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A new method for determination of serum cholestanol by high-performance liquid chromatography with ultraviolet detection

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Abstract

We developed a method for the determination of serum 5α-cholestan-3β-ol (cholestanol). The sterols were derivatized to the 4′-bromobenzenesulfonyl esters and heated in isopropanol. The cholesterol-4′-bromobenzenesulfonate was solvolyzed to cholesteryl isopropyl ether, but the derivatized cholestanol did not change and could be measured in a high-performance liquid chromatographic system equipped with a UV detector at 235 nm. On the other hand, the resulting cholesteryl isopropyl ether, having different absorbance and chromatographic mobility was not detected. This method was used for measuring cholestanol levels in patients with cerebrotendinous xanthomatosis (CTX), liver cirrhosis and serum from healthy control subjects. Reproducibility, linearity and recovery tests were done on 0.3 ml of serum samples containing >2 μg/ml cholestanol, using stigmastanol as an internal standard (I.S.). Determining cholestenol by this method can be used for diagnosis and follow-up of patients with CTX and various liver diseases. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

The elevation of cholestanol (5α-cholestan-3β-ol) in the serum is known to be one of the biochemical markers of cerebrotendinous xanthomatosis (CTX) [1,2] and liver and biliary tract diseases [3–6]. A definite diagnosis of CTX is based on the molecular identification of homozygosity to mutant CYP27 alleles [7]. The determination of serum cholestanol [3,7] and bile alcohols together with cholestanol has been used for monitoring treatment efficacy in CTX [8–13]. Cholestanol measurement was also used for the follow-up of patients with cirrhosis, primary biliary cirrhosis, or after liver transplantation [4–6].

Methods for the simultaneous analysis of cholestanol and cholesterol include gas chromatography (GC) [14,15], capillary GC [3,6,16], gas chromatography–mass spectrometry (GC–MS) [17], high-performance liquid chromatography (HPLC) with fluorescence detection [18–20], and HPLC with UV detection [21–24]. The simultaneous quantification of cholesterol (5-cholesten-3β-ol) and cholestanol in the serum, and comparison of the concentration ratio,

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provide useful information on the disease-state [3,16,19–21,25]. However, setting up a method for determination of cholestanol and cholesterol in serum is difficult, due to similarity of these steroids and the large concentration differences [3], which in most cases exceeds 1:500 [2,16,18].

In the studies cited above, special derivatizations and/or chromatographic systems were used to separate the metabolites. Some methods included pretreatment of the samples to enhance separation of the metabolites by thin-layer chromatography (TLC) [14], or oxidation with *m*-chloroperbenzoic acid and estimation of the resulting 5,6-epoxide and cholestanol by GC [26–28] or HPLC [18,27] directly or after separation [26]. The epoxidation prior to chromatography improved the separation of the metabolites but, the resulting chromatographic baselines were unstable [18,28]. The fluorescence detection methods are very sensitive, however the derivatization reagents are not available commercially, and their preparation is involved with elaborated synthetic methods [18–20]. When GC, capillary GC or HPLC with UV detection were used the baseline separation was generally not perfect [3,21–24]. An undefinitive peak separation may considerably influence the results of the minor component (cholestanol), especially when very high cholesterol/cholestanol ratio samples are tested. Therefore, all of the existing methods do not have the necessary degree of accuracy under all conditions.

In this paper we offer to measure cholestanol alone. In this way we overcome the difficulty of separating the two metabolites. Since the cholesterol level is included in the lipoprotein profile, which is routinely analyzed by the enzymatic colorimetric method [25], making the chromatographic measurement of cholesterol less important for the calculation of the concentration ratio of the two metabolites.

The purpose of the present study was to develop a method for measuring microgram amounts of cholestanol in human serum by modifying Stoll reaction [29,30] to eliminate cholesterol from the system, using commercially available reagents and HPLC with UV detection. This new method enables us to measure microgram amounts of cholestanol in an accurate way which is not affected by the cholesterol/cholestanol ratio.

2. Materials and methods

2.1. Chemicals

All chemicals and solvents were of analytical reagent grade. Double distilled water was used. Cholesterol, cholestanol, 5β-cholestan-3α-ol, 5β-cholestan-3β-ol, lathosterol, stigmasterol (24-ethyl-5,22-cholestadien-3β-ol), stigmastanol (24α-ethyl-5α-cholestan-3β-ol) and campesterol (24α-methyl-5-cholesten-3β-ol), were purchased from Sigma (St. Louis, MO, USA) and 4-bromobenezenesulfonyl chloride from Aldrich (Milwaukee, WI, USA). A mixture of 3.7% 5α-cholestan-3α-ol in cholestanol was prepared as described previously [31]. Solutions of 1 mg/ml of the sterol or internal standard (I.S.) (stigmastanol) in toluene in glass culture tubes with screw caps were used. The solutions were stored at 4°C in the dark and could be used for at least 2 months without measurable change. The derivatizations were performed in the same type of tubes. Serum samples were kept at −20°C.

2.2. Apparatus

UV spectra were recorded with a Kontron Unikon 930 spectrophotometer. Mass and nuclear magnetic resonance (NMR) spectra were obtained using a LKB Bromm-2091 and a Varian VXR 300S. TLC was performed on Silica gel S (Schleicher & Schuell), in the system hexane–chloroform (1:1) and visualized by iodine vapor or concentrated sulfuric acid. A Kontron HPLC system Model 400 equipped with a spectrophotometric detector 430 was used.

3. Subjects

Six molecularly diagnosed CTX Jewish patients of North African origin were examined. CTX 215-5 and CTX 224-1 are homozygous for a guanosine to adenosine substitution at the 3′ splice acceptor site intron 4 of CYP27. CTX 216-6 and 206-3 are homozygous for a frameshift mutation resulting from a deletion of thymidine in exon 4 of the gene. The siblings CTX 208-1 and CTX 208-5 are homozygous for a threonine to methionine substitution at residue

306. The three mutations previously described in CTX patients of Jewish North African origin, result in null alleles [25,32]. The first two mutations are mapped to exon 4 and the third one to exon 5. Screening of the CTX families was carried out by polymerase chain reaction–single-strand conformational polymorphism (PCR–SSCP) analysis, using oligonucleotides that flank these regions. For the detection of exon 4 and 5 mutations, oligonucleotides 4a, 4b and 5a, 5b were used as described previously [25,32].

The following siblings of CTX patient were examined: (1) a brother of the patients CTX 208-1 and 208-5, who was found to be heterozygote. (2) Two sisters and one brother of the patient CTX 224-1, who showed no clinical manifestations of the disease; they were not typed genetically.

Serum cholestanol levels were also measured in two females (aged 54 and 75 years) with end-stage liver cirrhosis, and control subjects.

The control samples were taken from a cohort of healthy individuals who came for a check up at the ''Institute of Medical Screening and Assessment, at the Chaim Sheba Medical Center''. All the control subjects (23 males and 11 females aged 27–59 years) had normal liver function test and serum cholesterol levels below 240 mg/dl.

Serum samples from 30 healthy subjects were randomly pooled and divided into 0.3-ml aliquots in stoppered tubes. The samples were kept at −20°C and thawed before use for the recovery experiments.

4. Procedure

Venous blood was collected in the fasting state for lipoprotein typing [25]. Aliquots (0.3 ml) of serum were used for cholestanol analysis. I.S. (3–10 μg) was added and the samples were saponified with five volumes of 90% ethanolic 1 *M* sodium hydroxide at 60°C for 60 min. Water (one-fifth of the total volume) and light petroleum (3 ml) were added. The test tube was thoroughly shaken and the lower phase was removed. The upper phase was further washed with 80% aqueous methanol, and anhydrous sodium sulfate (0.1–0.2 g) was added. The solution was vortex-mixed, decanted to a screw-capped test tube and the solvent was evaporated to dryness under a stream of nitrogen at room temperature. 4-Bromobenzensulfonyl chloride in dry pyridine (0.3 ml, 70 mg/ml) was added, and the stoppered tubes were kept for 4 h at room temperature in the dark. Excess reagent was destroyed with ice (approx. 0.1 g), and after 30 min water (3 ml) and light petroleum (5 ml) were added. The tubes were vigorously shaken and the upper phase was washed with 1 *M* hydrochloric acid (3 ml) and 80% methanol (3 ml). The solvent evaporated and isopropanol (0.4 ml) was added. The tubes were heated at 75–80°C for 60 min in the water bath. Light petroleum (3 ml) was added and the upper phase was washed with 85% aqueous methanol. The solvent was evaporated and the residue was dissolved in isopropanol (0.4 ml) for analysis with a YMC-Pack Pro C_{18} (Wilmington, DE, USA), 250× 4.6 mm, S-5 μm, 120 A column, protected with a YMC Pro C_{18}, 10×4.6 pre-column. Volumes of 60 μl were automatically injected. The product was eluted with acetonitrile–isopropanol–0.5% aqueous acetic acid (62:34:4, v/v/v) at a flow-rate of 1.4 ml/min. The bromobenzene sulfonates were detected by absorbance at 235 nm.

5. Results

5.1. Sterol detection after derivatization with and without solvolysis

Derivatization and solvolysis of the sterols were performed with 4-bromobenzensulfonyl chloride and isopropanol as described in the Section 4. The results are given in Fig. 1.

4′-Bromobenzenesulfonyl esters of cholesterol and cholestanol were detected at 235 nm after 9 and 10 min, respectively. After solvolysis the cholestanol was not changed but cholesterol was converted to cholesteryl-3β-isopropyl ether.

The separation capacity of the 4′-bromobenzenesulfonyl derivatives performed with 10 μg of sterols in 0.3 ml saline (I.S. and cholestanol 20 μg) is shown in Fig. 1A. The lathosterol (peak 1) had the lowest retention time value of the sterol tested. The 5β-cholestan-3β-ol (peak 2) had the lowest mobility of the four cholestanol-3,5-epimers; the 5β-choles-

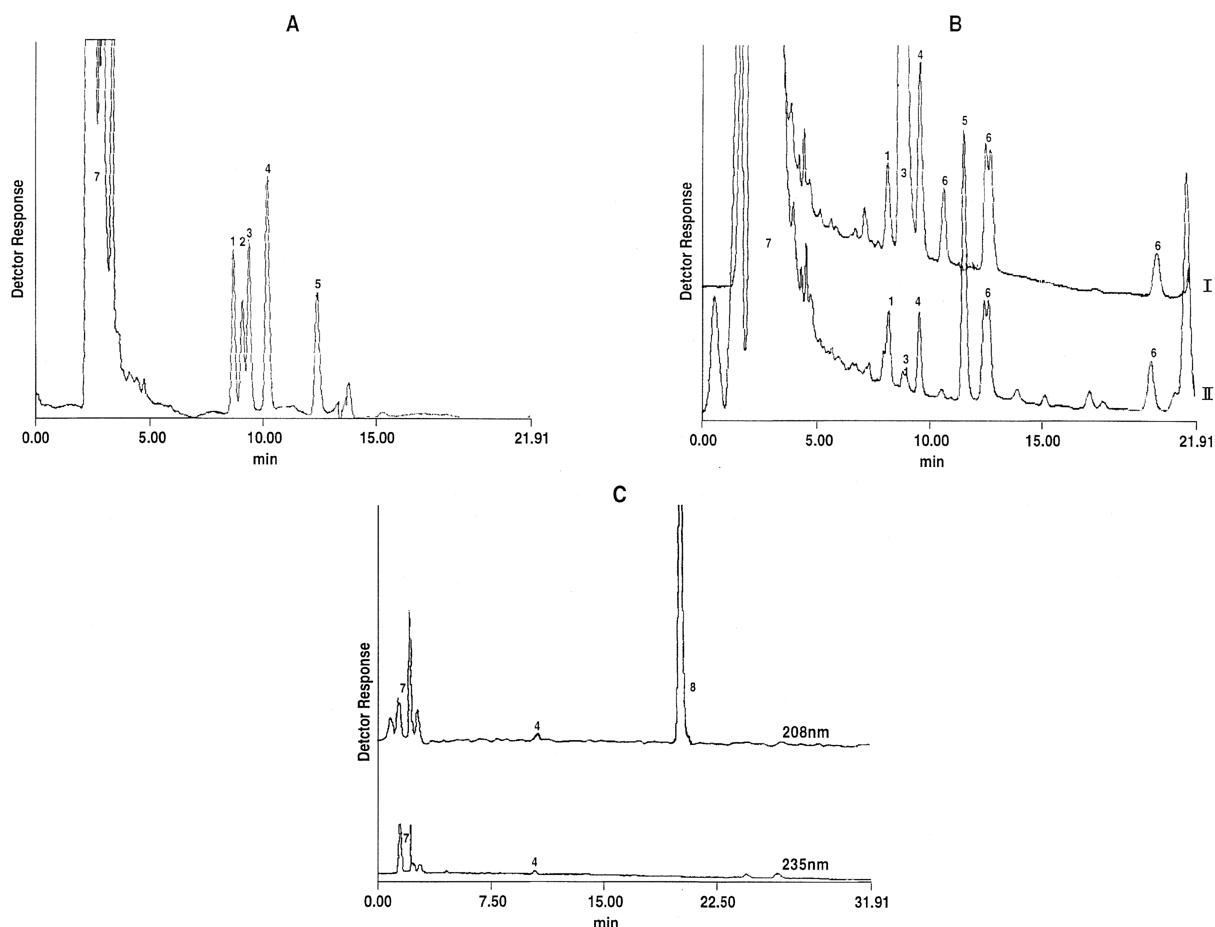

Fig. 1. HPLC of cholesterol and cholestanol after derivatization or derivatization and solvolysis. (A) The chromatographic separation of synthetic sterol-4-bromobenzenesulfonates. (B) Chromatography of sterols from pooled serum (0.3 ml) after derivatization (I) and after addition of I.S. derivatization and solvolysis (II). (C) HPLC of cholesterol (100 μg) after derivatization and solvolysis, visualized at 208 nm and 235 nm. Peaks corresponding to cholesteryl-isopropyl ether (8), and trace amount of impurity with mobility similar to cholestanol-4′-bromobenzenesulfonate (4) was detected at 208 nm. Peak 8 was not detected at 235 nm. The chromatography was performed with a YMC-Pack Pro C_{18}, 250×6 mm, S-5 μm 120 A column and monitored at UV 235 nm, unless otherwise stated, in the system acetonitrile–isopropanol–0.5% aqueous acetic acid (62:34:4, v/v/v) at a flow-rate of 1.4 ml/min. Peaks: 1=lathosterol; 2=3β,5β-cholestanol; 3=cholesterol and 3α,5α- or 3α,5β-cholestanol-epimers; 4=cholestanol, stigmasterol and campesterol; 5=I.S. (internal standard); 6=unidentified; 7=blank components; 8=cholesteryl-isopropyl ether.

tan-3α-ol, and 5α-cholestan-3α-ol peaks emerged together with cholesterol (peak 3), all the five compounds were separated from cholestanol (peak 4), which had the lowest mobility of the six sterols but was not separated from the C-5-unsaturated sterols stigmasterol and campeseterol. The I.S. stigmastanol emerged after 12 min.

A definite separation of cholestanol was achieved in pooled serum samples (0.3 ml) with excess of endogenous cholesterol (cholesterol/cholestanol ratio of 690) only after derivatization and solvolysis. The separation of cholestanol from cholesterol is illustrated in Fig. 1B (I) – after derivatization and Fig. 1B (II) – after derivatization and solvolysis.

The removal of cholesterol from the measuring system is illustrated in Fig. 1C. Cholesterol (100 μg) was derivatized, then solvolyzed, and the product was chromatographed and visualized at 208 nm and

235 nm. Peak 8 corresponding to cholesteryl isopropyl ether appeared at 19.5 min when detected at 208 nm but did not appear at 235 nm. A trace amount of impurity with mobility similar to cholestanol-4′-bromobenzenesulfonate could be seen at the two wavelengths.

5.2. Validation studies

5.2.1. Linearity

In order to test the validity of the method, cholestanol (12, 25, 50, 100, 150 nmol/l) was added to 0.3 ml saline samples containing 80 and 500 nmol/l I.S. and cholesterol, respectively. The measurements were repeated on 10 different days. The correlation of cholestanol/I.S. peak area ratios represented a calibration curve with the calculated linear regression of $y>0.97$ (range 0.97–0.99), $r>0.98$ (range 0.98–0.99), $P<0.0001$.

5.2.2. Recovery tests

Recovery tests were performed by adding I.S. (10 μg) into 0.3 ml of pooled serum containing known amounts of cholestanol (0.6, 1.1, 2.2, 6.1, 16.1, 21.1, 26.1 and 31.1 μg). The first concentration was achieved by saline dilution of the pooled serum, to the other aliquots the appropriate amounts of cholestanol were added (0–30 μg). The first sample contained 380 μg and the others 760 μg of cholesterol, respectively. The levels of cholestanol were measured on 6 different days, by which the within- and between-day recovery values were 101.5±4.58% and 101.2±4.72%, respectively.

5.2.3. Reproducibility

The reproducibility of the method was studied in four patients with different cholestanol concentrations during 6 different days (Table 1). The precision of the measurements was higher in the high cholestanol level samples (i.e., lower RSD values).

5.2.4. Sensitivity

The limit of detection with peak/noise ratio of 2 was achieved by 1:5 dilution of the pooled serum with saline, and the measured cholestanol value was 0.7 μg/ml [Fig. 2B (I)]. In two cases of Gaucher's disease and Polycythemia Vera, respectively, the cholestanol peak was not seen.

Table 1
Precision of serum cholestanol determination[a]

Case	Mean ($n=6$)	SD	RSD (%)
CTX 208-5	42.0	0.44	1.0
CTX 216-6	34.3	0.62	1.8
Patient with cirrhosis (54 years)	11.9	0.54	4.6
Pooled serum	3.7	0.23	6.2

[a] Aliquots (0.3 ml) were taken from samples of different endogenous cholestanol concentrations. The levels given in μg/ml, were measured on six different days.

5.3. Cholestanol levels in CTX and non-CTX subjects

The mean levels of cholestanol in the control subjects was 3.6±2.1 μg/ml (Table 2). The highest levels of cholestanol were found in the medically untreated CTX patients (Table 2). In one treated patient (CTX 206-3) the values of cholestanol did not deviate from the control range, however the cholesterol/cholestanol ratio was significantly lower, but higher compared to the untreated cases. A similar result was obtained when a brother of the patients CTX 208-1 and 208-5 was tested. He was genetically typed as a heterozygote with cholestanol level of 5.4 μg/ml and ratio of 322 (Table 2). Two sisters and one brother of the patient CTX 224-1 (30–36 years old) were also investigated. These siblings had no clinical manifestations and were not analyzed genetically, their cholestanol levels were normal (3.5±1 μg/ml) with cholesterol/cholestanol ratio of 445±9. The concentration of cholestanol in two patients with liver cirrhosis (Table 2) was lower compared to the untreated CTX patients, but significantly higher than the normal levels.

6. Discussion

The elimination of cholesterol from cholestanol was based on the Stoll reaction [29], who esterified cholesterol with *p*-toluenesulfonyl chloride and solvolyzed the product to cholesteryl-3β-methyl ether by heating with methanol, but cholestanol like other C-5-saturated sterols, was stable under the same conditions [30]. In order to achieve a more rapid derivatization and solvolysis of the product in an

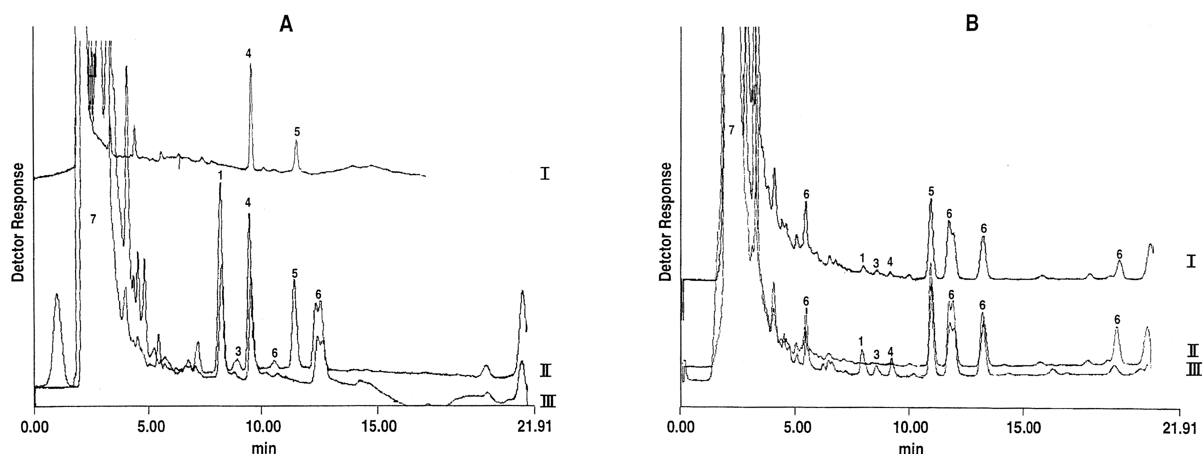

Fig. 2. Determination of sterols in CTX patients and pooled serum. (A) Chromatographic detection of cholestanol in CTX cases. I. CTX 215-5; II and III CTX 208-5 with and without addition of I.S., respectively. (B) The limit of cholestanol detection was achieved by 1:5 dilution of the pooled serum with saline (I, 0.7 μg/ml). For comparison, the determination of cholestanol in a seven-fold diluted (beyond detection limit, II) and undiluted serum (3.7 μg/ml) is shown (III). The chromatographic conditions and the peak numbers are as in Fig. 1.

open tube, 4-bromobenzenesulfonyl chloride and isopropanol, respectively, were used. The sterols carrying the 4-bromobenzenesulfonyl moiety absorbed at 235 nm but the sterols converted to isopropyl ethers could not be detected at this wavelength, but could be visualized at 208 nm. The retention time of cholesteryl isopropyl ether was 95% higher compared to cholestanol-4′-bromobenzenesulfonate. These differences in the absorbance and chromatographic mobilities made it possible to get a clear separated peak of cholestanol even when samples with high cholestrol/cholestanol ratio were analyzed. The disappearance of cholesterol eliminated the difficulty of baseline separation [3] and the "carry-over" effect caused by excess of cholesterol. Our results are comparable to the HPLC benzoate

Table 2
Comparison of serum sterol concentrations and cholesterol/cholestanol ratios in patients and control subjects

No.	Case	Sex	Age (years)	Cholestanol (μg/ml)	Cholesterol (mg/dl)	Lathosterol (μg/ml)	Cholesterol/ cholestanol ratio
1	CTX 215-5	M	25	57.0	144	<1.0	25
2	CTX 216-6	F	13	34.3	158	21.4	46
3	CTX 224-1	M	35	29.4	168	19.6	57
4	CTX 208-1[a]	M	33	59.0	224	27.3	38
5	CTX 208-5[a]	F	31	42.0	215	62.1	51
6	CTX 206-3 (treated)	F	30	4.9±1.3[b]	186	5.4±1.6	379
7	Cirrhosis	F	75	15.4	62	5.2	40
8	Cirrhosis	F	54	11.9	134	2.1	113
9	A brother of CTX 208-1 and 5[a,c]	M	3.5	5.4	174	4.2	322
10	Three siblings of CTX 224-1[d]		30–36	3.5±1	127± 9	3.8±1.5	445
11	Control (n=34)	–	27–59	3.6± 2.1	202.2±45	3.8±2.7	561

[a] Patients 4, 5 and 9 are siblings.
[b] Two years of follow-up (four measurements).
[c] Heterozygote.
[d] Genetically unspecified.

derivatization method with UV detection [21]. However, in this communication no results were given for samples of very high cholesterol/cholestanol ratio. On the present study a definite cholestanol peak was observed when the ratio exceeded 1000 in four of the control subjects (Table 2).

In some cases of low cholestrol/cholestanol ratio a good separation of the two metabolites were also observed when the solvolysis step was omitted (i.e., the treated CTX patient 206-3, Table 2). However it can not be assumed that the solvolysis step is unnecessary at these cases since their serum may contain interfering C-5-unsaturated sterol like stigmasterol or campesterol [4,6,23,24] that can be eliminated in the solvolysis step.

The solvolysis step made it possible to also use columns of lower separation capacity (i.e., Hypersil ODS 5 μm, Altech Econospher C_{18} 5 μm or Waters μBondapak C_{18}). Stigmastanol was found to be a suitable I.S. (peak 5) for the following reasons: (i) due to the identical steroid moiety and the minor difference in the side chain of cholestanol and the I.S., it can be assumed that the derivatization reaction rate is similar in the two compounds. (ii) It is an available compound. (iii) The difference in the retention time values (20%) is suitable for peak area comparison (Fig. 1A, peaks 4 and 5). (iv) It was not found in the serum of sitosterolemia and xanthomatosis patients [4,23] and in the cases tested in this study (Figs. 1B and 2A).

The recovery tests were performed with cholestanol concentrations of 0.6 μg (2 μg/ml) to 30 μg (100 μg/ml). This range of concentrations covers the possibilities from healthy cases up to the chronic billiary cirrhosis patients with the highest cholestanol levels [3]. The precision of the method was measured on 6 different days using serum samples of different cholestanol concentrations. As expected, the higher the metabolite concentration the lower the RSD values were (Table 1). For the entire assay the recovery of added cholestanol in serum was 101.5% within-day, and 101.2% between-days.

The levels of the control subjects in our study were 3.6 ± 2.1 (μg/ml). These results are compatible with most of the other communicated methods, i.e.,: GC 1.4–9.5 [14]; GC–MS 3.36 ± 0.54 [17]; capillary GC 2 ± 0.2 and 0.2–4.3 [3,16]; GC after epoxidation 3.15 ± 0.74 [28]; HPLC with UV detection 3.37 ± 1.55 and 3.9 ± 1.8 [23,24]; HPLC with fluorescence detection 4.32 ± 0.7, 2.8 ± 1.7 and 2.2 ± 0.6 [18,20,21].

The highest cholestanol concentrations and lowest cholesterol/cholestanol ratios were measured in the untreated CTX cases (Table 2). In the treated patient the levels of cholestanol were similar to the control but the ratio was lower than the normal subjects values. Similar results were also obtained in the heterozygous siblings of the CTX patients. Definite diagnosis can be achieved only by molecular genetic typing. However, the observations of the present study are compatible with previous publications in which the levels of cholestanol in cases of CTX were dependent on the clinical manifestations of the disease, which was well expressed by the cholesterol/cholestanol ratio [3,20,28].

In our study liver cirrhosis patients had a moderate cholestanol elevation but a very low ratio.

Since reagent for cholestanol determination are readily available and the measurement of cholesterol/cholestanol ratio provides useful information about the disease state, it may serve as an alternative method to bile alcohols measurement [9,10,33–35], especially in the long term follow up of treated CTX patients.

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