Placental Transport of Bile Acids: Analysis of Bile Acids in Maternal Serum and Urine, Umbilical Cord Blood, and Amniotic Fluid

KOSUKE USHIJIMA, AKIHIKO KIMURA, TAKAHIRO INOKUCHI*, YASUHIKO YAMATO, KOHJI MAEDA, YASUHIRO YAMASHITA, EISUKE NAKASHIMA AND HIROHISA KATO

Summary: To investigate the role of placental transport of bile acids in fetal bile acid metabolism, such as with regard to synthesis of the unusual bile acids (1β- and 6α-hydroxylated and unsaturated bile acids), we measured the concentrations of bile acids in umbilical cord blood, amniotic fluid, maternal serum and maternal urine at delivery by means of gas chromatography-mass spectrometry. Serum and urine from healthy nonpregnant women were used as controls. We detected large amounts of unusual bile acids, especially hyocholic acid and 3β-hydroxy-Δ5 bile acids, in amniotic fluid and umbilical cord blood. The concentration of total bile acids in maternal serum was less than that of control serum and umbilical cord blood, and the concentration of total bile acids in maternal urine was higher than that of control urine and amniotic fluid. In conclusion, the fetus synthesized large amounts of unusual bile acids, and these compounds were transported from fetus to mother by placental transfer. We suggest that pregnant women may excrete large amounts of bile acids into the urine to control serum concentration of bile acids in fetus.

Key words umbilical cord blood, amniotic fluid, 1β-hydroxylated bile acid, hyocholic acid, 3β-hydroxy-Δ5 bile acid

INTRODUCTION

A variety of unusual bile acids have been detected in urine of newborn infants [1-3], amniotic fluid [4,5], meconium [1], and in the bile from the fetal gallbladder [6], e.g., 3β-hydroxy-5-cholenoic acid (Δ5-3β-ol), hyocholic acid (HCA), and 1β, 3α,7α,12α-tetrahydroxy-5β-cholan-24-oic acid (CA-1β-ol). These findings suggested the existence of some alternative pathways of bile acid metabolism in the fetal liver.

Large amounts of unusual bile acids are synthesized by the fetal liver in late gestation. These compounds are mostly transferred from the fetus to mother [7], with some being excreted into the amniotic fluid [4,5]. Polyhydroxylated bile acids are more abundant in the body fluids of the fetus than in adults [4]. Analysis of umbilical cord blood and amniotic fluid indicates that the bile acid levels are generally elevated in the fetus [4,7]. Serum bile acid concentrations may also be increased in pregnant women, with higher levels being observed in late gestation [8].

We suggest that placental transport of bile acids from fetus to mother may be related to fetal bile acid metabolism, such as synthesis of the unusual bile acids, especially 1β- and 6α-hydroxylated and unsaturated bile acids. To test this hypothesis we measured the concentrations of bile acids in umbilical cord blood, amniotic fluid, and maternal serum and urine. By the analysis of this data, we hope to elucidate some aspects of fetal bile acid metabolism.
in late gestation.

**MATERIALS AND METHODS**

**Subjects and study design**

Samples were collected from healthy pregnant women who later had healthy newborn infants (3 males and 4 females, mean birth weight: 3048 g, range 2702-3488 g) at 36-41 weeks of gestation. Maternal serum, umbilical cord blood, and amniotic fluid were obtained from 7 pregnant women (mean age: 26 years, range 22-35 years) at delivery. Maternal urine was also obtained from 5 pregnant women (mean age: 26 years, range 22-35 years) at delivery. As control subjects, we obtained serum and urine from 4 healthy nonpregnant women (mean age: 29 years, range 26-35 years). All samples were stored at -25°C before analysis. Informed consent was obtained from the pregnant women.

We divided bile acids into four groups: usual bile acids: cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), and lithocholic acid (LCA), 1α-hydroxylated bile acids: CA-1α-ol, 1α, 3α, 7α-trihydroxy-5β-cholan-24-oic acid (CACA-1α-ol), 1α, 3α,12α-trihydroxy-5β-cholan-24-oic acid (DCA-1α-ol), and 1α, 3α-dihydroxy-5β-cholan-24-oic acid (LCA-1α-ol), 6α-hydroxylated bile acid: HCA, and unsaturated bile acids: Δ5-3β-ol and 3β, 12α-hydroxy-5-cholenoic acid (Δ5-3β, 12α-diol). The concentration of each bile acid group was correlated between control serum, maternal serum and umbilical cord blood, and was also correlated between control urine, maternal urine and amniotic fluid.

**Materials and reagents**

CA, CDCA, DCA, LCA, HCA and Δ5-3β-ol were obtained from Sigma Chemical Co. (St Louis, MO, USA). CA-1α-ol, CACA-1α-ol, DCA-1α-ol, LCA-1α-ol, and Δ5-3β, 12α-diol were synthesized at the Faculty of Pharmaceutical Science of Health Sciences University in Hokkaido.

**Derivatization of bile acids for gas chromatography-mass spectrometry (GC-MS)**

Each bile acid or mixture of bile acids was converted to the methyl ester by incubation with diazomethane ether (1 ml) at room temperature for 10 min. After evaporation, the trimethylsilyl ethers were prepared by heating the residue with N-trimethylsilylimidazole (50 µl) (Tokyo Kasei, Tokyo, Japan) in acetonitrile at 38°C for 60 min. Excess reagents were evaporated in a stream of N2, and the residue was dissolved in acetone before analysis by GC-MS with selected ion monitoring (SIM).

**Quantitative analysis of bile acids in umbilical cord blood, amniotic fluid, and maternal serum and urine**

An internal standard ([2,2,3,4,4,23,23-2H6] CA) was added to each sample. All samples were applied to a Bond Elut C18 cartridge (6 ml) (Analytichem, Harbor City, CA, USA). The cartridge was washed with 5 ml of water and the bile acid conjugates were eluted with 5 ml 90% ethanol. The solvent was evaporated and the residue treated at pH 1 with 2 mol/L HCl in ethanol-acetone (1:9) (4 ml) at 38°C for 60 min. After being neutralized with 1 mol/L NaOH and evaporated, the sample was incubated with 4 ml of 50% methanol at 80°C for 16 hrs and then applied again to the Bond Elut C18 cartridge. The cartridge was washed with 5 ml of water and the bile acid conjugates were eluted with 5 ml 90% ethanol. The free bile acids were excreted with Piperidino-hydroxypropyl Dextran Gel (Shimadzu, Co., Kyoto, Japan), eluted with 5 ml of 0.1 mol/L acetic acid in 90% ethanol and converted to the methyl ester-trimethylsilyl ether (50 µl) for GC-MS analysis. After conversion of the methyl ester-trimethylsilyl ether (50 µl) the sample was added to 50 µl of acetone. Next, 1 µl of the sample was injected into the splitless injection port of the GC-MS system. The mean recovery of unconjugated bile acids was 97.8% (range 89.2-113.2%); the lowest recovery rate was observed with hyocholic acid.

**GC-MS of bile acids and related compounds**

GC-MS was performed using a Hitachi-M-80B instrument equipped with a data processing system (Hitachi M-0101; Hitachi Ltd., Tokyo, Japan). A Megabore DB-1 GC capillary column (25 m by 0.53 mm, internal diameter, glass coil; J and W Scientific, Folsom, CA, USA) was used. The temperature of the column oven was programmed to rise from 220 to 280°C at 2°C/minute; the temperature of both the injection port and the detector was 290°C. The flow rate of helium gas was 25 ml/minute. Ionization energy was set at 70 eV, multiplier voltage at 1,300 V, acceleration voltage at 3,000 V, source slit at 500 µm, and collector slit at 450 µm.

The GC-MS data for bile acid derivatives and related compounds were summarized, and a chromatogram was obtained using SIM of the characteristic fragments of the methyl ester-trimethylsilyl ether derivatives of a mixture of reference bile acids.
PLACENTAL TRANSPORT OF BILE ACIDS

TABLE 1.

Analysis of bile acids in maternal serum and umbilical cord blood

<table>
<thead>
<tr>
<th>µmol/L [%]</th>
<th>Control serum (n=4)</th>
<th>Maternal serum (n=7)</th>
<th>Umbilical cord blood (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Usual bile acids</td>
<td>9.36 ± 1.83 a, b [99.45 ± 1.10]</td>
<td>2.64 ± 1.50 [85.27 ± 20.19]</td>
<td>2.43 ± 0.50 [37.21 ± 4.93 A, B]</td>
</tr>
<tr>
<td>13-Hydroxylated bile acids</td>
<td>0.04 ± 0.08 c, d [0.05 ± 1.10 C]</td>
<td>0.16 ± 0.06 e [6.53 ± 4.64]</td>
<td>0.26 ± 0.05 [4.17 ± 1.35]</td>
</tr>
<tr>
<td>Hyocholic acid</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.22 ± 0.29 f [3.21 ± 4.18 D]</td>
</tr>
<tr>
<td>Unsaturated bile acids</td>
<td>n.d.</td>
<td>0.44 ± 1.16 [8.20 ± 21.70]</td>
<td>3.71 ± 1.40 g, h [55.34 ± 7.47 E, F]</td>
</tr>
<tr>
<td>Total bile acids</td>
<td>9.40 ± 1.75 i, j</td>
<td>3.24 ± 1.78</td>
<td>6.62 ± 1.68 k</td>
</tr>
</tbody>
</table>

a: vs. maternal serum p<0.0001, b: vs. umbilical cord blood p<0.0001, c: vs. maternal serum p=0.0041, d: vs. umbilical cord blood p<0.0001, e: vs. umbilical cord blood p=0.0094, f: vs. maternal serum p<0.0001, g: vs. control serum p=0.0001, h: vs. maternal serum p<0.0001, i: vs. maternal serum p<0.0001, j: vs. umbilical cord blood p=0.0217, k: vs. maternal serum p=0.0024, A: vs. control serum p<0.0001, B: vs. maternal serum p<0.0001, C: vs. maternal serum p=0.0076, D: vs. maternal serum p=0.0379, E: vs. control serum p<0.0001, F: vs. maternal serum p<0.0001.

TABLE 2.

Analysis of bile acids in maternal urine and amniotic fluid

<table>
<thead>
<tr>
<th>µmol/L [%]</th>
<th>Control urine (n=4)</th>
<th>Maternal urine (n=5)</th>
<th>Amniotic fluid (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Usual bile acids</td>
<td>2.83 ± 1.06 [70.45 ± 19.99]</td>
<td>6.04 ± 6.62 a [76.80 ± 6.57]</td>
<td>0.55 ± 0.17 [61.49 ± 13.59]</td>
</tr>
<tr>
<td>13-Hydroxylated bile acids</td>
<td>0.73 ± 0.62 b [15.43 ± 12.67]</td>
<td>0.63 ± 0.55 [8.98 ± 1.99]</td>
<td>0.15 ± 0.04 [16.66 ± 4.72]</td>
</tr>
<tr>
<td>Hyocholic acid</td>
<td>n.d.</td>
<td>0.04 ± 0.08 c [1.46 ± 3.27]</td>
<td>0.13 ± 0.10 [14.40 ± 10.72 A, B]</td>
</tr>
<tr>
<td>Unsaturated bile acids</td>
<td>0.64 ± 0.43 d [14.08 ± 10.46]</td>
<td>0.78 ± 0.50 e [12.80 ± 5.93]</td>
<td>0.09 ± 0.15 [7.47 ± 12.87]</td>
</tr>
<tr>
<td>Total bile acids</td>
<td>4.20 ± 1.63</td>
<td>7.49 ± 7.46 f</td>
<td>0.92 ± 0.28</td>
</tr>
</tbody>
</table>

a: vs. amniotic fluid p=0.0252, b: vs. amniotic fluid p=0.0475, c: vs. control urine p=0.0192, d: vs. amniotic fluid p=0.0305, e: vs. amniotic fluid p=0.0063, f: vs. amniotic fluid p=0.0196, A: vs. control urine p=0.0091, B: vs. maternal urine p=0.0114.

(see reference 1).

Calibration curves for the determination of bile acids were obtained by plotting the peak area ratio that corresponded to the monitored ion for each bile acid and the corresponding internal standard versus the amount of each bile acid. A linear relation (r > 0.985) was obtained over a range of 1.5 to 10 ng for each bile acid. These chromatographic responses are appropriate for the assay of bile acids in urine with the addition of adequate amounts of internal standard.

Statistical analysis

Data are reported as mean ± SD. One-way ANOVA was used to determine the significance of differences between groups. Comparisons between groups of categorical data were made using Student’s t test. A p value of less than 0.05 was accepted as statistically significant.
RESULTS

The results of this study are shown in Tables 1 and 2. Total bile acids in control serum were significantly increased as compared with that of maternal serum and umbilical cord blood ($p < 0.05$). The main bile acids in control and maternal serum were usual bile acids (94% and 85% of total bile acids, respectively), while the main bile acids in umbilical cord blood were unusual bile acids, especially unsaturated bile acids (55% of total bile acids). Unusual bile acids were also detected in maternal serum, such as 1β-hydroxylated and unsaturated bile acids.

Total bile acids in maternal urine were higher than in control urine and amniotic fluid. In control and maternal urine and amniotic fluid, the main bile acids were usual bile acids (>61% of total bile acids). We detected 6α-hydroxylated bile acid (HCA) in maternal urine and amniotic fluid, however we did not detect any in control urine.

DISCUSSION

Our results demonstrated that the profiles of the various bile acids in control serum and urine were quite different from those of maternal serum and urine and those of umbilical cord blood and amniotic fluid. Unusual bile acids in umbilical cord blood and amniotic fluid accounted for almost 60% and 40% of total bile acids, respectively. Therefore we speculate that large amounts of unusual bile acids, especially HCA, Δ^3-3β-ol, and Δ^5-3β, 12α-diol, are synthesized by the fetal liver and are then transported from the fetus to mother by placental transfer [7].

Comparing the profiles of bile acids of maternal serum with those of umbilical cord blood, we speculate that 1β-hydroxylated and unsaturated bile acids may pass through the placenta more easily than 6α-hydroxylated bile acid, HCA. On the other hand 6α-hydroxylated bile acid may be excreted into the amniotic fluid by the human fetal kidney more easily than other unusual bile acids. Most of the bile acids synthesized by the human fetal liver were transported from the fetus to mother by placental transfer, because fetal capacity to excrete bile acid into the amniotic fluid remains immature until near fullterm. However, it is said that production of urine by the human fetus increased after 30 weeks of gestation [9,10]. We suggest, therefore, that the large amount of usual, 1β-hydroxylated and unsaturated bile acids that were transferred from the fetus to mother were excreted into the urine by the mother.

Based on the concentrations of total bile acids, we suggest that pregnant women excrete large amounts of bile acids into urine and that these consisted bile acids synthesized by these own hepatocytes as well as those transferred from fetus to mother by placenta. In fact, we detected large amounts of total bile acids in maternal urine and small amounts of total bile acids in maternal serum (Tables 1 and 2). Therefore, we speculate that serum concentration of bile acid in fetus is controlled by this placental transfer system. Certainly pregnant women also excrete bile acids into the biliary bile. If a pregnant woman has liver and/or kidney functional disorder at late gestation, fetus may develop fetal distress because of elevated maternal serum concentration of bile acids [11].

In conclusion, the fetus synthesized large amounts of unusual bile acids, such as 1β- and 6α-hydroxylated and unsaturated bile acids, and these compounds were transported from fetus to mother by placental transfer. We suggest that pregnant women may excrete large amounts of bile acids into the urine to control the serum concentration of bile acids in the fetus.

REFERENCES


