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Biliary Bile Acid Composition of the Human Fetus in Early Gestation

C. COLOMBO, G. ZULIANI, M. RONCHI, J. BREIDENSTEIN, AND K. D. R. SETCHELL

Department of Pediatrics and Obstetrics, University of Milan, Milan, Italy [C.C., G.Z., M.R.] and Department of Pediatric Gastroenterology and Nutrition, Children's Hospital Medical Center, Cincinnati, Ohio 45229 [J.B., K.D.R.S.]

ABSTRACT. Using analytical techniques, which included capillary column gas-liquid chromatography and mass spectrometry, detailed bile acid profiles were obtained for 24 fetal bile samples collected after legal abortions were performed between the 14th and 20th wk of gestation. Qualitatively, the bile acid profiles of all fetal bile samples were similar. The predominant bile acids identified were chenodeoxycholic and cholic acid. The presence of small but variable amounts of deoxycholic acid and traces of lithocholic acid suggested placental transfer of these bile acids from the maternal circulation. 3β-Hydroxy-5-cholenoic acid was detected at higher levels than lithocholic acid. A conspicuous feature of the profiles was the presence of bile acids with hydroxyl groups at positions C-1 and C-6, and one other nuclear position of unknown origin, indicating fetal hepatic synthesis via pathways different from those normally seen in the adult. Quantitatively total biliary bile acid concentrations were extremely low (<0.05 mM) before wk 17 of gestation, but thereafter concentrations markedly increased reflecting a possible surge in bile acid synthesis; however, the ratio of cholic:chenodeoxycholic acids remained relatively constant over this period (mean ± SD = 0.85 ± 0.36) and different from that reported for the healthy newborn (*ca.* 2.5) and adult (*ca.* 1.6). These data indicate an immaturity in hepatic 12α-hydroxylation of bile acids during early development and may explain why other pathways, in particular 1β and 6α-hydroxylation, are activated at this stage of life. (*Pediatr Res* 21: 197–200, 1987)

Abbreviations

Me-TMS, methyl ester-trimethylsilyl
GLC, gas-liquid chromatography
MS, mass spectrometry

Only limited studies have been performed in the human infant to define the development of bile acid synthesis and metabolism during early life (1–10) and these have been the subject of reviews, (11, 12). A greater insight of the ontogeny of bile acid metabolism is essential to aid in the understanding of nutritional problems related to fat malabsorption in the normal, and above all, the premature newborn. A state of physiologic cholestasis has been defined during the first months of life (13–15), but the exact mechanisms responsible are not completely understood, save the demonstration in animal models of an immaturity of several of the key processes of the enterohepatic circulation of bile acids (16–19). As a result of physiologic cholestasis, the newborn infant has a tendency to develop a true neonatal cholestasis when subjected to such stresses as sepsis, hypoxia, and total parenteral nutrition (15).

Abnormalities in bile acid metabolism have been suggested to be a factor in the development of certain forms of cholestasis in the newborn (20) and in animal models the hepatotoxic nature of bile acids has been well established (21–24). A greater understanding of the etiology of these conditions requires a better knowledge of bile acid metabolism in the human fetus and in the newborn infant.

Previous studies of bile acid concentrations of maternal and fetal serum in early gestation suggest that the synthesis of bile acids is reasonably well developed. This study extends these previous observations to examine the bile acid composition of human fetal bile during the same period of gestation, but utilized more sophisticated analytical techniques, including capillary GLC-MS (26–32).

EXPERIMENTAL

Collection of fetal bile. Gallbladders were obtained from 24 human fetuses immediately after legal abortion, performed between the 14th and 20th wk of gestation in Italy. All of the pregnancies were uncomplicated until the time they were interrupted and none of the mothers had obvious organic diseases (including gastrointestinal and hepatic abnormalities). Fetal age was assessed on the basis of clinical history as well as by means of ultrasound. All abortions were performed through suction and none of the mothers received prostaglandins, phenobarbital, or other drugs know to influence bile acid metabolism. After the liver was removed, the cystic duct was tied and the gallbladder was immediately isolated. Gallbladder bile was then aspirated using a 100-μl syringe and the volume of bile was recorded in each case and frozen within 5 min of completion of the abortion. Bile acids were isolated from the bile samples by liquid-solid extraction (26) as described below and analyzed by previously reviewed techniques (26–29).

Analysis of fetal bile. Fetal bile (10–100 μl) was diluted in 1 ml with distilled water and 0.1 M sodium hydroxide (4 ml) was added. The sample was heated to 64° C and bile acids and their conjugates were extracted using reverse phase octadecylsilane bonded silica cartridges (Bond Elut) as described previously (26, 29). Conjugated bile acids were hydrolyzed, first by solvolysis at 37° C for 24 h in a mixture of methanol (9 ml):acetone (1 ml):6 N HCl, 3 drops followed by an overnight enzymatic hydrolysis at 37° C with cholylglycine hydrolase from *Clostridia perfringens* (50 U in 2 ml of 0.1 M phosphate buffer pH 5.8). The pH of the hydrolysate was adjusted to pH 3–4 and passed through a column of Lipidex 1000, where unconjugated (or hydrolyzed) bile acids are retained by the gel (26, 30). Elution of the gel with 68%

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methanol (20 ml) afforded a means of recovering bile acids, separately from monohydroxy sterols which are retained in this reverse phase solvent system. After addition of an internal standard (1–10 μg coprostanol depending on sample size) the Me-TMS ether derivatives were prepared and the derivative purified by passage through a small column of Lipidex 5000 as detailed elsewhere (26).

Capillary column GLC. Bile acids were analyzed as their Me-TMS ethers by capillary column GLC on a 30-m DB-1 chemically bonded fused silica column using temperatured programed conditions from 225–295° C with increments of 2° C/min after an initial isothermal period of 5 min. Helium was used as carrier gas (2 ml/min) and samples were injected using an all glass dropping needle injector.

Biliary bile acids were quantified by capillary GLC from the ratio of peak height response to the peak height response for the internal standard, coprostanol (26). The sensitivity of the technique is sufficient to detect a bile acid with a concentration of 0.01–0.02 μmol/liter (26).

Capillary column GLC-MS. GLC-MS was performed using a Finnigan 4635 Quadrupole GC-MS system, housing an identical GLC column. The GLC effluent was continuously scanned over the mass range 50–800 D (2 s/cycle) and spectra were generated by electron impact ionization (70 eV). Identification of a bile acid was made on the basis of its complete mass spectrum as compared with an authentic standard and from the GLC retention data. Where authentic compounds were unavailable the identification was based on the predicted fragmentation patterns for the structure (31, 32).

RESULTS

Qualitative analysis. Figure 1 shows a typical GLC profile of the biliary bile acids from the gallbladder bile of an 18-wk fetus. Qualitatively all samples of fetal bile analyzed revealed similar profiles. The predominant bile acids identified were chenodeoxycholic and cholic acids. The principal secondary bile acids of adult man, deoxycholic and lithocholic acids, were not present in all samples and when detected, were in low and variable amounts. 3β-Hydroxy-5-cholenoic acid was always present at low levels but in excess of lithocholic acid.

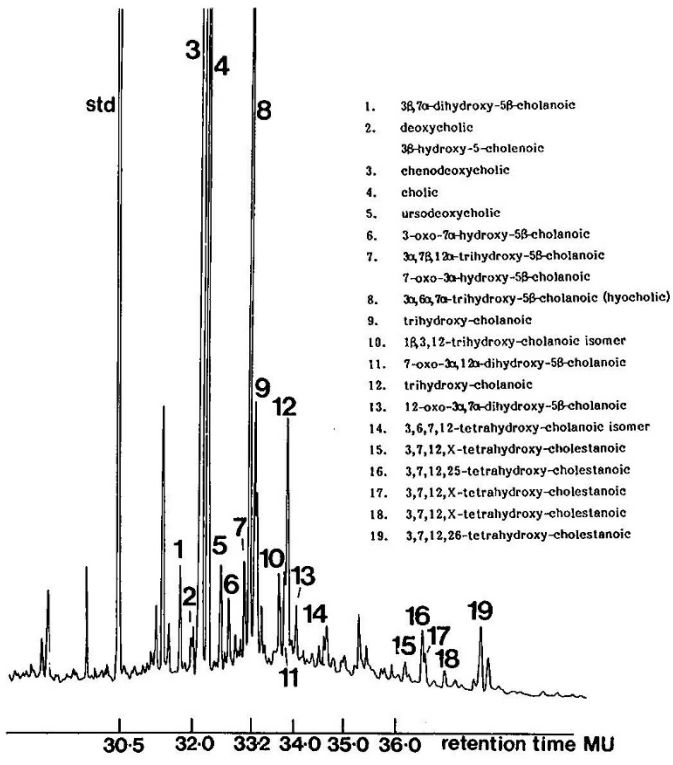

Fig. 1. A typical GLC profile for the total bile acids of human fetal bile obtained at the 18th wk of gestation. Bile acids were analyzed as their Me-TMS ether derivatives on a 30 m DB-1 chemically bonded open tubular capillary column by temperature programed operation.

A conspicuous feature of all profiles was the presence of a wide range of atypical bile acids of which hyocholic (3α,6α,7α-trihydroxy-5β-cholanoic) and a trihydroxy-bile of unknown structure occurred in significant amounts, and together these frequently exceeded the amounts of cholic acid. The mass spectra, the Me-TMS ether derivative of this unknown bile acid is shown in Figure 2. The ions at *m/z* 548, 458, 368, and 253 are characteristic of a trihydroxy-5β-cholanoic structure and the significant ion at *m/z* 369 suggests that two of the hydroxyls are on adjacent carbons (31, 32). The significant and prominent ion at *m/z* 181 may arise from a disubstituted A or B ring structure, but the spectra is not typical of 1-hydroxylated structures, all of which exhibit a diagnostically significant ion at *m/z* 217, nor is it similar to reported spectra of 2β-hydroxy bile acids (7, 32). Bile acids with 3,6,7-trihydroxy structures have characteristic ions at *m/z* 195 and 285, not seen in this mass spectrum. An unidentified bile acid with the same series of ions was found in the gastric aspirate from newborn infants (7, 8). This mass spectra does not match any of the 98 spectra of bile acids recently compiled (32) and further work is required to elucidate the structure of this quantitatively important compound.

A number of trihydroxy and tetrahydroxy bile acids were present but lack of available authentic compounds makes it difficult to determine their exact structures. Finally, a series of oxo bile acids and tetrahydroxy-cholestanoic acids, the latter with side chain hydroxy groups, were evident.

Quantitative analysis. The concentration of the principal bile acids, cholic and chenodeoxycholic acids measured in fetal bile, obtained between the 14th and 20th wk of gestation, are shown in Figure 3. Values for both bile acids prior to wk 16 of gestation were relatively constant and below 0.05 mM (mean ± SD values for cholic acid, 0.020 ± 0.016 mM, and for chenodeoxycholic 0.031 ± 0.023 mM, $n = 6$); however, between the 17th and 20th wk there was a marked increase in the biliary concentration of both bile acids (range 0.026–0.317 mM for cholic acid and 0.017–0.563 mM for chenodeoxycholic acid) reflecting an increase in the range of 20-fold by the 20th wk of gestation.

In general chenodeoxycholic acid concentrations exceeded those of cholic acid in bile, but the ratio of the cholic:chenodeoxycholic acid concentrations although variable between samples, remained relatively constant over this period of gestation (Fig. 4). Excluding the two samples obtained from wk 14, which were small in volume and concentration, the mean ratio (±SD) for all bile samples irrespective of the gestational age was 0.85 ± 0.36.

DISCUSSION

There are very little data on biliary bile acid composition in the human fetus, and studies which have examined this issue (2, 4) were carried out in the early 1960s when analytical techniques were less advanced than those now available. Poley *et al.* (2) in 1964 described for the first time the biliary bile acid composition of three human fetuses at age 22–28 wk of gestation and reported a predominance of chenodeoxycholic acid compared with cholic acid up to the 28th wk of gestation. Furthermore, bile acids were predominantly conjugated to taurine while no secondary or unconjugated bile acids were found. The presence of other compounds in the bile was recognized but with the relatively crude procedures used in this study (2) their identification was not possible. Sharp *et al.* (4) later confirmed these findings using a conventional GLC technique, but the gestational age of the 14 fetuses considered in this study was not defined.

At birth, in the newborn infant, cholic acid has been reported to be the major biliary bile acid (1, 3). This is also true for adults

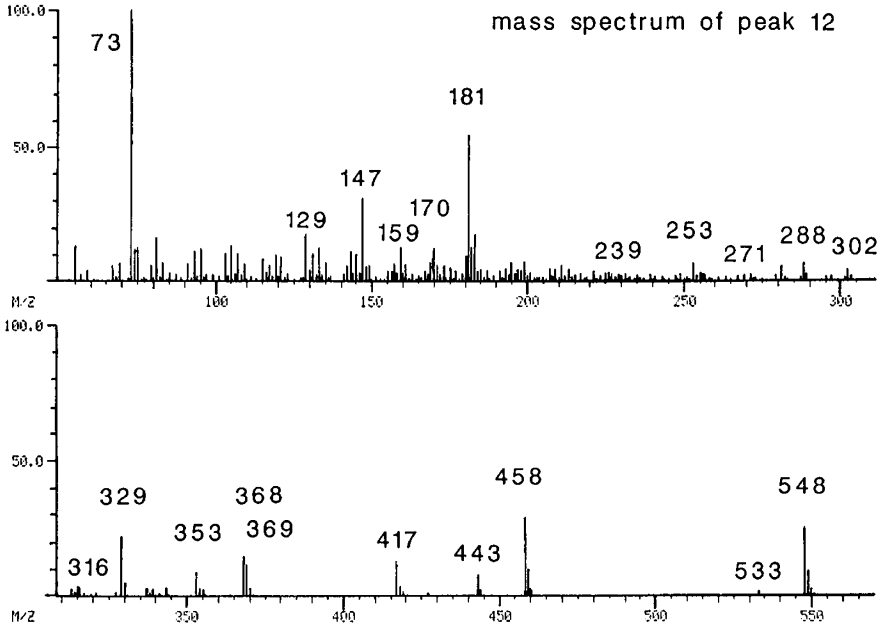

Fig. 2. MS (electron impact ionization, 70eV) for the Me-TMS ether derivative of a trihydroxy bile acid derivative eluted from the GLC column with retention time 32.73 MU.

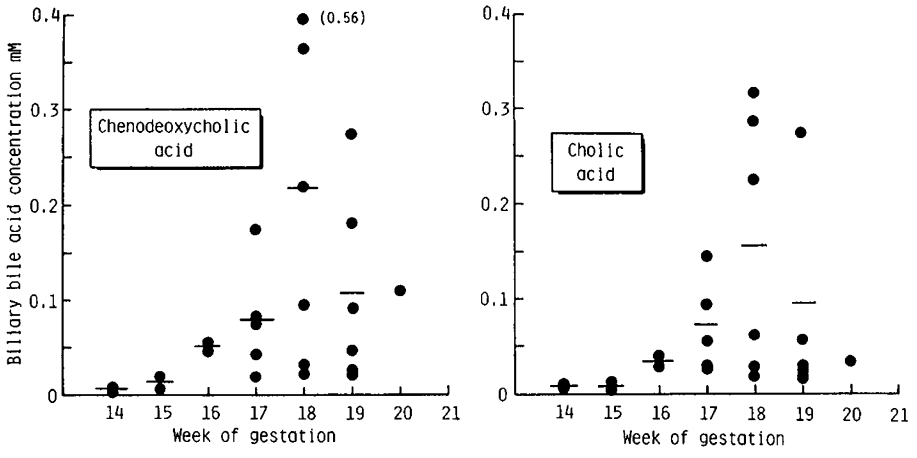

Fig. 3. Concentrations for chenodeoxycholic acid and cholic acid in samples of human fetal bile obtained between wk 14 and 20 of gestation.

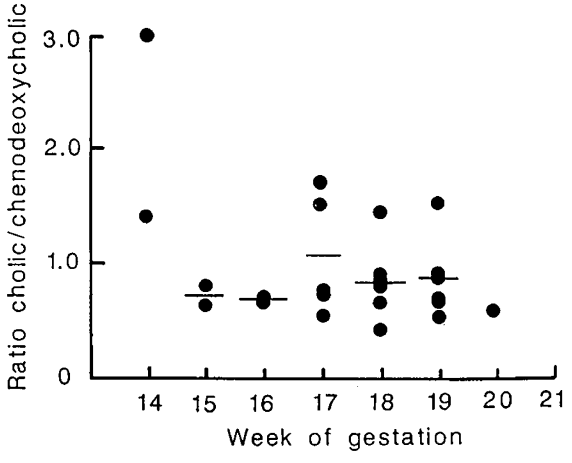

Fig. 4. The ratio of cholic:chenodeoxycholic acid concentrations in human fetal bile obtained between wk 14 and 20 of gestation.

(33). Our data are in agreement with the gross findings of these previous studies. Chenodeoxycholic acid was the predominant bile acid in fetal bile obtained from the 14th to 20th wk of gestation and secondary bile acids were not present in large amounts during this period. In our study, however, owing to the high sensitivity and chromatographic resolution of the analytical procedure, the bile acid profile of fetal bile is described for the first time. It is evident when these chromatograms (see Fig. 1) are compared with those obtained from low resolution conventional packed column GLC analysis that the application of these procedures is necessary to provide a more accurate definition of metabolism during development.

These preliminary observations revealed that 3β-hydroxy-5-cholenoic acid and lithocholic acid were present only in trace amounts in fetal bile, despite these two bile acids having been found to be major components of meconium (34–36), but nevertheless consistent with a previous report using radioimmunoassay, which found relatively low concentrations of glycolithocholic sulfate in fetal serum (26).

Furthermore, fetal bile is characterized by the presence of many "atypical bile acids," which possess hydroxyl groups at positions C1, C6, and one other position of unknown origin but in the steroid nucleus. 1-Hydroxy and 6-hydroxy bile acids are not normally found in the bile of healthy adults, but have been found in meconium (35, 36) and are therefore most likely to be of fetal hepatic origin. This is supported from a recent study where fetal hepatic microsomes *in vitro* were found to efficiently 1β-hydroxylate deoxycholic acid (10). Hyocholic acid (3α,6α,7α-trihydroxy-5β-cholanoic acid) was the major atypical bile acid

identified in fetal bile and this is also the case for meconium (35, 36). In this study, no attempt was made to search for short-chain bile acids which have been recently identified in meconium and in the serum of patients with cholestatic syndromes (37–39) or to determine the mode of conjugation of the individual bile acid species; this is the focus of ongoing studies. It is of interest to note that bile acid metabolism in adults with severe cholestatic liver disease, which has been well documented (40), shows striking similarities to that seen in this study of the normal fetus.

From a quantitative point of view these data reveal for the first time in the human fetus a marked increase in biliary bile acid concentration from the 17th wk of gestation which may be accounted for by an increase in hepatic synthesis. It would be of interest to know what the key determinants are in triggering the surge in biliary bile acid concentrations in early gestation. In a previous report, no correlation between serum bile acid concentrations and gestational age was found and this may be explained by a rapid equilibration of bile acids between the fetal and maternal compartments (25).

Finally, the finding of a lower ratio of cholic:chenodeoxycholic acid [mean = 0.85 ± 0.36 (SD)] in fetal bile compared with the bile of newborn infants [approximate ratio 2.5 (1)] and adults (approximate ratio 1.6) appears to indicate an immaturity of the 12α-hydroxylase enzyme system in the fetal liver (9). Since the cholic:chenodeoxycholic acid ratio remained relatively constant over the period of gestation considered in the study, this suggests that the activity of this enzyme must increase significantly in late gestation to account for the finding of an excess of cholic acid in newborn infant bile. This immaturity may account for the activation of other biosynthetic pathways, in particular 1β- and 6α-hydroxylation of bile acids in fetal life. Studies in animals have shown a marked increase in hepatic enzymes concerned with bile acid synthesis during the perinatal period (41) and a rapid expansion of the bile acid pool (42). Our studies confirm that the development of bile acid synthesis in the human fetus proceeds quantitatively in a similar fashion and that qualitatively when compared to the adult human there are significant differences in the pathways of bile acid synthesis.

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