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Levels of 7α-hydroxy-4-cholesten-3-one in plasma reflect rates of bile acid synthesis in man

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A method for analysis of 7α-hydroxy-4-cholesten-3-one in plasma is described. Following solid-phase extraction/purification the compound is determined by high-performance liquid chromatography using a UV detector. The median concentration in healthy subjects was 12 ng/ml (range 3–40). The levels were lower in diseases associated with a low bile acid production: extrahepatic cholestasis, < 1.5 ng/ml (range < 0.9–3); liver cirrhosis < 1.5 ng/ml (range < 0.9–38), and higher in diseases associated with a high bile acid production: cholestyramine treatment, 188 ng/ml (range 54–477); ileal resection 397 ng/ml (range 128–750). The levels were essentially normal in patients with colon resection. The results are consistent with a strong positive correlation between the levels of 7α-hydroxy-4-cholesten-3-one in plasma and the rate of bile acid synthesis.

Sterol metabolism; Bile acid synthesis; Solid-phase extraction; HPLC; (Human plasma)

1. INTRODUCTION

The development of cardiovascular disease is positively correlated to the levels of cholesterol in plasma [1]. Among factors regulating this level are the rates of synthesis and removal of cholesterol. Formation of bile acids constitutes a major pathway of elimination [2]. Rates of bile acid synthesis in vivo may be determined by isotope dilution methods or analysis of fecal bile acid excretion. Relative rates may also be evaluated in vitro by determination of cholesterol 7α-hydroxylase [3,4]. Recently the levels of 7α-hydroxycholesterol in plasma were found to correlate positively to the activity of the 7α-hydroxylase in cases with increased bile acid synthesis [5,6]. In the course of studies on sterols and bile acids in plasma, the levels of 7α-hydroxy-4-cholesten-3-one, the next intermediate in bile acid biosynthesis, were found to be increased in patients with ileal resection, a condition in which bile acid production is increased. This paper describes a simple liquid-chromatographic method for the analysis and reports on the concentrations of 7α-hydroxy-4-cholesten-3-one in plasma from patients with diseases known to be associated with an abnormal bile acid production. The results are consistent with a strong positive correlation between the levels of the sterol in plasma and the rate of bile acid biosynthesis.

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2. MATERIALS AND METHODS

2.1. *Patients*

Blood was obtained from patients with liver diseases, intestinal diseases or under treatment with cholestyramine. The patients were divided into the following groups. (A) Three men and eight women, 50–87 years old (median age 69), with large bile duct obstruction secondary to carcinoma or gallstones and with grossly elevated bile acid levels in blood. (B) Five men and three women, 54–60 years old (median age 58), with moderate or severe forms of alcoholic liver cirrhosis. (C) Two men and eleven women, 41–75 years old (median age 65), with primary biliary cirrhosis. The diagnosis of patients in groups B and C was made on the basis of full clinical and chemical investigation including liver biopsy. Most of these patients had elevated levels

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of bile acids in blood. (D) Three men and three women, 28–50 years old (median age 37), subjected to a complete colectomy due to polyposis coli. One patient had ileostomy whereas the others had an ileo-rectal anastomosis. (E) One man and seven women, 39–70 years old (median age 62) with hypercholesterolemia and treated with 8 g cholestyramine (Questran®, Bristol) twice daily for 6–8 weeks prior to sampling. (F) Two men and three women, 30–59 years old (median age 39), subjected to a resection of terminal ileum (>60 cm) with or without hemicolectomy. (G) Two men, 38 and 42 years old, subjected to a resection of terminal ileum (<100 cm) and the entire colon. (H) Two men and one woman, ages 32, 43 and 50 years, respectively, subjected to a resection of the terminal ileum (>100 cm) and the entire colon. The patients in groups F–H had Crohn's disease. The operations were performed more than 2 years before this investigation.

2.2. *Control material*

Blood from 20 apparently healthy men and women, 21–48 years old (median age 37) was collected in tubes with or without heparin. Following centrifugation, plasma/serum was separated and stored at −20°C until analyzed. All subjects were not fasting, but most samples were collected in the morning.

2.3. *Glassware and chemicals*

All glassware was silanized and cleaning was carried out in an ultrasonic bath. Solvents were of analytical reagent grade. 7α-Hydroxy-4-cholesten-3-one and 25-hydroxyvitamin D_3 were kind gifts from Professor I. Björkhem, Huddinge Hospital and Roche-Produkter, Sweden, respectively. 25-Hydroxy[26,27-*methyl*-^{3}H]vitamin D_3 (176 Ci/mmol) was from the Radiochemical Centre (Amersham, England) and radioactivity was determined in an LKB/Wallac 1215 Rackbeta scintillation counter with Picofluor as the scintillation liquid. Octadecylsilane (ODS)-bonded silica (Preparative C_{18}, particle size 55–105 μm) was from Waters Associates (Milford, MA). Column beds (1.0 × 0.8 cm, dry wt about 0.34 g) of this material were prepared in jacketed glass columns connected to a water bath and equipped with gauze-covered valves of Teflon. The sorbent was washed with 5 ml each of methanol, methanol/chloroform (1:1, v/v), methanol and water prior to use. Appropriate flow rates were obtained by application of nitrogen pressure.

2.4. *Analytical procedure (see also [7])*

Following addition of ^{3}H-labelled 25-hydroxyvitamin D_3 (about 10000 cpm), plasma or serum (usually 2 ml) was diluted with 2 vols isotonic saline (when bile acids were also to be analyzed, 0.25 M aqueous triethylamine sulphate, pH ~7, was used instead of saline [8]). The solution was heated to 64°C for 5 min and then extracted on the column of ODS-bonded silica at the same temperature (flow rate about 1 ml/min). The column was washed with 10 ml water (at 64°C) and 10 ml of 65% aqueous methanol (at room temperature). Prior to elution of steroids with 8 ml hexane/chloroform (95:5, v/v) or 6 ml hexane/chloroform (1:1, v/v), a gentle stream of nitrogen was passed through the column for 0.5 min. The eluate, collected in a stoppered tube, was then taken to dryness under a stream of nitrogen and the residue was redissolved in 0.1 ml hexane.

2.5. *High-performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC/MS)*

HPLC was carried out on a column (250 × 4.5 mm) of LiChrospher (Hibar, Si 100, 5 μm, Merck-Darmstadt) connected to a pump (Constametric III) and a fixed-wavelength (254 nm) detector (LDC/Milton Roy, Riviera Beach, FL). The mobile phase was hexane/isopropanol (95:5, v/v). The amount of 7α-hydroxy-4-cholesten-3-one in plasma was determined by comparing its peak area with that of known amounts of the reference compound. Since a radioactive version was not available, ^{3}H-labelled 25-hydroxyvitamin D_3, added prior to extraction and determined in the effluent from the HPLC column, served to correct for losses and differences in injection volumes. The extraction yield and polarity of 25-hydroxyvitamin D_3 were very similar to those of 7α-hydroxy-4-cholesten-3-one (see section 3). The molar response of 7α-hydroxy-4-cholesten-3-one at 254 nm was 0.33–0.40 relative to that of 25-hydroxyvitamin D_3 in the system used. For identification purposes, the HPLC column was connected to a Waters 990, photodiode array detector (Waters Associates) monitoring wavelengths from 220 to 300 nm. GC/MS analyses of trimethylsilyl ether derivatives [9] were performed on a Finnigan 1020 instrument housing a fused-silica column (30 m × 0.32 mm) coated with SE-30 DB-1 [9]. The conditions were those used in previous studies [9] and repetitive scanning covering the *m/z* range 50–800 was started after suitable delay.

3. RESULTS AND DISCUSSION

3.1 *Analysis of 7α-hydroxy-4-cholesten-3-one*

The analytical method is essentially that described previously for analysis of 25-hydroxyvitamin D_3 [7]. When levels of the latter were studied in patients with small bowel resection, a large peak of an unknown compound was observed (fig.1). Analyses using the photodiode array detector showed an absorption maximum at about 240 nm. Upon GLC and GC/MS the retention index (Kovats) of the trimethylsilyl derivative on a methyl silicone column was 3233 and the mass spectrum showed a molecular ion at *m/z* 472 and fragment ions typical of the derivative of 7α-hydroxy-4-cholesten-3-one [9–11]. This identification was confirmed by comparison with the authentic compound.

The HPLC method is simple compared to analysis by selected ion monitoring GC/MS [11]. HPLC is preceded by a single sorbent extraction/purification step. Protein binding of the steroids is counteracted by dilution of the plasma/serum with saline [7,8,12] or triethylamine sulphate [8] and performing the extraction at 64°C [7,12]. Under these conditions polar material as well as most (>80–90%) of the hydrophobic lipids (e.g. cholesterol) are not sorbed. Recoveries of

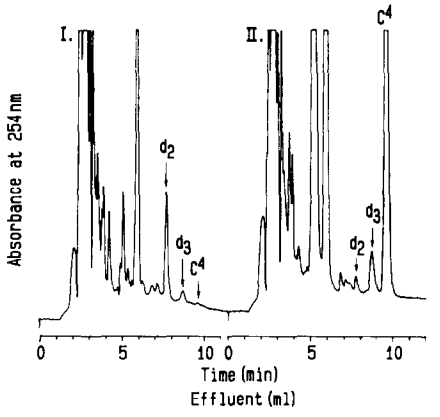

Fig.1. HPLC analyses of 7α-hydroxy-4-cholesten-3-one (C^4) in plasma from (I) a 69-year-old woman with primary biliary cirrhosis and (II) a 50-year-old woman with resection of about 2 m terminal ileum and the entire colon. The equivalents of about 400 μl (I) and 200 μl (II) of plasma were injected and the concentrations of the compound were <1 ng/ml (I) and 732 ng/ml (II). The peaks d_2 and d_3 represent 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3, respectively. The concentrations of the latter compound were 2.4 ng/ml (I) and 21 ng/ml (II). Both patients were treated with vitamin D.

added 7α-hydroxy-4-cholesten-3-one (table 1) were essentially quantitative, i.e. like those of 25-hydroxyvitamin D_3 reported previously [7]. Thus, commercially available ^{3}H-labelled 25-hydroxyvitamin D_3 could be used to correct for losses and variations in injection volumes. Extraction yields were the same whether saline or aqueous triethylamine sulphate was used as diluent. The latter has to be used for quantitative extraction of bile acids [9]. Although labile in acidic and alkaline solutions, the 7α-hydroxy-Δ^4-3-oxo structure was stable under the conditions of the analytical method. In contrast to 7α/β-hydroxy-, 7-oxo-, 25-hydroxy- and other oxygenated forms of cholesterol, 7α-hydroxy-4-cholesten-3-one is not formed as an artefact from cholesterol [13]. Thus, the problems encountered in analysis of 7α-hydroxycholesterol are avoided. The absence of artefactual formation is supported by the very low levels found in some patients.

The precision of the method was evaluated by analysis of 8 samples from the same plasma pool. The coefficient of variation was about 5% at a concentration of about 25 ng/ml plasma. The detection limit was usually about 0.5–1.5 ng/ml. If necessary, this could be lowered by increasing

Table 1

Precision and recoveries in the analysis of 7α-hydroxy-4-cholesten-3-one

7α-Hydroxy-4-cholesten-3-one in 2 ml plasma		
Amount added (ng)	Amount measured (ng)	Recovery (%)[a]
0	48.8 ± 2.6[b]	–
13	62	100
31	80	100
62	100	82
110	163	104
540	621	106
1100	1045	91
5400	5144	94

[a] Per cent of added amount
[b] Mean ± SD, CV = 5%

A pool containing plasma from 20 subjects was analyzed eight times. Recoveries were determined after addition of the compound to 2-ml samples prior to extraction

the volume of plasma used (sorbent capacity about 10 ml plasma) and optimizing the HPLC conditions. A variation of the retention time relative to 25-hydroxyvitamin D_3 (usually about 1.1) was sometimes observed depending on solvent composition and column conditions. In the present form the method is about 10-times more sensitive than the reported GC/MS procedure [11]. The analysis requires no specialized instrumentation and a technician can readily analyze 10–15 samples in a day.

3.2. *Concentrations of 7α-hydroxy-4-cholesten-3-one in plasma*

The occurrence of 7α-hydroxy-4-cholesten-3-one in liver and plasma at levels of 50 ± 10 ng/ml plasma in healthy subjects has been reported by Björkhem et al. [10,11]. When we found highly elevated levels in patients with ileal resection, a possible relationship to the increased bile acid synthesis in these patients [14] was suspected. To test this hypothesis, the concentrations of 7α-hydroxy-4-cholesten-3-one were measured in plasma from patients with diseases known to be associated with increased or decreased bile acid production. The results are shown in fig.2. Healthy controls usually had levels between 6 and 30 ng/ml (median 12 ng/ml, range 3–40 ng/ml). A possible sex difference (fig.2) was not statistically significant. The highest

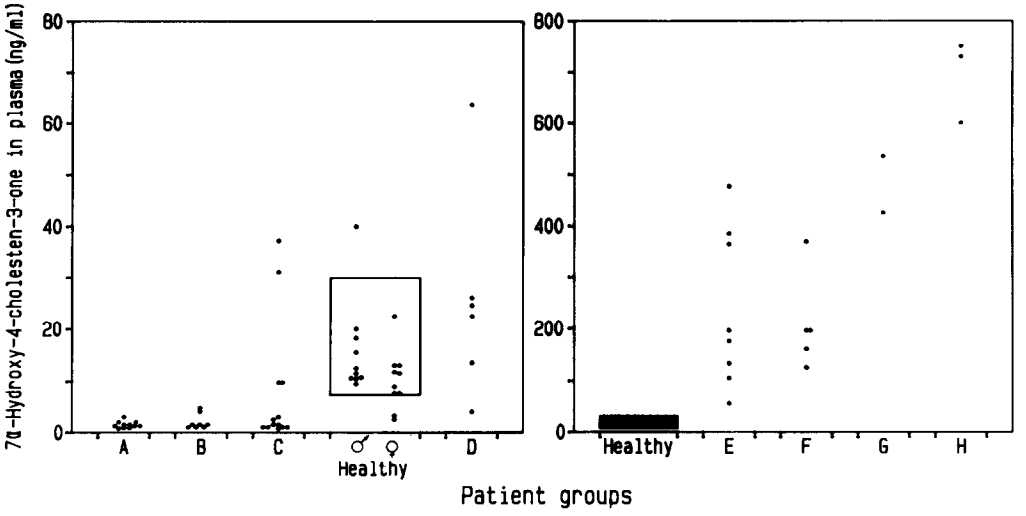

Fig.2. Concentrations of 7α-hydroxy-4-cholesten-3-one in plasma from patients belonging to groups known to have a decreased (groups A–C; liver diseases) or increased (groups E–H; cholestyramine, ileal resection) production of bile acids. Group D included patients with resection of the entire colon. For patient classification see section 2. The concentration range between 6 and 30 ng/ml was arbitrarily used to represent values for healthy subjects. The values are transformed to nmol/l by multiplication with 2.5.

levels were seen in patients with ileal resection (median 397 ng/ml, range 128–750 ng/ml) who have a defective feed-back inhibition of the bile acid synthesis due to inefficient reabsorption of bile acids [14]. Patients treated with cholestyramine, also affecting bile acid reabsorption, all had high concentrations (median 188 ng/ml, range 54–477 ng/ml), supporting the conclusion that interference with bile acid absorption and not removal of ileum per se affects the concentration of 7α-hydroxy-4-cholesten-3-one.

As shown in fig.2 the levels of 7α-hydroxy-4-cholesten-3-one in patients with ileal resection depended on the presence or absence of colon. Thus, the levels were lower (median 195 ng/ml, range 128–370 ng/ml) when half of the colon was intact than when a shorter part of ileum but the entire colon had been removed (median 600 ng/ml, range 424–750 ng/ml). Patients with an intact ileum had essentially normal levels also in the absence of a colon (median 24 ng/ml, range 4–64 ng/ml). Taken together, these results indicate that reabsorption of bile acids from colon plays an important role in patients with ileal resection but is of minor importance under normal conditions.

Patients with extrahepatic cholestasis and cirrhosis, conditions associated with a decreased bile acid production [15], in most cases had very low levels of 7α-hydroxy-4-cholesten-3-one (median < 1.5 ng/ml; figs 1,2). This indicates that when its rate of formation is low, there is no accumulation of this steroid in blood. A 2-year-old boy with cirrhosis and ileal resection had greatly elevated levels (249 ng/ml) in agreement with the hypothesis that the decreased bile acid production in cirrhosis is due to bile acid-induced inhibition of cholesterol 7α-hydroxylase and not to a decreased amount of liver tissue.

7α-Hydroxy-4-cholesten-3-one has previously been determined in patients with cerebrotendinous xanthomatosis [11]. These patients lack the normal feed-back inhibition of bile acid formation because of a defect in the side chain hydroxylation required for normal bile acid formation [2,16–18]. The levels of 7α-hydroxy-4-cholesten-3-one were extremely high (4500–8500 ng/ml). While this may be due to an accumulation secondary to the lack of mitochondrial 26-hydroxylase [17], an increased formation due to the absence of feed-back inhibition may be an additional reason.

Many factors will determine the levels of 7α-hydroxy-4-cholesten-3-one in plasma. It is reasonable to assume that the rate of formation is

one determinant. The rate-limiting step in bile acid biosynthesis is the 7α-hydroxylation of cholesterol [2]. When the rate of this reaction increases, there will also be an increased formation of 7α-hydroxy-4-cholesten-3-one. Unless 12α-hydroxylated, this intermediate will leave the endoplasmic reticulum for subsequent metabolic transformations [2]. It is reasonable to assume that the leakage to plasma can increase when the rate of formation is increased. A rate limitation of the 5β-reduction or 26-hydroxylation cannot be excluded. A similar explanation has been given for the paradoxical increase of 7α-hydroxycholesterol in plasma when the activity of cholesterol 7α-hydroxylase is increased [5].

3.3. *Possible significance of the method*

Methods for determination of bile acid production by analytical or isotope-kinetic methods are time-consuming, as is the measurement of cholesterol 7α-hydroxylase. There is a need for a simple method to monitor relative changes in bile acid formation during dietary, hormonal and pharmacological manipulations of plasma cholesterol levels. While further studies of the quantitative relationships between levels of 7α-hydroxy-4-cholesten-3-one, cholesterol 7α-hydroxylase and bile acid production are required, the present results indicate that analysis of 7α-hydroxy-4-cholesten-3-one may be such a method. It is simpler than analysis of 7α-hydroxycholesterol which has the additional problem with autooxidation and uncertainties in analyses of the lower levels when the activity of 7α-hydroxylase is low. Analysis of 7α-hydroxy-4-cholesten-3-one in plasma may also be a complementary method in the evaluation of patients with fat malabsorption.

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