic or intestinal diseases and had not evacuated the meconium from their bowels. The bile-acid profiles of meconium

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and the gallbladder were obtained from 9 infants (2 full-term and 7 premature; cases 1–9) who died from abortion or from respiratory failure within 72 h after birth. Furthermore, the profiles of meconium, the first content evacuated from the rectum, from seven full-term live births were obtained for comparison (cases 10–16). Characteristics of the 16 infants are shown in Table 1. In cases 1 to 9, samples were collected from the following eight locations: gallbladder, ileum terminal, ileocecum, ascending colon, transverse colon, descending colon, sigmoid colon, and rectum. Meconium and bile samples were stored at −20 °C until the time of assay. The concentration and composition of bile acids differ with excretion time and collection location of samples. The concentrations of individual bile acids are expressed as micromoles per gram of intestinal content and meconium, and as millimoles per liter of bile. This study was approved by the Juntendo University Institutional Review Board, and informed consent was obtained from patients' parents prior to study enrollment.

2.2. Materials and reagents

Authentic reference bile acids (see Appendix A) were purchased from Sigma Chemicals (St. Louis, MO, USA) as follows: CA, glycocholic acid (GCA), taurocholic acid (TCA), CDCA, glycochenodeoxycholic acid (GCDCA), taurochenodeoxycholic acid (TCDCA), ursodeoxycholic acid (UDCA), glycoursodeoxycholic acid (GUDCA), tauroursodeoxycholic acid (TUDCA), LCA, glycolithocholic acid (GLCA), taurolithocholic acid (TLCA), DCA, glycodeoxycholic acid (GDCA), taurodeoxycholic acid (TDCA), and HCA. [2,2,4,4-d_4]-CA (d_4-CA, internal standard (IS) for unconjugated bile acids), [2,2,4,4-d_4]-GCA (d_4-GCA, IS for glycine-conjugated bile acids), and [2,2,4,4- d_4]-TCA (d_4-TCA, IS for taurine-conjugated and double-conjugated bile acids) were obtained from CDD Isotopes Inc. (Quebec, Canada). The 3-sulfates for CA, CDCA, UDCA, DCA, LCA, GCA, GCDCA, GUDCA, GDCA, GLCA, TCA, TCDCA, TUDCA, TDCA, and TLCA were synthesized using a previously reported method [14,15]. Ethanol, methanol, acetonitrile and water were of HPLC grade, ammonium acetate was analytical grade, and all were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan).

2.3. Preparation of standards

Individual stock solutions of bile acids in ethanol (10 μmol/ml) were prepared separately and stored at −20 °C. These solutions were mixed in equal amounts for the analysis of unknown samples, and 5-point calibration standard solutions (30, 100, 300, 1000, and 3000 pmol/ml) were prepared in 50% ethanol containing IS (d_4-CA, d_4-GCA and d_4-TCA 100 pmol/ml). The calibration standard solutions were stable for 4 weeks at 4 °C in analytical glass vials.

Table 1
Characteristics of infants studied.

Case	Age (days)	Sex	Gestational age	Weight (g)	Underlying disease
1	1	Unknown	15w4d	70	Acrania
2	1	Unknown	18w1d	200	Thanatophoric dysplasia
3	1	Unknown	20w6d	400	Monosomy 7
4	1	M	20w0d	360	Cerebellar hypoplasia
5	3	M	25w5d	484	Severe toxemia of pregnancy, IUGR
6	1	M	28w3d	946	21 trisomy, oligoamnios
7	1	M	31w2d	1476	Cystic kidney, oligoamnios
8	1	F	38w0d	1464	Lethal short-limbed dwarfism
9	2	F	39w0d	3090	Pulmonary hemorrhage
10	1	F	39w1d	3030	No disease
11	1	M	39w6d	3590	No disease
12	1	F	41w0d	3074	No disease
13	1	F	41w0d	3180	No disease
14	1	F	41w0d	3924	No disease
15	1	M	41w2d	3616	No disease
16	1	M	41w3d	3220	No disease

w, week; d, day; M, male; F, female; IUGR, intrauterine growth restriction.

2.4. Preparation of samples

Samples of intestinal content were thoroughly mixed then snap frozen at −20 °C until analysis. The specimen was lyophilized before use. The lyophilized intestinal contents (3–6 mg, weighed exactly) were suspended in 50% ethanol (1.0 ml). IS (0.5 ml), containing d_4-CA, d_4-GCA and d_4-TCA (200 pmol/ml in 50% ethanol), was added and the mixture heated at 90 °C for 2 h in a screw-capped glass tube. The bile acids were thoroughly extracted from the intestinal matrix by ultrasonication at room temperature for 15 min. After centrifugation, the supernatant was transferred to a glass test tube, and the pellet washed again with 50% ethanol (1 ml). Water (2.5 ml) was added to the combined extract of intestinal content. IS (0.5 ml) and water (0.75 ml) were added to bile (1 μl). Both were applied to an Agilent Bond ELUT C18 cartridge (500 mg sorbent), which had been primed with methanol (5 ml); the cartridge was successively washed with 10% ethanol (15 ml). Retained bile acids were eluted with 90% ethanol (4 ml) and evaporated to dryness under an N_2 stream at <40 °C. For LC-ESI-MS/MS analysis, the dried samples were dissolved in 50% ethanol (1.0 ml). Precipitated solids were moved by filtration through a 0.45 μm Millipore filter (Millex®-LG, Billerica, MA, USA). Aliquots (20 μl) of the above filtrate were injected directly into the LC-ESI-MS/MS instrument.

2.5. LC-ESI-MS/MS

The LC-ESI-MS/MS system is composed of a TSQ Quantum Discovery Max mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) equipped with an ESI probe and Surveyor HPLC system (Thermo Fisher Scientific). A chromatography separation column, InertSustain C18 (150 mm × 2.1 mm ID, 3 μm particle size; GL Sciences Inc., Tokyo, Japan), was employed at 40 °C. A mixture of 5 mM ammonium acetate and acetonitrile was used as the eluent, and the separation carried out by linear gradient elution at a flow rate of 0.2 ml/min. The mobile phase composition was gradually changed as follows: ammonium acetate–acetonitrile (90:10, v/v) for 0.5 min, ammonium acetate–acetonitrile (78:22, v/v) for minutes 0.5–5, ammonium acetate–acetonitrile (40:60, v/v) for minutes 5–36, ammonium acetate–acetonitrile (2:98, v/v) for minutes 36–46, and then ammonium acetate–acetonitrile (2:98, v/v) for 4 min. The total run time was 50 min.

To operate the LC-ESI-MS/MS, the spray voltage and vaporizer temperature were set at 3500 V and 330 °C, respectively. The sheath and auxiliary gas (nitrogen) pressures were set at 50 and 10 arbitrary units, respectively, and the ion transfer capillary temperature was 330 °C. The collision gas (argon) pressure and the collision energy were kept at 1.3 mm Torr and 27–55 eV, respectively, all in the negative ion mode [16].

The LC-ESI-MS/MS data for unconjugated and conjugated acids are summarized in Table 2. Typical selected reaction monitoring chromatograms of standard unconjugated and conjugated bile acids are shown in Fig. 1. Peak numbers and compounds correspond to those in Table 2.

3. Results

3.1. Concentration of total bile acids

The total bile acid concentration in each sample is shown in Table 3. The concentration of total bile acid in gallbladder samples from cases 1, 4, 6 and 8 was >600 μmol/l, but was low in the other cases. The concentration in intestinal contents was <35 μmol/g in each case. The concentration in all meconium samples from cases 10 to 16 was <35 μmol/g (Table 4).