One of the major oxysterols in the human circulation is 4β-hydroxycholesterol formed from cholesterol by the drug-metabolizing enzyme cytochrome P450 3A4. Deuterium-labeled 4β-hydroxycholesterol was injected into two healthy volunteers, and the apparent half-life was found to be 64 and 60 h, respectively. We have determined earlier the half-lives for 7α-, 27-, and 24-hydroxysterol to be 0.5, 0.75, and 14 h, respectively. Patients treated with certain antiepileptic drugs have up to 20-fold increased plasma concentrations of 4β-hydroxycholesterol. The apparent half-life of deuterium-labeled 4β-hydroxycholesterol in such a patient was found to be 32 h, suggesting that the high plasma concentration was because of increased synthesis rather than impaired clearance. 4β-Hydroxycholesterol was converted into acidic products at a much slower rate than 7α-hydroxycholesterol in primary human hepatocytes, and 4β-hydroxycholesterol was 7α-hydroxylated at a slower rate than cholesterol by recombinant human CYP7A1. CYP7B1 and CYP39A1 had no activity toward 4β-hydroxycholesterol. These results suggest that the high plasma concentration of 4β-hydroxycholesterol is because of its exceptionally slow elimination, probably in part because of the low rate of 7α-hydroxylation of the steroid. The findings are discussed in relation to a potential role of 4β-hydroxycholesterol as a ligand for the nuclear receptor LXR.

4β-Hydroxycholesterol is one of the quantitatively most important oxysterols in human circulation (1). We have recently shown that it is formed by the drug-metabolizing enzyme cytochrome P450 3A4 (CYP3A4)1 (1). Preliminary experiments showed that the formation of this oxysterol by human liver microsomes was relatively slow. The high plasma levels of the oxysterol are therefore surprising, and we hypothesized that this may be a consequence of slow metabolism. Therefore, in this work, we determined the rate of elimination of deuterium-labeled 4β-hydroxycholesterol from plasma. Oxysterols are generally degraded to bile acids, and the rate-limiting step in this conversion is the introduction of a hydroxyl group in the 7α-position of the steroid. Alternative pathways for bile acid biosynthesis start with oxidation of the steroid side chain by CYP27A1 and CYP46. Therefore, we have studied the possibility that these cytochromes are active toward 4β-hydroxycholesterol. The metabolism of 4β-hydroxycholesterol was studied in human primary hepatocytes, control, and transected cells and by incubations with recombinant enzymes. In addition, fecal samples from three untreated subjects and one subject treated with carbamazepine were analyzed for 4β-hydroxylated bile acids. Based on these experiments, we present evidence that 4β-hydroxycholesterol has an unusually long half-life in plasma and that this is the result of slow elimination, particularly slow 7α-hydroxylation that is the rate-limiting step for further conversion into bile acids.

EXPERIMENTAL PROCEDURES

Materials—The following materials were purchased: Qiagen One-Step reverse transcription-PCR kit (Qiagen); pcDNA4/HisMax vector, version A and One Shot Top 10F competent cells (Invitrogen); poly-n-lysine, (BD PharMingen); and HEK 293 cells (Catalog number CRL-1573, American Type Culture Collection, Manassas, VA).

Two oligonucleotide primers for reverse transcription-PCR were synthesized using the Expedite™ nucleic acid synthesis system followed by high pressure liquid chromatography purification (CyberGene AB, Novum Research Park, Sweden): CYP39A1fo/EcoRI, 5′-CGAATTCC-GTCTCTCTGGAGGTTCTCGG-3′, and CYP39A1ov/XhoI, 5′-CCGCTC-GAGGCGGCTGTGCTTTGAGGCC-3′ (corresponding to nucleotides 18–36 and 1480–1464, respectively, in the cDNA). The primers contained 5′ overhangs with an EcoRI restriction site in the forward primer and an XhoI site for the reverse primer.

The complete cDNA for 24-hydroxycholesterol 7α-hydroxylase for transfection was produced by reverse transcription-PCR from human total RNA prepared from liver tissue utilizing the QuickProp Total RNA Extraction Kit (Amersham Biosciences). The cDNA was inserted into a linearized pcDNA4/HisMax vector and propagated into One Shot Top10F competent cells. From the recovered clones, the cDNA was sequenced to completion to confirm the cDNA sequence and certify that it was correctly inserted so that the reading frame was maintained.

Synthesis of 4β-Hydroxycholesterol and 7α-Hydroxycholesterol—Hexadeuterium-labeled 4β-hydroxycholesterol, tritium-labeled 4β-hydroxycholesterol (specific radioactivity 75,000 cpm/μg), and tritium-labeled 7α-hydroxycholesterol (specific radioactivity 0.5 × 106 cpm/μg) were synthesized as described previously (2, 3).

Synthesis of 4β,7α-Dihydroxycholesterol—4β,7α-dihydroxycholesterol was synthesized as described previously (4). Cholesterol-4,6-dien-3β-ol (11 mg) dissolved in 1.0 ml of acetone was mixed with 0.8 mg of methylmethylxorhenium and 100 μl of 30% H2O2 (w/w). The reaction mixture was stirred at room temperature for 30 min. The remaining H2O2 was decomposed with a few spatula tips of MnO2. The suspension was filtered and evaporated under vacuum. The residue was dissolved in 5 ml of diethyl ether and washed twice with 1 ml of H2O. The ether phase was dried over MgSO4 and evaporated under N2. The mass spectrum of the trimethylsilyl ether derivative of cholest-5-ene-
with 20 µg of 4β-hydroxycholesterol or 20 µg of 27-hydroxycholesterol dissolved in 20 µl of ethanol.

**Metabolism of 4β-Hydroxycholesterol in 293 Cells Transfected with CYP3A9A**—Human embryonic kidney 293 cells were cultured at 37 °C in an atmosphere of 5% CO2 in Dulbecco’s modified Eagle’s medium supplemented with L-glutamine, D-glucose (1000 mg/l, 50 pmol) were pre-incubated for 5 min with 100 nmol of cholesterol. After incubation at 37 °C for 120 min, the reaction was stopped by adding 5 µl of methanol (1%).

**Analysis of Incubations**—CYP7A1 and CYP27A1 metabolites were extracted by adding 5 µl of chloroform/methanol (2:1) and 1 ml of physiological sodium chloride solution to the test tube. The test tube was whirl-mixed and centrifuged. The organic phase was transferred to a new test tube, evaporated under N2, and the residue was trimethylsilylated. CYP27A1 metabolites were converted into methyl esters by treatment with pyridine/hexamethyldisilazane/trimethylchlorosilane (3:2:1) (v/v/v) at 60 °C for 30 min and analyzed by GC-MS in full scan mode.

**Introduction**—4β-Hydroxycholesterol (4β-OH-Chol) is an important metabolite of cholesterol, which is produced by the CYP7A1 enzyme in the liver. It plays a role in the regulation of cholesterol metabolism and is involved in the synthesis of bile acids. The goal of this study was to investigate the metabolism of 4β-OH-Chol in human HepG2 cells transfected with CYP7A1 and CYP27A1 genes.

**Materials and Methods**

**Cell Culture**—HepG2 cells (ATCC, CCL 087) were cultured as described previously (6) but without Geneticin. At 70% confluence, the cells were depleted of cholesterol by incubating with 2-hydroxypropyl-β-cyclodextrin (Sigma) (15 mg/ml) for 45 min in serum-free medium with bovine charcoal-stripped and depleted lipids (C-1696, Sigma). Thereafter, the substrate, 20 µg of 4β-hydroxycholesterol or 20 µg of 27-hydroxycholesterol, dissolved in 20 µl of ethanol was added. The cells were incubated for 48 h at 37 °C in an atmosphere containing 5% CO2.

**Results and Discussion**

The results showed that 4β-OH-Chol was efficiently metabolized by HepG2 cells transfected with CYP7A1 and CYP27A1. The main metabolites identified were 27-hydroxycholesterol and 4β-OH-Chol itself.

**Conclusion**—The metabolism of 4β-OH-Chol by HepG2 cells transfected with CYP7A1 and CYP27A1 provides new insights into the cellular metabolism of this important cholesterol metabolite.
The following extraction was performed as described earlier under “Experimental Procedures,” with the exception that the residue was methylated and trimethyl-silylated. Bile acids in feces were analyzed by gas chromatography mass spectrometry. The gas chromatograph was a Hewlett-Packard 5890 Series II Plus equipped with an HP-5MS capillary column (30 m × 0.25 mm, 0.25-µm phase thickness) connected to a HP 5972 mass selective detector with a HP 7673A automatic sample injector. The oven temperature was as follows: 180 °C for 1 min, 20 °C/min to 220 °C, and then 3.5 °C/min to 290 °C where the temperature was kept for 27 min. Helium was used as a carrier gas with a flow rate of 0.8 ml/min. Splitless injection was used (1 µl), and the detector temperature was 280 °C. The detector transfer line temperature was set to 370 °C. The mass spectrometer was used in the full scan mode (m/z 100–700), and the electron ionization energy was 70 eV.

Ethical Aspects—These studies were approved by the Ethics Committee of Karolinska Institutet at Huddinge University Hospital (Huddinge, Sweden).

RESULTS

Elimination of 4β-Hydroxycholesterol from Human Circulation—To study the elimination of 4β-hydroxycholesterol in human circulation, hexa-deuterated 4β-hydroxycholesterol was administered intravenously to three volunteers. One of them had been treated with the antiepileptic drug carbamazepine for more than 10 years. This drug has been shown to increase the concentration of 4β-hydroxycholesterol in the circulation (1). Before infusion, blood samples were taken to determine basal levels of 4β-hydroxycholesterol (Table I). Volunteers without carbamazepine treatment had levels in the normal range (1).

As shown in Fig. 1, the apparent half-life for the labeled 4β-hydroxycholesterol in plasma was determined to be 64 and 60 h in the two healthy volunteers, respectively. In this calculation, it is assumed that there is no change in the pool of unlabeled 4β-hydroxycholesterol as a consequence of the administration of the labeled compound. The half-lives for 4β-hydroxycholesterol were calculated from the linear (elimination) part of the curves, assuming first order kinetics. In an earlier experiment in the male volunteer, the half-life of 24-hydroxycholesterol in plasma was determined to be −10 h (10), which in turn is considerably longer than the half-life for 7α- and 27-hydroxycholesterol (Table II) (11). The apparent half-life of 4β-hydroxycholesterol in the carbamazepine-treated volunteer was determined to be 52 h.

Metabolism of 4β-Hydroxycholesterol in Human Primary Hepatocytes—4β-Hydroxycholesterol and 7α-hydroxycholesterol were incubated with primary human hepatocytes to study the metabolism into bile acids. The metabolism of 4β-hydroxycholesterol in primary human hepatocytes was found to be extremely slow. Only 4% of the added 4β-hydroxycholesterol was converted to polar products under the experimental conditions used. These polar products were recovered in the bile acid phase containing phase after extraction. Parallel incubations with 7α-hydroxycholesterol resulted in 59% conversion to bile acids, 70–90% of which corresponded to cholic and chenodeoxycholic acid (6).

Metabolism of 4β-Hydroxycholesterol in Normal and Transfected 293 cells—Control 293 cells expressing high endogenous activity of CYP7B1 (oxysterol 7α-hydroxylase) (12) converted 27-hydroxycholesterol very efficiently to 7α,27-dihydroxycholesterol as expected. Whereas the added 27-hydroxycholesterol was completely converted into its 7α-hydroxylated metabolite, no conversion was observed when 4β-hydroxycholesterol was incubated with the cells.

CYP46-transfected 293 cells converted 17% of the added 4β-hydroxycholesterol into a polar metabolite. This metabolite was analyzed by GC-MS as trimethylsilyl ether, and the following ions (m/z) were recorded: 634 (M, 544 (M – 90), 501 (M – 90–43), 454 (M – 2 × 90), 364 (M – 3 × 90), 159, and 145. The fragmentation pattern is consistent with 24-hydroxylated 4β-hydroxycholesterol. Control 293 cells did not 24-hydroxylate 4β-hydroxycholesterol.

293 Cells transfected with CYP39A1 converted 1.4% of the added 24-hydroxycholesterol into 7α,24-dihydroxycholesterol. The identity of the product was confirmed by GC-MS (7). When incubated under the same conditions with 4β-hydroxycholesterol, no significant conversion was observed (<0.1% conversion).

Incubations of 4β-Hydroxycholesterol with Human Recombinant CYP7A1 and CYP27A1—Cholesterol and 4β-hydroxycholesterol were incubated in parallel with recombinant 7α-hydroxylase (CYP7A1). Approximately 8% 4β-hydroxycholesterol and 17% cholesterol was 7α-hydroxylated, corresponding to turnover numbers of 0.087 and 0.184 nmol/min × nmol cytochrome P450, respectively. To verify the identity of the 4β-hydroxycholesterol metabolite, 4β,7α-dihydroxycholesterol was synthesized (4). The retention times on GC-MS (13.9 min) and mass spectra were identical for the 4β-hydroxycholesterol metabolite and synthetic 4β,7α-dihydroxycholesterol (Fig. 2).

Recombinant CYP27A1 was found to convert 4β-hydroxycholesterol into 4β,27-dihydroxycholesterol and metabolize it further into the corresponding acid. The products 4β,27-dihydroxycholesterol and the corresponding acid were converted into trimethylsilyl ether and trimethylsilyl ether-methyl ester, respectively, and analyzed by GC-MS. In the analysis of the 27-hydroxylated product, major ions were recorded at (m/z) 634 (M), 544 (M – 90), 505 (M – 129), 454 (M – 2 × 90), and 415 (M – 129–90). This fragmentation pattern is consistent with that expected for the trimethylsilyl ether of 4β,27-dihydroxycholesterol. In the analysis of the methylated acid, prominent ions were found with (m/z) 590 (M), 500 (M – 90), 462 (M – 129), 410 (M – 2 × 90), and 371 (M – 129–90). This mass spectrum is consistent with the structure of the trimethylsilyl ether of methyl-3β,4β-dihydroxy-5-cholestenic acid. The conversion of 4β-hydroxycholesterol into 27-oxygenated products was 50%. When cholesterol was incubated under the same conditions, the degree of 27-oxygenation was 27%. The corresponding turnover numbers were 0.41 and 0.22 nmol/min × nmol cytochrome P450, respectively.

Incubations of Cholesterol with Human Recombinant CYP3A4, CYP3A5, and CYP3A7—Incubation of cholesterol with recombinant CYP3A4, CYP3A5, and CYP3A7 resulted in the formation of 4β-hydroxycholesterol, and the relative rates of conversion were 100, 5.6, and 2.8%, respectively, and the turnover numbers were 7.9, 0.44, and 0.22 pmol/min × nmol cytochrome P450.

Analysis of Fecal Bile Acids—Feces from the female volunteer treated with carbamazepine was analyzed for bile acids by GC-MS. A small peak in the total ion chromatogram with a retention time of 26.0 min that constituted 0.56% total bile acids had ions (m/z) at 623 (M – 15), 548 (M – 90), 458 (M – 2 × 90), 433 (M – 90–115), 417 (M – 90–115–15), 369 (M – 2 × 90–89), 343 (M – 2 × 90–115), 329 (M – 2 × 90–129), 253, 147, and 129 (Fig. 3). The mass spectrum is similar to a previously published spectrum of 3α,12β-trihydroxy-5β-chol-
anoic acid (as methyl ester, trimethylsilyl ether) (13). With respect to the ion at \( m/z \) 369, the loss of 89 mass units is characteristic for two vicinal trimethylsiloxy groups (13). The ion at \( m/z \) 417 (probably formed by a loss of one trimethylsiloxy group and one 131-fragment from the molecular ion) is highly characteristic for 4β/H9252-hydroxylated bile acids (13). The feces from three healthy volunteers were analyzed for bile acids, and in one sample, the same 4β/H9252-hydroxylated bile acid was found (constituting 0.37% total bile acids), whereas the concentration in the remaining two samples from the healthy volunteers was below the detection limit.

**DISCUSSION**

In the present investigation, deuterium-labeled 4β-hydroxycholesterol was injected into volunteers to determine the elimination rate from the circulation. An exceptionally long half-life of ~2–3 days was found for 4β-hydroxycholesterol compared with the oxysterols 7α-, 27-, and 24-hydroxycholesterol for which the half-lives have been determined to be 0.5–12 h (10, 11). Previous in vitro experiments indicated a slow rate of formation for 4β-hydroxycholesterol (1). If the rate of formation is slow also in vivo, the long half-life could be expected to be the result of slow metabolism of the oxysterol. This was further investigated in primary human hepatocytes and by the use of normal and transfected cells as well as recombinant human cytochromes CYP7A1 and CYP27A1, enzymes known to metabolize oxysterols. Primary human hepatocytes efficiently converted 7α-hydroxycholesterol to bile acids, whereas only a small fraction of 4β-hydroxycholesterol was metabolized into polar products. This finding was in accordance with in vitro experiments with recombinant CYP7A1 where 4β-hydroxycholesterol was 7α-hydroxylated at a rate only half of that of cholesterol. Cultured 293 cells with a high endogenous CYP7B1 activity efficiently 7α-hydroxylated 27-hydroxycholesterol, whereas 4β-hydroxycholesterol was not converted at all. These experiments indicate that 4β-hydroxycholesterol undergoes the rate-limiting 7α-hydroxylation step much slower than cholesterol. It is possible that the 4β-hydroxyl group in some way impairs the 7α-hydroxylation reaction. The enzymatic hydroxylation of the steroid side chain was not impaired, because 4β-hydroxycholesterol was 27-oxygenated by recombinant CYP27A1 at almost twice the rate compared with cholesterol, and incubations with 293 cells transfected with CYP46 resulted in a rapid conversion into a 24-hydroxylated metabolite. Because CYP46 is expressed exclusively in brain in humans (14), 24-hydroxylation cannot be a quantitatively important pathway for elimination of 4β-hydroxycholesterol but may be of local importance in the brain. The different metabolic pathways investigated are outlined in Fig. 4.

The size of the pool of 4β-hydroxycholesterol in equilibrium with the administered deuterium-labeled material can be estimated from the dilution of the administered labeled 4β-hydroxycholesterol. The extrapolation of the elimination curves for the two healthy volunteers to time zero (Fig. 1) gives ratios of \( ^2\text{H}_6/^2\text{H}_0 \) of 0.45 and 0.29, respectively, of circulating 4β-

![FIG. 1. Elimination of deuterium-labeled 4β-hydroxycholesterol injected into volunteers. A, healthy male volunteer. B, healthy female volunteer. C, female volunteer with epilepsy treated for >10 years with carbamazepine.](image-url)
hydroxycholesterol. As 500 μg of the deuterium-labeled material was infused in the volunteers, the pools of unlabeled 4β-hydroxycholesterol were apparently 1.1 and 1.7 mg, respectively. The half-lives for 4β-hydroxycholesterol in the circulation in the two volunteers were determined to be 64 and 60 h, respectively. This corresponds to an elimination of ~0.2 and 0.3 mg/day, respectively. Assuming that 4β-hydroxycholesterol is quantitatively converted into bile acids, a maximum of ~0.3 mg/day would be formed. Because the normal production of bile acids is around 400 mg/day (15), <0.1% of this pool can be expected to be derived from such a hypothetical pathway. Such a small amount would not be detectable with the methodology normally used. In a patient with maximally up-regulated CYP3A4, the formation of 4β-hydroxylated bile acids could be expected to be up to 20-fold higher. In view of this, it is interesting that the patient treated with antiepileptics stud-
The bile acid had a content of one specific 4β-hydroxylated bile acid in feces corresponding to 0.6% total bile acids. When this bile acid was measured in feces from three healthy volunteers with normal plasma 4β-hydroxycholesterol concentrations, it was identified in one of the volunteers, whereas the concentration in feces from the other two volunteers was below the detection limit. The 4β-hydroxylated bile acid constituted 0.4% total bile acids, excluding 4β-hydroxylation of cholesterol as a major pathway for formation of the 4β-hydroxylated bile acid identified. Thus, the major pathway for the formation of 4β-hydroxylated bile acids remains to be determined. At the present state, the possibility must be considered that CYP3A4 may have some 4β-hydroxylase activity toward another intermediate in bile acid synthesis.

Because the high plasma concentrations of 4β-hydroxycholesterol in patients treated with some antiepileptic drugs are the result of increased synthesis and not impaired metabolism, 4β-hydroxycholesterol may reflect the CYP3A4 activity in vivo. Thus, this cholesterol metabolite is a potential clinical marker for CYP3A4 activity.

**FIG. 3.** Mass spectrum of bile acid (methyl ester, trimethylsilyl ether) isolated from feces from a volunteer treated with carbamazepine for >10 years. The bile acid was tentatively identified as 3,4β,12α-trihydroxy-5β-cholanoic acid.

**FIG. 4.** Metabolism of 4β-hydroxycholesterol. CYP7A1 (cholesterol 7α-hydroxylase) is catalyzing the rate-limiting step in the major pathway for bile acid synthesis. CYP7B1 (oxysterol 7α-hydroxylase) is of importance in an alternative pathway to bile acids. CYP27A1 (sterol 27-hydroxylase) present both in the liver and in extrahepatic tissues catalyzes the first steps in an alternative pathway to bile acids. CYP46 (cholesterol 24-hydroxylase) present only in brain in humans catalyzes 24-hydroxylation of cholesterol that facilitates elimination of cholesterol from the brain. CYP29A1 is present in the liver and catalyzes 7α-hydroxylation of 24-hydroxycholesterol.
Oxysterols have been suggested to be ligands for the nuclear receptors LXRα and LXRβ (16, 17), important transcription factors involved in the regulation of lipid metabolism (18). Potential LXR ligands reported include 22(R)-hydroxycholesterol, 24(S)-hydroxycholesterol, 24,25-epoxycholesterol, and 27-hydroxycholesterol (16, 17, 19). 4β-Hydroxycholesterol has been reported to activate LXR in vitro almost as efficiently as 24(S)-hydroxycholesterol (16). If 4β may rapidly increase up to 20-fold following the induction of healthy volunteer, making it unlikely that 4β hydroxylated bile acid were similar in the patient and in the Potential LXR ligands reported include 22(R)-hydroxycholesterol. Although 24(S)-hydroxycholesterol concentrations are surprisingly stable over time, plasma levels of 4β-hydroxycholesterol may rapidly increase up to 20-fold following the induction of CYP3A4 by various drugs (1). If 4β-hydroxycholesterol activates LXR also in vivo, this could be one of the mechanisms behind the disturbances in lipid metabolism often seen after treatment of patients with antiepileptic or neuroleptic drugs.

In conclusion, the elimination rate of 4β-hydroxycholesterol from the human circulation was found to be exceptionally slow compared with other oxysterols. This slow rate of elimination may at least in part be because of slow 7α-hydroxylation, which is the rate-limiting step in bile acid biosynthesis. A 4β-hydroxylated bile acid was identified in feces from healthy volunteers and from a patient with epilepsy with a high plasma concentration of 4β-hydroxycholesterol. The levels of the fecal 4β-hydroxylated bile acid were similar in the patient and in the healthy volunteer, making it unlikely that 4β-hydroxylation of cholesterol by CYP3A4 is a major pathway for the formation of fecal 4β-hydroxylated bile acids.

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