SYNTHESIS OF 6-HYDROXYLATED BILE ACIDS AND IDENTIFICATION OF 3α,6α,7α,12α-TETRAHYDROXY-5β-CHOLAN-24-OIC ACID IN HUMAN MECONIUM AND NEONATAL URINE

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Three 6-hydroxylated bile acids, 3α,6α,7α,12α-, 3α,6β,7α,12α- and 3α,6β,7β,12α-tetrahydroxy-5β-cholan-24-oic acids, were synthesized from methyl cholate, and a sensitive method was developed for analyzing them by gas chromatography-mass spectrometry for the stoichiometric study of fetal bile acids. 3α,6α,7α,12α-Tetrahydroxy-5β-cholan-24-oic acid (6α-hydroxylated cholic acid) was identified from human meconium and healthy neonatal urine by comparison with the mass spectrum of the reference compound. In human meconium, 6α-hydroxylated cholic and chenodeoxycholic acids were determined in 1.2% and 29.0% of the total bile acids, respectively. We discuss the significance of hydroxylation at the C-1β and C-6α positions of bile acids and their elimination in fetal and neonatal periods.

KEYWORDS 6-hydroxylated bile acid synthesis; 3α,6α,7α,12α-tetrahydroxy-5β-cholan-24-oic acid; 1β-hydroxylated bile acid; fetal bile acid; GC-MS; methyl ester-trimethylsilyl ether; human meconium; neonatal urine

There are reports of the presence of 6-hydroxylated cholic acids (CA-6-ol) in the urine of patients with liver diseases[1] and in the gastric contents of neonates with duodenal atresia.[2] This suggests that cholic acid (CA) can be metabolized to 6α-hydroxylated cholic acid (CA-6α-ol) through a pathway similar to that of chenodeoxycholic acid (CDCA) metabolized to hyocholic acid (HCA).[3] Back *et al.*[4] isolated a small amount of CA-6α-ol from the urine of patients with intrahepatic cholestasis, and studied its structure by nuclear magnetic resonance spectroscopy. However, the study of the biosynthesis and significance of CA-6α-ol in hepatobiliary diseases has been hindered by the unavailability of the reference compounds. Chemical synthesis of CA-6α-ol was first reported by Bremmelgaard and Sjövall[5] in micro scale but no data were given except its mass spectrum. Here we wish to report the synthesis of CA-6α-ol (3α,6α,7α,12α-tetrahydroxy-5β-cholan-24-oic acid) and its stereoisomers, and the identification of the bile acid in human meconium and healthy neonatal urine by gas chromatography-mass spectrometric (GC-MS) analysis.

Three CA-6-ols were synthesized from methyl cholate (**1**) as a starting material according to the reaction scheme in Chart 1. First, the 6α-bromo-7-ketone (**2**)[6] was substituted for a hydroxyl group with potassium hydroxide in methanol to give the α-ketol (**3**), and subsequent reduction with sodium borohydride and alkaline hydrolysis afforded CA-6α-ol, mp 121-124°C/161-163°C, $[\alpha]_D^{26}$ +23.3° (c=0.47, MeOH). The other isomers were also synthesized by the following procedure. The bromoketone (**2**) was converted into the bromohydrin (**4**) with sodium borohydride, and then treated with zinc-powder in acetic acid to give the Δ^6-cholenate (**5**). Epoxidation of **5** with m-chloroperbenzoic acid gave two isomeric α- and β-epoxides (**6**), which were easily converted into the desired 3α,6β,7α,12α-tetrahydroxy-5β-cholan-24-oic acid (CA-6β-ol), mp 165-167°C, $[\alpha]_D^{26}$+36.3° (c=0.52, MeOH), with the trans-fission of both the epoxides by acid catalyzed hydrolysis and subsequent alkaline hydrolysis. Finally, treatment of the Δ^6-cholenate (**5**) with silver acetate-bromine and successive hydrolysis in the presence of silver ion gave 3α,6β,7β,12α-tetrahydroxy-5β-cholan-24-oic acid (**7**),[7] mp 240-242°C, $[\alpha]_D^{26}$ +68.5° (c=0.64, MeOH) in a reasonable yield after alkaline hydrolysis. The structures of CA-6α-ol, CA-6β-ol and **7** were determined by the comparing the proton nuclear magnetic resonance spectra in pyridine-d_5: 1.01, 1.52 and 1.45 ppm for 19-CH_3 indicated the deshielding effect of the 6β-hydroxyl group, and 4.22 (bs), 4.30 (bs) and 3.85 ppm (dd, J=3 and 11

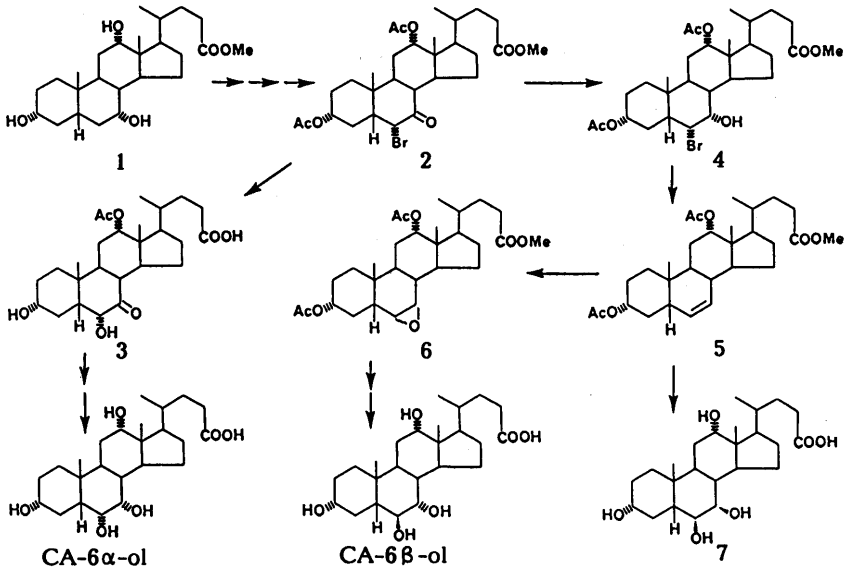

Chart 1

Hz) for 7-H coupled with 8β-axial-H, respectively, and the mass spectra also supported their structures as the methyl ester-trimethylsilyl ether derivatives (Fig. 1). The mass spectra of CA-6α-ol and 6β-isomeric derivatives are almost identical with those of the 6-hydroxylated bile acids proposed by Bremmelgaard and Sjövall.[5]

The glycine, taurine and sulfuric acid conjugates of these bile acids were also prepared according to the previous method[8] in order to develop a quantitative assay of them.

A sensitive and specific quantitative assay for these bile acids and related compounds in human biological fluids has been developed by selected ion monitoring of the characteristic fragments at m/z 367 for CA-6α-ol and m/z 341 for CA-6β-ol and m/z 285 for the 6β,7β-isomer, and the fragment ions assigned previously for the related compounds in GC-MS analysis (Fig. 2), using [2,2,3,4,4,23,23-$^2H_7$] CA and three other deuterated bile acids as internal standards.[9] Prior to GC-MS analysis, biological samples were treated in the similar manner reported previously and the extracted bile acids were derivatized into the methyl ester-trimethylsilyl ethers. This method gave good linearity on the calibration curve over the range of 0.1-10 ng for each bile acid, and reasonable recoveries of 90-101% of the free and conjugated bile acids.

Profile analysis of the bile acids in human meconium and healthy neonatal urines were performed by the developed GC-MS method, and the results are summarized in Table I. CA-6α-ol in meconium was determined in only a small percentage (1.2%) of the total bile acids, accompanied with a trace of CA-6β-ol, whereas HCA (CDCA-6α-ol) appeared predominantly (29.0%) together with the 1β-hydroxylated bile acids,[10] CA-1β-ol (6.3%) and CDCA-1β-ol (6.7%). In neonatal urine, CA-6α-ol and HCA have been found respectively in trace amounts

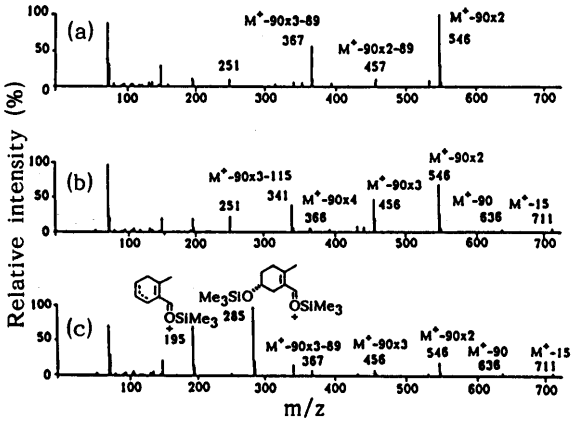

Fig. 1. Mass Spectra of the Methyl-Trimethylsilyl Derivatives

(a) 6α-Hydroxycholic acid.
(b) 6β-Hydroxycholic acid.
(c) 3α,6β,7β,12α-Tetrahydroxy-5β-cholan-24-oic acid (7).

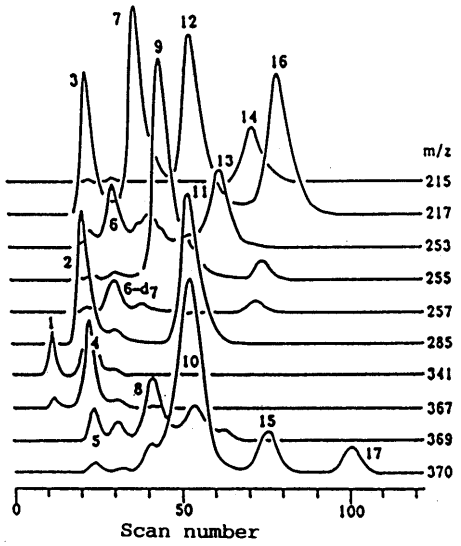

TABLE I. Bile Acids in Fetus and Neonates

Bile acid	Meconium μg/mg (%)	Urine μg/ml (%)
CA	0.70 (27.0)	0.34 (15.2)
CDCA	0.42 (10.3)	0.27 (11.8)
CA-6α-ol	0.03 (1.2)	trace
CDCA-6α-ol (HCA)	0.75 (29.0)	0.12 (5.2)
CA-6β-ol	trace	n.d.
CDCA-6β-ol	n.d.	n.d.
3α,6β,7β,12α-ol	n.d.	n.d.
CA-1β-ol	0.16 (6.3)	1.00 (44.5)
CDCA-1β-ol	0.17 (6.7)	0.27 (12.1)
Total ± S.D.	2.59 ± 1.36	2.25 ± 0.78

Fig. 2. GC-MS of the Methyl-Trimethylsilyl Derivatives of the Bile Acid Standards

GC-MS was performed on a Shimadzu-LKB 9000-PAC 300M. GC column packed with 1.5% Poly I-110 on Gas Chrom Q (2m x 2.5mm i.d. glass coil) was used at 250°C and MS were recorded at 70eV. 1. CA-6β-ol, 2. 3α,6β,7β,12α-ol, 3. CA-1β-ol, 4. CA-6α-ol, 5. CDCA-6β-ol, 6. CA, 7. DCA-1β-ol, 8. HCA, 9. DCA, 10. CDCA, 11. UDCA-6β-ol, 12. CDCA-1β-ol, 13. Δ^5- 3β,12-diol, 14. LCA, 15. UDCA, 16. LCA-1β-ol, 17. Δ^5-3β-ol.

and only 5.2% of the total bile acids. But CA- and CDCA-1β-ols were the predominant bile acids, accounting for 44.5% and 12.1%, respectively. These observations are recognized by assuming that the 6α-hydroxylation of CA was inhibited in contrast to the formation of HCA from CDCA, and CA was predominantly hydroxylated at the C-1β position in fetal and neonatal liver.

These results suggest that the hydroxylation at the C-1β and C-6α positions of the usual bile acids in liver might be carried out to eliminate the bile acids in fetus-neonatal periods. Further, the enzymes concerned with these hydroxylations have different specificities for their substrates, and the 1β- and 6α-hydroxylated products tend to be excreted selectively into meconium (feces) or urine. A more detailed investigation will be reported in the near future.

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