Short Communication

Simultaneous determination of cholesterol precursors, plant sterols, and oxysterols in plasma using one-round pretreatment

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Abstract Cholesterol is biosynthesized mainly in the liver, absorbed from diets in the small intestine, and metabolized to oxysterols or cholestanol. The measurement of cholesterol, its precursors, plant sterols, cholestanol, and oxysterols in plasma is useful in the diagnosis of inherited disorders of cholesterol biosynthesis, malformation syndromes, familial hypercholesterolemia, cerebrotendinous xanthomatosis or sitosterolemia, and the evaluation of drug administration. Herein, we report the development of a new method for the determination of cholesterol, its related sterols, and oxysterols in plasma by a one-round pretreatment using gas chromatography coupled with mass spectrometry. Plasma pretreatments were performed as follows: lipid extraction, sterol ester saponification, extraction with *n*-hexane, and trimethylsilyl (TMS) derivatization. In the case of oxysterol, pretreatment requires additional solid-phase extraction before TMS to remove a large amount of cholesterol. Cholesterol, five cholesterol precursors, three plant sterols, cholestanol, and twelve oxysterols were simultaneously pretreated using this method. The recoveries of the plasma samples were 88.8 to 103.4%. The mean relative standard deviation values of the intra- and inter-day precision were less than 14.2%. This new development method appeared to be congruent with the conventional results, and its accuracy ranged from 85.0 to 115.5%. The concentrations of cholesterol and related sterols determined in our study were within the same range as those reported in the previous reports. The developed pretreatment procedure is simple, useful for application to human plasma, and contributes to the diagnosis of inherited disorders of cholesterol biosynthesis, cholesterol absorption, and other related diseases, and evaluation of drug administration.

Key words: cholesterol, oxysterols, plant sterols, cholesterol precursors, simultaneous determination

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Introduction

Cholesterol is an essential structural component of all mammalian cells and is obtained from endogenous and exogenous sources[1)]. Endogenous cholesterol is synthesized mainly in the liver from acetyl-coenzyme A and is converted to squalene and lanosterol[1, 2)]. There are two pathways from lanosterol to cholesterol: via lathosterol and 7-dehydrocholesterol and via desmosterol[2)] (Fig. 1). Cholesterol and some plant sterols (campesterol, stigmasterol, and *β*-sitosterol) are absorbed from the food in the small intestine[1)] (Fig. 1). Cholesterol is oxygenized by cytochrome

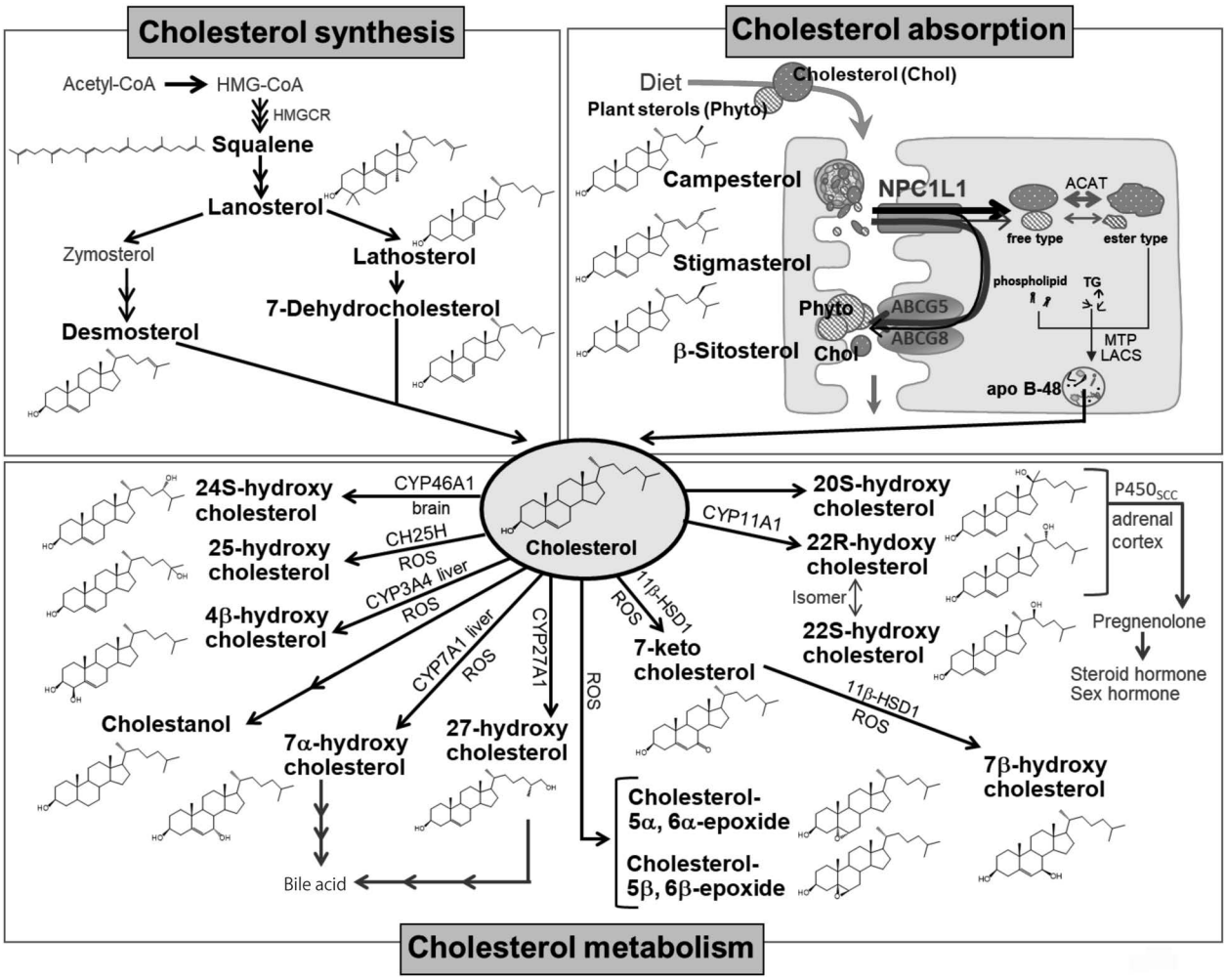

Fig. 1. Schematic of cholesterol biosynthesis, absorption from the intestine, and metabolic pathways (HMG-CoA; 3-hydroxy-3-methylglutaryl-coenzyme A, HMGCR; HMG-CoA reductase, NPC1 L1; Niemann-Pick C1-like protein 1, ABCG5; ATP-binding cassette transporter G5, ABCG8; ATP-binding cassette transporter G8, ACAT; acyl-coenzyme A:cholesterol acyltransferase, TG; triglyceride, MTP; microsomal triglyceride transfer protein, LACS; long-chain acyl-coenzyme A synthetase, apo B-48; apolipoprotein B-48, ROS; reactive oxygen species, CYP46A1; Cholesterol 24*S*-hydroxylase, CH25H; Cholesterol 25-hydroxylase, CYP7A1; Cholesterol 7-Alpha-hydroxylase, CYP27A1; Cholesterol 27-hydroxylase, 11β-HSD1; 11β-hydroxysteroid dehydrogenase type 1).

P450 (CYP), radical reactions, etc., and produces oxysterols first[1] (Fig. 1). Oxysterols are related to several diseases; for example, 27-hydroxycholesterol, 7-ketocholesterol, 24*S*-hydroxycholesterol and 7β-hydroxycholesterol could be used as markers of cerebrotendinous xanthomatosis (CTX)[3], diabetes mellitus[4], Alzheimer's disease[5] and lung cancer[6], respectively.

Several human malformation syndromes, such as lathosterolosis, Smith-Lemli-Opitz syndrome (SLOS), and desmosterolosis, have been associated with defects in cholesterol biosynthesis[7]. Xanthoma of the Achilles tendon (ATX) is a sign of long-term exposure to high blood cholesterol in patients with familial hypercholesterolemia (FH), which is associated with cardiovascular disease[8]. However, ATX is also a common symptom other conditions, such as Achilles tendon thickening, sitosterolemia, and CTX[9]. Sitosterolemia is an inherited metabolic disorder characterized by increased levels of plant sterols such as sitosterol[1]. Patients with sitosterolemia require dietary measures (restriction of phytosterols in food) and treatment with bile acid sequestrates (colestipol, cholestyramine) or ezetimibe, which are cholesterol absorption inhibitors[9]. CTX has been reported to not only decreases plasma levels of 27-hydroxycholesterol but to also increases plasma levels of cholestanol, lathosterol, campesterol, and sitosterol[3]. Treatment with CTX was used for chenodeoxycholic acid and statins,

which are 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors[9]. Patients with FH frequently have hypercholesterolemia, whereas patients with CTX and sitosterolemia show normocholesterolemia[3, 9]. FH, sitosterolemia, and CTX have similar symptoms as ATX; however, they differ in the treatment and behavior of cholesterol precursors, plant sterols, and oxysterols. Therefore, it is important to distinguish these diseases.

Furthermore, statins have been reported to decrease plasma oxysterols[10], decrease the level of lathosterol, and increase the levels of campesterol and sitosterol compensatory[11] in hypercholesterolemic patients. In addition, we previously reported that ezetimibe decreased the levels of campesterol, sitosterol, and stigmasterol, increased levels of compensatory lathosterol, and decreased serum 7β-hydroxycholesterol in Japanese hypercholesterolaemia[12]. Therefore, the simultaneous determination of cholesterol, cholesterol precursors, plant sterols, cholestanol, and oxysterols in plasma plays an important role not only in the diagnosis of inherited disorders of cholesterol biosynthesis[1, 7] but also in the evaluation of drug administration[10–12].

Several methods for sterol analysis have been proposed[3, 10, 12–17]. However, to measure cholesterol, its precursors, plant sterols, cholestanol, and oxysterols, it is necessary to use different pretreatment methods and machines. For instance, Mignarri et al.[3] used a commercial assay kit (colorimetric) to measure total plasma cholesterol levels, high performance liquid chromatography (HPLC) and gas chromatography coupled to mass spectrometry (GC-MS) to measure plasma levels of cholestanol, and GC-MS to measure plasma levels of cholesterol precursors, plant sterols, and oxysterols. This method required three pretreatments and three types of machines. Matysik et al.[18] developed an analytical method for the simultaneous determination of four cholesterol precursors, three plant sterols, and four oxysterols in human plasma. This method could not distinguish between CTX, sitosterolemia, and FH because cholesterol could not be measured simultaneously. Oxysterols are present in very low concentrations in all mammals and are almost invariably accompanied by a 10^3- to 10^6-fold excess of cholesterol[1, 13]. Therefore, the simultaneous determination of oxysterols requires the removal of a large amount of cholesterol at pretreatment before trimethylsilyl (TMS) derivatization.

This study aimed to determine the levels of cholesterol, its precursors, plant sterols, cholestanol, and oxysterols in plasma by a one-round pretreatment using GC-MS.

Materials and Methods

Reagents

Methanol, ethyl acetate, *n*-hexane, potassium hydroxide, phosphoric acid, squalene and 5α-cholestane {an internal standard (IS) for cholesterol, its precursors, plant sterols, and cholestanol)} were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Lanosterol was manufactured by the Nagara Science Corporation (Gifu, Japan). Lathosterol, 7-dehydrocholesterol, desmosterol, cholesterol, 7α-hydroxycholesterol, 7β-hydroxycholesterol, 4β-hydroxycholesterol, 22*S*-hydroxycholesterol, cholesterol-5β, 6β-epoxide, 22*R*-hydroxycholesterol, cholesterol-5α, 6α-epoxide, 20*S*-hydroxycholesterol, and 25-hydroxycholesterol were purchased from Sigma-Aldrich Corporation (St. Louis, USA). 7-Ketocholesterol and 27-hydroxycholesterol were purchased from Avanti Polar Lipids Incorporated (Birmingham, England). 24*S*-hydroxycholesterol and 27-hydroxycholesterol-26, 26, 26, 27, 27-D_5 (27d_5-OHC) (IS for oxysterols) were purchased from Medical Isotopes Incorporated (Pelham, USA). Campesterol and β-sitosterol were purchased from Tama Biochemical Corporation Ltd. (Tokyo, Japan) and stigmasterol and β-cholestanol were purchased from Tokyo Chemical Industry Corporation, Ltd. (Tokyo, Japan). Tri-Sil HTP reagent (hexamethyldisilazane [HMDS] : trimethylchlorosilane [TMCS] : pyridine=2 : 1 : 10) and a trimethylsilyl derivatizing agent, was purchased from Thermo Fisher Scientific Corporation (Waltham, USA). A Bond Elut SI cartridge (100 mg, 1 mL) was purchased from Agilent Technologies Japan (Tokyo, Japan), Isolute Biotage SI cartridge (100 mg, 10 mL) was purchased from Biotage Japan (Tokyo, Japan), and human plasma (sample A: Lot. No. R201529M; sample B**:** Lot. No. R201537M; sample C: Lot. No. R201557M; sample D: Lot. No. N113881F, sample E: N113882F, sample F: N113886F) were purchased from Tennessee Blood Service (Memphis, USA).

Sample preparation for the conventional method

The method presented here was compared with a conventional method of measuring cholesterol precursors, plant sterols, and oxysterols concentrations, as reported by Hirayama et al.[12] Briefly, to measure markers of cholesterol precursors and plant sterols, 50 μL of plasma containing 250 ng of 5α-cholestane (IS) was saponified for 1 h with

2 mL of 10 mol/L potassium hydroxide and 5 mL of methanol. After adjusting the pH to 7.0 with 50% phosphoric acid, 5 mL of H_2O was added to the mixture and extracted twice with 10 mL of *n*-hexane. The *n*-hexane layer was dried under a stream of nitrogen and then derivatized for 30 min at 60°C with the Tri-Sil HTP reagent. The derivatized cholesterol precursors and plant sterols were resuspended in 100 μL of *n*-hexane for GC-MS analysis.

For oxysterols, 250 μL of plasma containing 150 ng 27d_5-OHC (IS) was saponified for 15 min and extracted with *n*-hexane. The *n*-hexane layer was loaded onto a Bond Elut SI cartridge (100 mg, 1 mL), which was preconditioned with *n*-hexane. Excess cholesterol was removed by washing with *n*-hexane (2 mL) and ethyl acetate (10%) with *n*-hexane. The oxysterols retained on the cartridge were eluted using ethyl acetate (2 mL). The ethyl acetate eluate was dried under nitrogen and derivatized for 30 min at 60°C using Tri-Sil HTP reagent. The derivatized oxysterols were resuspended in 50 μL *n*-hexane for GC-MS analysis.

Sample preparation for the newly developed method

The new sample preparation method was based on the method described by Hirayama et al. [12] with modifications. Human plasma (250 μL) (samples A–F) containing 250 ng of 5α-cholestane and 150 ng of 27d_5-OHC (IS) was saponified for 22 h with 2 mL of 10 mol/L potassium hydroxide and 10 mL of methanol. After adjusting the pH to 7.0 with 50% phosphoric acid, 5 mL of H_2O was added to the mixture and extracted twice with 10 mL of *n*-hexane. For cholesterol, its precursors, plant sterols, and cholestanol, 2 mL of the *n*-hexane layer was dried under a stream of nitrogen and then derivatized for 30 min at 60°C with Tri-Sil HTP reagent. These were resuspended in 100 μL *n*-hexane for GC-MS analysis (Fig. 2). For oxysterols, 18 mL of the *n*-hexane layer was loaded onto an Isolute Biotage SI cartridge (100 mg, 10 mL) that was preconditioned using *n*-hexane. Excess cholesterol was removed by washing with *n*-hexane (2 mL) and ethyl acetate (10%) with *n*-hexane. The oxysterols retained on the cartridge were eluted using

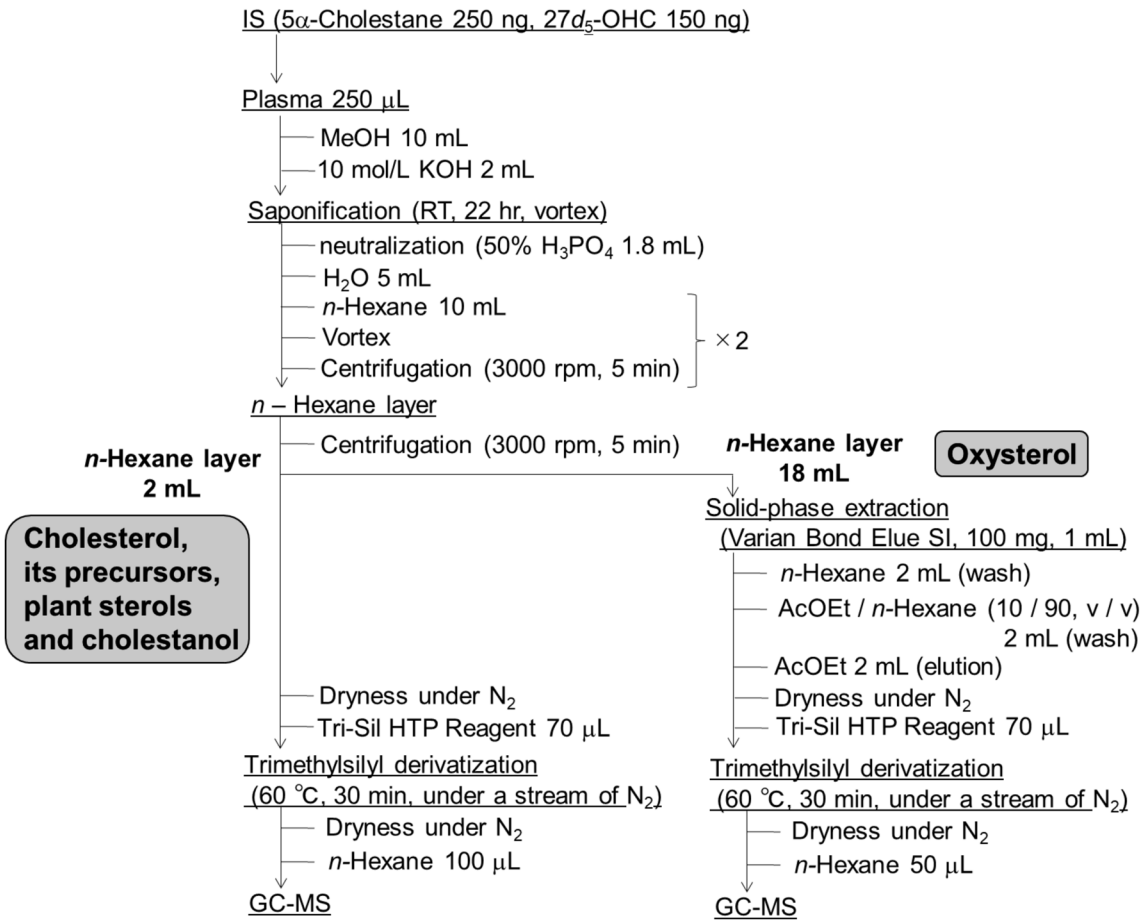

Fig. 2. Pretreatment procedure for human plasma for GC-MS analysis (IS, internal standard; 27d_5-OHC, 27-hydroxycholesterol-26,26,26,27,27-D_5; MeOH, methanol; KOH, potassium hydroxide; RT, room temperature; H_3PO_4, phosphoric acid; AcOEt, ethyl acetate; GC-MS, gas chromatography-mass spectrometry).

Table 1. Selected ion monitoring (SIM) of oxysterol derivatives by GC-MS

Sterols	*m/z*	
	Quantities ion	Certificate ion
Cholesterol	368	458, 329
Cholestanol	445	355, 460
Cholesterol precursors		
Squalene	69	81
Lanosterol	393	498, 483
Lathosterol	458	459, 255
7-dehydrocholesterol	325	351
Desmosterol	343	456, 253
Plant sterols		
Campesterol	382	343
Stigmasterol	83	484, 485
β-sitosterol	396	357
Oxysterols		
7*α*-hydroxycholesterol	456	233
7*β*-hydroxycholesterol	456	233
4*β*-hydroxycholesterol	456	356
Cholesterol-5*β*,6*β*-epoxide	457	385, 459
Cholesterol-5*α*,6*α*-epoxide	474	366, 384
24*S*-hydroxycholesterol	413	145
25-hydroxycholesterol	131	271, 327, 456
7-ketocholesterol	472	367, 382
27-hydroxycholesterol	456	417
22*S*-hydroxycholesterol	173	546, 531
22*R*-hydroxycholesterol	173	546, 531
20*S*-hydroxycholesterol	201	461
IS		
5*α*-cholestane	217	357
27d_5-OHC	461	422

GC-MS, gas chromatography-mass spectrometry; IS, internal standard; 27d_5-OHC, 27-hydroxycholesterol-26,26,26,27,27-D_5

ethyl acetate (2 mL). After drying the elute under a stream of nitrogen, the collected oxysterols were derivatized for 30 min at 60˚C with Tri-Sil HTP reagent. The derivatized oxysterols were resuspended in 50 μL *n*-hexane for GC-MS analysis (Fig. 2).

GC-MS Analysis

The conditions for GC-MS measurements were as follows: capillary column, DB-5MS (30 m × 0.25 mm, 0.25 μm particle size); carrier gas, helium (1 mL/min); column temperature, 180˚C for 1 min → 20˚C/min → 250˚C → 5˚C/min → 280˚C → 3˚C/min → 300˚C for 12 min; inlet temperature, 230˚C; ion source temperature, 250˚C; interface temperature, 250˚C; injection volume, 1 μL (for cholesterol, its precursors, plant sterols, and cholestanol) or 5 μL (for oxysterols); detection mode, selected ion monitoring (SIM); GC-MS instrument, GCMS-QP2010Plus (Shimadzu Corporation, Kyoto, Japan); ion quantities: *m/z* 69 for squalene, *m/z* 393 for lanosterol, *m/z* 458 for lathosterol, *m/z* 325 for 7-dehydrocholesterol, *m/z* 343 for desmosterol, *m/z* 368 for cholesterol, *m/z* 445 for cholestanol, *m/z* 382 for campesterol, *m/z* 83 for stigmasterol, *m/z* 396 for *β*-sitosterol, *m/z* 217 for 5*α*-cholestane (IS), *m/z* 456 for 7*α*-hydroxycholesterol, *m/z* 456 for 7*β*-hydroxycholesterol, *m/z* 456 for 4*β*-hydroxycholesterol, *m/z* 173 for 22*S*-hydroxycholesterol, *m/z* 474 for cholesterol-5*β*, 6*β*-epoxide, *m/z* 173 for 22*R*-hydroxycholesterol, *m/z* 474 for cholesterol-5*α*, 6*α*-epoxide, *m/z* 201 for 20*S*-hydroxycholesterol, *m/z* 413 for 24*S*-hydroxycholesterol, *m/z* 131 for 25-hydroxycholesterol, *m/z* 472 for 7-ketocholesterol, *m/z* 456 for 27-hydroxycholesterol, and *m/z* 461 for 27d_5-OHC (IS) (Table 1).

Results and Discussion

Chromatograms of cholesterol, its precursors, plant sterols, cholestanol, and oxysterols are shown in Supplementary Fig. S1. The peaks corresponding to ten analytes (cholesterol, its precursors, plant sterols, and cholestenol) and 5 *α*-cholestane (IS) were clearly separated at a concentration of 5 μg/mL (Fig. S1A) except for 7-dehydrocholesterol, whose peak shape differed from that of the other sterols. The smaller ion intensity of 7-dehydrocholesterol, as indicated by its mass spectrum, presumably led to its higher limit of quantitation than that of the other sterols. Therefore, the chromatogram of 7-dehydrocholesterol corresponded to a concentration of 10 μg/mL (Fig. S1A). Similarly, the peaks corresponding to twelve analytes (oxysterols) and 27d_5-OHC (IS) were clearly separated at a concentration of 200 ng/mL (Fig. S1B). Table 2 shows the recorded linear regression, respective correlation coefficients, recovery rates, and mean relative standard deviation (RSD) values of the intra- and inter-day precision for each component in the present study. The 22*S*-hydroxycholesterol, 22*R*-hydroxycholesterol, and 20*S*-hydroxycholesterol concentrations were below the limit of detection in plasma. The correlation coefficients of the calibration curves were greater than 0.997 for all the compounds. Recovery rates ranged from 88.8 to 103.4%. The intra-day precision (RSD) ranged from 2.3 to 11.0% and the inter-day precision (RSD)

Table 2. Validation data for cholesterol, its precursors, plant sterols, oxysterols, and cholestanol in spiked human plasma

Sterols	Calibration curve	Correlation coefficient	Calibration range	Recovery (%)	Intra-day precision (RSD, %)	Inter-day precision (RSD, %)
Cholesterol	y=0.0362x+0.2049	0.999	0.3−3 mg/mL	—	3.5	10.7
Cholestanol	y=0.0138x+0.0329	0.999	0.10−10 μg/mL	99.7±6.2	4.6	3.6
Cholesterol precursors						
Squalene	y=0.3384x+0.0591	0.999	0.12−10 μg/mL	100.1±0.02	4.3	12.3
Lanosterol	y=0.0363x+0.0062	0.999	0.12−10 μg/mL	98.1±1.3	7.5	14.2
Lathosterol	y=0.0034x+0.0040	0.999	0.12−10 μg/mL	101.0±5.5	6.4	2.6
7-dehydrocholesterol	y=0.0046x+0.0022	0.999	0.25−10 μg/mL	97.5±1.6	4.3	2.3
Desmosterol	y=0.0268x+0.0158	0.999	0.12−10 μg/mL	102.0±1.8	2.6	4.6
Plant sterols						
Campesterol	y=0.0108x+0.0222	0.999	0.12−10 μg/mL	102.5±4.3	11.0	3.8
Stigmasterol	y=0.0475x+0.0076	0.999	0.10−10 μg/mL	88.8±6.0	2.7	12.6
β-sitosterol	y=0.0083x+0.0159	0.999	0.15−10 μg/mL	100.3±1.0	9.2	11.1
Oxysterols						
7α-hydroxycholesterol	y=0.0322x+1.3891	0.999	1.72−200 ng/mL	97.8±2.4	5.0	8.7
7β-hydroxycholesterol	y=0.0246x+0.5180	0.999	1.72−200 ng/mL	98.3±0.7	6.2	3.5
4β-hydroxycholesterol	y=0.0152x+0.3356	0.999	1.72−200 ng/mL	97.7±3.4	5.6	10.0
Cholesterol-5β,6β-epoxide	y=0.00111x+0.0241	0.997	15−200 ng/mL	97.0±1.0	3.1	3.4
Cholesterol-5α,6α-epoxide	y=0.00084x+0.02162	0.999	12−200 ng/mL	102.4±3.8	4.7	7.3
24S-hydroxycholesterol	y=0.0030x+0.0704	0.999	5−200 ng/mL	100.1±0.4	10.0	4.5
25-hydroxycholesterol	y=0.0670x+0.3575	0.999	1.72−200 ng/mL	97.3±0.1	7.5	1.5
7-ketocholesterol	y=0.0009x+0.0552	0.999	1.72−200 ng/mL	100.1±0.9	5.2	13.4
27-hydroxycholesterol	y=0.0017x+0.1668	0.999	1.72−200 ng/mL	103.4±3.2	2.3	5.6

Recovery is represented as the mean±standard deviation (n=3).

ranged from 1.5 to 14.2% (Table 2). This method was highly specific, sensitive, and reproducible.

This method was applied to measure plasma cholesterol, its precursors, plant sterols, cholestenol, and oxysterols in six samples. The newly developed method appeared to be congruent with the conventional results[12], and the accuracy ranged from 85.0 to 115.5%. The concentrations of cholesterol and related sterols determined in our study were within the same range as those published in the previous reports[3, 10, 12−17, 19] (Table 3).

Several methods for sterol analysis from various biological matrices have been described, including commercial assay kits (colorimetric)[3], HPLC[3, 14], LC-MS/MS[10, 17], and GC-MS[3, 12, 13, 15, 16, 18]. However, to measure cholesterol, its precursors, plant sterols, cholestanol, and oxysterols, it is necessary to use different pretreatment methods and machines. Matysik et al.[18] developed an analytical method for the simultaneous determination of four cholesterol precursors, three plant sterols, and four oxysterols in the human plasma. This method alone could not distinguish between CTX, sitosterolemia, and FH because cholesterol could not be measured simultaneously. In contrast to CTX and sitosterolemia, FH is associated with hypercholesterolemia[3, 9]. Therefore, oxysterols are found in cholesterol-rich tissues, usually at concentrations several orders of magnitude lower than cholesterol[1, 13]. Cholesterol is present in large amounts in human plasma, and the normal value for cholesterol is 1.6−2.3 mg/mL[10, 14], whereas oxysterol is present at markedly lower levels, with normal values of 2−200 ng/mL[10−12, 15−17], which is less than 0.01% of cholesterol. Thus, to develop a method for the simultaneous determination of oxysterols, a large amount of cholesterol must be removed before TMS derivatization. Our newly designed method is superior to the conventional method[18] in the following respects: this method could be measured using only two ISs, whereas the conventional methods used five types of IS[18], the amount of plasma was low (250 μL vs. 400 μL[18] by one-round pretreatment), and our method could determine a wider variety of oxysterols to remove the large amount of cholesterol with solid-phase extraction at

Table 3. Determination of plasma levels of cholesterol, its precursors, plant sterols, oxysterols, and cholestenol

		sample A	sample B	sample C	sample D	sample E	sample F	Mean±S.D.	Accuracy (%)	Nomal values
Cholesterol (mg/mL)	a	0.847±0.152	1.589±0.232	1.154±0.060	0.955±0.102	1.467±0.317	0.467±0.050	1.080±0.418	N.D	1.578−2.300[10, 14]
	b	N.D	N.D	N.D	N.D	N.D	N.D	N.D		
Cholestanol (μg/mL)	a	1.256±0.045	1.515±0.151	1.473±0.048	2.082±0.057	0.918±0.134	1.054±0.025	1.383±0.395	N.D	1.838±0.999[15]
	b	N.D	N.D	N.D	N.D	N.D	N.D	N.D		
Cholesterol precursors (μg/mL)										
Squalene	a	0.340±0.038	0.782±0.096	0.417±0.006	0.143±0.015	0.330±0.111	0.181±0.101	0.366±0.224	91.6	0.610±0.380[12]
	b	0.369±0.137	0.670±0.108	0.431±0.096	0.262±0.031	0.427±0.043	0.237±0.081	0.399±0.165		
Lanosterol	a	0.221±0.039	0.126±0.084	0.446±0.129	1.637±0.089	0.147±0.011	0.735±0.563	0.552±0.580	N.D	0.00853−0.213[16]
	b	N.D	N.D	N.D	N.D	N.D	N.D	N.D		
Lathosterol	a	0.843±0.082	0.740±0.066	2.140±0.206	0.550±0.021	0.794±0.073	2.013±0.316	1.180±0.674	104.2	2.056±0.792[3]
	b	1.068±0.651	0.863±0.420	1.668±0.552	0.566±0.166	0.862±0.105	1.769±0.590	1.113±0.597		
7-dehydrocholesterol	a	0.341±0.166	0.296±0.180	0.261±0.008	0.519±0.214	0.591±0.165	0.423±0.163	0.405±0.149	N.D	0.039−0.385[14, 19]
	b	N.D	N.D	N.D	N.D	N.D	N.D	N.D		
Desmosterol	a	0.603±0.516	0.845±0.370	0.684±0.099	0.770±0.346	0.998±0.351	0.559±0.263	0.743±0.329	113.4	0.740±0.420[12]
	b	0.451±0.233	0.655±0.115	0.759±0.100	0.689±0.230	0.850±0.016	0.527±0.211	0.655±0.199		
Plant sterols (μg/mL)										
Campesterol	a	2.084±0.903	4.639±2.589	2.005±0.184	4.574±0.373	3.230±0.326	0.910±0.094	2.907±1.710	106.8	3.160±1.710[12]
	b	1.622±0.884	3.553±0.332	1.654±0.688	3.941±1.106	4.783±0.420	0.777±0.313	2.722±1.598		
Stigmasterol	a	0.112±0.008	0.114±0.071	0.129±0.049	0.245±0.119	0.294±0.063	0.113±0.034	0.172±0.057	110.3	0.140±0.110[12]
	b	0.109±0.058	0.135±0.019	0.133±0.085	0.214±0.130	0.250±0.010	0.092±0.025	0.156±0.055		
β-sitosterol	a	0.765±0.106	1.095±0.147	1.637±0.240	3.138±2.173	3.148±0.945	0.807±0.485	1.765±1.338	94.0	2.050±1.050[12]
	b	0.979±0.546	1.687±0.470	1.596±0.519	3.029±0.976	3.377±0.441	0.602±0.245	1.878±1.147		
Oxysterols (ng/mL)										
7α-hydroxycholesterol	a	35.95±8.72	50.74±16.64	71.72±28.45	22.05±5.20	33.16±1.92	16.00±3.47	38.27±22.49	110.7	43±48[13]
	b	27.80±5.90	43.76±5.76	72.14±3.06	15.33±2.91	31.22±4.60	17.23±5.00	34.58±21.20		
7β-hydroxycholesterol	a	23.13±9.25	29.72±1.71	68.54±21.05	27.70±0.97	40.99±17.07	18.92±3.29	34.83±19.70	115.5	14−206[10]
	b	18.25±3.75	36.02±3.18	55.64±4.85	17.53±5.23	33.44±10.75	20.02±9.45	30.15±15.06		
4β-hydroxycholesterol	a	28.48±3.81	14.13±2.88	43.19±4.85	33.72±7.08	45.43±9.37	7.01±1.49	28.66±15.28	N.D	24±11[17]
	b	N.D	N.D	N.D	N.D	N.D	N.D	N.D		
Cholesterol-5β,6β-epoxide	a	46.70±34.68	19.37±10.75	118.63±31.98	31.64±5.71	59.33±22.01	44.67±9.78	53.39±37.60	N.D	26±18[13]
	b	N.D	N.D	N.D	N.D	N.D	N.D	N.D		
Cholesterol-5α,6α-epoxide	a	35.11±2.57	20.73±5.35	61.36±10.66	22.24±3.06	15.27±4.54	27.08±6.03	30.30±16.40	N.D	6±8[13]
	b	N.D	N.D	N.D	N.D	N.D	N.D	N.D		
24*S*-hydroxycholesterol	a	18.58±1.02	48.68±9.18	37.76±0.78	32.73±1.21	10.77±0.83	11.79±1.66	26.72±14.82	N.D	31±4[10]
	b	N.D	N.D	N.D	N.D	N.D	N.D	N.D		
25-hydroxycholesterol	a	6.28±0.21	8.34±1.20	9.15±0.14	4.66±0.31	4.23±0.17	3.34±1.04	6.00±2.28	N.D	2±3[13]
	b	N.D	N.D	N.D	N.D	N.D	N.D	N.D		
7-ketocholesterol	a	52.79±11.96	127.91±20.61	140.44±9.92	63.68±4.68	37.70±11.72	32.32±7.37	74.59±46.30	N.D	69±18[10]
	b	N.D	N.D	N.D	N.D	N.D	N.D	N.D		
27-hydroxycholesterol	a	51.44±13.50	68.91±9.45	93.63±5.23	61.63±2.90	137.23±15.21	52.61±10.33	77.58±32.22	85.0	101.8±22.8[12]
	b	67.40±4.15	111.18±5.93	130.70±6.63	58.34±6.55	124.96±8.71	54.99±11.27	91.26±33.30		

Data are expressed as mean±standard deviation (n=3). a: Results obtained using the newly developed method; b: Results obtained using a conventional method[12]; N.D: no data; Accuracy was determined as the mean percentage of the concentration in plasma measured using the newly developed method with respect to that evaluated using a conventional method[12].

pretreatment. Furthermore, using our method, the volume of the organic solvent was reduced by approximately 36% compared to the conventional method[12]. In clinical practice, cholesterol is typically measured using the enzyme method with an autoanalyzer. However, this method may also detect other related sterols (cholesterol precursors, plant sterols, or oxysterols)[20, 21]. Therefore, the GC-MS method was developed to facilitate specific, accurate separation of cholesterol.

This new method of measuring plasma cholesterol, its precursors, plant sterols, cholestanol, and oxysterols, is a useful tool in the diagnosis of inherited disorders of cholesterol biosynthesis, malformation syndromes, FH, CTX, or sitosterolemia[1, 3, 7–9] and evaluation of drug administration[10–12]. This method combines the excellent power of separation performed by GC with the high sensitivity of the mass spectrometric detection system operating in the SIM mode. In addition, the SIM mode significantly increases the specificity of the analysis, minimizing the risk of erroneous quantitative determination of sterols due to the co-elution of impurities.

Conclusions

We developed a method to determine cholesterol, its related sterols, and oxysterols in plasma by a one-round pretreatment using GC-MS. This method has been fully validated and is specific, accurate, precise, and reproducible. It contributes to the diagnosis of inherited disorders of cholesterol biosynthesis, malformation syndromes, FH, CTX, or sitosterolemia by a one-round pretreatment. Furthermore, this method can be used to evaluate the effects of new drugs used to treat dyslipidemia.

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Conflict of Interest

The authors declare no conflict of interest.

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