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# HIGHLY SENSITIVE AND SIMPLE DETERMINATION OF CHOLESTEROL AND CHOLESTANOL IN HUMAN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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## SUMMARY

A simple and highly sensitive high-performance liquid chromatographic method for the determination of cholesterol and cholestanol in human serum is described. After extraction of serum with *n*-hexane, these compounds and 1-eicosanol (internal standard) are converted into the corresponding fluorescent carbamic esters by treatment with 3,4-dihydro-6,7-dimethoxy-4-methyl-3-oxo-quinoxaline-2-carbonyl azide in benzene. The derivatives are separated within 32 min on a reversed-phase column (YMC Pack $C_8$) with acetonitrile–methanol–water (81:9:10, v/v/v) as eluent and detected fluorimetrically. The detection limits are 2 pg (5 fmol) and 3 pg (7 fmol) for cholesterol and cholestanol, respectively, at a signal-to-noise ratio of 2 in a 20-$\mu$l injection volume. This sensitivity permits precise determination of cholesterol and cholestanol in 1–5 $\mu$l of normal human serum.

## INTRODUCTION

The concentration of cholestanol, a metabolite of cholesterol, is increased in sera of patients with cerebrotendious xanthomatosis (CTX), obstructive jaundice and cholestatic hepatitis, whereas the cholesterol concentration in sera from patients with CTX remains in the normal range [1]. Therefore, the ratio of cholestanol to cholesterol is greatly increased in sera from patients with CTX, and has been widely used as a biological diagnostic index for CTX [2]. Furthermore, the determination of the ratio has also been used successfully for the follow-up of some liver diseases [3]. Thus, the simultaneous determination of cholesterol and cholestanol is very useful for the diagnosis and therapy of these diseases.

Several methods, including gas chromatography–mass spectrometry (GC–MS) [4] and high-performance liquid chromatography (HPLC) with fluorimetric detection [5], have been reported for the simultaneous determination of choles-

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terol and cholestanol in human serum. The GC–MS method has been widely used, but it requires expensive instrumentation and a large volume of serum (0.5–1 ml). Although the fluorimetric HPLC method is sensitive, it requires a rather tedious derivatization procedure including epoxidation with *m*-chloroperbenzoic acid and fluorescence derivatization with 4-chloroformyl-3-methoxycoumarin. Recently, a simple and rapid GC method has been developed [2]. The method is highly sensitive, and thus requires only a small amount of human serum (5 $\mu$l). However, it is difficult to determine cholestanol precisely in normal human serum using this method.

We have therefore investigated the use of 3,4-dihydro-6,7-dimethoxy-4-methyl-3-oxo-quinoxaline-2-carbonyl azide (DMEQ-$CON_3$) as a highly sensitive and reactive fluorescence derivatization reagent for alcoholic compounds in HPLC [6]. Recently, we found that DMEQ-$CON_3$ reacts with cholesterol and cholestanol to form the corresponding highly intense fluorescent products, which are separated by reversed-phase HPLC. Thus, we have developed a simple, rapid and highly sensitive method for the simultaneous determination of cholesterol and cholestanol in serum by HPLC with pre-column fluorescence derivatization with DMEQ-$CON_3$. 1-Eicosanol, which does not occur in biological fluid, was used as an internal standard (I.S.).

## EXPERIMENTAL

### *Reagents and solutions*

All chemicals and solvents were of analytical-reagent grade unless noted otherwise. Deionized and distilled water was used. Cholesterol (5-cholesten-3$\beta$-ol) and cholestanol (5$\alpha$-cholestan-3$\beta$-ol) were purchased from Sigma (St. Louis, MO, U.S.A.). DMEQ-$CON_3$ was prepared as described previously [6]. DMEQ-$CON_3$ (3.0 m*M*) and 1-eicosanol (2.5 m*M*) solutions were prepared in benzene and acetone, respectively. The DMEQ-$CON_3$ solution could be used for two days when stored in a refrigerator at 4 °C in the dark. Reversed-phase columns, Radial Pak cartridge $C_{18}$, $C_8$, phenyl and CN (all 100×8 mm I.D.; 10 $\mu$m particle size), YMC Pak $C_8$ (150×6 mm I.D.; 10 $\mu$m particle size) and LiChrosorb RP-8 (150×4 mm I.D.; 10 $\mu$m particle size) were purchased from Waters Assoc. (Milford, MA, U.S.A.), Yamamura Chemical Labs. (Kyoto, Japan) and Merck (Darmstadt, F.R.G.), respectively.

Human serum samples were obtained from healthy volunteers in our laboratory and kept at −20 °C until analysis.

### *Apparatus and HPLC conditions*

Uncorrected fluorescence spectra and intensities were measured with a Hitachi 650-60 spectrofluorimeter (Tokyo, Japan) in 10×10 mm quartz cells; spectral bandwidths of 10 nm were used for both the excitation and the emission monochromators.

Infrared (IR) spectra were recorded with a Shimadzu 430 IR spectrophotometer (Kyoto, Japan) in potassium bromide pellets. $^1H$ Nuclear magnetic resonance (NMR) spectra were obtained with a Hitachi R-90H spectrometer at 90

MHz using ca. 5% (w/v) solution in [$^2$H]chloroform containing tetramethylsilane as an internal standard. Splitting patterns were designated as follows; s, singlet; m, multiplet. Field desorption mass spectra were taken with a JEOL DX-300 spectrometer (Tokyo, Japan).

A Waters 510 high-performance liquid chromatograph equipped with a U6K universal injector (10-$\mu$l loop) and a Hitachi F1100 fluorescence spectromonitor fitted with a 12-$\mu$l flow-cell operated at excitation and emission wavelengths of 360 nm and 440 nm, respectively, were used. The column was a YMC Pack $C_8$. The mobile phase was acetonitrile–methanol–water (81:9:10, v/v/v). The flow-rate was 2.0 ml/min (ca. 70 kg/cm$^2$). The column temperature was ambient (ca. 25°C). The column can be used for more than 1000 injections with only a small decrease in the theoretical plate number if it is washed with aqueous methanol (1:1, v/v) at a flow-rate of 2.0 ml/min for ca. 30 min at the end of each working day.

When uncorrected fluorescence excitation and emission spectra of the eluates needed to be measured, a Hitachi 650-60 fluorescence spectrophotometer fitted with a 20-$\mu$l flow-cell was used; the spectral bandwidths were 5 nm for both the excitation and the emission wavelengths.

*Fluorescent derivatives of cholesterol and cholestanol*

*Cholesterol.* DMEQ-$CON_3$ (200 mg, 0.69 mmol) and cholesterol (230 mg, 0.59 mmol) were dissolved in 10 ml of benzene, and the solution was refluxed for 1 h at 100°C. The reaction mixture was evaporated to dryness. The residue, dissolved in 5 ml of chloroform, was chromatographed on a silica gel 60 (ca. 130 g, 70–230 mesh; Merck) column (2.5×2.7 cm I.D.) with *n*-hexane–ethyl acetate (1:1, v/v). The main fraction was evaporated to dryness, and the residue was recrystallized from methanol as pale blue needles (I; m.p. 219.6–220.6°C; yield, 70 mg). Analytical data were as follows: IR $\nu_{max}^{KBr}$ (cm$^{-1}$): 1740 [N(C=O)]; 1645 (C=O); 1630 (C=C and/or C=N); 2900 (NH); $^1$H NMR ($C^2HCl_3$) $\delta$ (ppm): 0.60–2.55 (45H, m, $C_{25}H_{45}$); 3.75 (3H, s, N–$CH_3$); 3.93 and 3.98 (3H each, s each, O–$CH_3$); 4.48–4.78 (1H, m, NHC*H*); 5.30–5.65 (1H, m, C=CH); 7.26 and 6.69 (1H each, s each, aromatic H); 8.51 (1H, s, NH). Analysis calculated for $C_{39}H_{57}N_3O_5$: C, 72.03; H, 8.87; N, 6.49; found: C, 72.58; H, 8.57; N, 6.30. Mass spectra, $m/z$=647 ($M^+$).

*Cholestanol.* Cholestanol (230 mg, 0.59 mmol) was treated in the same way as cholesterol. Pale blue needles (II; m.p. 225.0–226.3°C; yield, 50 mg) were obtained. Analytical data were as follows: IR $\nu_{max}^{KBr}$ (cm$^{-1}$): 1740 [N(C=O)]; 1645 (C=O); 1630 (C=C and/or C=N); 2900 (NH); $^1$H NMR ($C^2HCl_3$) $\delta$ (ppm): 0.60–2.55 (46H, m, $C_{27}H_{46}$); 3.74 (3H, s, N–$CH_3$); 3.93 and 3.98 (3H each, s each, O–$CH_3$); 4.58–4.82 (1H, m, NHC*H*); 7.26 and 6.69 (1H each, s each, aromatic H); 8.49 (1H, s, NH). Analysis calculated for $C_{39}H_{59}N_3O_5$: C, 72.08; H, 9.15; N, 6.47; found: C, 72.21; H, 9.05; N, 6.50. Mass spectra, $m/z$=649 ($M^+$).

*Procedure*

Human serum (5 $\mu$l) was mixed with 5 $\mu$l of the eicosanol (I.S.) solution and 0.5 ml of 0.2 *M* sodium hydroxide in 95% ethanol, and the mixture was hydrolysed

at 100°C for 15 min. To the resulting solution, 1 ml of water and 3 ml of *n*-hexane were added. The resulting mixture was vortexed for ca. 10 min and centrifuged at 1000 *g* for 2 min. The organic layer (ca. 2 ml) was transferred to a PTFE screw-capped 10-ml vial and evaporated to dryness under a stream of nitrogen. The residue was dissolved in 200 $\mu$l of the DMEQ-$CON_3$ solution, and the mixture was heated at 100°C for 60 min in the dark. A 20-$\mu$l portion of the reaction mixture diluted with 0.8 ml of methanol was analysed by HPLC.

The calibration graphs were prepared according to the procedure without alkaline hydrolysis, except that 5 $\mu$l of the I.S. solution were replaced by the I.S. solution containing cholesterol [2.5–20 $\mu$g (6.5–51.7 nmol) per 5 $\mu$l] and cholestanol [2–100 ng (5.1–257 pmol) per 5 $\mu$l]. The net peak-height ratios of cholesterol (or cholestanol) and 1-eicosanol were plotted against the concentrations of cholesterol (or cholestanol).

## RESULTS AND DISCUSSION

### *HPLC conditions*

The separation of the DMEQ derivatives of cholesterol, cholestanol and 1-eicosanol was studied on the reversed-phase columns described in Experimental, using methanol, acetonitrile, water and their mixtures as mobile phases. The best separation was achieved using a YMC Pack $C_8$ and methanol–acetonitrile–water (81:9:10, v/v/v) as the eluent. A typical chromatogram obtained with a mixture of the three compounds is shown in Fig. 1. The peaks for cholesterol, cholestanol and 1-eicosanol (retention times: 19.2, 26.8 and 31.2 min, respectively) were completely separated from those for the blank components (peaks 4 and 5) within 32 min. Even when concentration differences between cholesterol and cholestanol were large, these compounds could be baseline-separated (Fig. 1B). Cholesterol, cholestanol and 1-eicosanol gave single peaks in the chromatogram.

### *Derivatization conditions*

Maximum and constant peak heights for cholesterol, cholestanol and 1-eicosanol are attained at DMEQ-$CON_3$ concentrations greater than 0.5 m*M* in the reaction mixture; 3.0 m*M* was selected as a sufficient concentration. The derivatization reaction of the three compounds with DMEQ-$CON_3$, which apparently occurred even at moderate temperatures, was accelerated at higher temperatures. An example for cholesterol is shown in Fig. 2. However, at 140°C, the peak heights for all the compounds decreased with heating time. At 100 and 120°C, the peak heights reached almost maximum after heating for 15 and 55 min, respectively. Thus, heating at 100°C for 60 min was recommended for convenience in the procedure.

The effect of reaction solvents on the derivatization reaction was examined using benzene, toluene, acetone, acetonitrile, ethyl acetate, diethyl ether, chloroform, dimethyl sulphoxide and N,N-dimethylformamide. Benzene and toluene gave the most intense peaks, therefore the derivatization reaction was performed in benzene. Water interfered with the reaction.

The fluorescence excitation (maximum, 360 nm) and emission (maximum,

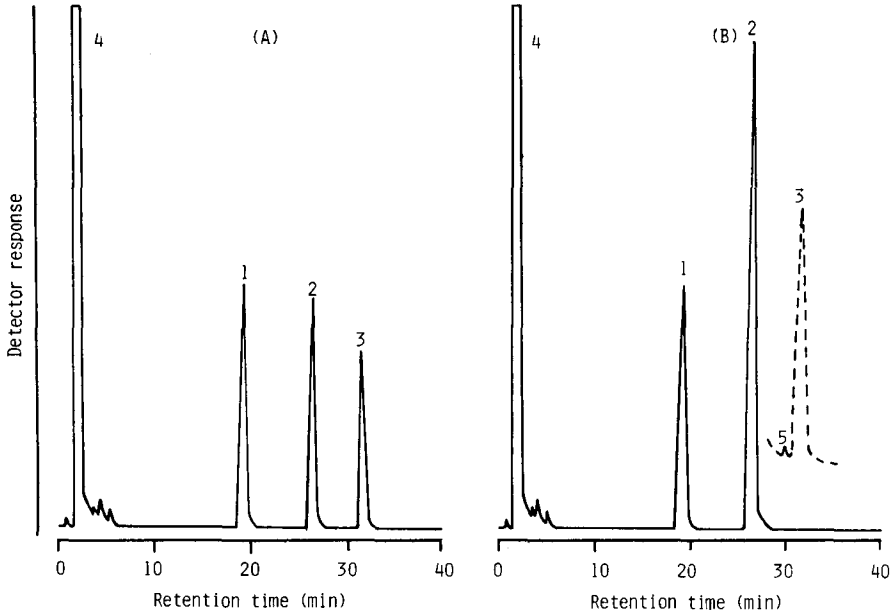


Fig. 1. Chromatogram of the DMEQ derivatives of cholesterol, cholestanol and 1-eicosanol. A portion (5 $\mu$l) of standard mixtures of cholesterol, cholestanol and 1-eicosanol in acetone (A = 12.5 nmol each per 5 $\mu$l; B = 12.5 nmol, 25.0 nmol and 50.0 pmol, respectively, per 5 $\mu$l) were treated according to the procedure. Peaks: 1 = 1-eicosanol; 2 = cholesterol; 3 = cholestanol; 4 and 5 = blank components. Detector sensitivity: —, 0.05; ----, 20.

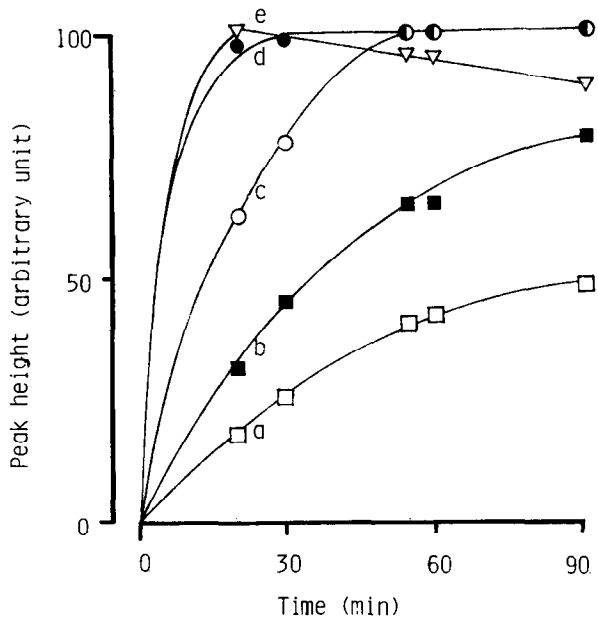


Fig. 2. Effect of reaction time and temperature on the fluorescence derivatization of cholesterol. Portions (5 $\mu$l) of cholesterol solution in acetone (12.5 nmol per 5 $\mu$l) were treated as in the procedure at various temperatures for various periods. Temperatures: a, 60°C; b, 80°C; c, 100°C; d, 120°C; e, 140°C.

440 nm) spectra of the eluates from peak 1 (1-eicosanol), peak 2 (cholesterol) and peak 3 (cholestanol) were identical. The efficiency of conversion of cholesterol and cholestanol into the DMEQ derivatives was examined by comparing the peak heights obtained under the present conditions with those given by the corresponding reaction products (compounds I and II); the extents of conversion (mean ± S.D., $n=5$) were 82.2 ± 2.9% for cholesterol and 70.6 ± 2.7% for cholestanol. The DMEQ derivatives of all the compounds in the final mixture were stable for at least 72 h in daylight at room temperature.

*Validation of the method*

The precision of the method was determined for ten repeated determinations using a standard mixture of cholesterol, cholestanol and 1-eicosanol (12.5 nmol each per 5 μl). The coefficients of variation (C.V.) did not exceed 2% for all the compounds. The detection limits were 2 pg (5 fmol) for cholesterol and 3 pg (7 fmol) for cholestanol at a signal-to-noise ratio of 2 in in a 20-μl injection volume. This sensitivity is at least 30 times higher than that afforded by the fluorimetric HPLC method using 3-chloroformyl-7-methoxycoumarin [5].

Many biogenic substances examined gave no fluorescent derivatives under the described conditions, at a concentration of 5 nmol per 5 μl; i.e. sugars (D-xylose, D-ribose, 2-deoxy-D-ribose, D-fucose, D-glucose, D-galactose, D-fructose, D-glucosamine, D-galactosamine, glucuronic, mannuronic, iduronic and galacturonic acids, maltose, cellobiose, gentiobiose, and lactose), hydroxycarboxylic acids (lactic and malic acids), seventeen different L-α-amino acids. On the contrary, some hydroxysteroids (11-dehydrocorticosterone, deoxycorticosterone, pregnenolone and dehydroisoandrosterone) gave highly fluorescent products [6]. However, under the recommended HPLC conditions, the products were co-eluted with DMEQ-$CON_3$. Thus, these substances did not interfere with the determination of cholesterol and cholestanol.

*Fluorescent derivatives in the determination of cholesterol and cholestanol*

We previously reported that DMEQ-$CON_3$ reacts with alcoholic compounds to give the fluorescent carbamic acid esters [6]. Thus, the reaction products from cholesterol and cholestanol were identified as the corresponding carbamic acid esters by the analytical data described under Experimental. The fluorescence excitation (maximum, 360 nm) and emission (maximum, 440 nm) spectra of both the products in acetone–methanol–water (81:9:10, v/v/v) were almost identical with those of the eluates from peaks 2 and 3 for cholesterol and cholestanol, respectively.

*Determination of cholesterol and cholestanol*

Esters of cholesterol and cholestanol in serum were hydrolysed at 100°C in aqueous ethanolic sodium hydroxide solution, and the resulting free cholesterol and cholestanol were extracted with *n*-hexane in the usual manner [4,5].

A typical chromatogram obtained with a normal human serum is shown in Fig. 3A. The peaks for cholesterol and cholestanol were characterized on the basis of the retention times and fluorescence excitation and emission spectra, compared

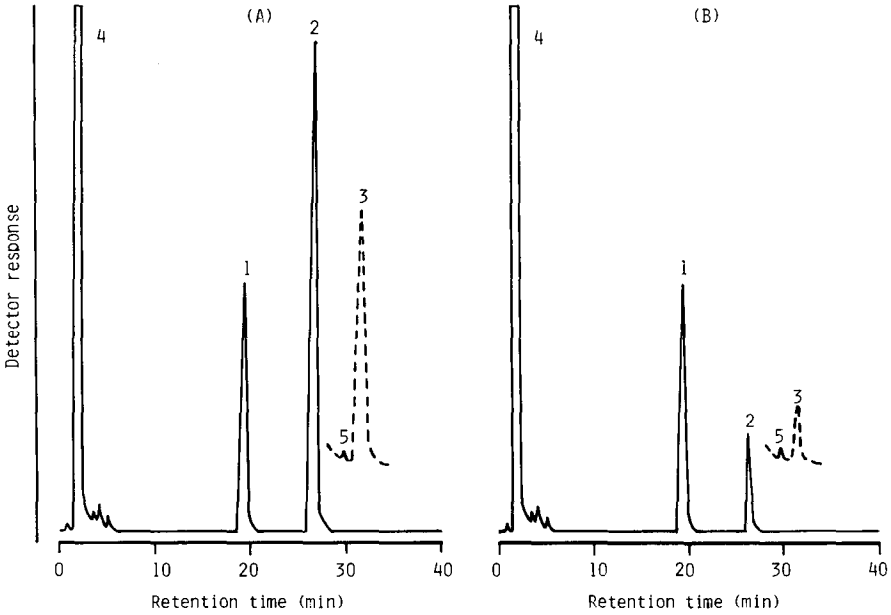


Fig. 3. Chromatograms obtained with (A) normal human serum and (B) serum diluted five-fold with water. Portions (5 $\mu$l) of the samples were treated according to the procedure. Concentration of cholesterol and cholestanol in serum: 2.0 mg (5.2 $\mu$mol) and 2.9 $\mu$g (7.5 nmol) per ml, respectively. Peaks and detector sensitivity are as in Fig. 1. Peak 3 in Fig. 3B corresponds to ca. 100 fmol of cholestanol in a 20-$\mu$l injection volume.

with those of the reference compounds, and co-chromatography of the reference compounds and sera with aqueous 85–100% methanol as mobile phase.

Linear relationships were observed between the ratios of the peak heights of cholesterol and cholestanol to that of 1-eicosanol and the amounts of cholesterol [2.5 $\mu$g (6.5 nmol) to 20 $\mu$g (51.7 nmol)] and cholestanol [2 ng (5.1 pmol) to 100 ng (257 pmol)] added to 5 $\mu$l of serum. The linear regression equations (the linear correlation coefficients in parentheses) of the curves were $y=0.3982x+0.10212$ ($r=0.999$) for cholesterol and $y=0.3068x+0.00004$ ($r=0.999$) for cholestanol; where $y$ and $x$ are the peak-height ratios and the concentrations ($\mu$mol/ml) of the individual compounds, respectively.

The within-day precision was determined from repeated analyses ($n=10$) of a normal human serum containing 1.8 mg (4.7 $\mu$mol) and 2.6 $\mu$g (6.7 nmol) per ml of cholesterol and cholestanol, respectively. The coefficients of variation were 3.2 and 3.5% for cholesterol and cholestanol, respectively. The between-day precision was obtained by performing the analyses ($n=3$ each day) using the calibration graphs prepared on that day during ten days with the same sample kept frozen at $-40$°C. The coefficients of variation were 3.8 and 3.9% for cholesterol and cholestanol, respectively.

Recovery tests were performed by adding known amounts of cholesterol [15 $\mu$g (38.8 nmol)] and cholestanol [20 ng (51.7 pmol)] to serum (5 $\mu$l). The recoveries of cholesterol and cholestanol (mean $\pm$ S.D., $n=10$) were $99.8\pm2.8$% and $98.6\pm3.0$%, respectively.

The concentrations of cholesterol and cholestanol and their concentration ratios in human serum are given in Table I. The individual mean values were in good

TABLE I

CONCENTRATION OF CHOLESTANOL AND CHOLESTEROL AND THEIR RATIO IN NORMAL HUMAN SERA

| Age | Sex* | Cholestanol [μg (nmol)/ml] | Cholesterol [mg (μmol)/ml] | Cholestanol/cholesterol [(mol/mol) × 100] |
|---|---|---|---|---|
| 29 | M | 1.2 ( 3.1) | 1.2 (3.1) | 0.10 |
| 22 | M | 6.0 (15.5) | 1.6 (4.1) | 0.38 |
| 22 | M | 4.6 (11.9) | 1.9 (4.9) | 0.24 |
| 20 | M | 3.9 (10.1) | 1.2 (3.1) | 0.33 |
| 20 | M | 2.9 ( 7.5) | 1.4 (3.6) | 0.21 |
| 20 | M | 1.8 ( 4.7) | 1.2 (3.1) | 0.15 |
| 27 | F | 3.0 ( 7.8) | 1.2 (3.1) | 0.26 |
| 22 | F | 3.8 ( 9.8) | 3.2 (8.3) | 0.12 |
| 22 | F | 3.6 ( 9.5) | 2.5 (6.5) | 0.14 |
| 22 | F | 2.9 ( 7.5) | 2.0 (5.2) | 0.15 |
| 22 | F | 10.8 (27.9) | 2.4 (6.2) | 0.45 |
| 22 | F | 4.2 (10.9) | 1.2 (3.1) | 0.37 |
| 22 | F | 4.0 (10.3) | 1.5 (3.9) | 0.28 |
| 22 | F | 1.4 ( 3.6) | 1.2 (3.1) | 0.12 |
| 22 | F | 2.1 ( 5.4) | 0.9 (2.3) | 0.23 |
| Mean | | 3.7 ( 9.6) | 1.6 (4.1) | 0.24 |
| S.D. | | 2.3 ( 5.9) | 0.6 (1.6) | 0.11 |

*M = male; F = female.

agreement with those obtained by other workers [1–5].

The present HPLC method using DMEQ-$CON_3$ with fluorimetric detection gives a satisfactory sensitivity. In this study, 5 μl of human serum were used for precise sampling. A chromatogram (Fig. 3B) obtained with a serum sample diluted five-fold with water indicates that if serum is accurately taken, the method may permit the determination of cholesterol and cholestanol in only 1.0 μl or less. Thus, the method should be useful in cases where only an extremely small amount of serum is available. This method is rapid and simple enough for twenty samples to be assayed in a day, and should be useful for physiological and pharmacological investigations of cholesterol and cholestanol.

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