REDUCTIVE BIOTRANSFORMATION OF 3-OXO BILE ACIDS IN HUMAN BLOOD

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7α,12α-Dihydroxy-3-oxo-5β-cholanoic acid labeled with ^{18}O atoms was incubated with human blood, and the biotransformation products were separated and characterized by gas chromatography-mass spectrometry as the pentafluorobenzyl ester-trimethylsilyl and -dimethylethylsilyl ether derivatives. 3β,7α,12α-Trihydroxy-5β-cholanoic acid was identified as a main metabolite. When 3-oxo bile acid was incubated with human blood denatured at 70°C for 2 min, no metabolites were formed. The enzymic reduction activity proved to be localized in the red blood cell fraction.

KEYWORDS 3-oxo bile acid; 3β,7α,12α-trihydroxy-5β-cholanoic acid; dehydrocholic acid; enzymic reduction; extrahepatic biotransformation; red blood cell; gas chromatography-mass spectrometry; negative ion detection; ^{18}O-labeled bile acid

It is sufficiently substantiated in the literature that 3,7,12-trioxo-5β-cholanoic acid (dehydrocholic acid), widely used as a choleretic, is metabolized by the hepatic enzyme into mono-, di- and trihydroxy bile acids when administered to humans.[1] Recently, we disclosed the rapid elevation of unconjugated 7,12-dioxo-3α-hydroxy-5β-cholanoic acid and the delayed occurrence of conjugated 3α,7α-dihydroxy-12-oxo-5β-cholanoic acid in serum after intravenous administration of dehydrocholic acid. In bile, on the other hand, conjugated 3α,7α-dihydroxy-12-oxo bile acid was a major metabolite, beside the conjugated 3α-monohydroxy bile acid in an extremely low level.[2] These results suggest the existence of an extrahepatic reduction system for the 3-oxo group of 5β-cholanoic acid. Therefore, in the present study, 7α,12α-dihydroxy-3-oxo-5β-cholanoic acid labeled with ^{18}O atoms as a model compound was incubated with human blood, and the biotransformation products were separated and characterized by gas chromatography-mass spectrometry (GC-MS).

The highly purified ^{18}O-labeled bile acid was synthesized by the usual method. Methyl 7α,12α-dihydroxy-3-oxo-5β-cholanoate, readily obtainable from methyl cholanoate by selective oxidation with silver carbonate-Celite, was transformed into the 3,3-ethylene ketal, which in turn was subjected to oxidation with pyridinium chlorochromate in dichloromethane followed by alkaline hydrolysis. The yielded 7,12-dioxo-3,3-ethylenedioxy-5β-cholanoic acid was converted to the potassium salt using carboxymethyl Sephadex LH-20 (K^+ form) in the manner previously reported.[3] A 20-mg portion of the salt was dissolved in $H_2{}^{18}O$ (isotopic purity of 98 atom %, 300 μl) and heated at 90°C for 50 h. The ^{18}O-labeled substrate was reduced with $NaBH_4$ and the product was then treated with p-toluenesulfonic acid in aqueous acetone for removal of the protecting group at C-3. The ^{18}O-labeled 3-oxo bile acid thus obtained was purified by column chromatography on silica gel, high-performance liquid chromatography on a reversed-phase column, and subsequent recrystallization from dichloromethane-hexane. After derivatization into the pentafluorobenzyl (PFB) ester-dimethylethylsilyl (DMES) ether, the ^{18}O content in the labeled bile acid was determined by GC-selected ion monitoring with negative ion chemical ionization (NICI) detection using a characteristic ion $[M-181]^-$.[3,4] It was confirmed that a theoretical number of ^{18}O atoms was incorporated into bile acid. The ratio of the unlabeled fragment to the fully labeled fragment was found to be less than 1/1000.

[7,12-$^{18}O_2$]7α,12α-Dihydroxy-3-oxo-5β-cholanoic acid was incubated with human blood taken from healthy subjects. To a heparinized blood sample (1 ml) preincubated at 37°C for 5 min was added the ^{18}O-labeled substrate (1 μg), and the whole was incubated at 37°C for 1 h. After addition of [3,7,12-$^{18}O_3$-$^2H_3$]3α,7α,

12α-trihydroxy-5β-cholanoic acid (cholic acid)[3] as an internal standard (IS), the incubation mixture was acidified with 0.1 N HCl and extracted with ethyl acetate. Bile acids were purified by ion-exchange chromatography on piperidinohydroxypropyl Sephadex LH-20 and then treated to produce the PFB ester-trimethylsilyl (TMS) or -DMES ether derivatives.[3]

Capillary GC-MS was carried out using a VG Analytical MM12030 quadrupole mass spectrometer equipped with a cross-linked 5% phenylmethylsilicone fused-silica capillary column (20 m x 0.3 mm i.d.)(J & W Scientific, Folsom, CA, U.S.A.) and a Van den Berg's solventless injector. Isobutane was used as a reagent gas and helium as a carrier gas. The injection port, column oven and ion source were kept at 280°C, 260-280°C and 270°C respectively. The ionization energy was 30 eV for the electron impact ionization (EI) mode or 70 eV for the NICI mode, and the emission current was 400 μA.

A typical NICI-selected ion recording of bile acids in the incubation mixture as the PFB ester-TMS ether derivatives is illustrated in Fig. 1. Each bile acid was unequivocally identified by comparing the relative retention times (RRT) to cholic acid and the mass spectra with those of reference compounds. In NICI-MS, the PFB ester-TMS and -DMES ether derivatives corresponding to peak c on the chromatogram afforded prominent negative ions at m/z 627 and 669, respectively, indicating the trihydroxylated bile acid structure.[4] These derivatives of the product formed from the unlabeled substrate (50 μg) with human blood (10 ml) were entirely identical with those of the authentic 3β,7α,12α-trihydroxy-5β-cholanoic acid with regard to the RRT values (0.925 for TMS ether, 0.923 for DMES ether) and EI mass spectra. No transformed product was detected when 3-oxo bile acid was incubated with human blood denatured at 70°C for 2 min. These results indicate that 3-oxo bile acid is enzymatically reduced to the corresponding 3β-hydroxy compound in human blood.

Finally, a mixture of the ^{18}O-labeled and unlabeled substrates was incubated with the red blood cell, white blood cell and platelet fractions respectively, obtained by the discontinuous density gradient method using a lymphocyte separation solution (Nacalai Tesque Inc., Kyoto, Japan), and the biotransformed metabolites were separated and characterized by NICI-GC-MS using the isotope cluster technique. As shown in Fig. 2, the 3β-hydroxy metabolite at a yield of 30% was formed exclusively with the red blood cell

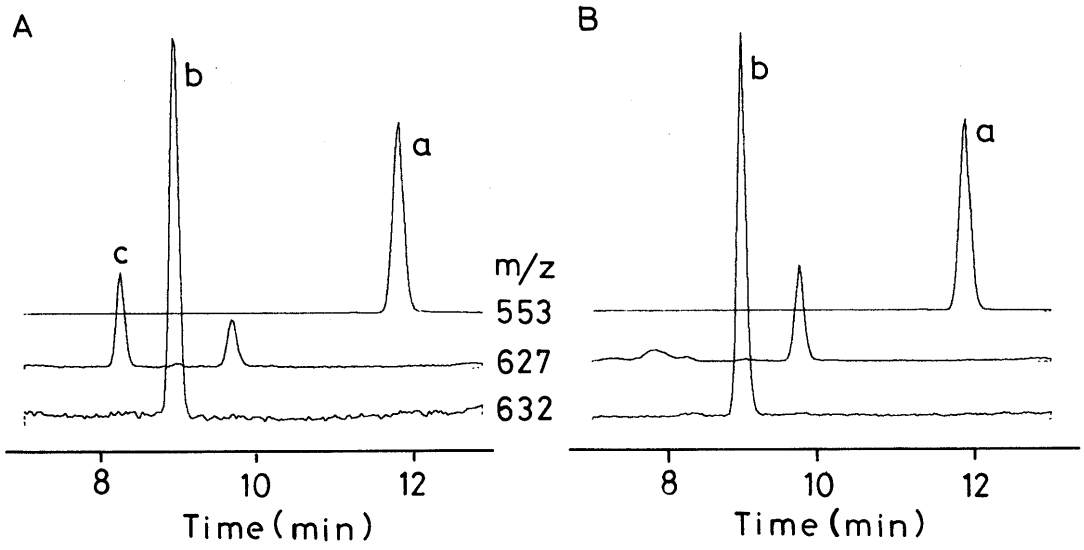

Fig. 1. NICI-Selected Ion Recording of PFB Ester-TMS Ether Derivatives of the ^{18}O-Labeled Bile Acids Formed by Incubation with Intact Whole Blood (A) and Denatured Blood (B)
a: 7α,12α-dihydroxy-3-oxo-5β-cholanoic acid (substrate), b: [3,7,12-$^{18}O_3$-$^{2}H_3$]-cholic acid (IS), c: 3β,7α,12α-trihydroxy-5β-cholanoic acid.

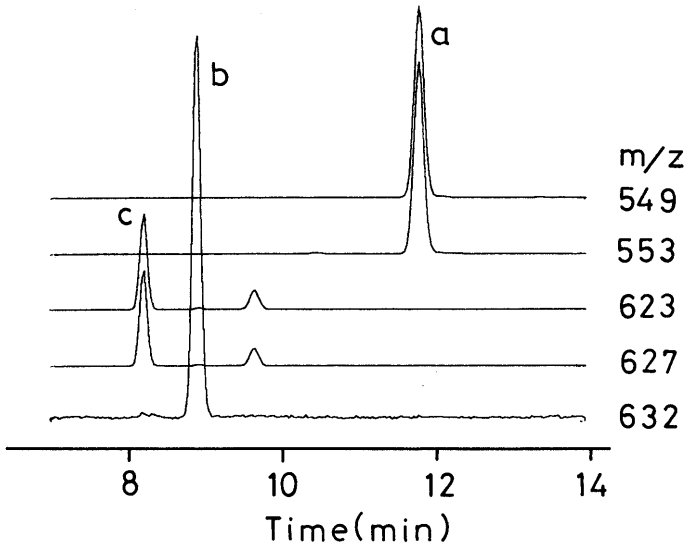

Fig. 2. NICI-Selected Ion Recording of PFB Ester-TMS Ether Derivatives of the ^{18}O-Labeled and Unlabeled Bile Acids Formed by Incubation with Red Blood Cell Fraction a: 7α,12α-dihydroxy-3-oxo-5β-cholanoic acid (substrate, m/z 549, 553), b: [3,7,12-$^{18}O_3$-$^{2}H_3$]cholic acid (IS, m/z 632), c: 3β,7α,12α-trihydroxy-5β-cholanoic acid (m/z 623, 627).

fraction. In this biotransformation, the isotope effect was not observed for ^{18}O-labeled bile acid.

We have demonstrated the existence of an enzyme system catalyzing the reduction of the 3-oxo group to the 3β-hydroxy function in human red blood cells. Maeda et al. reported that 3α,7β-dihydroxy-5β-cholanoic acid (ursodeoxycholic acid) administered to human subjects underwent epimerization at C-3 in the intestinal tract, providing unconjugated 3β,7β-dihydroxy-5β-cholanoic acid in serum.[5] The present result strongly implies that ursodeoxycholic acid is oxidized in part in the intestinal tract into 3-oxo bile acid which is further metabolized into 3β,7β-dihydroxy bile acid in red blood cells. In our previous study, we disclosed the occurrence of the 3α-hydroxy compound as a main metabolite in serum after intravenous administration of dehydrocholic acid.[2] In the present work we also detected a trace amount of cholic acid formed from 7α, 12α-dihydroxy-3-oxo-5β-cholanoic acid.

Further studies are being conducted in these laboratories for clarifying the metabolic pathways of 3-oxo bile acids, and the details will be reported elsewhere.

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