Serum concentration of 7α-hydroxycholesterol as an indicator of bile acid synthesis in humans

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Abstract The serum concentration of 7α-hydroxycholesterol as an indicator of total bile acid synthesis was investigated under different experimental conditions in humans. 7α-Hydroxycholesterol was measured by gas–liquid chromatography–mass spectrometry, using [$^2H_7$]7α-hydroxycholesterol and/or 5α-cholestane-3β,6β-diol as internal standards, and bile acid synthesis was estimated by the fecal balance method. Intraindividual variation was small when the concentration of 7α-hydroxycholesterol was determined twice in the same subject 2 days to 11 months apart (7.3 ± 6.5%, n = 52). In patients with advanced cirrhosis of the liver (n = 22) 7α-hydroxycholesterol was 3.4-fold lower (22 ng/ml ± 8) compared to matched controls (75 ng/ml ± 19). Administration of cholestyramine (4 g b.i.d.) for 14 days increased 7α-hydroxycholesterol concentration in five healthy volunteers from 40 ± 11 ng/ml to 181 ± 95 ng/ml ($P = 0.02$) and fecal excretion of acidic sterols from 254 ± 60 mg/d to 1336 ± 344 mg/d ($P < 0.01$). Although a significant correlation was found between 7α-hydroxycholesterol in serum and bile acid synthesis in patients with hypercholesterolemia ($r = 0.847$, $P < 0.001$, n = 17), it was impossible to accurately determine bile acid synthesis from the serum levels of 7α-hydroxycholesterol. Thus, determination of 7α-hydroxycholesterol concentrations in serum can be used to assess changes in bile acid synthesis rates over short and long term periods under various experimental conditions, but not to calculate bile acid synthesis correctly.–**Hahn, C., C. Reichel, and K. von Bergmann.** Serum concentration of 7α-hydroxycholesterol as an indicator of bile acid synthesis in humans. *J. Lipid Res.* 1995. **36:** 2059–2066.

Supplementary key words stable isotopes • isotope dilution • mass spectrometry • selected ion monitoring • cholestyramine

Hepatic conversion of cholesterol to bile acids followed by biliary secretion and subsequent fecal loss is a major metabolic step for the elimination of cholesterol from the body (1). Therefore, measurements of bile acid synthesis are important for understanding the regulation of overall cholesterol homeostasis. Several methods have been developed for determining the amount of bile acids synthesized in the liver. The isotope dilution technique of Lindstedt (2) was the first method described, but the patient had to take a radioactive marker. The fecal balance method (3, 4) and the stable isotope dilution technique in plasma (5, 6) represent improvements in methodology, but they are time-consuming and the intake of markers is still necessary. Measuring the activity of cholesterol 7α-hydroxylase, the rate-limiting enzyme of bile acid synthesis, under different therapeutic regimens indicates indirectly the bile acid synthesis (7, 8), but this method is restricted by a requirement for liver biopsies. 7α-Hydroxycholesterol and 7α-hydroxy-4-cholesten-3-one concentrations in human serum have been shown to reflect the enzyme activity of cholesterol 7α-hydroxylase in the liver (9–13). In a recent report it was shown that the serum 7α-hydroxycholesterol level reflects biliary bile acid output in patients with obstructive jaundice after percutaneous transhepatic biliary drainage (14). The analysis of one of these intermediates in the bile acid synthesis pathway is a favorable method for the determination of the relative synthesis rate from just one serum sample and for detecting short-term intraindividual modulations in synthesis rates or differences among different groups of patients.

Therefore, 7α-hydroxycholesterol was measured in the same subject twice at different time intervals and in patients with advanced cirrhosis of the liver. Bile acid synthesis and 7α-hydroxycholesterol were also measured before and during treatment with cholestyramine. Patients with severe liver disease were studied as well as the effect of cholestyramine because in both circumstances bile acid synthesis is altered (15, 16). In addition, the concentration of 7α-hydroxycholesterol in serum was compared with direct measurement of total bile acid synthesis in patients with hypercholesterolemia.

Abbreviations: GLC, gas–liquid chromatography; GLC–MS, gas–liquid chromatography–mass spectrometry; BHT, butylated hydroxytoluene; TMSi, trimethylsilyl; SIM, selected ion monitoring; LDL, low density lipoproteins; HDL, high density lipoproteins.

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MATERIALS AND METHODS

Materials

We purchased 7α-hydroxycholesterol and 5α-cholestane-3β,6β-diol from Steraloids, Inc. (Wilton, NH), and [25,26,26,26,27,27,27-$^2H_7$]7α-hydroxycholesterol was generously donated by I. Björkhem (Huddinge University Hospital, Stockholm, Sweden). The purity of the sterols was confirmed by gas–liquid chromatography by means of their trimethylsilyl (TMSi)-ether derivatives and proved to be better than 98%. Bond Elut silica cartridge columns (Bonded Phase, SI, size 1 cc) were obtained from Varian, Harbor City, CA.

Patients

We determined the fecal excretion of acidic sterols in nine patients with hypercholesterolemia (eight of them were studied twice), and in five normolipemic male volunteers before and during treatment with cholestyramine (4 g b.i.d.) for 14 days using the fecal balance method. All subjects gave their informed consent; the study was in accord with the Declaration of Helsinki and approved by the local ethical committee.

Experimental procedure

For the determination of the fecal excretion of acidic sterols, the subjects received sitostanol (30 mg t.i.d.) for 7 days as a fecal marker and they kept a dietary protocol during this time. Fecal samples were collected before and on days 5 to 7 during the fecal marker intake. Eight of the subjects repeated the procedure a second time after a period of 4 weeks and five healthy volunteers after 2 weeks of treatment with cholestyramine. Blood samples were collected in the morning after an overnight fast. After centrifugation, 2,6-di-tert-butyl-4-methyl-phenol (BHT) was added as antioxidant to a final concentration of 50 μg/ml serum. The samples were frozen immediately at -20°C.

To investigate storage stability, blood samples from ten patients were divided into several aliquots. BHT was added to only one of these aliquots and all samples were stored at -20°C. The first aliquot was analyzed within 2 weeks, the next after 6, 7, and 8 months until an autoxidation process was observable. The sample with a supplement of BHT was then measured to compare the serum concentration of 7α-hydroxycholesterol to the other samples without BHT.

For the determination of the intra-individual variation of 7α-hydroxycholesterol concentration in serum, blood samples from 52 patients were collected twice at different times (in 33 patients 2 to 3 days apart, in 8 patients 1 to 3 weeks apart, and in 11 patients 2 to 11 months apart). Serum samples from 22 male patients with advanced liver disease and biopsy-proven cirrhosis of the liver were collected for measurement of 7α-hydroxycholesterol, and the results were compared to those of 22 healthy subjects matched for sex, age, and weight. All serum samples were drawn in the morning and were handled as noted above.

Sample preparation for determination of 7α-hydroxycholesterol

The sample preparation was carried out according to Yamashita, Kuroki, and Nakayama (17) and Oda et al. (12) with minor modifications. Two different internal standards were used, one was deuterated 7α-hydroxycholesterol, the second was 5α-cholestane-3β,6β-diol. The internal standard solutions were dissolved in cyclohexane to a final concentration of 1 μg/ml of [$^2H_7$]7α-hydroxycholesterol and 5 μg/ml of 5α-cholestane-3β,6β-diol and were stabilized with BHT (50 μg/ml). In contrast to Yamashita et al. (17) and Oda et al. (12), a smaller size of silica cartridge columns was used (100 mg instead of 500 mg), the solvent volume was reduced, and toluene was used instead of benzene as a solvent for the clean-up procedure with the silica columns. Fifty ng [$^2H_7$]7α-hydroxycholesterol and 250 ng 5α-cholestane-3β,6β-diol were added to 500 μl of serum. After a 1-h alkaline hydrolysis with 1 ml 1 N NaOH in 90% ethanol at 67°C, 100 μl of water was added and the sterols were extracted three times with 3 ml cyclohexane. The combined organic phases were evaporated to dryness under a stream of dry nitrogen. The residue was dissolved in 200 μl toluene–ethyl acetate 9:1(v/v) and applied to a silica cartridge column that had been pre-washed with 1.8-ml portions each of methanol, ethyl acetate, toluene–ethyl acetate 2:3(v/v), and toluene–ethyl acetate 9:1(v/v). The column was eluted with 1.2 ml of toluene–ethyl acetate 9:1 to remove most of the cholesterol and then eluted with 1.2 ml of toluene–ethyl acetate 2:3 to recover the 7α-hydroxycholesterol and the internal standards. The latter fraction was evaporated to dryness under nitrogen and the sterols were converted to trimethylsilyl (TMSi)-ethers by adding 1 ml TMSi-reagent (pyridine–hexamethyldisilazan–trimethylchlorosilane 9:3:1 (v/v/v)) and heating the mixture at 65°C for 75 min. The silylating reagent was evaporated to dryness under nitrogen and the residue was dissolved in 0.5 ml of n-decane. Four hundred μl was transferred into vials for gas–liquid chromatography–mass spectrometry (GLC–MS).

Gas–liquid chromatography–mass spectrometry

The samples were analyzed by GLC–MS on a Hewlett-Packard (HP) gas chromatograph 5890 equipped with an automatic sample injector (HP 7673A) using a splitless injection technique. The gas chromatograph was connected with an HP 5971 quadrupole mass spec-

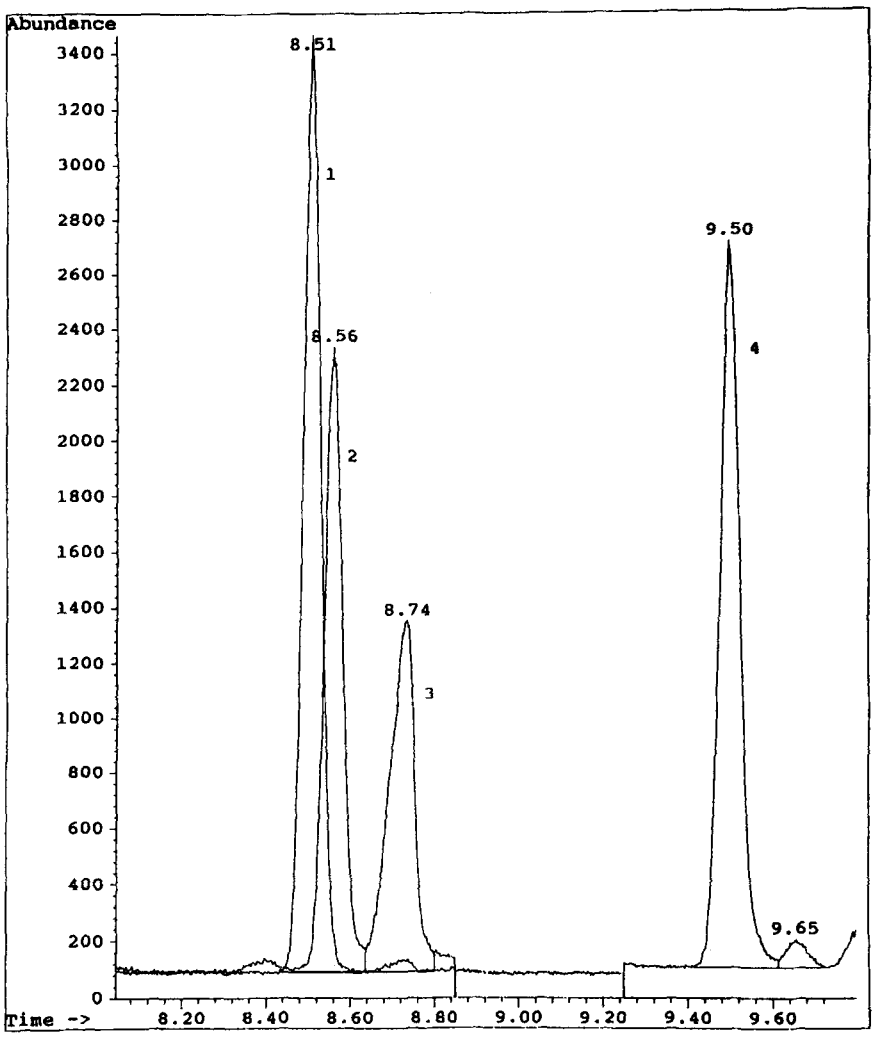

Fig. 1. Selected ion monitoring of the ions at *m/z* 456, 463, and 458 of the TMSi-ether derivatives extracted from a serum sample: *m/z* 456 was chosen to detect 7α-hydroxycholesterol (2) and cholesterol (3); *m/z* 463 for [$^2H_7$]7α-hydroxycholesterol (1) and *m/z* 458 for 5α-cholestane-3β,6β-diol (4).

trometer. The GLC–MS instrument was controlled by an HP Vectra 386s/20 computer equipped with a DOS Chem Station data system. The TMSi-ether derivatives were separated on a 12-m fused silica capillary column (HP Ultra 1, 0.2 mm i.d. and 0.32 μm film thickness). Helium was used as a carrier gas with a column head pressure of 5 psi. The injector temperature was 280°C. The temperature program was as follows: start at 180°C for 1.5 min; rate A: 30°C min^{-1} up to 280°C; hold for 0.83 min; rate B: 3°C min^{-1} up to 290°C. The temperature of the transfer line was kept at 280°C. Electron impact ionization was applied with 70 eV. Selected ion monitoring (SIM) was performed by cycling the quadrupole mass filter between the chosen *m/z* values at a rate of 6.94 cycles per second for 7α-hydroxycholesterol and [$^2H_7$]7α-hydroxycholesterol and 7.62 cycles per second for 5α-cholestane-3β,6β-diol. In the SIM mode the ion of *m/z* 456 (M–90) was monitored for 7α-hydroxycholesterol, *m/z* 463 (M–90) for [$^2H_7$]7α-hydroxycholesterol, and *m/z* 458 (M–90) for 5α-cholestane-3β,6β-diol derivatives, respectively. From the ratio between the peak areas from these three tracings, the amount of unlabeled 7α-hydroxycholesterol was calculated by use of a standard curve.

Determination of fecal excretion of acidic sterols

Total bile acid synthesis was measured by the fecal balance method as described in the literature (4).

Determination of serum lipoproteins

Serum total cholesterol, HDL-cholesterol, and triglycerides were measured by enzymatic methods (Boehringer, Mannheim, Germany). LDL-cholesterol concentration was calculated by the formula of Friedewald et al. (18).

Statistical analysis

Data are expressed as mean ± standard deviation (SD) to show variation within a group. The reproducibility of two measurements in one subject at different time intervals was assessed by precision. Precision was calculated by the following equation: precision (%) = $[(M_1 - M_2) / 2 \times M \text{ mean}] \times 100$, where $M_1 - M_2$ is the difference between the two measurements and M is the mean. Differences between two groups were calculated by independent samples *t*-test and were considered significant at the level of $P < 0.05$. The change in 7α-hydroxycholesterol concentration within the group treated with cholestyramine was analyzed with a paired Student's *t*-test. Linear regression was calculated by the method of least squares. The correlation between parameters was tested by Pearson's correlation coefficient. All calculations were done with the statistical software SPSS/Windows (SPSS Inc.).

RESULTS

Sample preparation

In contrast to Oda et al. (12) and Yamashita et al. (17), toluene was used instead of benzene because it had less toxicity. The smaller sized silica cartridge columns (100 mg instead of 500 mg) allowed a faster sample elution, and less solvent was needed for sample preparation. The removal of cholesterol with this procedure was between 90 and 95% (data not shown), which was sufficient for an accurate and precise measurement of 7α-hydroxycholesterol. **Figure 1** shows selected ion chromatograms of a serum sample with the TMSi-ether derivatives of 7α-hydroxycholesterol, [$^2H_7$]7α-hydroxycholesterol, and 5α-cholestane-3β,6β-diol.

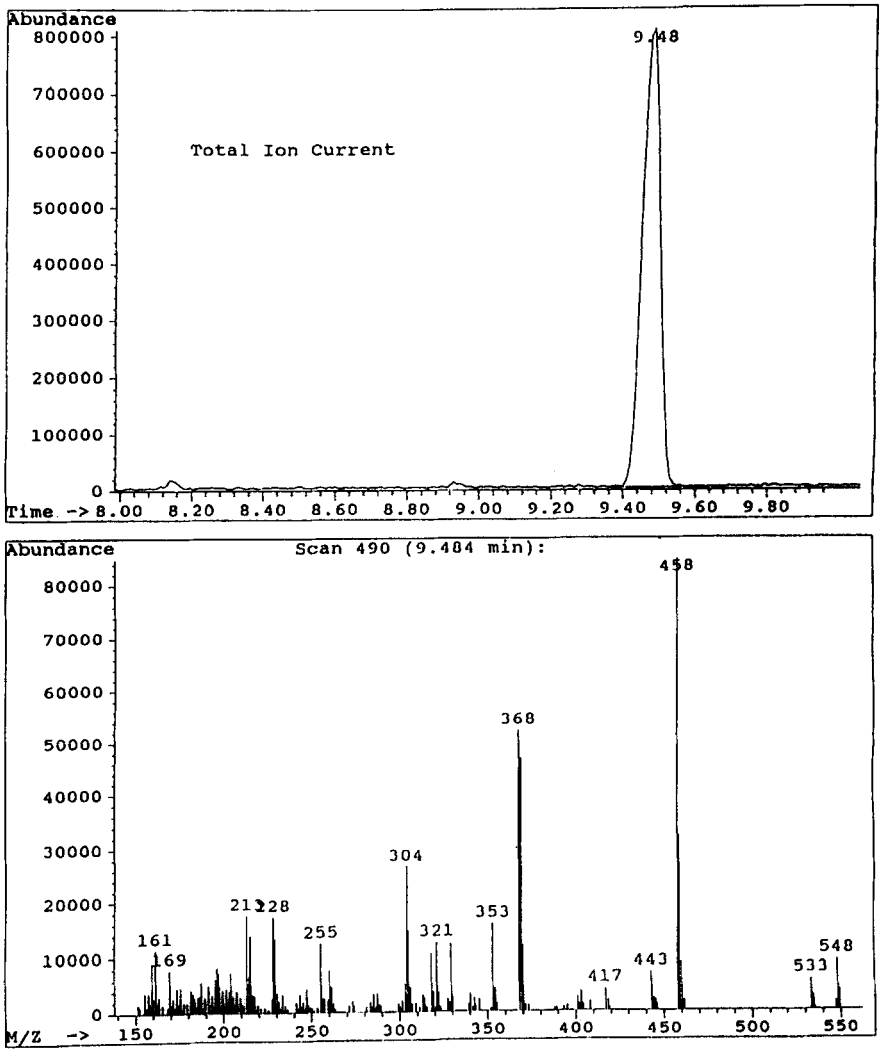

Fig. 2. Total ion current chromatogram obtained from GLC–MS analysis of the TMSi-ether derivative of 5α-cholestane-3β,6β-diol (upper panel). The electron impact mass spectrum of the compound is shown in the lower panel with the molecular ion *m/z* 548 and the base peak at *m/z* 458 (M - 90).

5α-Cholestane-3β,6β-diol as an internal standard

Deuterated 7α-hydroxycholesterol or 5α-cholestane-3β,7β-diol have been the internal standards used up to now for the quantification of 7α-hydroxycholesterol in human serum. The disadvantage of both is that they are not commercially available and need to be synthesized. Therefore, we tried 5α-cholestane-3β,6β-diol. Because of its chemical structure we expected it to behave similarly to 7α-hydroxycholesterol during sample preparation. We then showed that the compound is not present in human serum and, second, that it could be separated from 7α-hydroxycholesterol by GLC. The TMSi-ether derivative of 5α-cholestane-3β,6β-diol produces a high-intensity fragment ion at *m/z* 458 (M - 90) on GLC–SIM. **Figure 2** shows the total ion chromatogram and the mass spectrum of the TMSi-ether derivative of 5α-cholestane-3β,6β-diol. To demonstrate that both internal standards give identical results for the determination of 7α-hydroxycholesterol, 32 serum samples were analyzed. In **Figure 3** the serum concentrations with [$^2H_7$]7α-hydroxycholesterol as internal standard are plotted against the results obtained with 5α-cholestane-3β,6β-diol. Slope (1.02) and intercept (-0.9) calculated by linear regression analysis show that the values are identical (r = 0.995).

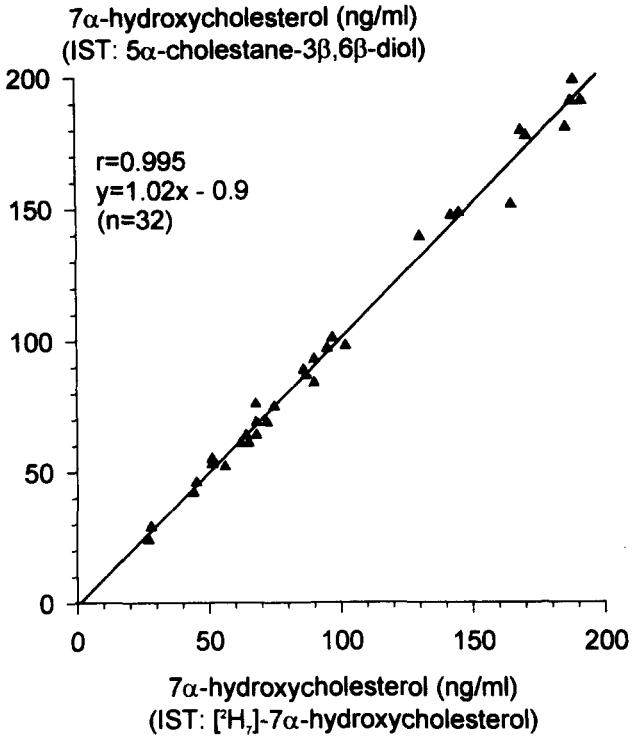

Fig. 3. Comparison of 7α-hydroxycholesterol concentrations in serum samples calculated with [$^2H_7$]7α-hydroxycholesterol and 5α-cholestane-3β,6β-diol as internal standards.

TABLE 1. Recovery of different amounts of 7α-hydroxycholesterol added to serum containing 23.5 ng/0.5 ml 7α-hydroxycholesterol

Amount Added	Amount Found	Recovery[a]
ng	*ng*	%
0	23.5 ± 0.46	
20	40.7 ± 0.91	86 ± 5
40	63.3 ± 1.25	99 ± 3
80	97.3 ± 6.09	92 ± 8

Values given as mean ± SD; n = 3.
[a]Recovery (%) = [(amount found - basic concentration) / amount added] × 100.

Reproducibility and recovery

The precision of the method was assessed by fivefold analysis of one serum sample. The coefficient of variation for sample preparation with [$^2H_7$]7α-hydroxycholesterol as internal standard was about 1.6%, with 5α-cholestane-3β,6β-diol as internal standard about 6.3%. For the recovery studies, different amounts of 7α-hydroxycholesterol were added to aliquots of the same serum sample. [$^2H_7$]7α-hydroxycholesterol was used as external and 5α-cholestane-3β,6β-diol as internal standard. Every amount added and the blank value of the serum were prepared threefold. The recovery was calculated by dividing the amount recovered by the amount added. The overall recovery was approximately 93% (**Table 1**). Adding varying amounts of 7α-hydroxycholesterol to serum produced corresponding parallel curves compared to the standard curves. The lower limit of quantification was about 10 ng/ml, and the limit of detection was found to be approximately 3 ng/ml. The linearity was in the range of 10 to 600 pg per injection.

Autoxidation of cholesterol in frozen serum samples

Because the autoxidation of cholesterol is a restricting factor in the analysis of 7α-hydroxycholesterol in serum, we examined the effectiveness of BHT as an antioxidant. After storing the samples between 6 and 8 months at -20°C, 7α-hydroxycholesterol concentration in serum increased. Samples to which BHT was added did not show higher 7α-hydroxycholesterol levels, indicating that autoxidation of cholesterol can be delayed by the addition of BHT (data not shown).

Intra-individual variation

7α-Hydroxycholesterol concentrations in serum were measured in 52 patients on 2 different days. The results showed a slight intra-individual variation (**Fig. 4**). Comparison of 7α-hydroxycholesterol concentrations on the two occasions gave a correlation coefficient of 0.977 and the slope was close to 1. The precision of the two measurements at different time intervals is given in **Table 2.** No difference between the subgroups was

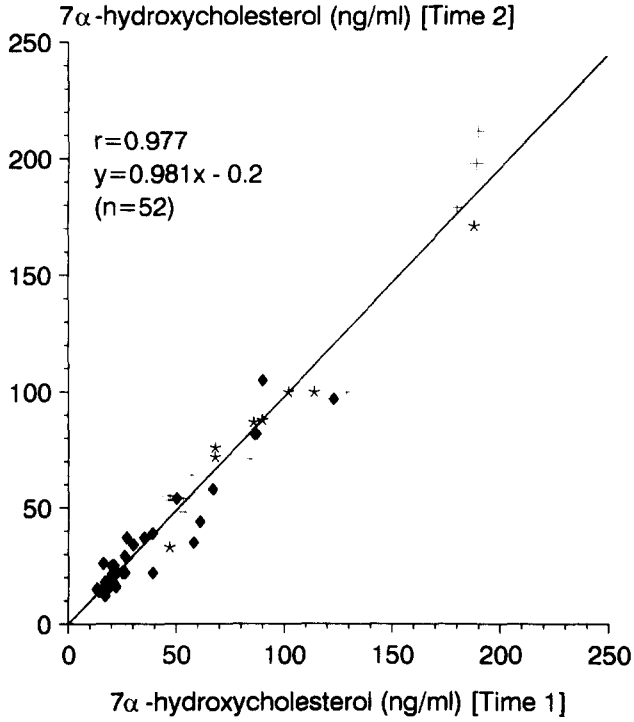

Fig. 4. Comparison of the serum concentration of 7α-hydroxycholesterol measured in the same subject on two different occasions (◆, 2 to 3 days apart; *, 1 to 3 weeks apart; and +, 2 to 11 months apart). The correlation was highly significant ($P < 0.001$).

noted, and the precision averaged 7.3% (range 0 to 28%). However, it should be noted that in some cases the difference was about 40%, and in one patient an increase in 7α-hydroxycholesterol concentration of about 63% was observed.

7α-Hydroxycholesterol in patients with cirrhosis of the liver

7α-Hydroxycholesterol concentrations in serum of 22 male patients with biopsy-proven cirrhosis of the liver were significantly lower than values obtained in healthy volunteers (22 ± 8 ng/ml vs. 75 ± 19 ng/ml; $P < 0.001$) who were matched for sex, age, and weight (**Table 3**).

7α-Hydroxycholesterol in serum and bile acid synthesis

Treatment with cholestyramine reduced mean LDL-cholesterol concentration in five healthy volunteers from 107 mg/dl to 77 mg/dl (-28%). 7α-Hydroxycholesterol concentration averaged 40 ± 11 ng/ml before treatment and increased to 181 ± 95 ng/ml ($P = 0.02$) during treatment with cholestyramine. The mean fecal excretion of acidic sterols was 254 ± 60 mg/d and 1336 ± 344 mg/dl, respectively. Thus, both parameters increased almost to the same extent (4.5- and 5.3-fold). The correlation between the concentration of 7α-hydroxycholesterol and acidic sterols was highly significant ($r = 0.931$, $P < 0.001$, **Fig. 5**).

Serum levels of 7α-hydroxycholesterol were compared with the fecal excretion of acidic sterols (n = 17). Although mean bile acid synthesis measured with the fecal balance method on 3 consecutive days is correlated with the 7α-hydroxycholesterol concentration measured in one serum sample ($r = 0.847$, $P < 0.001$) (data not shown), and the intercept of the regression line was not different from zero, the variation was too large to conclude that serum levels of 7α-hydroxycholesterol accurately determine bile acid synthesis.

DISCUSSION

Previous methods for the determination of bile acid synthesis rates in humans are well established but time- and material-consuming procedures and are not suited to detecting changes in bile acid synthesis occurring within a few days. Therefore, several teams of investigators have tried to establish an accurate and simple method for the evaluation of changes in bile acid synthesis in a large number of patients. Quantification of the bile acid precursor 7α-hydroxycholesterol in serum seems to be a good parameter that has been shown to reflect the activity of cholesterol 7α-hydroxylase, the rate-limiting enzyme of bile acid synthesis (12, 13). However, it has been reported that the analysis of 7α-hy-

TABLE 2. Precision of measurements in 52 patients on two different occasions

Measurement on Two Different Occasions	Precision[a]
	%
2 to 3 days apart (n = 33)	8.3 ± 7.3
1 to 3 weeks apart (n = 8)	5.0 ± 5.5
2 to 11 months apart (n = 11)	6.1 ± 3.9
Whole group (n = 52)	7.3 ± 6.5

Values given as mean ± SD.
[a]For calculation of precision see Methods.

TABLE 3. Comparison of 7α-hydroxycholesterol concentrations in serum in male patients with biopsy-proven cirrhosis of the liver and controls matched for sex, age, and weight

	Cirrhosis (n = 22)	Controls (n = 22)
	ng/ml	
Age (yrs)	55 ± 11	55 ± 10
Weight (kg)	80 ± 17	81 ± 10
7α-Hydroxycholesterol (ng/ml)	22 ± 8[a]	75 ± 19

Values given as mean ± SD.
[a]$P < 0.001$.

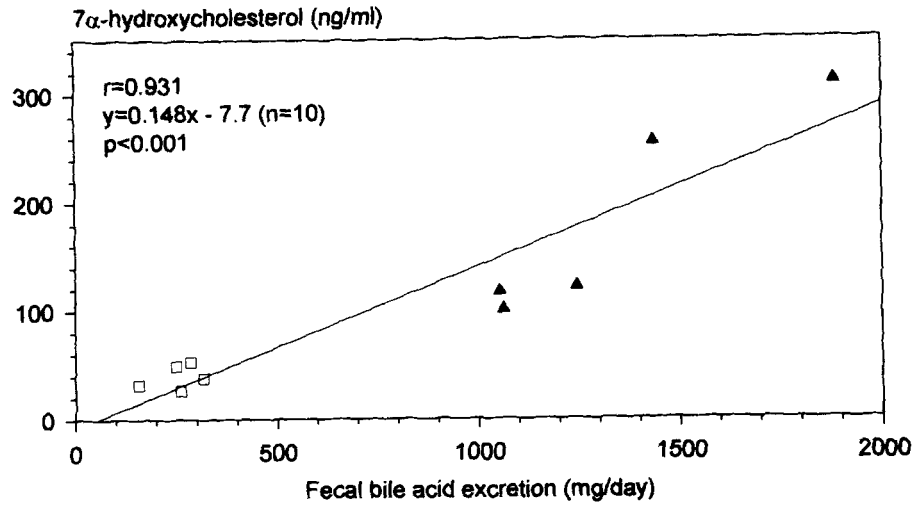

Fig. 5. Comparison of 7α-hydroxycholesterol concentration in serum and fecal bile acid excretion on 3 consecutive days in five healthy volunteers before and during treatment with cholestyramine (4 g b.i.d.) (□, before treatment, ▲, during treatment).

droxycholesterol is complicated by the autoxidation of cholesterol and that 7α-hydroxycholesterol may be produced while handling serum samples or during prolonged storage (11, 19). Therefore, we added BHT as an antioxidant before freezing the samples and were able to show that samples without BHT are stable for at least 6 months. Autoxidation can be delayed by the addition of BHT. Thus values obtained for the same sample were reproducible (intra- or inter-day variation). Another difficulty is the availability of a suitable internal standard. Both compounds in use up to now have the disadvantage that they need to be synthesized. The commercially available compound, 5α-cholestane-3β,6β-diol, has now proved to be an alternative. It is not present in human serum, behaves the same way as 7α-hydroxycholesterol during the sample preparation, gives a high-intensity fragment ion at *m/z* 458, and leads to identical results compared to those calculated with [$^2H_7$]7α-hydroxycholesterol as internal standard. Serum concentrations of 7α-hydroxycholesterol obtained with our modified method are consistent with the previous findings of Oda et al. (12) and Breuer and Björkhem (19). Taking all this into account and with an overall recovery rate of 93%, we claim this simplified method to be an improved alternative for the routine measurement of 7α-hydroxycholesterol as an indicator of changes in bile acid synthesis in humans. Determination of 7α-hydroxy-4-cholesten-3-one, another bile acid precursor, might be an alternative possibility for this purpose. Indeed, both bile acid precursors show a close relationship with the activity of cholesterol 7α-hydroxylase in liver biopsies (9, 12).

The intraindividual variations of the serum levels of 7α-hydroxycholesterol are low when blood was drawn at two different time intervals (from 2 to 3 days to 2 to 11 months apart) (Fig. 4 and Table 2). Therefore, for short term changes in bile acid synthesis 7α-hydroxycholesterol is a suitable indicator. Indeed, short term treatment with a bile acid-binding resin confirms a strong relationship between changes in bile acid synthesis and the serum concentration of 7α-hydroxycholesterol (Fig. 5). During treatment with the bile acid resin, 7α-hydroxycholesterol increased almost to the same extent (4.5-fold) as fecal excretion of bile acid (5.3-fold). Further evidence for the close relationship between bile acid synthesis and serum concentrations of 7α-hydroxycholesterol was obtained by our data and showed that patients with advanced cirrhosis of the liver have a more than 3-fold lower concentration of the bile acid precursor than matched controls (Table 3). These results are in line with previous studies from Vlahcevic et al. (15) who found a 75% reduction in cholic acid and a 30% reduction in chenodeoxycholic acid compared to patients without liver disease using the isotope dilution technique of Lindstedt (2).

The results prompted us to compare total bile acid synthesis with the concentration of 7α-hydroxycholesterol in patients with hypercholesterolemia without drug treatment. Although the concentration of 7α-hydroxycholesterol in serum was significantly correlated with fecal bile acid excretion, it was impossible to accurately calculate bile acid synthesis from serum levels of 7α-hydroxycholesterol. It should also be pointed out that the level of 7α-hydroxycholesterol does not correspond to total bile acid synthesis in patients with the rare lipid storage disease of cerebrotendinous xanthoma-

tosis. These patients with a lack of C_{27}-steroid-27-hydroxylase (20) showed extremely high values of 7α-hydroxycholesterol (unpublished results).

In conclusion, we were able to establish a simplified method with easily prepared samples and to establish 5α-cholestane-3β,6β-diol as a new internal standard for the determination of 7α-hydroxycholesterol in serum. Thus, analysis of the bile acid precursor after a simple blood sampling can be used as complementary method in studies of short-term changes in bile acid synthesis in humans. In addition, screening large populations for the effects of drugs that might influence bile acid or cholesterol metabolism can be performed easily to elucidate possible disturbances in bile acid synthesis.

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