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Simultaneous determination of cholesterol and cholestanol in human serum by high-performance liquid chromatography using 3-(5,6-methylenedioxy-2-phthalimidyl)benzoyl azide as precolumn fluorescent labelling reagent

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ABSTRACT

A fluorescent labelling reagent, 3-(5,6-methylenedioxy-2-phthalimidyl)benzoyl azide, designed for the determination of alcohols by precolumn high-performance liquid chromatography, has been applied to the simultaneous determination of cholesterol and cholestanol in human serum. The reagent reacts with cholesterol and cholestanol at 140°C for 10 min to produce the fluorescent derivatives, which can be separated on a reversed-phase column with acetonitrile–ethanol–water (60:35:7.5, v/v) as eluent. The detection limits for cholesterol and cholestanol were 45 and 50 fmol per injection (20 μl), respectively. The values of cholesterol and cholestanol in normal human sera were 135–212 mg/dl and 137–928 μg/dl, respectively.

INTRODUCTION

The remarkable elevation of serum cholestanol with normal levels of cholesterol is known to be one of the symptoms of biliary obstruction and other liver diseases, especially cerebrotendinous xanthomatosis, which is a rare familial disease due to an autosomal-recessive inheritance [1,2]. The simultaneous detection of cholestanol and cholesterol in serum, and the comparison of the concentration ratio of cholestanol to cholesterol, give very useful information for the biomedical diagnosis and therapy of these diseases. For this purpose, gas chromatographic–mass spectrometric (GC–MS) [3], GC [4,5] and high-performance liquid chromatographic (HPLC) with fluorimetric detection [6,7] methods have been reported. Recent developments in HPLC, including column packings and fluorescent derivatization reagents for alcoholic compounds, make it possible to separate and detect a small amount of cholestanol and a 400 times larger amount of cholesterol in serum at the same time.

During our research into fluorescent labelling reagents for HPLC [8–10], we designed 3-(5,6-methylenedioxy-2-phthalimidyl)benzoyl azide (MPB-N_3; Fig. 1) as a labelling reagent for alco-

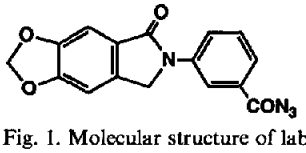

Fig. 1. Molecular structure of labelling reagent.

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holic compounds. We found that MPB-N_3 reacts with cholesterol and cholestanol to give the corresponding fluorescent carbamic acid esters quantitatively. Thus, we have applied this reagent for the simultaneous determination of cholesterol and cholestanol in small amounts of serum by HPLC with precolumn fluorescence derivatization, using 1-heneicosanol as an internal standard. We compared the concentration ratio of cholestanol to cholesterol in hypocholesterolemia, hypercholesterolemia and normal sera.

EXPERIMENTAL

Chemicals and solvents

All chemicals were of analytical-reagent grade, unless stated otherwise. 4,5-Methylenedioxyphthalaldehyde was prepared from helioalcohol according to the method of Dallacker *et al.* [11]. Cholesterol and cholestanol were purchased from Tokyo Kasei (Tokyo, Japan). Other alcohols and Cholesterol CII-Test Wako were obtained from Wako (Osaka, Japan). Deionized, distilled water was used. Organic solvents were purified by distillation prior to use.

Synthesis of MPB-N_3

m-Aminobenzoic acid (1.56 g) in dioxane (50 ml) and 4,5-methylenedioxyphthalaldehyde (2.0 g) in dioxane (150 ml) were mixed and stirred at room temperature for 2 days. The precipitate, 3-(5,6-methylenedioxy-2-phthalimidyl)benzoic acid, was recrystallized from dioxane. To a solution of 3-(5,6-methylenedioxy-2-phthalimidyl)-benzoic acid (0.5 g) and triethylamine (0.2 g) in dimethylformamide (45 ml), diphenylphosphoryl azide (1.7 g) in dimethylformamide (5 ml) was added at 0°C. The mixture was stirred for 2 h at 0°C, then 5% sodium bicarbonate aqueous solution (100 ml) was added. The resulting mixture was shaken with diethyl ether (100 ml). The organic layer and precipitates floating around between the organic and aqueous layers were collected and washed with cold water (100 ml, twice). The precipitates and the residue of the organic layer evaporated under the reduced pressure were combined, washed with a small amount of acetone (*ca.* 2 ml) and then dissolved in acetone at room temperature. MPB-N_3 was obtained as white needles on standing the acetone solution for 24 h at room temperature (yield 0.38 g, m.p. 180°C (dec.)). Anal. Calcd. for $C_{16}H_{10}N_4O_4$: C, 59.63; H, 3.13; N, 17.38%; Found: C, 59.59; H, 3.15; N, 17.14%. MS *m/z*: 322 (M^+). ^{1}H NMR (δ, ppm, in $CDCl_3$–DMSO-d_6 (9:1, v/v)): 4.82 (2H, s, CH_2 of phthalimidine), 6.12 (2H, s, OCH_2O), 6.98 and 7.22 (1H each, s each, aromatic H of phthalimidine), 7.43–8.36 (4H, m, H of benzene ring).

Preparation of MPB derivatives of cholesterol and cholestanol

The isolation of the MPB derivative of cholesterol was achieved as follows. MPB-N_3 (200 mg) and cholesterol (250 mg) were dissolved in acetone (100 ml). The mixture was divided into fifty portions; these were heated in fifty vials without seals at 140°C for 15 min. The resulting residues were washed with *n*-hexane and then suspended with acetone. The suspended mixtures were combined and filtered. The filtrate was evaporated to dryness under the reduced pressure. The residue, dissolved in chloroform, was chromatographed on a silica gel column (25 × 3 cm I.D.) packed with Wakogel C-200 (Wako) with chloroform–hexane (5:5, v/v) 200 ml, (6:4, v/v) 800 ml, and (7:3, v/v) 400 ml, successively. A part of the main fraction was evaporated to dryness under reduced pressure. The MPB derivative of cholesterol was recrystallized from acetone–dichloroethane (6:4, v/v) (yield 28 mg, m.p. 270°C (dec.)). Anal. Calcd. for $C_{43}H_{56}N_2O_5$: C, 75.84; H, 8.29; N, 4.11%; Found: C, 75.76; H, 8.28; N, 4.17%. ^{1}H NMR (δ, ppm, in $CDCl_3$): 0.52–2.32 (43H, m, cholesterol skeleton $C_{25}H_{43}$), 4.40–4.80 (1H, m, cholesterol 3-CH), 4.75 (2H, s, CH_2), 5.30–5.50 (1H, m, cholesterol 6-CH=), 6.08 (2H, s, OCH_2O), 6.65 (1H, s, CONH), 6.89 and 7.25 (1H each, s each, aromatic H of phthalimidine), 7.00–8.10 (4H, m, benzene ring H).

The MPB derivative of cholestanol was prepared from MPB-N_3 (200 mg) and cholestanol (250 mg) as described above, and recrystallized from acetone–dichloroethane (8:2, v/v) (yield 25

mg, m.p. 272°C (dec.)). Anal. Calcd. for $C_{43}H_{58}N_2O_5$: C, 75.62; H, 8.56; N, 4.10%. Found: C, 75.56; H, 8.59; N, 4.29%. ^{1}H NMR (δ, ppm, in $CDCl_3$–DMSO-d_6 (9:1, v/v)): 0.66–3.80 (47H, m, cholestanol skeleton $C_{27}H_{47}$), 4.78 (2H, s, CH_2), 6.12 (2H, s, OCH_2O), 6.97 and 7.28 (1H each, s each, aromatic H of phthalimidine), 7.2–8.20 (4H, benzene ring H).

Instrumentation

The HPLC system consisted of a HLC-803D pump (Tosoh, Tokyo, Japan), a DG-980-51 line degasser (Jasco, Tokyo, Japan), a Rheodyne 7161 injector, a FS-8000 fluorescence detector (Tosoh) and a C-R6A Chromatopac integrator (Shimadzu, Kyoto, Japan). An ERC-ODS-1161 column (100 × 6 mm I.D., Erma, Tokyo, Japan), a TSK guardgel ODS-80T_M column (15 × 3.2 mm I.D., Tosoh) and acetonitrile–ethanol–water (60:35:7.5, v/v) as mobile phase were used at ambient temperature. The flow-rate was 1.0 ml/min. The fluorescence intensities were monitored at excitation and emission wavelengths of 312 nm and 427 nm, respectively. Uncorrected fluorescence spectra were measured with a Hitachi 650-10S spectrofluorimeter (Hitachi, Tokyo, Japan) using a quartz cell (optical path length 10 mm). A Heating block HF-21 (Yamato, Tokyo, Japan) was used.

Analytical procedure of human serum samples

To human serum (5 μl) were added water (100 μl) and potassium hydroxide in 90% ethanol (0.2 *M*, 1 ml). The mixture was hydrolysed at 100°C for 20 min in the heating block, and then water (0.4 ml), *n*-hexane (3 ml) and 1-heneicosanol (0.2 m*M* in acetone, 50 μl, I.S.) were added. The mixture was vortexed for *ca.* 2 min and centrifuged at 400 *g* for 2 min. The organic layer (1.5 ml) was transferred to a vial and evaporated to dryness at *ca.* 40°C under a stream of nitrogen. To the residue was added pregnenolone (0.2 m*M* in acetone, 50 μl), and the mixture was treated with MPB-N_3 (5 m*M* in acetone, 250 μl) at 140°C for 10 min without a seal in the heating block. An aliquot (20 μl) of the resulting residue dissolved in acetone (200 μl) was subjected to HPLC.

RESULTS AND DISCUSSION

MPB-N_3 and its reactivity

MPB-N_3 was synthesized from 3-(5,6-methylenedioxy-2-phthalimidyl)benzoic acid, which was obtained from the reaction of 3,4-methylenedioxyphthalaldehyde with 3-aminobenzoic acid. MPB-N_3, which was stable at room temperature for more than 6 months, reacted with primary and secondary alcohols but not with tertiary alcohols. The primary and secondary alcohols reacted under heating to produce the corresponding highly fluorescent derivatives, which were separated by reversed-phase HPLC. For example, the fluorescent derivatives of hexanol, heptanol, cyclohexanol and *d*-menthol treated with MPB-N_3 in screw-capped vials at 140°C for 60 min were eluted at the retention times of 5.6, 7.5, 5.8 and 11.8 min, respectively, on the reversed-phase column with acetonitrile–water (7:3, v/v). MPB-N_3 also reacted with cholesterol and cholestanol under the same conditions.

Because carbonyl azides react with alcohols to form carbamic acid esters, the reaction products of MPB-N_3 with cholesterol and cholestanol should be the corresponding carbamic acid esters; this was confirmed by the elemental analyses and ^{1}H NMR spectral data.

The fluorescence spectra of both MPB derivatives in acetonitrile–ethanol–water (60:35:7.5, v/v) showed excitation and emission maxima at 312 nm and 427 nm, respectively.

Chromatographic separation and identification

The separation of cholesterol, cholestanol and 1-heneicosanol (I.S.) labelled with MPB-N_3 was examined on the reversed-phase column ERC-ODS-1161 by eluting with acetonitrile, methanol, ethanol, water or their mixture as mobile phase. With acetonitrile–ethanol–water (60:35:7.5, v/v) as the mobile phase, the fluorescent derivatives of cholesterol, cholestanol and 1-heneicosanol were eluted at the retention times of 20.3, 23.4 and 17.2 min, respectively, and their peaks were completely separated from those due to the reagent blank and other components of human serum. Typical chromatograms obtained with a stan-

dard solution and a human serum sample are shown in Fig. 2. Because the concentrations of cholestanol in sera were very low, the sensitivity of the detector was raised to 128-fold and the chart speed of the integrator was doubled at the retention time of 22 min as shown in Fig. 2B.

The wavelengths of the fluorescence excitation and emission maxima of the eluates corresponding to the fluorescent derivatives of cholesterol, cholestanol and 1-heneicosanol were 312 nm and 427 nm, respectively, which were identical with those of the MPB derivatives.

The peaks due to the fluorescent derivatives of cholesterol and cholestanol in human serum were identified by comparing the retention times and fluorescence spectra with those of the prepared MPB derivatives and the standard solutions.

Selection of internal standard

Various alcohols (5 nmol each in the labelling reaction mixture) were examined to select an internal standard. The peaks of aliphatic primary alcohols, $C_nH_{2n+1}OH$ (n = 1–9), labelled with MPB-N_3 overlapped with that of the reagent blank under the present HPLC conditions. The peaks for 1-octadecanol, 1-nonadecanol, 1-eicosanol, 1-heneicosanol, 1-docosanol, stigmasterol and phytol labelled with MPB-N_3 eluted at 9.9, 11.7, 14.2, 17.2, 21.0, 24.5 and 15.8 min, respectively. The peaks for 1-docosanol and stigmasterol partially overlapped with those of cholesterol and cholestanol, respectively. Phytol labelled with MPB-N_3 had a very low peak (*ca.* one tenth in comparison with the others). The peaks for 1-octadecanol, 1-nonadecanol, 1-eicosanol and 1-heneicosanol were successfully separated from those of cholesterol, cholestanol and other serum components. 1-Heneicosanol was preferred as the I.S. because its peak eluted close to the retention times of the cholesterol and cholestanol derivatives, as shown in Fig. 2.

The peak heights of the primary alcohols labelled with the reagent were observed to decrease with decreasing amounts of cholesterol (less than 10 nmol) in the labelling reaction mixture. This decrease did not occur at amounts of more than 10 nmol of cholesterol. To prevent the peak height of the I.S. from decreasing at amounts of cholesterol lower than 10 nmol, some hydroxysteroids were examined. In the presence of more

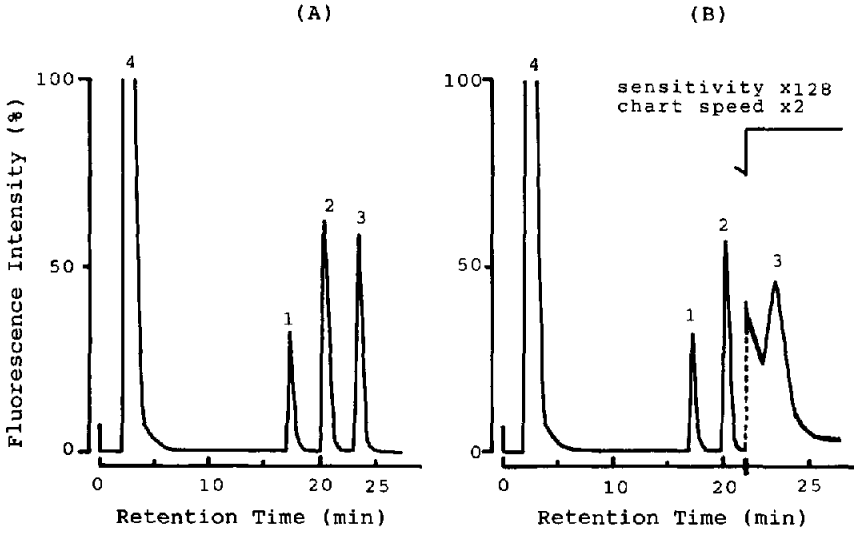

Fig. 2. Chromatograms of (A) standard solution and (B) human serum. Both 5-μl samples were treated according to the procedure. The concentrations of cholesterol and cholestanol were both 4 mM in the standard solution, and 3.63 mM and 15.6 μM, respectively, in serum; the concentration of 1-heneicosanol was 0.2 mM (50 μl) in both. Peaks: 1 = 1-heneicosanol (I.S.); 2 = cholesterol; 3 = cholestanol; 4 = reagent blank and pregnenolone.

than 10 nmol of pregnenolone in the reaction mixture the peak height of the I.S. remained constant regardless of the amount of cholesterol. Incidentally, the derivative of pregnenolone was eluted at the same time as the reagent blank. Therefore, pregnenolone was used to compensate for the fluctuation of the peak height of the I.S., though the peak heights of cholesterol and cholestanol labelled with the reagent were unaffected by the presence of pregnenolone.

Reaction conditions

The labelling reactions of cholesterol and cholestanol with the reagent were examined both in vials without a seal and in screw-capped vials. The solvents without seals were completely distilled away during the reaction, and the residues dissolved in acetone showed maximum and constant peak heights. However, the peak heights of the reaction mixtures in the screw-capped vials varied, because of irregular leaks of the solvents from the vials.

The effect of temperature on the labelling reactions of cholesterol with MPB-N_3 is shown in Fig. 3. At 120 and 140°C, the peak heights reached a maximum after 20 and 10 min, respectively. Similar results were obtained with cholestanol. Consequently, the labelling reactions with MPB-N_3 were performed at 140°C for 10 min without seals. The resulting residue and its acetone solution were stable for at least 24 h at room temperature in stoppered vials.

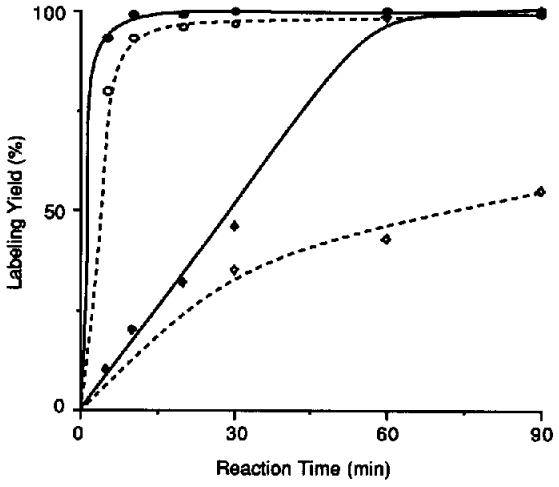

Fig. 3. Effects of reaction time and temperature on the labelling reaction of cholesterol with MPB-N_3: (◇) 80°C; (◆) 100°C; (○) 120°C; (●) 140°C.

The amount of MPB-N_3 (5 m*M* in acetone) was determined by the peak heights for cholesterol, cholestanol and 1-heneicosanol (I.S.) at the amounts of 25, 0.5 and 5 nmol, respectively, in the reaction mixture containing pregnenolone (10 nmol). The most intense and constant peak heights were obtained when more than 200 μl of the reagent solution were used. Therefore, 250 μl of MPB-N_3–acetone solution (5 m*M*) were used in the present procedure.

The efficiencies of conversion of cholesterol and cholestanol into the fluorescent derivatives were examined by comparing the peak heights under the reaction conditions with those of the prepared MPB derivatives of cholesterol and cholestanol. The extents of conversion (mean, $n = 5$) were 100.6% for cholesterol and 101.8% for cholestanol.

Calibration curves and detection limits

The peak heights of cholesterol and cholestanol labelled with MPB-N_3 were linear at concentration up to at least 12 m*M* (equivalent to 30 nmol per labelling reaction mixture). In the concentration range from 2.5 to 40 μ*M*, the peak heights of cholestanol labelled with the reagent were linear when the standard solutions containing cholesterol (2, 4 and 8 m*M*) were examined. The sensitivities at a signal-to-noise ratio of 3 of cholesterol and cholestanol were 45 and 50 fmol per injection, respectively.

Recoveries and precision

When recovery tests were carried out by adding cholesterol (10 and 30 nmol) and cholestanol (20 and 100 pmol) to sera (5 μl each), the recoveries were 99.6–105.3% for cholesterol and 96.6–104.9% for cholestanol (Table I). The precision was determined by separate analyses of hypo- and hypercholesterolemia and normal sera ($n = 8$ each). The coefficients of variation (C.V.) were 2.1%, 2.0% and 1.8% for cholesterol and 2.2%, 2.4% and 1.8% for cholestanol (Table II).

TABLE I

RECOVERIES OF CHOLESTEROL AND CHOLESTANOL ADDED TO SERUM

Cholesterol			Cholestanol		
Content (m*M*)	Added[a] (nmol)	Recovery (%)	Content (μ*M*)	Added[a] (pmol)	Recovery (%)
1.9	10	105.3	2.5	20	98.9
	30	105.3		100	99.8
4.8	10	102.5	9.1	20	104.9
	30	100.8		100	104.7
9.5	10	99.6	12.5	20	99.9
	30	103.8		100	96.6

[a] Various amounts of standard were added to 5 μl of serum.

Determination of cholesterol and cholestanol in human serum

Esters of cholesterol and cholestanol in serum were hydrolysed at 100°C for 20 min with aqueous ethanolic potassium hydroxide solution, and then the free forms were extracted with *n*-hexane according to the conventional procedure [6].

The concentrations of cholesterol and cholestanol in nine hypocholesterolemia, eleven normal and nine hypercholesterolemia sera were measured by the present HPLC method, and the concentration ratios of cholestanol to cholesterol are listed in Table III. The mean values of normal sera (135–212 mg/dl for cholesterol and 137–928 μg/dl for cholestanol) agreed with those previously reported [3–7]. The cholesterol values obtained by the present HPLC method were correlated with those of Cholesterol CII-Test Wako ($y = 0.9981x - 11.691$, $r = 0.9948$) (Fig. 4).

The mean value of cholestanol in hypocholesterolemia sera was lower than that in normal sera. The mean value in hypercholesterolemia sera was about double that in normal sera. However, the mean values of the concentration ratio of cholestanol to cholesterol in the three kinds of serum were almost the same.

The newly designed reagent, MPB-N_3, is highly sensitive and available for the determination of cholesterol and cholestanol in serum by HPLC with fluorescence detection. The present HPLC method should be useful for physiological and biomedical studies of cholesterol and cholestanol.

TABLE II

PRECISION OF SERUM CHOLESTEROL AND CHOLESTANOL DETERMINATIONS

Compound	Concentration (mean ± S.D., $n = 8$)	C.V. (%)
Hypocholesterolemia		
Cholesterol (m*M*)	1.9 ± 0.04	2.1
Cholestanol (μ*M*)	4.6 ± 0.10	2.2
Normal serum		
Cholesterol (m*M*)	4.5 ± 0.08	1.8
Cholestanol (μ*M*)	9.2 ± 0.17	1.8
Hypercholesterolemia		
Cholesterol (m*M*)	10.0 ± 0.20	2.0
Cholestanol (μ*M*)	26.5 ± 0.64	2.4

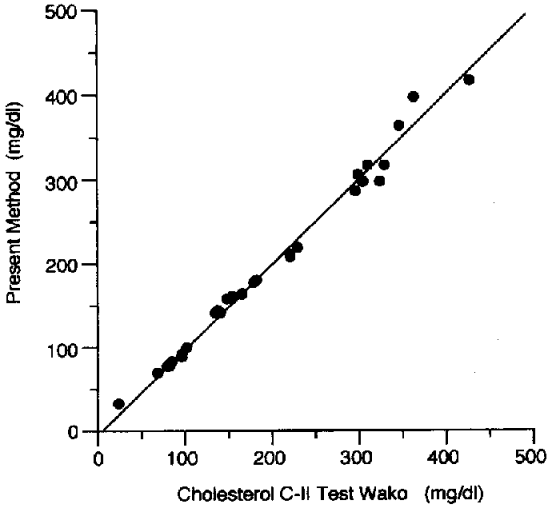

Fig. 4. Relationship between cholesterol amounts estimated by the present method and by Cholesterol CII-Test Wako.

TABLE III

CONCENTRATION RANGES OF CHOLESTEROL AND CHOLESTANOL AND THEIR CONCENTRATION RATIO

Serum	Cholesterol		Cholestanol		Ratio[a] ($M/M \times 100$)
	mM	mg/dl	μM	μg/dl	
Hypocholesterolemia					
1	2.0	78	4.0	154	0.20
2	1.6	62	4.8	186	0.30
3	1.9	72	4.6	180	0.25
4	2.1	82	7.6	295	0.36
5	2.2	84	3.1	119	0.14
6	0.7	26	1.9	72	0.28
7	1.9	75	6.3	243	0.32
8	1.8	71	3.9	152	0.21
9	2.4	93	7.7	299	0.32
Mean (n = 9)	1.84	71.4	4.88	188.9	0.264
S.D.	0.49	19.2	1.98	77.1	0.070
Normal sera					
10	4.1	157	11.4	444	0.28
11	3.5	135	3.6	139	0.10
12	4.5	172	9.0	351	0.20
13	3.9	151	3.5	137	0.10
14	3.6	139	23.9	928	0.67
15	4.0	154	6.7	260	0.17
16	4.5	174	9.2	356	0.20
17	3.5	135	16.5	642	0.47
18	5.3	205	16.3	634	0.31
19	5.5	212	7.1	276	0.13
20	5.1	201	6.1	239	0.12
Mean (n = 11)	4.32	166.8	10.30	400.5	0.250
S.D.	0.72	28.38	6.29	244.3	0.178
Hypercholesterolemia					
21	7.4	292	14.3	556	0.19
22	7.4	292	9.5	371	0.13
23	7.9	311	39.6	1511	0.49
24	7.1	280	15.2	590	0.21
25	7.6	299	13.4	522	0.18
26	10.0	410	26.5	1029	0.26
27	10.5	410	16.7	658	0.16
28	9.1	356	26.1	1014	0.29
29	7.9	310	25.3	982	0.32
Mean (n = 9)	8.32	326.9	20.73	803.7	0.248
S.D.	1.24	47.4	9.40	357.4	0.110

[a] Ratio of cholestanol to cholesterol.

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