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Assay of intermediates in bile acid biosynthesis using isotope dilution–mass spectrometry: hepatic levels in the normal state and in cerebrotendinous xanthomatosis

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Abstract The synthesis of $^{2}H_4$-labeled 5β-cholestane-3α, 7α-diol, 5β-cholestane-3α,7α,12α-triol, 7α-hydroxy-4-cholesten-3-one, and 7α,12α-dihydroxy-4-cholesten-3-one is described. A mixture of these compounds, together with $^{2}H_3$-labeled 5-cholestene-3β, 7α-diol, was added to extracts of different subcellular fractions of liver. After purification by high performance liquid chromatography and conversion into trimethylsilyl ethers, the amounts of different endogenous unlabeled steroids were determined by selected ion monitoring. In normal liver, the concentration of 5-cholestene-3β, 7α-diol (about 0.1-0.2 μg/ml protein) was higher than the concentration of the other steroids (about 0.01–0.05 μg/mg protein). The concentration of the different steroids was highest in the microsomal fraction of the liver homogenate. In a liver sample from a patient with cerebrotendinous xanthomatosis (CTX), the amounts of the 12α-hydroxylated steroids were considerably higher than in the normal liver. The levels of 7α-hydroxy-4-cholesten-3-one and 5β-cholestane-3α, 7α-diol were similar or only slightly higher than in the liver of the control patients. The concentration of 5-cholestene-3β, 7α-diol was very high in the mitochondrial fraction of the CTX-liver. The findings are in accordance with the previous demonstration that the basic metabolic defect in CTX is a lack of the mitochondrial 26-hydroxylase. The results are further compatible with the contention that 7α,26-dihydroxy-4-cholesten-3-one is an important intermediate in the normal bile acid biosynthesis.—**Björkhem, I., H. Oftebro, S. Skrede, and J. I. Pedersen.** Assay of intermediates in bile acid biosynthesis using isotope dilution–mass spectrometry: hepatic levels in the normal state and in cerebrotendinous xanthomatosis. *J. Lipid Res.* 1981. *22:* 191–200.

Supplementary key words cholesterol · selected ion monitoring · deuterated carrier · high performance liquid chromatography

The rate limiting step in bile acid biosynthesis in human liver is the 7α-hydroxylation of cholesterol. The further metabolism of 7α-hydroxycholesterol is rapid. As a consequence, the steady-state level of the different intermediates in the synthesis can be expected to be very low. However, very few attempts have been made to assay endogenous bile acid intermediates in human liver (1–3).

Recently we developed a sensitive and accurate assay for 5β-cholestane-3α,7α,12α-triol, based on isotope dilution–mass spectrometry (3). Using this assay, it was shown that the concentration of 5β-cholestane-3α,7α,12α-triol was very low in normal human liver, less than 3 ng/mg of protein in the two samples analyzed. In a liver sample from a patient with cerebrotendinous xanthomatosis, the concentration of this steroid was about 50-fold higher. This accumulation of 5β-cholestane-3α,7α,12α-triol could be explained by the demonstration that this patient was completely devoid of the hepatic mitochondrial 26-hydroxylase. The finding supports the previous contention that 5β-cholestane-3α,7α,12α-triol is an important substrate for the mitochondrial 26-hydroxylase in bile acid biosynthesis (4, 5).

In the absence of a mitochondrial 26-hydroxylase, cholic acid might be formed by a microsomal pathway, involving 5β-cholestane-3α,7α,12α,25-tetrol and 5β-cholestane-3α,7α,12α,24β,25-pentol as intermediates (6–8). In accordance with this, patients with CTX excrete considerable amounts of 5β-cholestane-3α,7α,12α,25-tetrol and 5β-cholestane-3α,7α,12α,-24α,25-pentol in urine and feces (6). It should be mentioned that the possibility has been discussed that the pathway involving 25-hydroxylation of 5β-cholestane-3α,7α,12α-triol might be the major pathway for cholic acid biosynthesis, and that patients with CTX have a decreased capacity to convert 5β-cholestane-3α,7α,12α,-

Abbreviations: CTX, cerebrotendinous xanthomatosis; MOPS, morpholinopropanesulfonic acid; HPLC, high performance liquid chromatography; GLC, gas–liquid chromatography.

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25-tetrol into 5β-cholestane-3α,7α,12α,24β,25-pentol (8, 9). The in vitro data referred to above (3), as well as some in vivo data (10), suggest that the pathway involving 25-hydroxylation of 5β-cholestane-3α,7α, 12α-triol is of minor importance under normal conditions.

If it is accepted that the basic metabolic defect in CTX is a lack of the mitochondrial 26-hydroxylase, determination also of the levels of other steroids in the liver of a patient with CTX would certainly provide more information concerning the mechanism of bile acid biosynthesis. In the present work we have developed a sensitive and accurate assay for 5β-cholestane-3α,7α-diol, 7α-hydroxy-4-cholesten-3-one, and 7α,-12α-dihydroxy-4-cholesten-3-one based on isotope dilution–mass spectrometry. Using these assays and a previously developed assay for 5-cholestene-3β, 7α-diol (2), the levels of the different intermediates in bile acid biosynthesis have been determined both in the normal human liver and in a liver sample from a patient with CTX.

MATERIALS AND METHODS

Synthesis of 6α,6β,8β,$^2H_3$, 7β-^2H,^3H-labeled 5β-cholestane 3α,7α-diol, and 7α-hydroxy-4-cholesten-3-one

5β-Cholestane-3α,7α-diol was prepared by electrolytic coupling of chenodeoxycholic acid and isovaleric acid as described by Bergström and Krabisch (11); it had a melting point of 83–85°C, reported 84–86°C (11). This compound, 200 mg, was treated with 109 mg of *N*-bromosuccinimide at room temperature for 17 hr in a mixture of acetone (5 ml) and water (1 ml) (12). The mixture was then heated for 1 hr at 80°C. After acidification with 0.1 M HCl and addition of water, the mixture was extracted with ether. The ether phase was washed with water until neutral, and the solvent was evaporated in vacuo.

The residue was chromatographed on a column of 25 g of aluminum oxide grade III (Woelm, Eschwege, Germany). The column was eluted with increasing concentrations of ethyl acetate in benzene. Ethyl acetate, 10% (v/v) in benzene, eluted the 3α-hydroxy-5β-cholestan-7-one. Crystallization from acetone–water gave 71 mg, which was pure as judged by thin-layer chromatography, using benzene–ethyl acetate 1:1 (v/v) as solvent. The material was then refluxed in 10 ml of CH_3O^2H and 2 ml of ^2H_2O together with 24 mmol of NaOH for 24 hr. The deuterated solvents were obtained from Merck (Darmstadt, West Germany) and had a purity of more than 99% with respect to ^2H. The mixture was diluted with ^2H_2O, acidified with ^2HCl (Merck, 99% pure with respect to ^2H), and extracted with diethyl ether. The ether phase was washed with ^2H_2O until neutral. The material was then immediately dissolved in CH_3O^2H and treated with ^3H-labeled sodium borohydride, 0.1 mg, 25 mCi (Radiochemical Centre, Amersham, England) for 30 min at room temperature. The tritium label was introduced in order to facilitate detection in connection with the high performance liquid chromatography (cf. Fig. 6).

Excess ^2H-labeled sodium borohydride was then added (Merck, 99% pure with respect to ^2H) and the mixture was allowed to stand an additional 2 hrs at room temperature. After acidification and dilution with water, the residue was extracted with ether. After removal of the ether by evaporation in vacuo, the residue was subjected to aluminum oxide chromatography, using the same conditions as above. Ethyl acetate, 20% (v/v) in benzene eluted 6α,6β,8β-$^2H_3$,7β-^2H, ^3H-labeled 5β-cholestane-3α,7α-diol. The material was pure as judged by thin-layer chromatography, using the same system as above. The specific radioactivity was 0.11 mCi/mg. Mass spectrometry of trimethylsilyl ether of the material confirmed the identity of the material as deuterated 5β-cholestane-3α,7α-diol. The material was a mixture of di-, tri-, and tetra-deuterated molecules (**Fig. 1**). The composition was the following when calculated according to Biemann (13) and using the fragments at m/e 368–m/e 372 (M-2 $\times$ 90) in the calculation: unlabeled molecules, 0%; mono-deuterium-labeled, 0%; di-deuterium-labeled, 4%; tri-deuterium-labeled, 31%; tetra-deuterium labeled, 65%.

Part of the ^2H,^3H-labeled 5β-cholestane-3α,7α-diol was oxidized according to Oppenhauer and the 7α-hydroxy-5β-cholestane-3-one thus formed further oxidized with selenium dioxide to yield 7α-hydroxy-4-cholesten-3-one. Details concerned with this sequence of reactions and purification of the products have been described in a previous publication (14). The final product, 6α, 6β, 8β-$^2H_3$, 7β-^2H, ^3H-labeled 7α-hydroxy-4-cholesten-3-one was purified by preparative thin-layer chromatography, using benzene–ethyl acetate 1:1 (v/v) as solvent. The product was pure as judged by thin-layer chromatography and gas–liquid chromatography (as trimethylsilyl ether using a SE-30 column). The specific radioactivity was 0.11 mCi/mg. Mass spectrometry (as trimethylsilyl ether) confirmed the identity of the compound as deuterium labeled 7α-hydroxy-4-cholesten-3-one (**Fig. 2**). The material was a mixture of mainly di-, tri-, and tetra-deuterium-labeled molecules. The composition was the following when calculated according to Biemann,

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using the fragments at m/e 382–m/e 386 (M-90) in the calculation: 5%, unlabeled molecules; 7%, mono-deuterated; 26%, di-deuterated; 43%, tri-deuterated; and 19%, tetra-deuterated. Thus the oxidation with selenium dioxide led to removal of some deuterium.

Synthesis of 6α,6β,8β-$^2H_3$, 7β-^2H, ^3H-labeled 5β-cholestane-3α,7α,12α-triol and 7α, 12α-dihydroxy-4-cholesten-3-one

5β-Cholestane-3α,7α,12α-triol was prepared by electrolytic coupling of cholic acid and isovaleric acid as described by Bergström and Krabisch (11) and had a melting point of 186–187°C (reported 187–188°C) (15). This compound, 200 mg, was oxidized with *N*-bromosuccinimide, using the same procedure as in the synthesis of 3α-hydroxy-5β-cholestan-7-one. The product obtained, 3α,12α-dihydroxy-5β-cholestan-7-one, was purified by aluminum oxide chromatography, using the same chromatographic conditions

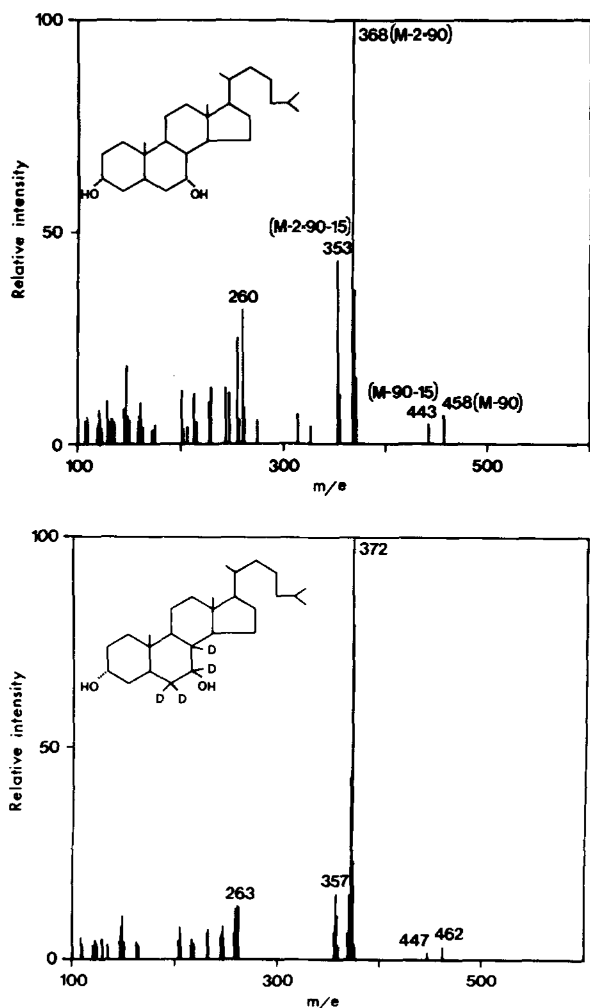

Fig. 1. Mass spectrum of trimethylsilyl ether of unlabeled (upper figure) and deuterium-labeled (lower figure) 5β-cholestane-3α,7α-diol.

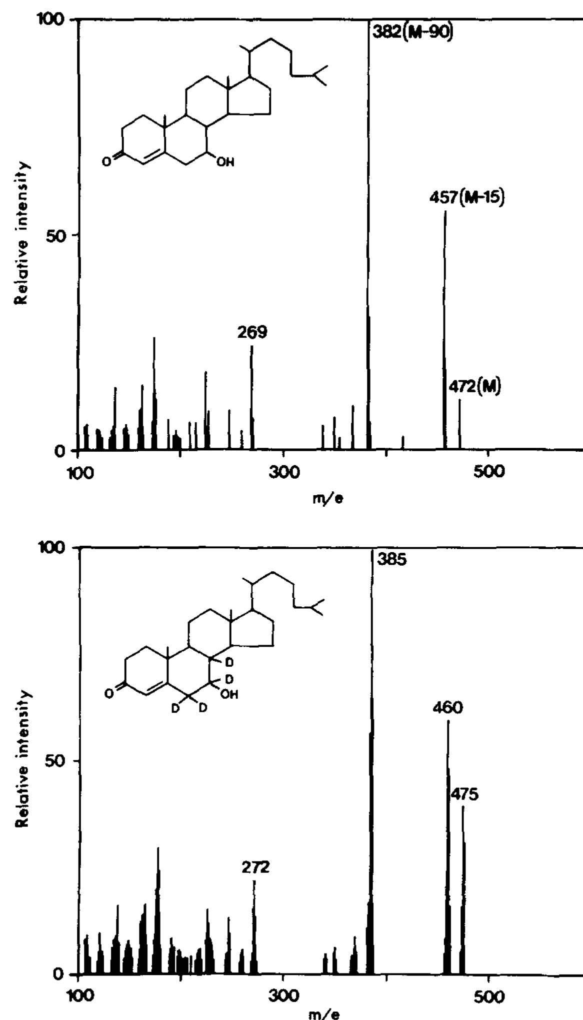

Fig. 2. Mass spectrum of trimethylsilyl ether of unlabeled (upper figure) and deuterium-labeled (lower figure) 7α-hydroxy-4-cholesten-3-one.

as above. Ethyl acetate (20%) in benzene eluted 3α,12α-dihydroxy-5β-cholestane-7-one, 130 mg. The compound was pure as judged by thin-layer chromatography using the same system as above. The material was directly enolized in deuterated alkaline medium, using the same conditions as above. The 6α,6β,8β-$^2H_3$-labeled 3α,12α-dihydroxy-5β-cholestan-7-one formed was immediately reduced, first with tritium-labeled sodium borohydride and then with deuterated sodium borohydride, as above. The reduced material was purified by aluminum oxide chromatography, using the same conditions as above. Ethyl acetate (40%) in benzene eluted 6α,6β,8β-$^2H_3$, 7β-^2H,^3H-labeled 5β-cholestane-3α,7α,12α-triol, 87 mg. The material was pure as judged by thin-layer chromatography, using ethyl acetate as solvent. The specific radioactivity was 0.14 mCi/mg. Mass spectrometry of trimethylsilyl ether of the material confirmed the

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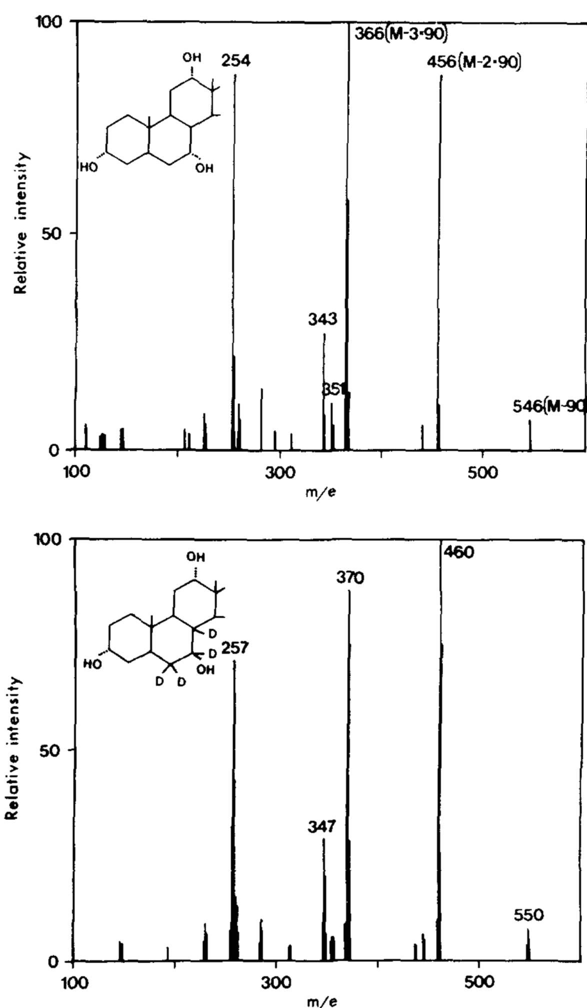

Fig. 3. Mass spectrum of trimethylsilyl ether of unlabeled (upper figure) and deuterium-labeled (lower figure) 5β-cholestane-3α,-7α-12α-triol.

identity as deuterated 5β-cholestane-3α,7α,12α-triol. The material was a mixture of mainly tri- and tetra-deuterated molecular species (**Fig. 3**). The composition was the following when calculated with use of the ions at m/e 456–m/e 460 (M-2 × 90): unlabeled molecules, 0%; mono-deuterated molecules, 0%; di-deuterated molecules, 8%; tri-deuterated molecules, 24%; tetra-deuterated molecules, 68%.

Part of the ^{2}H,^{3}H-labeled 5β-cholestane-3α,7α,12α-triol was oxidized according to Oppenhauer and the 7α,12α-dihydroxy-5β-cholestan-3-one thus formed was further oxidized with selenium dioxide to yield 7α,12α-dihydroxy-4-cholesten-3-one. Details concerned with this sequence of reaction and purification of the products have been described in a previous publication (15). The final product, 6α,6β,8β-$^2H_3$,7β-^{2}H,^{3}H-labeled 7α,12α-dihydroxy-4-cholesten-3-one was purified by thin-layer chromatography, using ethyl acetate as solvent. The product was pure as judged by thin-layer chromatography and gas–liquid chromatography (as trimethylsilyl ether using a SE-30 column). The specific radioactivity was 0.14 mCi/mg. Mass spectrometry (as trimethylsilyl ether) confirmed the identity of the compound as deuterium-labeled 7α,12α-dihydroxy-4-cholesten-3-one (**Fig. 4**). The material was a mixture of mainly di-, tri-, and tetra-deuterated molecular species. The composition was the following when calculated according to Biemann, using the fragments at m/e 380–m/e 384 (M-2 × 90) in the calculation: 0%, unlabeled molecules; 5%, mono-deuterium-labeled molecules; 27%, di-deuterium-labeled molecules; 48%, tri-deuterium-labeled molecules; and 20%, tetra-deuterium-labeled molecules.

$^2H_3$-labeled and 7β-^{3}H 5-cholestene-3β,7α-diol

These compounds were prepared as described previously (2, 16). The ^{3}H-labeled compound had a specific radioactivity of 10 mCi/mg.

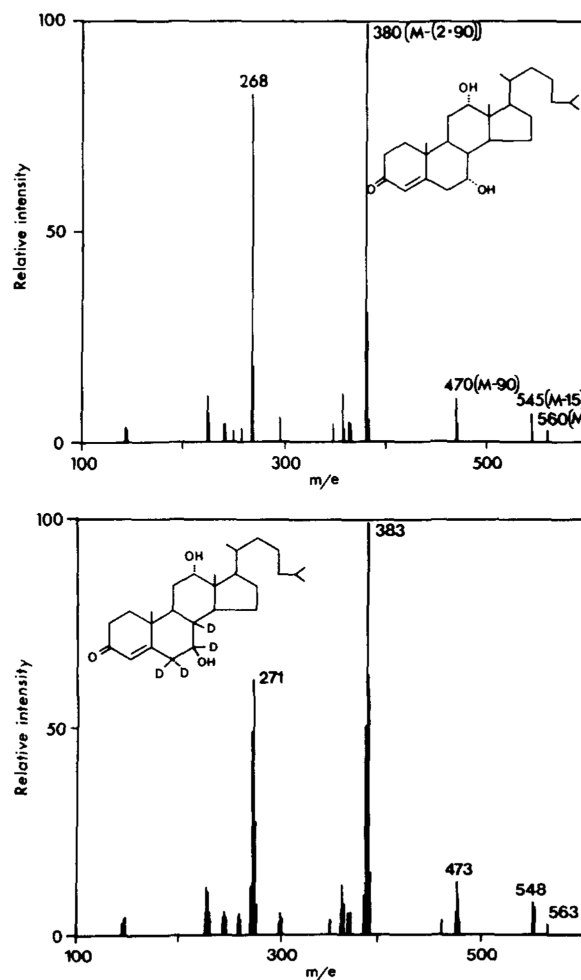

Fig. 4. Mass spectrum of trimethylsilyl ether of unlabeled (upper figure) and deuterium-labeled (lower figure) 7α,12α-dihydroxy-4-cholesten-3-one.

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TABLE 1. Levels of different intermediates in bile acid biosynthesis in subcellular fractions of human liver

Fraction	5-Cholestene 3β,7α-diol	7α-Hydroxy-4-cholesten-3-one	5β-Cholestane-3α,7α-diol	7α,12α-Dihydroxy-4-cholesten-3-one	5β-Cholestane-3α,7α,12α-triol
			ng/mg protein		
Whole homogenate, normal liver[a]	142 (117–198)	5 (0–19)	21 (8–44)	11 (3–27)	7 (0–27)
Whole homogenate, CTX	255	40	35	170	75
Microsomal fraction, normal liver[b]	215 (197–245)	7 (0–20)	43 (25–60)	7 (5–10)	6 (0–11)
Microsomal fraction, CTX	272	91	106	1590	276
Mitochondrial fraction, normal liver[c]	133–190	0–0	6	8–3	0–1
Mitochondrial fraction, CTX	1477	9	68	160	55
Cytosol, normal liver[c]	91–153	0–0	13–37	0–5	11–1
Cytosol, CTX	145	0	29	40	71

[a] The mean and range of results obtained from seven different liver samples are given.
[b] The mean and range of results obtained from three different liver samples are given.
[c] The results obtained from two different liver samples are given.

Chemicals

All chemicals and solvents used were standard commercial high purity materials.

Unlabeled steroids

The unlabeled steroids used (5-cholestene-3β,-7α-diol,5β-cholestane-3α,7α-diol, 7α-hydroxy-4-cholesten-3-one, 5β-cholestane-3α,7α,12α-triol, 7α,-12α-dihydroxy-4-cholesten-3-one were prepared as described previously (14–16).

Clinical

The patient with cerebrotendinous xanthomatosis was a woman born in 1935. Details concerning the clinical findings are given in previous work (3). With the informed consent of the patient and her family, a liver biopsy (1.4 g) was removed during cholecystectomy in June 1979. Normal human liver material was obtained from two renal transplant donors, dead according to officially adopted criteria. These two liver samples were used for preparation of the different subcellular fractions shown in **Table 1**. Needle biopsies taken routinely during abdominal surgery were made available from six patients free of liver diseases.

Preparation of subcellular fractions of liver biopsies

The biopsies were homogenized as quickly as possible in cold 0.25 M sucrose and 25 mM Mops buffer (morpholinopropanesulfonic acid), pH 7.4, using a Potter-Elvehjem homogenizer. The mitochondrial fraction, the microsomal fraction, and the cytosol were prepared according to standard procedures, described in the previous work (3). In most cases, the subcellular fraction was kept frozen at −70°C prior to extraction with ethyl acetate (3). In general, the amount of subcellular fraction extracted was equivalent to 0.5–2 mg of protein.

Protein was determined according to the procedure of Lowry et al. (17).

Assay of 5β-cholestane-3α,7α-diol, 7α-hydroxy-4-cholesten-3-one, 5β-cholestane-3α,7α,12α-triol, 7α,12α-dihydroxy-4-cholesten-3-one, and 5-cholestene-3β,7α-diol by isotope dilution–mass spectrometry

To each extract, a fixed amount of a mixture of the $^2H_4$-labeled steroids was added dissolved in acetone. The approximate amounts of tetra-deuterated steroids in the mixture added were the following: 5β-cholestane-3α,7α-diol, 220 ng; 7α-hydroxy-4-cholesten-3-one, 350 ng; 5β-cholestane-3α,7α,12α-triol, 150 ng; 7α,12α-dihydroxy-4-cholesten-3-one, 210 ng. In addition, the mixture contained $^2H_3$-labeled 5-cholestene-3β,7α-diol, 150 ng, and 7β-^{3}H-labeled 5-cholestene 3β,7α-diol, 2 ng. It should be pointed out that the amounts of deuterated steroids refer to the approximate amount of tetra-deuterated molecules (in the case of 5-cholestene-3β,7α-diol trideuterated molecules). Thus only these molecules are used as standards in the procedure (cf. below). It should futhermore be pointed out that exactly the same mixture of deuterated steroids as above was used in the preparation of the different standard curves (**Fig. 5**).

The extract, together with the deuterated steroids, was then subjected to high performance liquid chromatography using a Zorbax-ODS column (DuPont, 4.6 × 250 mm (particle size 5 μm)) and 7.5% water in methanol as eluting solvent. Part of the collected fractions was counted in a Packard Tri-Carb liquid scintillation counter. As shown in **Fig. 6**, there was a base-

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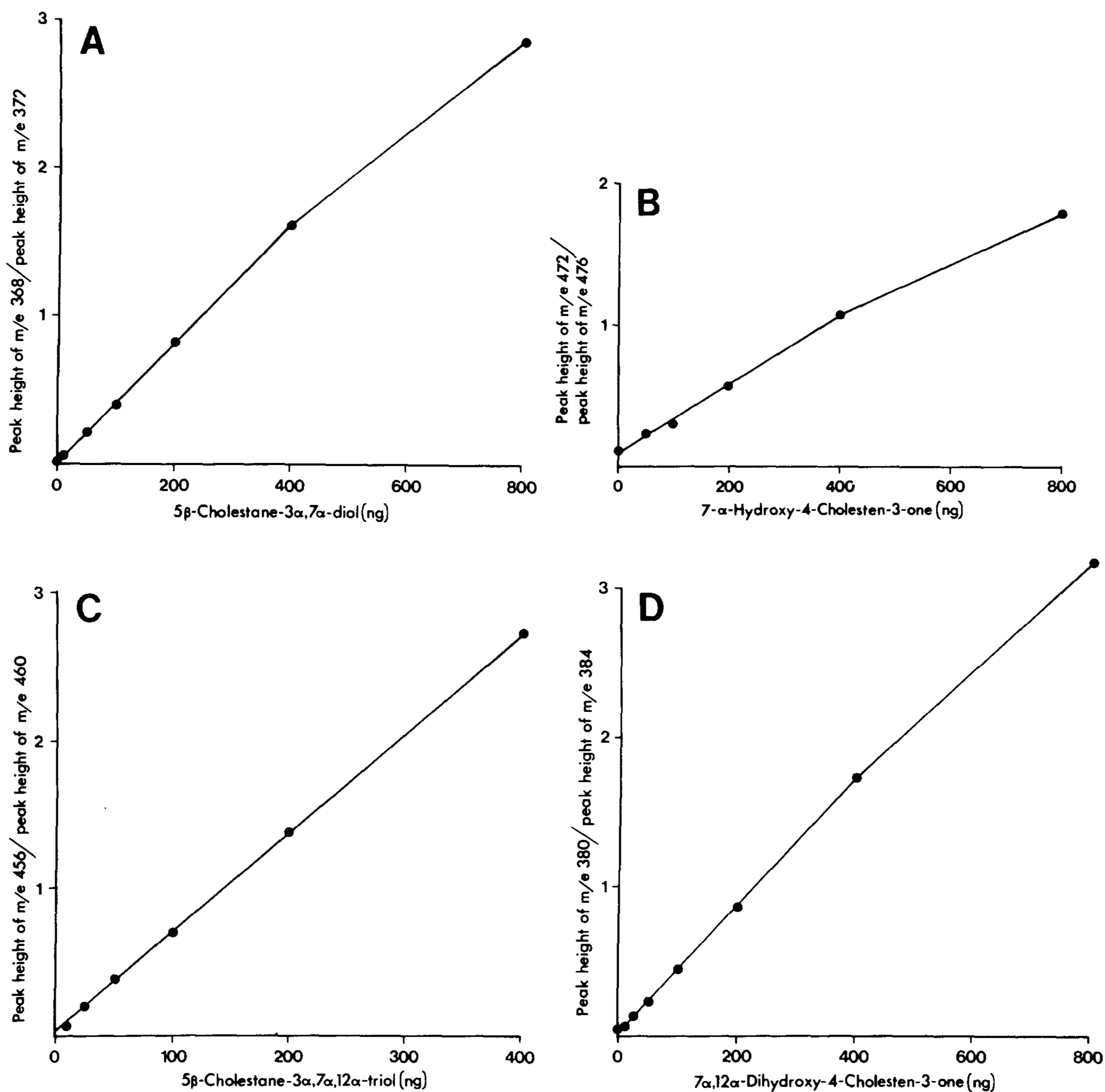

Fig. 5. Standard curve for assay of A, 5β-cholestane-3α,7α-diol; B, 7α-hydroxy-4-cholesten-3-one; C, 5β-cholestane-3α,7α,12α-triol; and D, 7α,12α-dihydroxy-4-cholesten-3-one, using isotope dilution–mass spectrometry.

line separation of each of the different steroids.

The peak fraction of each steroid was collected. After evaporation of the solvent and conversion into trimethylsilyl ether, the material was analyzed by combined gas–liquid chromatography–mass spectrometry. An LKB 9000 instrument equipped with a multiple ion detector was used. A column of 1.5% SE-30 was used for gas–liquid chromatography. In the assay of 5β-cholestane-3α,7α-diol, the multiple ion detector was focused on the ions at m/e 368 and m/e 372 (corresponding to M-2 × 90) in the mass spectrum of trimethylsilyl ether of unlabeled and tetra-deuterium-labeled 5β-cholestane-3α,7α-diol (Fig. 1). In the assay of 7α-hydroxy-4-cholesten-3-one, the two ions at m/e 472 and m/e 476 were used (corresponding to the molecular ion) in the mass spectrum of trimethylsilyl ether of unlabeled and tetra-deuterium-labeled 7α-hydroxy-4-cholesten-3 one, respectively (Fig. 2). In the assay of 5β-cholestane-3α,7α,12α-triol, the two ions at m/e 456 and m/e 460

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were used (corresponding to M-2 × 90) in the mass spectrum of trimethylsilyl ether of unlabeled and tetra-deuterium-labeled 5β-cholestane-3α,7α,12α-triol, respectively (Fig. 3). In the assay of 7α,12α-dihydroxy-4-cholesten-3-one, the two ions at m/e 380 and 384 were used (corresponding to M-2 × 90) in the mass spectrum of trimethylsilyl ether of unlabeled and tetra-deuterium labeled 7α,12α-dihydroxy-4-cholesten-3-one, respectively (Fig. 4). In the assay of 5-cholestene-3β,7α-diol, the two ions at m/e 456 and 459 were used (corresponding to M-90) in the mass spectrum of trimethylsilyl ether of unlabeled and tri-deuterium-labeled 5-cholestene-3β,-7α-diol (cf. ref. 2).

The amount of unlabeled steroid was calculated with the use of a standard curve, individual for each steroid (Fig. 5).

RESULTS

The results are summarized in the table. In the evaluation of the relative distribution of the steroids in the different subcellular fractions, it should be borne in mind that the protein content of the cytosol was 6–8 times higher than that of the particulate fractions.

Relatively high levels of 5-cholestene-3β,7α-diol were found in all subcellular fractions. The mitochondrial fraction of the CTX-liver contained appreciably higher amounts than the other fractions.

Only small amounts of 7α-hydroxy-4-cholesten-3-one were detected in normal homogenate, the cytosol, and the microsomal fractions. About five-fold higher amounts were determined in the microsomal fraction from the CTX-liver.

The concentration of 5β-cholestane-3α,7α-diol was also highest in the microsomal fraction. The microsomal fraction of the CTX-liver contained about twice the amount of 5β-cholestane-3α,7α-diol present in the microsomal fraction from normal liver.

7α,12α-dihydroxy-4-cholesten-3-one was present only in very low amounts in normal liver. The concentration was more than one hundred-fold higher in the microsomal fraction of the CTX-liver than in the corresponding fraction of the control liver. Also, the mitochondrial fraction of the CTX-liver contained appreciable amounts.

The level of 5β-cholestane-3α,7α,12α-triol was also very low in all fractions from normal human liver. Both in the microsomal and the mitochondrial fractions of the CTX-liver, however, the levels were almost 40- to 50-fold higher than in the corresponding fractions of the normal liver.

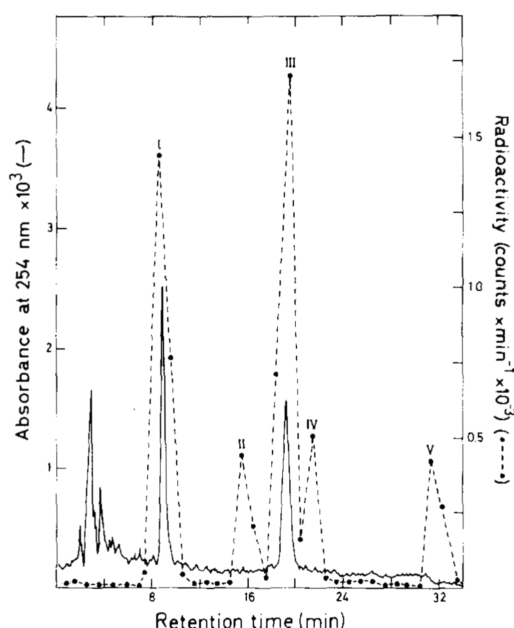

Fig. 6. High performance liquid chromatography of a standard mixture of bile acid intermediates. For chromatographic conditions see Experimental Procedure. Both absorbance at 254 nm (——) and radioactivity (O ---- O) were monitored. Trace amounts of the different labeled compounds were injected together with 23 ng of unlabeled 7α,12α-dihydroxy-4-cholesten-3-one and 21 ng of unlabeled 7α-hydroxy-4-cholesten-3-one. Peak I: 7α,12α-dihydroxy-4-cholesten-3-one; peak II: 5β-cholestane-3α,7α,12α-triol; peak III: 7α-hydroxy-4-cholesten-3-one; peak IV: 5-cholestene-3β,7α-diol; peak V: 5β-cholestane-3α,7α-diol.

DISCUSSION

The assays in the present work based on HPLC, GLC, and isotope dilution–mass spectrometry should be highly specific and accurate. The sensitivity was sufficient to permit quantitation of the different intermediates down to the nanogram level.

As pointed out in the introduction, the concentration of the different intermediates in bile acid biosynthesis can be expected to be very low. This was confirmed in the present work, and with the exception of 5-cholestene-3β,7α-diol, all the different intermediates were present in the whole homogenate of normal liver in a concentration of less than 50 ng/mg protein. It should be pointed out that 5-cholestene-3β,7α-diol, being the first intermediate in the biosynthesis, is also formed non-enzymatically (18). Thus, part of the 5-cholestene-3β,7α-diol present in the homogenate may have been formed by autoxidation. Also, our value of about 200 ng/mg protein in the microsomal fraction is higher than previously reported (1) (about 40 ng/mg).

The time interval and the handling of the samples prior to freezing might be critical for the metabolic activity in the liver sample studied and for the amounts

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of endogenous steroid intermediates determined. In addition, homogenization of the liver may cause redistribution of the intermediates between the different cell fractions.

From the above considerations, it is evident that it may be difficult to draw any firm conclusions from the amounts of different steroid intermediates or from their distribution in the subcellular fractions in the normal human liver when assayed as in the present work.

With the exception of the unexpected very high level of 5-cholestene-3β,7α-diol in the mitochondrial fraction of the CTX-liver, the highest concentration of the steroids was found in the microsomal fraction. In the case of 7α-hydroxy-4-cholesten-3-one and 7α,-12α-dihydroxy-4-cholesten-3-one, this may in part be due to the fact that these steroids are formed in this cell fraction. In the case of 5β-cholestane-3α,7α-diol and 5β-cholestane-3α,7α,12α-triol, the microsomal accumulation might be due to a redistribution in connection with preparation of the homogenate. After incubation of a whole homogenate of rat liver with the above steroids and subsequent centrifugation, most of the steroids are in fact recovered in the microsomal fraction.[1] Thus the subcellular distribution in vivo might be quite different from that in vitro.

In spite of all the above limitations, valid conclusions may be drawn from a comparison between the levels of the different steroids in normal livers and in the liver of the patient with CTX. With the exception of 5-cholestene-3β,7α-diol and 5β-cholestane-3α,7α-diol which were only slightly elevated, all other intermediates were present in considerably higher amounts in the whole homogenate of the CTX-liver than in the whole homogenate of the normal livers tested. To some extent this accumulation might be a consequence of a higher 7α-hydroxylase activity in the CTX-liver. Thus it has been reported that both HMG-CoA reductase activity and 7α-hydroxylase activity were increased in the liver of a patient with CTX (19, 20). The very high accumulation of the 12α-hydroxylated intermediates, 7α,12α-dihydroxy-4-cholesten-3-one and 5β-cholestane-3α,7α,12α,-triol however, must be more directly related to the metabolic block. The high accumulation of 5β-cholestane-3α,7α,12α-triol is in consonance with the previous finding (3), and is in accord with the contention that this steroid is an important substrate for the mitochondrial 26-hydroxylase (4–5) which is lacking in CTX (3).

The very high accumulation of 7α,12α-dihydroxy-4-cholesten-3-one in the microsomal fraction of the CTX-liver is surprising. In principle, there may be three different explanations: *1*. There might be a continuous transport of 7α,12α-dihydroxy-4-cholesten-3-one from the microsomal fraction into the cytosol, where it is rapidly converted into 5β-cholestane-3α,7α,12α-triol by the soluble reductase and dehydrogenase. If 5β-cholestane-3α,7α,12α-triol cannot be further metabolized by the mitochondrial 26-hydroxylase, it will accumulate in all cell fractions, including the cytosol. The mechanism for transport of 7α,12α-dihydroxy-4-cholesten-3-one into the cytosol will then be saturated, and as a consequence this steroid may accumulate in the microsomal fraction. *2*. 7α,12α-Dihydroxy-4-cholesten-3-one might be an important substrate per se for the mitochondrial 26-hydroxylase. *3*. 7α-Hydroxy-4-cholesten-3-one might be an important substrate for the mitochondrial 26-hydroxylase. The relatively low accumulation of this steroid in the microsomal fraction of liver of the CTX-patient might be explained by the fact that this cell fraction has a normal capacity to 12α-hydroxylate 7α-hydroxy-4-cholesten-3-one (21). Thus any accumulation of 7α-hydroxy-4-cholesten-3-one would lead to a rapid formation of 7α,12α-dihydroxy-4-cholesten-3-one.

At present none of the three hypotheses can be ruled out. The fact that the accumulation of 7α,12α-dihydroxy-4-cholesten-3-one was considerably higher than the accumulation of 5β-cholestane-3α,7α,12α-triol does not favor the first hypothesis. In a previous work from this laboratory, it was shown that the mitochondrial fraction of a human liver homogenate had a higher capacity to 26-hydroxylate 7α-hydroxy-4-cholesten-3-one than any other C_{27}-steroid tested (22). This finding is in accord with the third hypothesis. That 7α,26-dihydroxy-4-cholesten-3-one may be an important intermediate in biosynthesis of chenodeoxycholic acid is further supported by some very recent in vivo work in which the relative rates of conversion of different C_{27}-steroids into bile acids in bile fistula patients were studied (23). It should be pointed out, however, that such in vivo data are not fully conclusive per se, in view of the fact that the metabolic fate of endogenously formed steroids might be different from that of exogenously administered steroids.

Taken together with previous in vitro work with rat liver (24) and human liver (22), the in vivo studies (23) and the present work give strong support for the contention that 7α,26-dihydroxy-4-cholesten-3-one is an important intermediate in bile acid biosynthesis. Whether or not 7α,12α,26-trihydroxy-4-cholesten-3-one is also an intermediate is difficult to evaluate from the data presently available.

The accumulation of 5β-cholestane-3α,7α,-diol in

[1] Björkhem, I. Unpublished experiments.

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the CTX-liver was relatively low. Any accumulation of this steroid in the microsomal fraction would lead to a rapid formation of 5β-cholestane-3α,7α,12α-triol. Since 5β-cholestane-3α,7α,12α-triol in the absence of a mitochondrial 26-hydroxylase might be converted to cholic acid via a pathway involving 5β-cholestane-3α,7α,12α,25-tetrol, the biosynthesis of cholic acid will be favored. According to available in vitro data (22) as well as in vivo data (25), 5β-cholestane-3α,7α-diol cannot be converted into chenodeoxycholic acid in human liver via a corresponding pathway involving 5β-cholestane-3α,-7α,25-triol. 5β-Cholestane-3α,7α-diol was also found to be a very poor substrate for the microsomal 25-hydroxylase in the CTX liver (21). In accord with the above considerations, the bile of the CTX-patients contains only traces of chenodeoxycholic acid (3, 26).

APPENDIX

Calculation of isotope content

These calculations were performed according to Biemann (13). The example given here refers to calculation of deuterium content in $^2H_3$-labeled trimethylsilyl ether of 5-cholestene-3β,7α-diol (2) with use of the ions at m/e 456, m/e 457, m/e 458, and m/e 459 (M-90).

In this compound, there is no formation of fragments at M-90-1 (m/e 455), M-90-2 (m/e 454), or M-90-3 (m/e 453).

Peak heights (arbitrary units) in unlabeled standard were the following:

Mass:	456	457	458	
Intensity:	1.00	0.32	0.10	(A)

Peak heights in unlabeled sample were the following:

Mass:	456	457	458	459	460	
Intensity:	21	48	183	238	76	(B)

The entire mass at 456 must be due to unlabeled species. The contributions of the unlabeled species to m/e 456, m/e 457, and m/e 458 are calculated by multiplying the peak height at mass m/e 456 with the abundance at m/e 456, m/e 457, and m/e 458 in the analysis of the unlabeled standard (A).

$$21 \times 1.00 = 21 \quad 21 \times 0.32 = 8 \quad 21 \times 0.10 = 2 \qquad (C)$$

Then (C) are subtracted from (B)

21	48	183	238	76	
− 21	− 8	− 2			(D)
0	40	181	238	76	

The peak height due to single-labeled species is thus 40. The contributions of this species to m/e 457, m/e 458, and m/e 459 are the following:

$$40 \times 1.00 = 40 \quad 40 \times 0.32 = 13 \quad 40 \times 0.10 = 4 \qquad (E)$$

Then (E) are subtracted from (D)

40	181	238	76	
− 40	− 13	− 4		(F)
0	168	234	76	

The peak height due to double-labeled species is thus 168. The contributions of this species to m/e 458, m/e 459, and m/e 460 are thus the following:

$$168 \times 1.00 = 168 \quad 168 \times 0.32 = 54 \quad 168 \times 0.10 = 17 \qquad (G)$$

Then (G) are subtracted from (F)

168	234	76	
− 168	− 54	− 17	(G)
0	180	59	

The peak height due to the triple labeled species is thus 180. The contribution of this species to m/e 459 and m/e 460 are thus the following:

$$180 \times 1.00 = 180 \quad 180 \times 0.32 = 58 \qquad (H)$$

Then (H) are subtracted from (G)

180	59
− 180	− 58
0	1

No species containing more than four heavy isotopes are present. The sum of all corrected intensities is

$$21 + 40 + 168 + 180 + 1 = 410$$

The distribution in mole per cent is 21/410 = 5% for unlabeled species. Values of 10%, 41%, 44% and 0% are obtained for monodeuterated, dideuterated, trideuterated, and tetradeuterated species, respectively.

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