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DETERMINATION OF SOME HYDROXYCHOLESTEROLS IN HUMAN SERUM SAMPLES

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SUMMARY

The simultaneous determination of some hydroxycholesterols in human serum samples is described. The procedure is based on hydrolysis and extraction of these compounds in serum samples, followed by removal of especially cholesterol (making use of reversed-phase high-performance liquid chromatography) and derivatization of the purified compounds to their trimethylsilyl ethers and subsequent gas chromatography using flame ionization detection. Serum levels of 7α-hydroxycholesterol, 7β-hydroxycholesterol and 26-hydroxycholesterol were determined in several groups of patients: (a) normals, (b) untreated patients suffering from cerebrotendinous xanthomatosis, (c) patients suffering from cerebrotendinous xanthomatosis and treated with either chenodeoxycholic acid or cholic acid in an effective dose, (d) patients suffering from cerebro-hepato-renal syndrome, (e) patients suffering from hypercholesterolemia and treated with cholestyramine for prolonged periods and (f) one patient presumed to be suffering from an inborn error of metabolism in bile acid synthesis. It can be concluded that the 7α-hydyroxycholesterol concentration in serum is a good parameter for establishing disorders involving the metabolic conversion of 7α-hydroxycholesterol towards bile acids. In addition, 26-hydroxycholesterol levels in patients suffering from cerebrotendinous xanthomatosis are beyond detectable limits, even during treatment with bile acids in an effective dose, whereas in all other conditions this compound is substantially present.

INTRODUCTION

Bile acids are detergents,which are responsible for resorption of lipids in the gastro-intestinal tract [1]. Normal synthesis of the primary human bile acids chenodeoxycholic acid and cholic acid from cholesterol includes modifications in

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the steroid nucleus and degradation of the cholesterol side-chain [2]. A number of inherited diseases have been discovered, in which an enzyme deficiency is present in the synthetic pathway of bile acids. Examples of defects in the degradation of the cholesterol side-chain are cerebrotendinous xanthomatosis (CTX) [3], which includes a 26-hydroxylation or 24*S*-hydroxylation deficiency, and cerebro-hepato-renal syndrome (CHRS) [4,5] which is based on a deficiency in the removal of the terminal isopropyl group of the cholesterol side-chain owing to the absence of liver peroxisomes. Inborn errors of metabolism in bile acid synthesis involving the transformation of the steroid nucleus have not been described hitherto.

CTX can be detected by demonstrating a raised level of cholestanol [3,6], urinary and faecal excretion of abnormal so-called bile alcohols and abnormal bile acids (23-hydroxycholic acid and 23-norcholic acid) [3,7] and a decreased level of serum 26-hydroxycholesterol [8–10]. CHRS is indicated by the presence of coprostanoic acids in serum [4,5]. An interesting intermediate in the synthetic route of bile acids is 7α-hydroxycholesterol, because it represents the first, rate-limiting step in bile acid synthesis, formed by the action of liver 7α-hydroxylase [11]. The determination of 7α-hydroxycholesterol has been described in order to establish 7α-hydroxylase activity in the liver [12,13]. Moreover, analysis of 7α-hydroxycholesterol and 7β-hydroxycholesterol has also been performed in human serum samples as an illustration of the analysis of mixtures of polar steroids [14,15]. We assumed that the determination of this sterol in serum could be a useful parameter in studies concerning disturbances in bile acid synthesis, whether acquired, inherited or brought about by medical treatment. Such determinations could be even more worthwhile when combined with determinations of some other hydroxycholesterols known to be present in serum, such as 26-hydroxycholesterol [8–10]. Therefore we decided to set up a capillary gas chromatographic (GC) assay with the main objective of determining 7α-hydroxycholesterol in serum samples and, if possible, some other hydroxycholesterols of interest in one analytical run.

Difficulties in setting up a GC determination for hydroxycholesterols are caused by the close structural similarity and the large concentration differences of these compounds in comparison with cholesterol. Therefore, as described previously [8–10] for 26-hydroxycholesterol, sample preparation should include removal of cholesterol from more polar substances (hydroxycholesterols). Unfortunately, the described assay is less useful for routine clinical chemical analyses, as (a) the work-up procedure is rather laborious and large volumes of organic solvent have to be used, (b) the assay includes the use of scarce mass spectrometric (MS) instrumentation and (c) 2.5 ml of serum are necessary for the determination of just one substance.

Our assay is based on GC with flame ionization detection (FID) using trimethylsilyl derivatives after hydrolysis, extraction and removal of cholesterol by high-performance liquid chromatography (HPLC) of serum samples (1.0 ml) to which 20α-hydroxycholesterol was added as internal standard (endogenous 20α-hydroxycholesterol was not found in all the serum samples investigated). Normal values in serum for 7α-hydroxycholesterol, 7β-hydroxycholesterol and 26-

hydroxycholesterol were determined with this assay in 40 healthy volunteers. In a number of patients with known disturbances in bile acid metabolism, serum 7α-hydroxycholesterol, 7β-hydroxycholesterol and 26-hydroxycholesterol have been determined. The overall applicability of the assay in relation to cholesterol metabolism is discussed.

EXPERIMENTAL

Materials

7α-Hydroxycholesterol, 7β-hydroxycholesterol, 19-hydroxycholesterol, 20α-hydroxycholesterol, 22*R*-hydroxycholesterol and 22*S*-hydroxycholesterol were supplied by Steraloids (Wilton, NH, U.S.A.). 25-Hydroxycholesterol and 26-hydroxycholesterol were kindly provided by Professor N.B. Javitt (New York University Medical Center, New York, NY, U.S.A.). All other chemicals were purchased from Merck (Darmstadt, F.R.G.) and were of analytical grade. The HPLC guard column and analytical column RP-18 Spheri 5 were from Brownlee (Santa Clara, CA, U.S.A.). The fused-silica capillary column CP-Sil-19-CB (25 m$\times$0.22 mm I.D.) was supplied by Chrompack (Middelburg, The Netherlands).

Samples

Serum samples were analysed as soon as they arrived at the laboratory or stored at $-20°$C until analysis.

Controls

Serum samples of 40 healthy volunteers, aged 21–54 years, were collected for the determination of normal values of 7α-hydroxycholesterol, 7β-hydroxycholesterol and 26-hydroxycholesterol in serum.

Patients

Serum samples from several groups of patients were collected for the determination of 7α-hydroxycholesterol, 7β-hydroxycholesterol and 26-hydroxycholesterol: (a) nine untreated patients suffering from CTX; (b) eleven patients suffering from CTX treated either with chenodeoxycholic acid or cholic acid in effective doses for extended periods (see also refs. 16 and 17); (c) three patients suffering from CHRS; (d) fourteen patients suffering from hypercholesterolemia and treated daily with 12 g of Questran (cholestyramine) for prolonged periods; (e) one patient suffering from a malabsorption syndrome, as a consequence of a very low bile acid pool and therefore suspected from an inborn error of metabolism in bile acid synthesis, during fat loading and fasting [18].

Determination of 7α-hydroxycholesterol, 7β-hydroxycholesterol and 26-hydroxycholesterol in serum

Sample preparation. To 100 μl of an ethanolic internal standard solution, containing 4.9 mg/l 20α-hydroxycholesterol, 1.0 ml of serum and 2.5 ml of a freshly prepared mixture, consisting of 60% (w/v) aqueous potassium hydroxide solution–96% ethanol (5:95, v/v), were added. After vortexing, the solution was

incubated at 55°C for 45 min. After cooling, 1.5 ml of water and ca. 2 g of solid sodium chloride were added. After vortexing for 3 min, 5 ml of hexane were added. After shaking for 10 min, the two layers were separated by centrifuging for 1 min at 550 *g*. The hexane layer was transferred to another tube and evaporated to dryness under a stream of nitrogen at 60°C. The solution was redissolved in 230 μl of 2-propanol.

Sample clean-up (especially removal of cholesterol) was carried out by HPLC, using a Varian Model 5000 HPLC pump (Walnut Creek, CA, U.S.A.) equipped with a reversed-phase guard column and a reversed-phase analytical column (see *Materials*) and, in order to monitor the appearance of cholesterol, a Pye Unicam (Cambridge, U.K.) Model PU 4020 UV detector, set at a wavelength of 202 nm (absorbance maximum of cholesterol). The output filter of the UV detector was set at 2 s and the range at 0.08 a.u.f.s. The electrical output was fed to a Philips (Philips, Eindhoven, The Netherlands) Model PM 4100 recorder, which was set at 20 mV full scale. The mobile phase was a solution of acetonitrile–2-propanol–water (45:45:10, v/v/v). The flow-rate was 1.0 ml/min. A 200-μl sample was automatically injected on the column with a WISP 710B autosampler (Waters Assoc.). Following injection, the first 5-min fraction was collected with an LKB 2212 HeliRac fraction collector (Bromma, Sweden). The remainder of the eluate (a.o. containing cholesterol) was discarded.

The collected HPLC fraction was evaporated to dryness under a stream of nitrogen at 60°C. Derivatisation was carried out by addition of 100 μl of a mixture of bis(trimethylsilyl)acetamide–pyridine–trimethylbromosilane (4:5:1, v/v/v) and allowing to stand for 1 h at 40°C. Clean-up was performed by the addition of 5 ml of hexane and 3 ml of 0.1 *M* hydrochloric acid. The mixture was vortexed for 30 s. The aqueous layer was removed and the hexane layer was washed with 3 ml of water. The hexane layer was transferred to another tube and evaporated

TABLE I

METHYLENE UNIT VALUES OF HYDROXYCHOLESTEROLS (TRIMETHYLSILYL DERIVATIVES) ON THE CP-Sil-19CB CAPILLARY COLUMN

Methylene unit values were determined by previous injection of a hydrocarbon mixture containing C_{30}, C_{32}, C_{36} and C_{38} compounds under identical GC conditions and comparison of retention times of hydrocarbons and hydroxycholesterols.

Hydroxycholesterol	Methylene unit value CP-Sil-19CB
7α	31.86
0	32.23
7β	33.21
19	32.71
20α	34.00
22*S*	33.59
22*R*	33.78
25	35.04
26	35.70

to dryness under a stream of nitrogen at 50 °C. The residue was redissolved in 150 μl of hexane.

GC determination. A 2-μl sample was injected splitless into a 5790 Hewlett-Packard gas chromatograph equipped with a split/splitless injection device for capillary columns and a flame ionization detector. The gas chromatograph was connected to an interface, linked to an IBM computer, loaded with Nelson analytical chromatography software (Cupertino, CA, U.S.A.) as an integration device. Helium was used as carrier gas at a flow-rate of 0.33 ml/min. The injector and detector temperatures were 310 and 300 °C, respectively. Following injection, the oven temperature was kept at 120 °C for 2 min, then programmed at 30 °C/min to a temperature of 225 °C and subsequently at 1.7 °C/min to a final temperature of 300 °C. Quantification was done by comparison of peak areas relative to that of the internal standard.

RESULTS

Retention times of standard hydroxycholesterols (7α-, 7β-, 19-, 20α, 22*S*-, 22*R*, 25- and 26-hydroxycholesterol) were established on a CP-Sil-19CB capillary column (see Table I). A typical gas chromatogram derived from a serum sample from a normal person is shown in Fig. 1; this clearly shows peaks due to 7α-hydroxycholesterol, 7β-hydroxycholesterol, 20α-hydroxycholesterol (internal standard) and 26-hydroxycholesterol, but no peaks due to 19-hydroxycholesterol, 20α-hydroxycholesterol (if not used as internal standard), 22*S*-hydroxycholesterol, 22*R*-hydroxycholesterol and 25-hydroxycholesterol. The influence of the hydrolysis time (hydrolysis with ethanolic potassium hydroxide at 55 °C) on unesterified 7α-hydroxycholesterol, 7β-hydroxycholesterol and 26-hydroxycholesterol concentrations in a pooled serum was determined and the results are given in Fig. 2. Obviously, after 45 min at 55 °C, hydrolysis of esterified hydroxycholesterols is complete. Moreover, this figure clearly shows no indica-

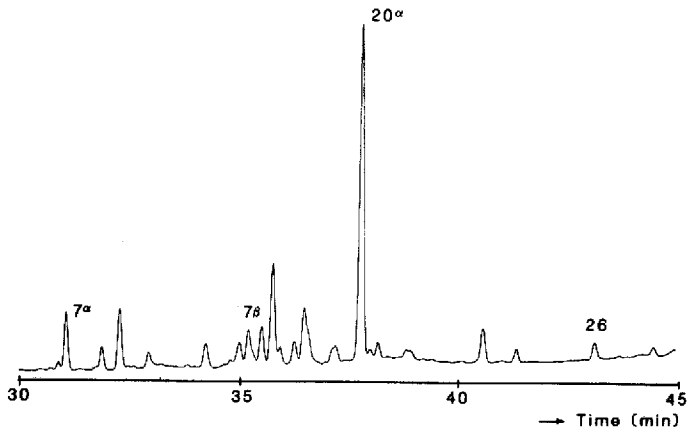

Fig. 1. Gas chromatogram of a serum sample of a healthy volunteer. Peaks: 7α = 7α-hydroxycholesterol; 7β = 7β-hydroxycholesterol; 20α = 20α-hydroxycholesterol (internal standard); 26 = 26-hydroxycholesterol.

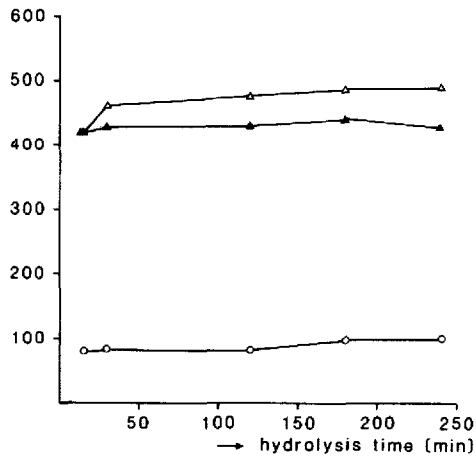

Fig. 2. Influence of hydrolysis time at 55 °C (in ethanolic potassium hydroxide) on unesterified 7α-hydroxycholesterol (▵), 7β-hydroxycholesterol (▲) and 26-hydroxycholesterol (○) concentrations (in nmol/l) in a pooled serum.

tion of the auto-oxidation of cholesterol, as described by others [11–13,17], during this step in the work-up.

Calibration curves for 7α-hydroxycholesterol, employing the area ratios of 7α-hydroxycholesterol and 20α-hydroxycholesterol, were found to be linear in the range 0–1300 nmol/l. The equation of the calibration curve was $y = 0.000590x - 0.0077$ ($n=6$), with a correlation coefficient of 0.9999, y being the peak-area ratio and x the 7α-hydroxycholesterol concentration in nmol/l. Calibration curves for 7β-hydroxycholesterol, employing the peak-area ratios of 7β-hydroxycholesterol and 20α-hydroxycholesterol, were found to be linear in the range 0–1300 nmol/l. The equation of the calibration curve was $y = 0.000591x - 0.0166$ ($n=6$), with a correlation coefficient of 0.9991, y being the peak-area ratio and x the 7β-hydroxycholesterol concentration in nmol/l. Calibration curves for 26-hydroxycholesterol, employing peak-area ratios of 26-hydroxycholesterol and 20α-hydroxycholesterol, were found to be linear in the range 0–1500 nmol/l. The equation of the calibration curve was $y=0.000431x-0.0124$ ($n=6$), with a correlation coefficient of 0.9971, y being peak-area ratio and x the 26-hydroxycholesterol concentration in nmol/l.

The within-day ($n=10$) and day-to-day ($n=10$) coefficients of variation (C.V.) in a pooled serum and the corresponding mean values were determined, being 321 nmol/l (C.V. 4.5%) and 307 nmol/l (C.V. 13.3%), respectively, for 7α-hydroxycholesterol; 262 nmol/l (C.V. 12.7%) and 280 nmol/l (C.V. 18.2%), respectively, for 7β-hydroxycholesterol; and 275 nmol/l (C.V. 5.1%) and 261 nmol/l (C.V. 8.6%), respectively, for 26-hydroxycholesterol. The results of recovery experiments are listed in Table II. Normal values in serum of 7α-hydroxycholesterol, 7β-hydroxycholesterol and 26-hydroxycholesterol were determined in serum samples from 40 healthy individuals and were in the ranges 49–410, 29–659 and 75–321 nmol/l, respectively. Finally, 7α-hydroxycholesterol, 7β-hydroxycholesterol and 26-hydroxycholesterol concentrations in sera of dif-

TABLE II

RECOVERIES OF 7α-HYDROXYCHOLESTEROL, 7β-HYDROXYCHOLESTEROL AND 26-HYDROXYCHOLESTEROL ADDED TO DIFFERENT POOLED SERA

Values in parentheses are recovery percentages.

Added (pmol per 0.5 ml)	Measured (pmol per 0.5 ml)			
7α-Hydroxycholesterol				
0	140	136	97	111
79	206 (84)	210 (94)	178 (103)	177 (84)
158	304 (104)	282 (92)	250 (97)	300 (120)
317	442 (95)	433 (94)	388 (92)	402 (92)
7β-Hydroxycholesterol				
0	163	111	149	119
81	262 (122)	189 (96)	234 (105)	196 (95)
161	343 (112)	251 (87)	305 (97)	288 (105)
322	430 (83)	392 (87)	448 (93)	413 (91)
26-Hydroxycholesterol				
0	120	98	153	82
94	207 (93)	199 (107)	261 (115)	171 (95)
188	275 (82)	297 (106)	334 (96)	275 (103)
377	443 (86)	450 (93)	460 (81)	469 (103)

ferent patient populations were determined. Gas chromatograms from such determinations are shown in Fig. 3 (A, an untreated patient suffering from CTX; B, the same patient during treatment with chenodeoxycholic acid in a daily dose of 750 mg; C, a patient suffering from CHRS; D, a patient suspected to be suffering from a yet unknown inborn error of metabolism in bile acid synthesis). Fig. 4 shows concentrations of 7α-hydroxycholesterol in sera of normals and different patient populations; Fig. 5 shows 7β-hydroxycholesterol concentrations in sera of the same patients and, finally, Fig. 6 shows 26-hydroxycholesterol concentrations in sera of the same patients.

DISCUSSION

The procedure described here was evaluated in the following way. The within-day and day-to-day variations in a pooled serum sample were determined, as were the corresponding mean values. In addition, various amounts of 7α-hydroxycholesterol, 7β-hydroxycholesterol and 26-hydroxycholesterol were added to aliquots of various pooled serum samples and the recovery was determined for all three steroids. The overall results established the reliability of the GC assay. Subsequently, serum concentrations of 7α-hydroxycholesterol, 7β-hydroxycholesterol and 26-hydroxycholesterol of (a) 40 healthy individuals, (b) nine untreated patients suffering from CTX, (c) eleven patients suffering from CTX treated for longer periods with either chenodeoxycholic acid or cholic acid in effective doses [16, 17], (d) three patients suffering from CHRS, (e) fourteen patients suffer-

ing from hypercholesterolemia and treated with 12 g of cholestyramine for prolonged periods and (f) one patient suspected to be suffering from an unknown inborn error of metabolism in bile acid synthesis during fat loading and fasting [18] were measured.

Fig. 4 shows the results of the 7α-hydroxycholesterol determinations. Clearly, untreated patients suffering from known inborn errors of metabolism in bile acid synthesis (CTX and CHRS) have high serum levels of 7α-hydroxycholesterol. These findings are to be expected in CTX patients, as such patients have an enhanced rate of cholesterol production [3] and metabolic conversion of cholesterol towards bile acids and other intermediates. They also have high levels of 7α-hydroxycholesterol in liver mitochondria [19,20]. One patient suffering from CHRS has only a moderately increased level, which is in accordance with declining levels of coprostanoic acid with advancing years [21]. Patients suffering from CTX and treated with effective doses of chenodeoxycholic acid or cholic acid still

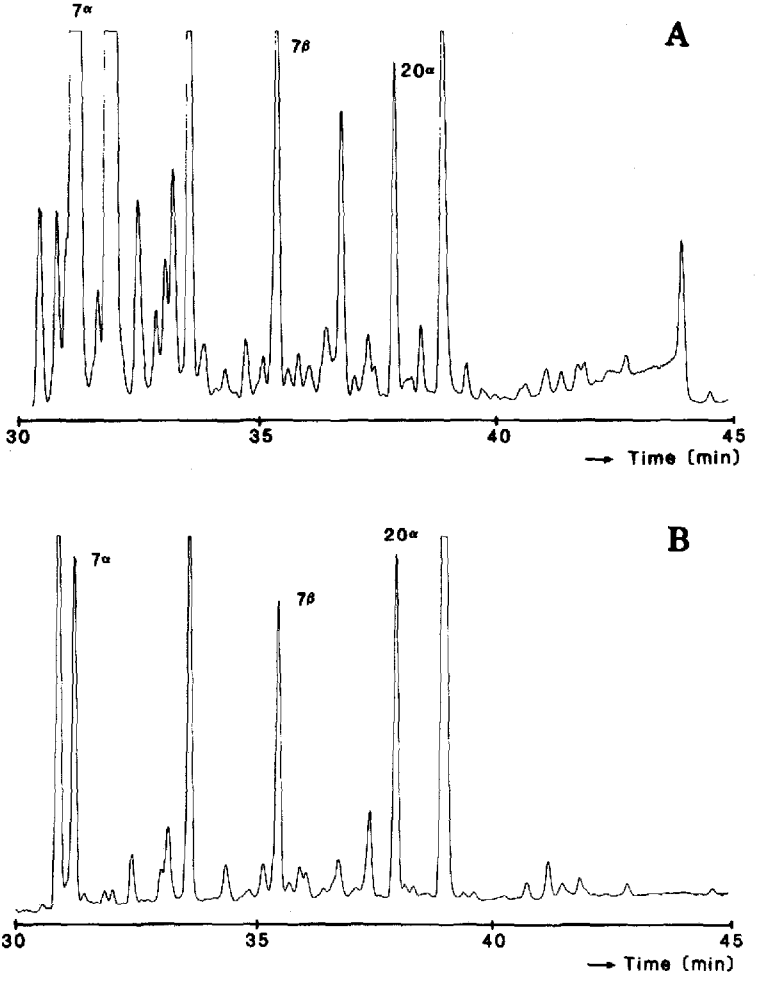

Fig. 3.

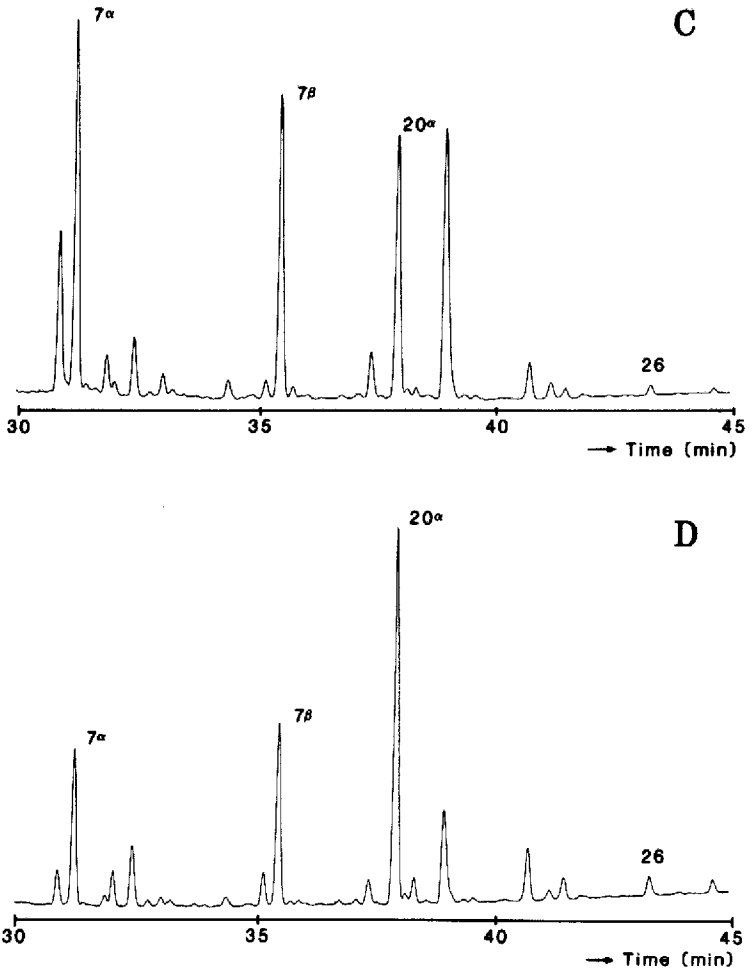

Fig. 3. Gas chromatograms of serum samples of patients. (A) An untreated CTX patient; (B) the same patient during treatment with chenodeoxycholic acid (750 mg/day); (C) a patient suffering from CHRS; (D) a patient suspected to have an inborn error of metabolism in bile acid synthesis. Peaks: $7\alpha = 7\alpha$-hydroxycholesterol; $7\beta = 7\beta$-hydroxycholesterol; $20\alpha = 20\alpha$-hydroxycholesterol (internal standard); 26 = 26-hydroxycholesterol.

have elevated levels in serum. Probably, endogenous bile acid synthesis is not completely suppressed during this treatment. An interesting question is whether higher doses of cholic acid or chenodeoxycholic acid are able to reduce the 7α-hydroxycholesterol concentration in serum to a non-detectable level. The patient with suspected inborn error of metabolism in bile acid synthesis as a result of very low endogenous bile acid pool size [18] has only slightly increased levels of 7α-hydroxycholesterol in serum. This finding does not indicate an enzyme deficiency at the level of the transformation of 7α-hydroxycholesterol to bile acids. Furthermore, a 7α-hydroxylase deficiency seems unlikely, as 7α-hydroxycholesterol levels are slightly elevated. However, this 7α-hydroxycholesterol level does not definitely exclude a 7α-hydroxylase deficiency, as it is possible that this sub-

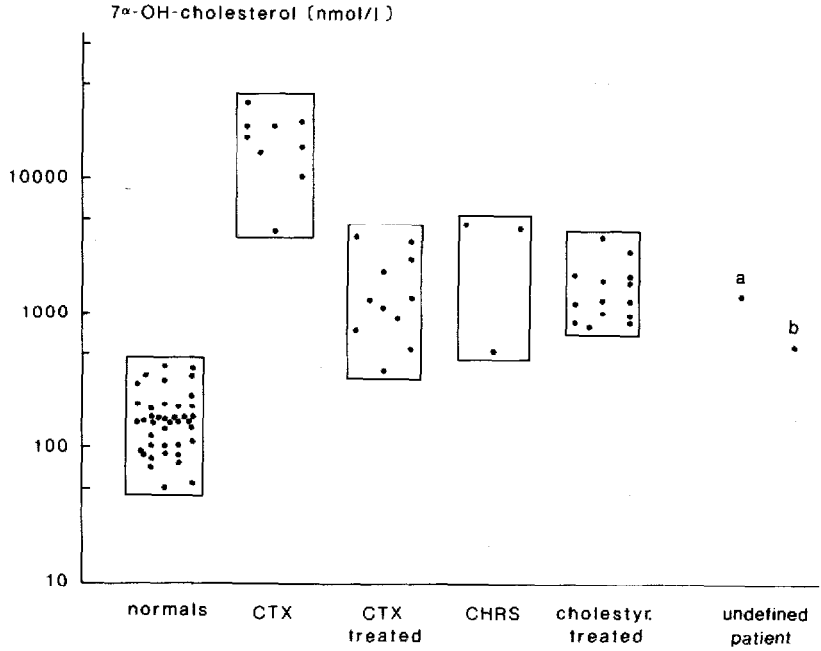

Fig. 4. 7α-Hydroxycholesterol levels in 40 normal patients, nine untreated CTX patients, eleven CTX patients treated with chenodeoxycholic acid or cholic acid, three CHRS patients, fourteen patients treated with cholestyramine and a patient suspected to have an inborn error of metabolism in bile acid synthesis during fat loading (a) and fasting (b).

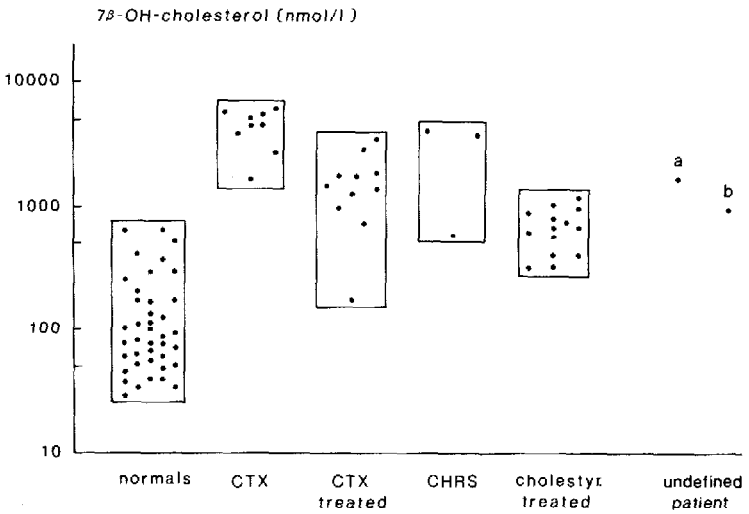

Fig. 5. 7β-Hydroxycholesterol levels in 40 healthy patients, nine untreated CTX patients, eleven CTX patients treated with chenodeoxycholic acid or cholic acid, three CHRS patients, fourteen patients treated with cholestyramine and a patient suspected to have an inborn error of metabolism in bile acid synthesis during fat loading (a) and fasting (b).

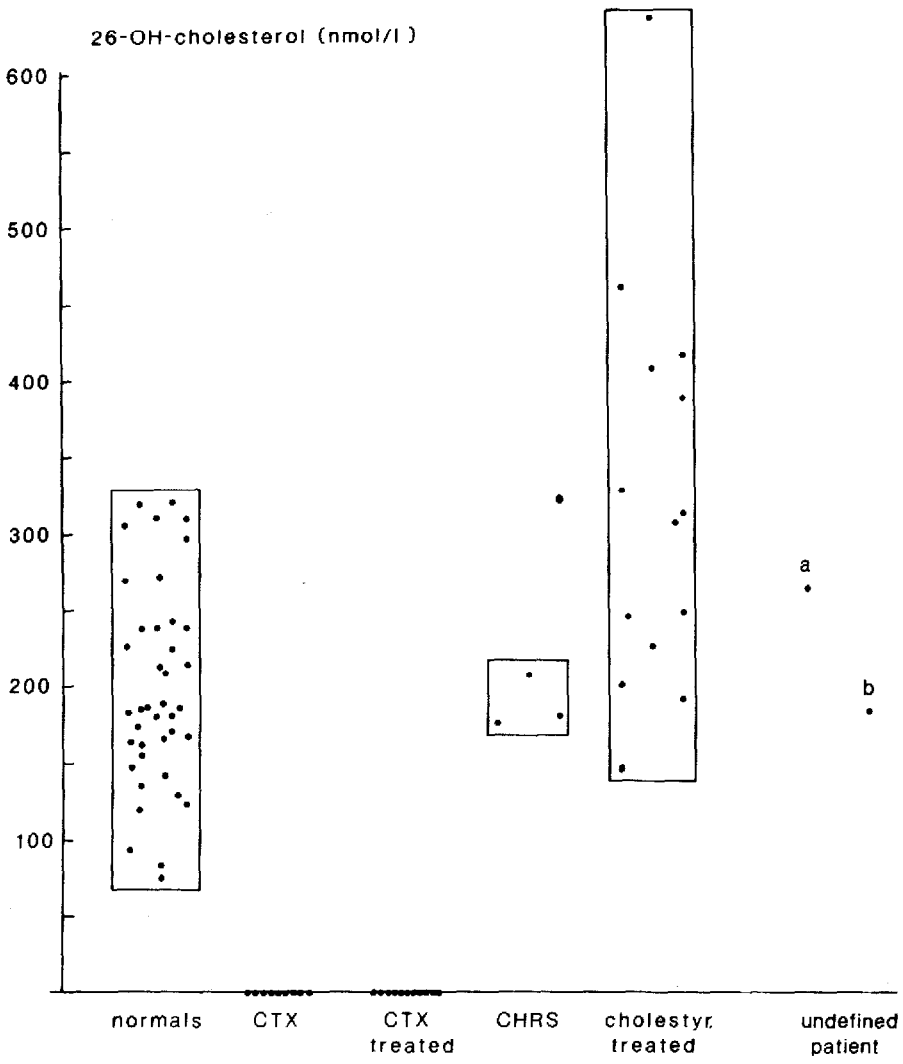

Fig. 6. 26-Hydroxycholesterol levels in 40 normal patients, nine untreated CTX patients, eleven CTX patients treated with chenodeoxycholic acid or cholic acid, three CHRS patients, fourteen patients treated with cholestyramine and a patient suspected to have an inborn error of metabolism in bile acid synthesis during fat loading (a) and fasting (b).

stance is formed in a "wrong" (i.e. an extrahepatic) compartment. Therefore measurements in liver material of the activity of the liver enzyme 7α-hydroxylase itself, as previously described by others [12,13], have to be performed in order to establish whether a 7α-hydroxylase deficiency is present.

Patients treated with cholestyramine also show increased levels of 7α-hydroxycholesterol, which is easily explained by increased endogenous cholesterol and bile acid synthesis, including 7α-hydroxylation.

Fig. 5 shows the results of the 7β-hydroxycholesterol determinations. Phenomena similar to those found with 7α-hydroxycholesterol, though less distinct, can be observed.

Fig. 6 shows the results of the 26-hydroxycholesterol determinations. In serum samples of normal patients, a 26-hydroxycholesterol concentration of 75–321 nmol/l (corresponding with 30–129 μg/l) was found, in close agreement with Javitt et al. [9], who found a normal range of 43–130 μg/l. Patients suffering from CTX show non-detectable levels of 26-hydroxycholesterol in serum (see also Fig. 3B), which is also in accordance with the results of Javitt et al. [9], suggesting a 26-hydroxylase deficiency in these patients. This also explains the interesting finding that 26-hydroxycholesterol levels in serum samples of CTX patients do not return to normal during effective bile acid treatment. Patients suffering from CHRS and the patient presumed to have an inborn error of metabolism in bile acid synthesis show normal levels, whereas patients treated with cholestyramine show a slightly elevated range.

The occurrence of additional peaks (see Fig. 3) in GC profiling of serum samples of patients suffering from CTX and patients suffering from CHRS is of interest from a metabolic point of view, with respect to bile acid synthesis. These substances seem to orginate from abnormal bile acid metabolism, occurring in both patient populations. As these peaks have not yet been identified we intend to study their nature more closely to obtain more information on the exact nature of both diseases. The results of this study will be presented elsewhere.

CONCLUSION

Diagnosis of inborn errors of metabolism in the metabolic break-down of 7α-hydroxycholesterol towards bile acid synthesis can be established using the assay presented here, as these patients have high serum levels of 7α-hydroxycholesterol. In addition, patients suffering from cerebrotendinous xanthomatosis also have non-detectable levels of 26-hydroxycholesterol, which provides a way of ascertaining the presence of this disease. The assay is also of use for evaluating the effect of bile acid treatment applied to such patients. An interesting question is whether higher doses of cholic acid or chenodeoxycholic acid are able to reduce 7α-hydroxycholesterol levels in serum to no-detectable limits.

ACKNOWLEDGEMENTS

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NOTE ADDED IN PROOF

At the International Bile Acid Meeting in Basel, Switzerland (October 19–21, 1986) Professor I. Björkhem presented his results of the GC–MS determination of 7α-hydroxycholesterol in human serum samples [22]. The outcome of these investigations is in close agreement with our conclusions.

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