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QUANTITATIVE ANALYSIS OF STEROLS IN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

APPLICATION TO THE BIOCHEMICAL DIAGNOSIS OF CEREBROTENDINOUS XANTHOMATOSIS

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SUMMARY

A method for the simultaneous determination of 5α-cholestan-3β-ol and cholesterol in serum by high-performance liquid chromatography was developed. After addition of internal standard (5β-cholestan-3α-ol) and saponification with ethanolic potassium hydroxide, the sterols were converted into their benzoyl derivatives, which were subjected to reversed-phase liquid chromatography with ultraviolet detection at 228 nm. Only 0.1 ml of serum was needed to give a reproducible result. This method has been used for the biochemical diagnosis of cerebrotendinous xanthomatosis, a hereditary disorder of cholesterol metabolism.

INTRODUCTION

Cerebrotendinous xanthomatosis (CTX) is a hereditary disorder resulting in an elevation in the concentration of 5α-cholestan-3β-ol in serum and tendon xanthomas. A definite diagnosis of this disease can be made by determination of the 5α-cholestan-3β-ol concentration. In 1976 we developed an analytical method for the determination of 5α-cholestan-3β-ol and cholesterol by gas chromatography–mass spectrometry (GC–MS)[1]; since then, more than 40 cases of CTX have been diagnosed in our laboratory. Several other methods have been reported for the determination of serum 5α-cholestan-3β-ol by gas chromatography (GC)[2,3] and thin-layer chromatography (TLC)[4,5]. For diagnostic purposes, however, a more accurate and easier method is desirable. In the present study, we report the simultaneous quantification of serum 5α-cholestan-3β-ol and cholesterol by high-performance liquid chromatography (HPLC), which is suitable for the clinical diagnosis of CTX.

EXPERIMENTAL

Benzoyl chloride reagent

The reagent for benzoyl derivatization was prepared before use by mixing 0.6

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ml of benzoyl chloride with 20 ml of 1,2-dichloroethane and 2 ml of pyridine with continuous swirling and cooling.

Synthesis of 5α-cholestan-3β-yl benzoate and 5-cholesten-3β-yl benzoate

5α-Cholestan-3β-ol (cholestanol, 0.315 g; Tokyo Kasei Kogyo Co., Tokyo, Japan) was dissolved in 30 ml of benzene in an erlenmeyer flask; 1.5 ml of pyridine and 0.581 g of benzoyl chloride were then added with continuous swirling in an ice-bath. The mixture was left overnight at room temperature in the dark, then poured into a separatory funnel and washed with 20 ml of 1 *M* hydrochloric acid. After washing twice with 20 ml of water, benzoic acid (a reaction by-product) was removed by washing with 20 ml of 0.5 *M* sodium carbonate. The aqueous layer was discarded, and the organic layer was washed with 20 ml of water until the washings became neutral. The organic layer was dried in a rotary evaporator. 5α-Cholestan-3β-yl benzoate (0.363 g) was obtained by recrystallization from ethanol. The yield was 91%. 5-Cholesten-3β-yl benzoate was synthesized according to the same procedure. Each benzoate showed a single peak in reversed-phase HPLC.

Sample preparation of 5α-cholestan-3β-ol and cholesterol in serum for routine HPLC assay

5β-Cholestan-3α-ol (10 μg; Sigma, St. Louis, MO, U.S.A.) as an internal standard was placed in a screw-capped test-tube, followed by 100 μl of serum and 1 ml of 1 *M* ethanolic potassium hydroxide. After vigorous stirring, the mixture was allowed to stand for 1 h at 80°C, after which 0.5 ml of water were added to the saponified mixture. The unsaponifiable material was extracted with two 2-ml portions of hexane. The extracts were pooled in another screw-capped test-tube and evaporated under a stream of nitrogen. The benzoyl chloride reagent (0.3 ml) was added to the test-tube containing the unsaponifiable material. After vigorous stirring, the solution was allowed to stand for 30 min at room temperature. After addition of 2 ml of 1,2-dichloroethane, the reaction mixture was washed successively with 2 ml of 0.1 *M* hydrochloric acid, and twice with 2 ml of water. The organic layer was evaporated under nitrogen and dried *in vacuo* for a few hours. The residue was dissolved in 0.5 ml of acetonitrile–1,2-dichloroethane (2:1, v/v), and 5 μl were injected into the HPLC system.

HPLC for sterol assay

The instrument was a LC-6A system, equipped with an autoinjector, a column oven (Shimadzu, Kyoto, Japan) and a chromatogram data processor (Chromatopac C-R3A with a floppy disk system, Shimadzu). The column was a SBC-ODS (15 cm × 2.5 mm I.D., Shimadzu), and the column-oven temperature was maintained at 50°C. The solvent was acetonitrile–water–acetic acid (97:3:0.2, v/v) at a flow-rate of 0.5 ml/min. Steryl benzoates were detected by their absorbance at 228 nm. They were automatically identified and quantitated by the Chromatopac C-R3A.

Determination of the recovery of 5α-cholestan-3β-ol from serum

Sera containing various concentrations of 5α-cholestan-3β-ol (0–40 μg/ml) were prepared by adding commercially available 5α-cholestan-3β-ol. A suitable amount of 5α-cholestan-3β-ol solution in chloroform was placed in a screw-capped

test-tube, and the solvent was removed under a stream of nitrogen. Serum (100 μl) was added and then treated for HPLC as mentioned above.

Examination of the reproducibility of the assay

The reproducibility of the entire HPLC assay was examined by analyzing the same serum obtained from a CTX patient over a 3-day period (five times per day). The reproducibility of the HPLC step was tested by injecting the same sample five times.

Gas chromatography–mass spectrometry

Mass fragmentographic analysis was performed according to the procedure described by Seyama *et al.*[1], using a GCMS-QP1000 (Shimadzu). The GC column (2 m × 3.2 mm I.D.) was packed with 3% QF-1. The column-oven temperature was maintained at 240°C, and the flow-rate of the carrier gas (helium) was 30 ml/min.

RESULTS AND DISCUSSION

The ultraviolet (UV) spectra of cholesteryl and 5α-cholestan-3β-yl benzoates have similar profiles, $\lambda_{max.}$ = 228 nm and $\varepsilon_{max.}$ = 12000 M^{-1} cm^{-1} in 1,2-dichloroethane–acetonitrile (1:1, v/v). The structural differences between the sterols did not affect the UV spectrum, which is derived only from the benzene ring. This is convenient for quantitating various sterols at a fixed wavelength (228 nm).

The time course of the alkaline hydrolysis of cholesteryl palmitate showed that it is completely hydrolyzed within 60 min at 80°C.

In the selection of the HPLC eluent, several solvents were examined. When eluent systems containing an alcohol (methanol, ethanol, 2-propanol) were used the chromatographic peak became broader. However, an eluent based on acetonitrile without alcohol gave a satisfactory chromatogram. A small amount of acetic acid was necessary to suppress the tailing of the front peak of benzoic acid derived from the excess of reagent. The elution time was controlled by the addition of secondary solvents. In order to speed up the elution, 1,2-dichloroethane was added. In order to delay the elution, water was added. A small-bore column (SBC-ODS, 15 cm × 2.5 mm I.D.) was used. This column allowed reduction of the solvent consumption to one-third, and gave a similar separation of sterols (Fig. 1) to that on the conventional column (25 cm × 4.6 mm I.D.). Under the conditions described in Experimental, the relative retention times of 5β-cholestan-3α-yl benzoate (I.S.), cholesteryl benzoate and 5α-cholestan-3β-yl benzoate were 1.00, 0.91 and 1.11, respectively (Table I).

The time course of benzoyl derivatization of sterol was examined. The reaction was completed in a few minutes at room temperature and no by-product was produced. Excess of benzoyl chloride was removed by washing with water. Hydrochloric acid, which shortens the column life in HPLC, was produced during this step, but it was removed as pyridine chloride by washing with water.

Excellent reproducibility of the HPLC analysis was observed. In HPLC the reproducibilities of the determinations of 5α-cholestan-3β-ol and cholesterol were 0.4 and 0.3% [coefficient of variation (C.V.), n = 5], respectively. When the reproducibility of the whole process was examined for three sequential days, using the same serum, the C.V.s for 5α-cholestan-3β-ol were 2.5 (day 1), 1.7 (day 2) and 2.1% (day

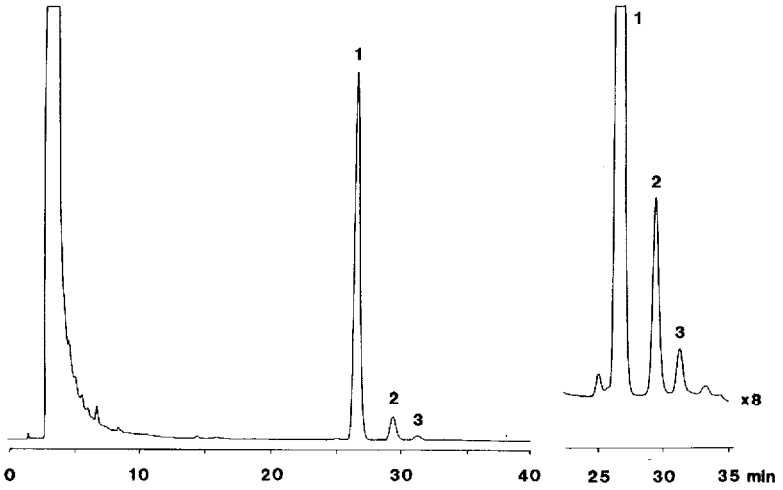

Fig. 1. HPLC chromatogram of the sterol fraction obtained from the serum of a CTX patient. Peaks: 1 = cholesterol; 2 = 5β-cholestan-3α-ol; 3 = 5α-cholestan-3β-ol. The right-hand side is an eight-fold magnification of the left between 25 and 35 min. Assay conditions as in Experimental.

3), and for cholesterol were 4.1 (day 1), 1.4 (day 2) and 1.0% (day 3). These values were much lower than those observed in mass fragmentography (*ca.* 10%). For the entire assay, the recovery of added 5α-cholestan-3β-ol in serum was 99.5% in the range of 0–40 μg/ml. The limit of detection of 5α-cholestan-3β-ol by HPLC (less than 0.1 μg/ml serum) was improved compared to that of the mass fragmentographic method (about 1 μg/ml serum).

A summary of the data on serum sterol concentrations, accumulated in our laboratory since 1975, is given in Table II. In most cases, CTX was definitevely diagnosed by examination of the 5α-cholestan-3β-ol concentration in serum, which is abnormally elevated in CTX patients. However, several CTX patients showed a normal concentration of 5α-cholestan-3β-ol. However, even in those cases the disease was diagnosed by means of the 5α-cholestan-3β-ol/cholesterol ratios, based on the finding[4] that the sera of CTX patients usually contain normal or even lower levels of cholesterol than those of healthy subjects. In the case of hypercholesterolemia, for

TABLE I

RELATIVE RETENTION TIMES OF STEROLS ON A SBC-ODS COLUMN

Values are expressed relative to 5β-cholestan-3α-ol.

Sterol	*Relative retention time*
5-Cholesten-5β-ol (cholesterol)	0.907
5β-Cholestan-3α-ol (epicoprostanol)	1.000
5α-Cholestan-3β-ol (cholestanol)	1.108
24α-Methyl-5-cholesten-3β-ol (campesterol)	1.051
24α-Ethyl-5-cholesten-3β-ol (β-sitosterol)	1.231

TABLE II

SERUM CONCENTRATIONS OF 5α-CHOLESTAN-3β-OL AND CHOLESTEROL OF CTX PATIENTS (44 CASES) IN JAPAN MEASURED BY GC–MS OR HPLC

No. (%)	*Age*	*Sex*	*5α-Cholestan-3β-ol*	*Cholesterol (μg/ml)*	*Ratio (mg/ml)*
1	32	M	19.6	1.78	1.10
2	33	F	16.5	1.79	0.92
3	25	F	31.0	2.08	1.49
4	27	F	26.6	1.33	2.00
5	29	F	44.0	1.88	2.34
6	22	F	28.5	1.29	2.21
7	43	M	6.4	1.22	0.52
8	39	M	24.0	2.11	1.13
9	55	F	8.0	1.62	0.49
10	44	M	18.3	1.97	0.93
11	50	M	17.0	1.26	1.35
12	56	F	10.3	1.81	0.57
13	45	F	13.0	1.58	0.82
14	40	F	14.9	1.65	0.90
15	32	F	26.4	1.46	1.81
16	35	M	22.8	1.52	1.50
17	39	M	36.0	1.83	1.97
18	52	F	22.9	1.34	1.71
19	42	M	47.5	1.52	3.13
20	41	F	10.1	1.39	0.73
21	43	M	71.3	0.72	9.90
22	33	M	49.2	0.91	5.41
23	32	F	26.8	1.48	1.81
24	36	M	40.7	1.01	4.02
25	55	F	39.9	1.43	2.79
26	31	M	68.8	1.73	3.97
27	27	F	21.9	0.90	2.43
28	34	F	48.4	1.51	3.20
29	31	F	27.1	1.47	1.85
30	38	F	23.2	1.86	1.25
31	43	M	29.2	1.02	2.86
32	47	F	43.9	1.86	2.36
33	44	M	22.4	1.86	1.20
34	16	F	11.7	1.73	0.67
35	25	M	41.1	2.09	1.97
36	39	M	38.9	1.31	2.97
37	42	F	40.3	1.90	2.12
38*	38	M	31.4	1.42	2.21
39*	47	M	36.4	1.76	2.07
40*	53	M	34.6	1.49	2.32
41*	61	M	13.0	2.15	0.60
42*	50	F	19.9	2.19	0.91
43*	46	F	23.1	1.35	1.71
44*	35	M	34.4	1.46	2.36
Other disease (n = 133)			4.0 ± 3.1	1.95 ± 0.73	0.20 ± 0.15
Control (n = 10)			2.2 ± 0.6	1.90 ± 0.16	0.15 ± 0.03

* Measured by HPLC.

example, the serum of a patient showed a high concentration not only of 5α-cholestan-3β-ol but also of cholesterol. In our examinations of several hundred patients, the 5α-cholestan-3β-ol/cholesterol ratio was greater than 0.003 for CTX, and in other diseases was less than 0.005. In the overlapping region, the diagnosis was usually established adequately by clinical findings.

Recently, a simultaneous determination of cholesterol and 5α-cholestan-3β-ol by HPLC with fluorescence detection was reported by Matsuoka *et al.*[6]. In their method, a good separation between cholesterol and 5α-cholestan-3β-ol was obtained by means of the epooxidation of cholesterol. The present method satisfies the demands by clinicians for simplicity, low cost and reliability.

Since 1975, we have developed and reported several methods for the determination of 5α-cholestan-3β-ol such as GC–MS[1], GC[3] and TLC[5] which were designed for the diagnosis of CTX. The HPLC method has several advantages over the earlier methods in terms of reproducibility and recovery. Of the four methods, only GC–MS and HPLC are able to quantify 5α-cholestan-3β-ol and cholesterol simultaneously. These methods are therefore proposed as standard methods for the diagnosis of CTX. However, when HPLC is used, the maintenance and operation of the instruments required are much easier than for the GC–MS method. Furthermore, the HPLC method has an higher sensitivity than the GC–MS method. High reproducibility and ease of operation facilitate the monitoring of the effect of experimental therapy by chenodeoxycholic acid administration[7]. In addition, this procedure can be applied for the diagnosis of other sterol metabolic diseases such as β-sitosterolemia, and for biochemical investigations of sterol metabolism. The sterol assay method described in this report is the best procedure, we believe, for the biochemical diagnosis of CTX.

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