methods

Highly sensitive analysis of sterol profiles in human serum by LC-ESI-MS/MS[S]

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Abstract We have developed a highly sensitive and specific method for the analysis of serum sterol profiles. Sterols in 1 μl of dried serum were derivatized into picolinyl esters (3β-picolinate) and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using the electrospray ionization (ESI) mode. In addition to cholesterol, 19 cholesterol precursors, cholestanol, campesterol, sitosterol, and sitostanol were identified simultaneously. Quantitative analyses for the picolinyl esters of 11 available sterols were performed, and detection limits were found to be less than 1 pg on-column. Reproducibilities and recoveries of 8 noncholesterol sterols were validated according to one-way layout and polynomial equation, respectively. The variances between sample preparations and between measurements by this method were calculated to be 1.6% to 8.2% and 2.5% to 16.5%, respectively. The recovery experiments were performed using 1 μl aliquots of normal human serum spiked with 1 ng to 6 ng of sterols, and recoveries of the sterols ranged from 88.1% to 102.5% with a mean recovery of 98.1%. The present method provides reliable and reproducible results for the identification and quantification of neutral sterols, especially in small volumes of blood samples, which is useful for serological diagnosis of inherited disorders in cholesterol metabolism and for noninvasive evaluation of cholesterol biosynthesis and absorption in humans.— Honda, A., K. Yamashita, H. Miyazaki, M. Shirai, T. Ikegami, G. Xu, M. Numazawa, T. Hara, and Y. Matsuzaki. **Highly sensitive analysis of sterol profiles in human serum by LC-ESI-MS/MS. *J. Lipid Res.* 2008.** 49: **2063–2073.**

Supplementary key words cholestanol • cholesterol precursors • congenital birth defect • liquid chromatography-electrospray ionization-tandem mass spectrometry • picolinic acid • plant sterols

Cholesterol is synthesized de novo in virtually all cells of humans and is an essential component of all plasma and intracellular membranes. Recent studies have shown that a number of human malformation syndromes are caused by gene mutations in enzymes for cholesterol biosynthesis after lanosterol (**Fig. 1**). The first malformation syndrome that was confirmed as a defect in cholesterol biosynthetic pathway was the Smith-Lemli-Opitz syndrome (SLOS) due to a deficiency of 3β-hydroxysteroid Δ^7-reductase (1). Afterwards, the cholesterol biosynthetic pathway was investigated in other malformation syndromes and the following deficiencies were discovered, i.e., desmosterolosis (3β-hydroxysteroid Δ^{24}-reductase deficiency) (2), Antley-Bixler syndrome (functional deficiency of lanosterol 14α-demethylase due to cytochrome P450 oxidoreductase gene mutations) (3, 4), hydrops-ectopic calcification-"moth-eaten" (HEM)/Greenberg skeletal dysplasia (3β-hydroxysteroid Δ^{14}-reductase deficiency) (5), congenital hemidysplasia with ichthyosis and limb defects (CHILD) syndrome or NAD(P)H steroid dehydrogenase-like (NSDHL) deficiency (deficiency of 3β-hydroxysteroid dehydrogenase in 4α-methylsterol-4-demethylase complex) (6, 7), CHILD syndrome, X-linked dominant *chondrodysplasia punctata* type 2 (CDPX2) or Conradi-Hünermann-Happle syndrome (3β-hydroxysteroid Δ^8,Δ^7-isomerase deficiency) (8–10), and lathosterolosis (3β-hydroxysteroid 5-desaturase deficiency) (11).

In addition to the deficiency of cholesterol biosynthesis, defects in cholesterol excretion and catabolism are well

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Abbreviations: CDPX2, X-linked dominant *chondrodysplasia punctata* type 2; CTX, cerebrotendinous xanthomathosis; ESI, electrospray ionization; GC, gas chromatography; LC-APCI-MS, liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry; LC-MS/MS, liquid chromatography-tandem mass spectrometry; SLOS, Smith-Lemli-Opitz syndrome; SRM, selected reaction monitoring.

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[S] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of three tables.

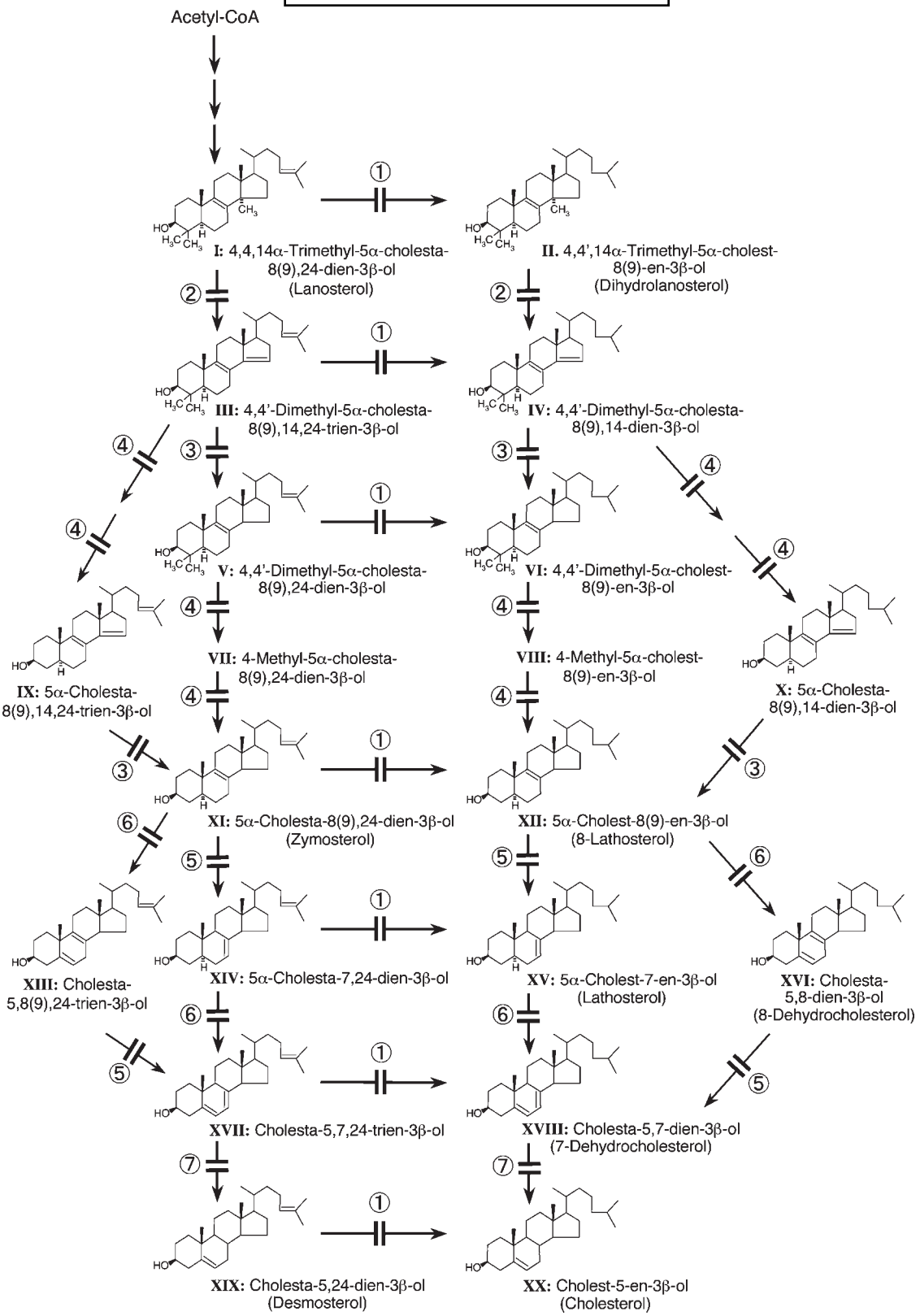

Fig. 1. Enzymatic steps and sterol intermediates in the cholesterol biosynthetic pathway. Biochemical blocks are indicated by the dashed lines. 1, desmosterolosis (3β-hydroxysteroid Δ^{24}-reductase deficiency); 2, Antley-Bixler syndrome (defective lanosterol 14α-demethylase activity due to cytochrome P450 oxidoreductase deficiency); 3, HEM/Greenberg skeletal dysplasia (3β-hydroxysteroid Δ^{14}-reductase deficiency; 4, CHILD syndrome or NSDHL deficiency (deficiency of 3β-hydroxysteroid dehydrogenase in 4α-methylsterol-4-demethylase complex); 5, CHILD syndrome, X-linked dominant *chondrodysplasia punctata* type 2 (CDPX2), or Conradi-Hünermann-Happle syndrome (3β-hydroxysteroid Δ^{8},Δ^{7}-isomerase deficiency); 6, lathosterolosis (3β-hydroxysteroid 5-desaturase deficiency); 7, Smith-Lemli-Opitz syndrome (3β-hydroxysteroid Δ^{7}-reductase deficiency).

known as sitosterolemia and cerebrotendinous xanthomathosis (CTX), respectively. The former is caused by mutations in the ATP-binding cassette transporter G5 or G8 gene (12) and the latter by mutations in the sterol 27-hydroxylase (CYP27A1) gene (13).

Most of the inherited disorders of cholesterol metabolism can be diagnosed by analysis of the sterol profiles in serum. In addition, the quantification of serum lathosterol (14, 15) and plant sterols (campesterol or sitosterol) (16) can be used as biomarkers for cholesterol biosynthesis and absorption, respectively. Thus, serum sterol analysis is a useful method for the diagnosis of inherited disorders in cholesterol metabolism and for noninvasive evaluation of cholesterol biosynthesis and absorption in humans.

Gas chromatography (GC) with flame ionization detection (3, 9, 14), GC-electron ionization-mass spectrometry (17, 18), and HPLC with ultraviolet detection (19, 20) have commonly been used for sterol analyses. Recently, liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (LC-APCI-MS) (21), LC-APCI-tandem mass spectrometry (LC-APCI-MS/MS) (22), and LC-atmospheric pressure photoionization-MS/MS (23) have been introduced as more sensitive, specific, and rapid quantification methods for nonpolar compounds, such as sterols. Electrospray ionization (ESI) is the most widely used ionization method for liquid chromatography-tandem mass spectrometry (LC-MS/MS) and sterols are also analyzed by LC-ESI-MS/MS (24). However, sterols are poorly ionized by electrospray and the sensitivity does not reach that obtained by APCI.

Recent developments of the methodology have demonstrated that the introduction of charged moieties markedly enhanced the ionization efficiency of neutral steroids in the ESI process. The aim of this study was to develop a simple, more sensitive and reliable method for the analysis of serum sterol profiles by LC-ESI-MS/MS. For this purpose, neutral sterols in 1 μl of dried serum were directly derivatized into their picolinyl esters (3β-picolinate) before LC-ESI-MS/MS analysis.

MATERIALS AND METHODS

Chemicals

7-Dehydrocholesterol (cholesta-5,7-dien-3β-ol) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Cholesterol (cholest-5-en-3β-ol), zymosterol [5α-cholesta-8(9),24-dien-3β-ol], desmosterol (cholesta-5,24-dien-3β-ol), lathosterol (5α-cholest-7-en-3β-ol), cholestanol (5α-cholestan-3β-ol), coprostanol (5β-cholestan-3β-ol), lanosterol [4,4′,14α-trimethyl-5α-cholesta-8(9),24-dien-3β-ol], dihydrolanosterol [4,4′,14α-trimethyl-5α-cholest-8(9)-en-3β-ol] were obtained from Steraloids (Wilton, NH). Sitosterol (24β-ethyl-cholest-5-en-3β-ol), sitostanol (24β-ethyl-5α-cholestan-3β-ol), and campesterol (24α-methyl-cholest-5-en-3β-ol) were kindly supplied by Dr. S. Shefer (UMDNJ-New Jersey Medical School, Newark, NJ). 8-Dehydrocholesterol (cholesta-5,8-dien-3β-ol) was synthesized according to the method of Wilson et al. (25) and purified by HPLC. Triparanol (MER-29) was a gift from Marion Merrell Dow Research Institute (Cincinnati, OH). Picolinic acid and 2-methyl-6-nitrobenzoic anhydride were purchased from Tokyo Kasei Kogyo (Tokyo, Japan), and 4-dimethylaminopyridine and triethylamine were obtained from Wako Pure Chemical Industries (Osaka, Japan). Additional reagents and solvents were of analytical grade.

Sample collection

Blood samples were collected from healthy human volunteers, and patients with SLOS, CDPX2, CTX, and sitosterolemia. After coagulation and centrifugation at 1,500 *g* for 10 min, serum samples were stored at −20°C until analysis. Informed consent was obtained from all subjects, and the experimental procedures were conducted in accordance with the ethical standards of the Helsinki Declaration.

Sample preparation

Coprostanol (10 ng/20 μl ethanol) was added as an internal standard to 1 μl of serum, and the mixture was evaporated to dryness at 80°C under a nitrogen stream. Derivatization to the picolinyl ester was performed according to the method of Yamashita et al. (26) with minor modifications. The reagent mixture for derivatization consisted of 2-methyl-6-nitrobenzoic anhydride (100 mg), 4-dimethylaminopyridine (30 mg), picolinic acid (80 mg), and tetrahydrofuran (1.5 ml). The freshly prepared reagent mixture (150 μl) and triethylamine (20 μl) were added to the dried serum, and the reaction mixture was allowed to stand at room temperature for 30 min. After evaporation at 80°C under nitrogen, the residue was redissolved in 100 μl of acetonitrile and centrifuged at 2,000 *g* for 1 min, and an aliquot (1 μl) of the supernatant was injected into the following LC-MS/MS system.

LC-MS/MS analysis

The LC-MS/MS system consisted of a TSQ Quantum Ultra quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA) equipped with an H-ESI probe and a Nanospace SI-2 HPLC system (Shiseido, Tokyo, Japan). Chromatographic separation was performed using a Hypersil GOLD column (150 × 2.1 mm, 3 μm, Thermo Electron) at 40°C, and the following gradient system was used at a flow rate of 300 μl/min: initially, the mobile phase consisted of acetonitrile-methanol-water (40:40:20, v/v/v) containing 0.1% acetic acid; then it was programmed in a linear manner to acetonitrile-methanol-water (45:45:10, v/v/v) containing 0.1% acetic acid over 20 min. The final mobile phase was kept constant for 20 additional min.

The general LC-MS/MS conditions were as follows: spray voltage, 1,000 V; vaporizer temperature, 350°C; sheath gas (nitrogen) pressure, 85 psi; auxiliary gas (nitrogen) flow, 60 arbitrary units; ion transfer capillary temperature, 350°C; collision gas (argon) pressure, 1.5 mTorr; and ion polarity, positive. Selected reaction monitoring (SRM) was conducted using the characteristic precursor-to-product ion transition under the optimized collision energy as listed in Tables 1 and 2.

Sterol analysis in human colonic adenocarcinoma cell line

Caco-2 cells, a human colonic adenocarcinoma cell line, were obtained from American Type Culture Collection (Rockville, MD). Stock cultures were grown and maintained in MEM Earle's (Invitrogen-Gibco Japan K.K., Tokyo, Japan) supplemented with 20% FBS. The cultures were incubated at 37°C in a humidified incubator containing 5% CO_2, 95% air. Cells were seeded at a density of $5 \times 10^5/9.6$ cm^2 tissue culture dish. After 3 days, when the cells were about 80% confluent, the medium was replaced with fresh medium with or without 10^{-5} M of Triparanol and 20 mM of mevalonolactone. After 48 h incubation, the medium from each dish was discarded and the attached cells were rinsed twice with PBS. Cells were then harvested by use of a cell scraper

TABLE 1. Positive ESI-MS, MS/MS, SRM, and HPLC data of the picolinyl ester derivative of each sterol

	Picolinyl Ester Derivatives[a]	MS Data $[M+Na+CH_3CN]^+$ (Relative Intensity)	MS/MS Data[b] (Collision Energy at Maximum Intensity)				SRM Data[c] Collision Energy	SRM Data[c] Precursor to Product	SRM Data[c] S/N[d]	HPLC Data[c] (RRT[e])
		m/z (%)	*m/z* (*V*)				*V*	*m/z*		
I	4,4′,14α-Trimethyl-5α-cholesta-8(9),24-dien-3β-ol (lanosterol)	595 (100)	554 (12)	146 (29)	–[f]	–[f]	12	595 → 554	10	1.01
II	4,4′,14α-Trimethyl-5α-cholest-8(9)-en-3β-ol (dihydrolanosterol)	597 (100)	556 (15)	146 (30)	–[f]	–[f]	15	597 → 556	10	1.25
XI	5α-Cholesta-8(9),24-dien-3β-ol (zymosterol)	553 (100)	512 (13)	146 (27)	–[f]	–[f]	12	553 → 512	10	0.82
XV	5α-Cholest-7-en-3β-ol (lathosterol)	555 (100)	514 (15)	146 (28)	–[f]	–[f]	15	555 → 514	10	0.96
XVI	Cholesta-5,8-dien-3β-ol (8-dehydrocholesterol)	553 (100)	512 (12)	146 (24)	–[f]	–[f]	12	553 → 512	5	0.88
XVIII	Cholesta-5,7-dien-3β-ol (7-dehydrocholesterol)	553 (100)	146 (20)	512 (10)	159 (34)	367 (23)	12	553 → 512	5	0.86
XIX	Cholesta-5,24-dien-3β-ol (desmosterol)	553 (100)	512 (11)	146 (23)	–[f]	–[f]	12	553 → 512	10	0.83
XX	Cholest-5-en-3β-ol (cholesterol)	555 (100)	514 (12)	146 (24)	–[f]	–[f]	15	555 → 514	30	1.00
	5α-Cholestan-3β-ol (cholestanol)	557 (100)	516 (14)	146 (29)	–[f]	–[f]	14	557 → 516	15	1.10
	5β-Cholestan-3β-ol (coprostanol)	557 (100)	516 (13)	146 (29)	–[f]	–[f]	14	557 → 516	15	1.06
	24α-Methylcholest-5-en-3β-ol (campesterol)	569 (100)	528 (12)	146 (30)	–[f]	–[f]	12	569 → 528	30	1.10
	24β-Ethylcholest-5-en-3β-ol (sitosterol)	583 (100)	542 (14)	146 (26)	–[f]	–[f]	14	583 → 542	30	1.23
	24β-Ethyl-5α-cholestan-3β-ol (sitostanol)	585 (100)	544 (12)	146 (27)	–[f]	–[f]	14	585 → 544	15	1.35

ESI, electrospray ionization; MS, mass spectrometry; MS/MS, tandem mass spectrometry; RRT, relative retention time; S/N, signal-to-noise ratio; SRM, selected reaction monitoring.

[a] Each sterol was derivatized to picolinyl ester. Roman numerals correspond to those in Fig. 1.

[b] $[M+Na+CH_3CN]^+$ was used as a precursor ion for each MS/MS analysis. Major product ions were arranged in the order of abundance from left to right.

[c] The same HPLC columns and flow rate described in Materials and Methods were employed.

[d] S/Ns were determined by injecting 1 pg of each derivative.

[e] RRTs are expressed relative to the retention time of cholesterol.

[f] Intense ion (>5% of base peak) was not observed.

and centrifugation at 2,000 *g* for 1 min. After the addition of 10 ng of coprostanol, cell lipids were hydrolyzed in 1 N ethanolic KOH at 37°C for 1 h and extracted twice with *n*-hexane. The extracted sterols were derivatized to their picolinyl esters and analyzed by LC-MS/MS as described above.

Statistics

Data are reported as the mean ± SD. Reproducibility was analyzed by one-way layout (JMP software, SAS Institute Inc., Cary, NC). Recovery was analyzed using a polynomial equation (27). Linearity of the calibration curves was analyzed by simple linear

TABLE 2. Predicted positive ESI-SRM and HPLC data of the picolinyl ester derivatives of sterols whose reference compounds were not available

	Picolinyl Ester Derivatives[a]	SRM Condition[b] Collision Energy	SRM Condition[b] Precursor to Product	HPLC Data[c] RRT[d]	HPLC Data[c] Reference SRM Chromatogram[e]
		V	*m/z*		
III	4,4′,-Dimethyl-5α-cholesta-8(9),14,24-trien-3β-ol	14	579 → 538	0.84	Caco-2
IV	4,4′,-Dimethyl-5α-cholesta-8(9),14-dien-3β-ol	14	581 → 540	1.01	CTX
V	4,4′,-Dimethyl-5α-cholesta-8(9),24-dien-3β-ol	14	581 → 540	0.97	Caco-2
VI	4,4′,-Dimethyl-5α-cholest-8(9)-en-3β-ol	14	583 → 542	1.19	CDPX2, CTX
VII	4-Methyl-5α-cholesta-8(9),24-dien-3β-ol	12	567 → 526	0.89	Caco-2
VIII	4-Methyl-5α-cholest-8(9)-en-3β-ol	12	569 → 528	1.07	CDPX2, CTX
IX	5α-Cholesta-8(9),14,24-trien-3β-ol	12	551 → 510	0.71	CDPX2, Caco-2
X	5α-Cholesta-8(9),14-dien-3β-ol	12	553 → 512	0.84	CDPX2
XII	5α-Cholest-8(9)-en-3β-ol (8-lathosterol)	15	555 → 514	0.98	CDPX2
XIII	Cholesta-5,8,24-trien-3β-ol	12	551 → 510	0.75	CDPX2, Caco-2
XIV	5α-Cholesta-7,24-dien-3β-ol	12	553 → 512	0.81	Caco-2
XVII	Cholesta-5,7,24-trien-3β-ol	12	551 → 510	0.73	Caco-2

Caco-2, Caco-2 cells treated with 10^{-5} M of Triparanol and 20 mM of mevalonolactone for 48 h; CTX, serum from a CTX patient; CDPX2, serum from a CDPX2 patient.

[a] Each sterol can be derivatized to its picolinyl ester. Roman numerals correspond to those in Fig. 1.

[b] The best conditions for SRM were provided from the data shown in Table 1.

[c] The same HPLC columns and flow rate described in Materials and Methods were employed.

[d] RRTs are expressed relative to the retention time of cholesterol. Each value was calculated from reference SRM chromatograms that gave a corresponding peak.

[e] The samples that showed a significantly elevated peak of the corresponding compound.

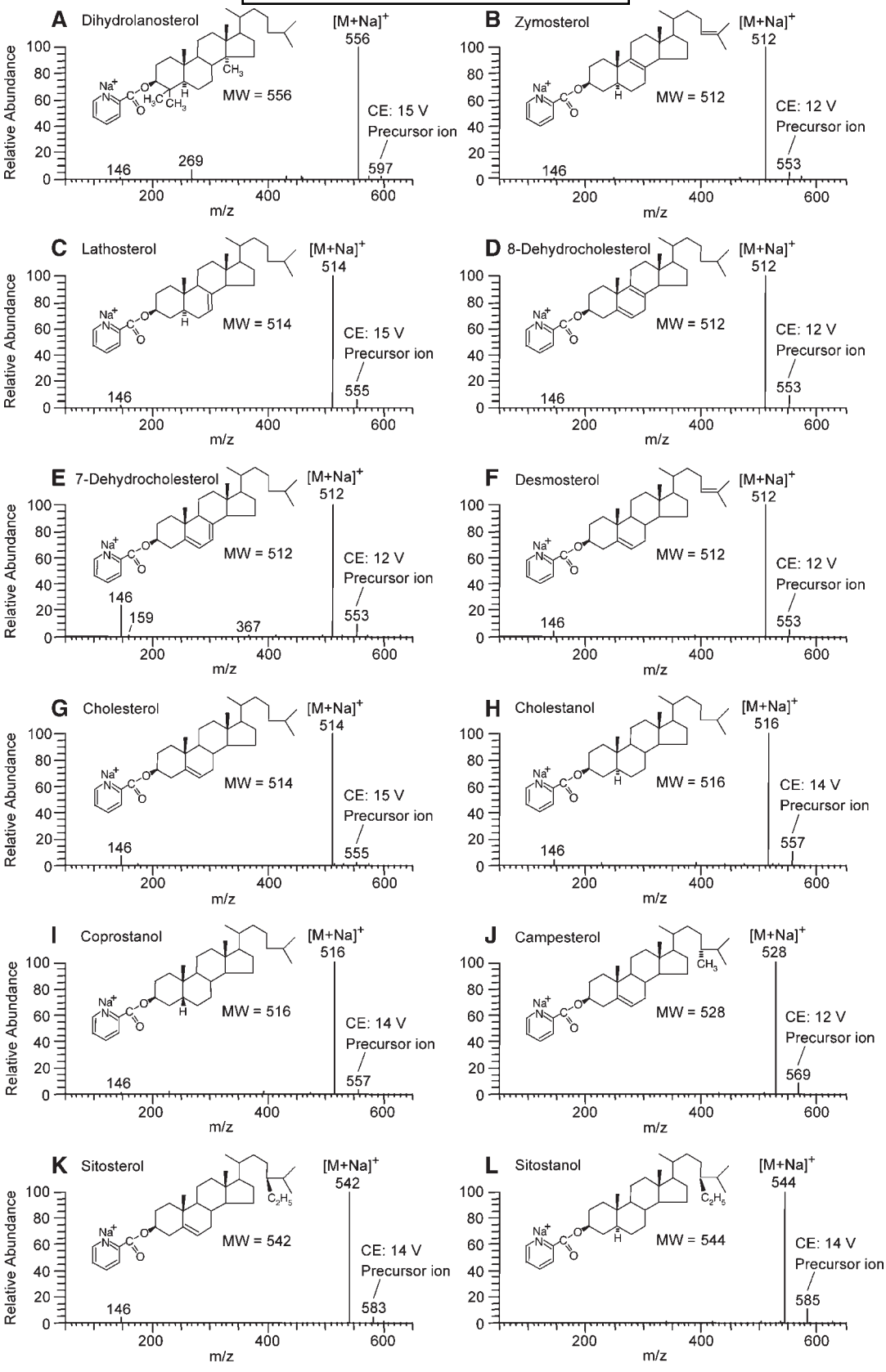

Fig. 2. Positive ESI product ion mass spectra of authentic 3β-picolinates of (A) dihydrolanosterol, (B) zymosterol, (C) lathosterol, (D) 8-dehydrocholesterol, (E) 7-dehydrocholesterol, (F) desmosterol, (G) cholesterol, (H) cholestanol, (I) coprostanol, (J) campesterol, (K) sitosterol, and (L) sitostanol. In all mass spectra, $[M+Na+CH_3CN]^+$ was used as a precursor ion. The general liquid chromatography-tandem mass spectrometry conditions were as follows: introducing solvent, acetonitrile-methanol-water (45:45:10, v/v/v) containing 0.1% acetic acid; flow rate, 300 μl/min; spray voltage, 1,000 V. CE, collision energy.

regression. The regression analysis was also used to calculate the estimated amount ± 95% confidence limit in the recovery study. For all analyses, significance was accepted at the level of $P < 0.05$.

RESULTS

Selection of monitoring ions for SRM

Thirteen sterols were converted into the corresponding picolinyl ester derivatives and positive ESI-MS, MS/MS, SRM, and HPLC data were obtained for each of them (**Table 1**). All picolinyl ester derivatives exhibited $[M+Na+CH_3CN]^+$ ions as the base peaks. The fragmentation pattern of the base peak ion of each derivative under various levels of collision energy was examined, and the $[M+Na]^+$ ion was observed as the most-abundant product ion (**Fig. 2**), and therefore it was selected as a monitoring ion for SRM.

Authentic compounds for the other intermediates in the cholesterol biosynthetic pathway were not available, but the best SRM conditions for the 3β-picolinates of the intermediates were easily estimated by calculating the molecular weights, because the fragmentation pattern of 13 reference sterol-3β-picolinates was very simple and common. The predicted data are shown in **Table 2**. The retention time of each sterol-3β-picolinate in this table was tentatively determined by analyzing sera from patients with CTX, SLOS, and CDPX2, and Caco-2 cells treated with Triparanol, an inhibitor of 3β-hydroxysteroid Δ^{24}-reductase. In sera from SLOS and CDPX2 patients, and Triparanol-treated cells, precursor sterols were accumulated markedly, whereas many intermediates in the cholesterol biosynthetic pathway after lanosterol were elevated moderately in CTX serum, as reported previously (28).

Calibration curves

A calibration plot was established for each sterol. Different amounts of authentic sterol were mixed with 10 ng of coprostanol, derivatized to the picolinyl ester, and quantified as described in the Materials and Methods. The amount of each sterol was plotted on the abscissa and the peak-area ratio of the sterol-3β-picolinate to the coprostanol-3β-picolinate measured by SRM was plotted on the ordinate. The linearity of the standard curves, as determined by simple linear regression, was excellent, as shown in **Table 3**.

Representative SRM

The separation of various authentic sterol-3β-picolinates by SRM is shown in **Fig. 3**. All sterol-3β-picolinates tested were successfully separated. **Figure 4** shows typical SRM chromatograms of several sterol-3β-picolinates obtained from 1 μl of sera from control (Fig. 4A), SLOS (Fig. 4B), CDPX2 (Fig. 4C), CTX (Fig. 4D) and sitosterolemia (Fig. 4E). SLOS, CDPX2, CTX, and sitosterolemia were easily diagnosed by the elevation of serum 7- and 8-dehydrocholesterols, 8-lathosterol, cholestanol, and sitosterol, respectively.

Precision and accuracy of the present method

The following studies were performed to determine the precision and accuracy of the present method using the same serum obtained from a normal human subject. Reproducibility was investigated by analyzing four samples in triplicate by LC-MS/MS (**Table 4**). The results were analyzed by a one-way layout, in which the analytical errors were divided into two sources: sample preparation and SRM measurement. The variances were considered to be attributable to the measurement, because the errors during sample preparation were negligible (see supplementary Tables I and II). The inter-assay coefficients of variation for the between- and within-sample variations were 1.6% to 8.2% and 2.5% to 16.5%, respectively.

For the recovery experiment, known amounts of sterols (a, 2a, 3a; a = 1.00–2.05 ng) were spiked into 1 μl aliquots of the serum samples (n = 2). After derivatization, LC-MS/MS was carried out in triplicate for each sample. The recoveries of the known spiked amounts of the sterols ranged from 88.1% to 102.5%, with a mean of 98.1% (**Table 5**). In addition, the amounts of each endogenous sterol found in 1 μl of unspiked serum were within the 95% confidence limit for the estimated amount of each sterol calculated by linear regression analysis; this also constituted an index for the

TABLE 3. Linearities of calibration plots for the amount of each sterol

Sterol	Range (n)	Linear Regression Equation[a]	Correlation Coefficient (r)
	ng		
Cholesterol	0.1 – 1000 (5)	$Y = 0.082X + 0.450$	1.000
Dihydrolanosterol	0.05 – 100 (5)	$Y = 0.044X + 0.004$	1.000
Zymosterol	0.1 – 100 (4)	$Y = 0.038X - 0.015$	1.000
Lathosterol	0.1 – 100 (4)	$Y = 0.056X + 0.002$	1.000
8-Dehydrocholesterol	0.1 – 100 (4)	$Y = 0.032X - 0.009$	1.000
7-Dehydrocholesterol	0.1 – 100 (4)	$Y = 0.030X - 0.024$	1.000
Desmosterol	0.1 – 100 (4)	$Y = 0.122X - 0.037$	1.000
Cholestanol	0.1 – 100 (4)	$Y = 0.162X + 0.023$	1.000
Campesterol	0.015 – 15 (4)	$Y = 0.148X + 0.004$	1.000
Sitosterol	0.02 – 200 (5)	$Y = 0.137X + 0.073$	1.000
Sitostanol	0.03 – 30 (4)	$Y = 0.208X + 0.000$	1.000

[a] X is the amount of each sterol (ng) and Y is the peak-area ratio calculated as the peak-area of the sterol-3β-picolinate divided by that of coprostanol-3β-picolinate (internal standard).

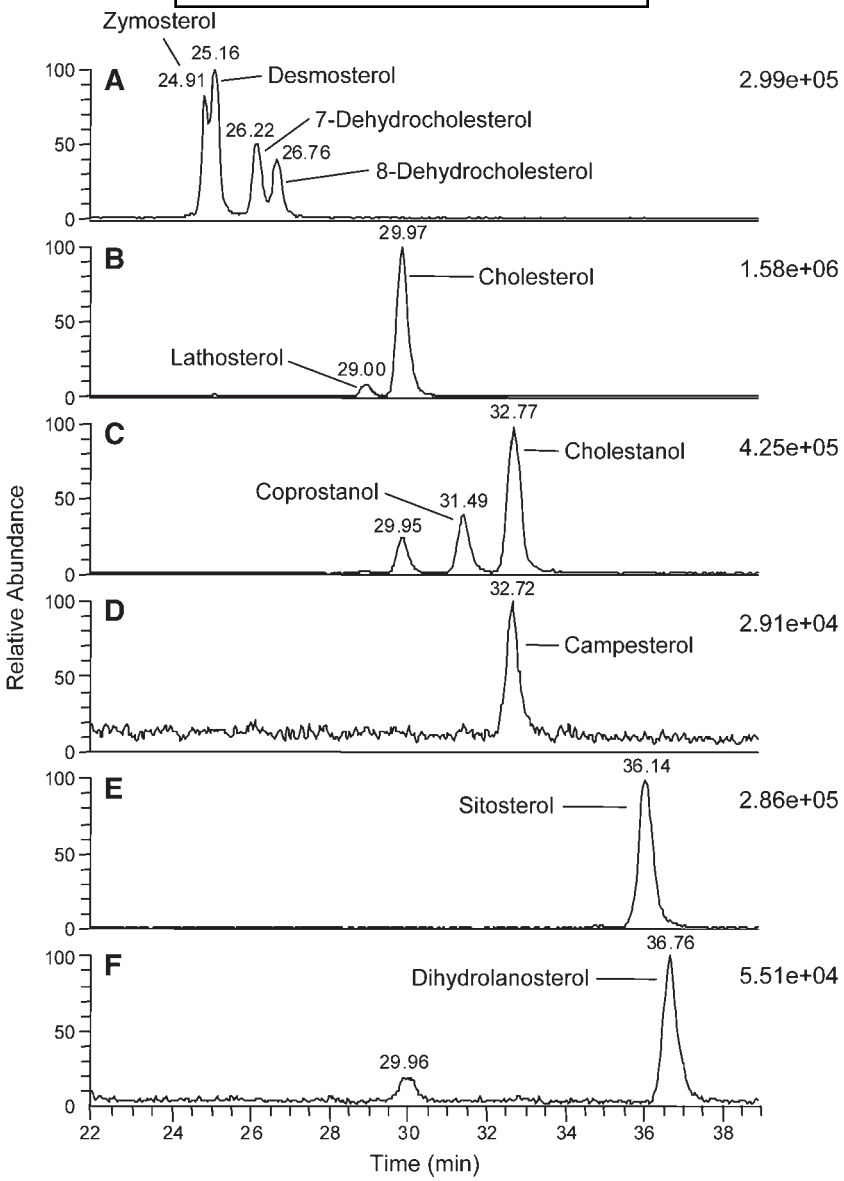

Fig. 3. Representative selected reaction monitoring chromatograms of authentic sterol-3β-picolinates. A: *m/z* 553 → 512 (collision energy: 12 V) for 3β-picolinate of cholestadien. B: *m/z* 555 → 514 (15 V) for 3β-picolinate of cholesten. C: *m/z* 557 → 516 (14 V) for 3β-picolinate of cholestan. D: *m/z* 569 → 528 (12 V) for 3β-picolinate of campesterol. E: *m/z* 583 → 542 (14 V) for 3β-picolinate of sitosterol. F: *m/z* 597 → 556 (15 V) for 3β-picolinate of dihydrolanosterol. The quantities of each peak are: zymosterol, ~500 pg; desmosterol, ~500 pg; 7-dehydrocholesterol, ~500 pg; 8-dehydrocholesterol, ~500 pg; lathosterol, ~500 pg; cholesterol, ~5 ng; coprostanol, ~500 pg; cholestanol, ~1 ng; campesterol, ~30 pg; sitosterol, ~410 pg; and dihydrolanosterol, ~500 pg. The numbers on the right side of the figure represent the full scale of each chromatogram.

precision and accuracy of the method (see supplementary Table III).

DISCUSSION

We describe a new, sensitive LC-ESI-MS/MS method for the simultaneous determination of more than 20 neutral sterols in human serum. This method requires only 1 μl of serum, and hydrolysis and extraction steps can be omitted for the purpose of serological diagnosis of inherited disorders. After a very simple derivatization step, an aliquot was injected directly into the LC-MS/MS system without any extraction steps. Because coprostanol was not detected in human serum, it was added to serum as a convenient internal standard. Although we did not use ideal internal standards labeled by any stable isotopes, the specificity and reproducibility of this method were highly satisfactory.

ESI is the most commonly used ionization method for the LC-MS/MS technique, and does not always require a derivatization step. However, because neutral sterols are poorly ionized by electrospray, the charged moieties were introduced into the 3β-hydroxyl group of the sterols as an

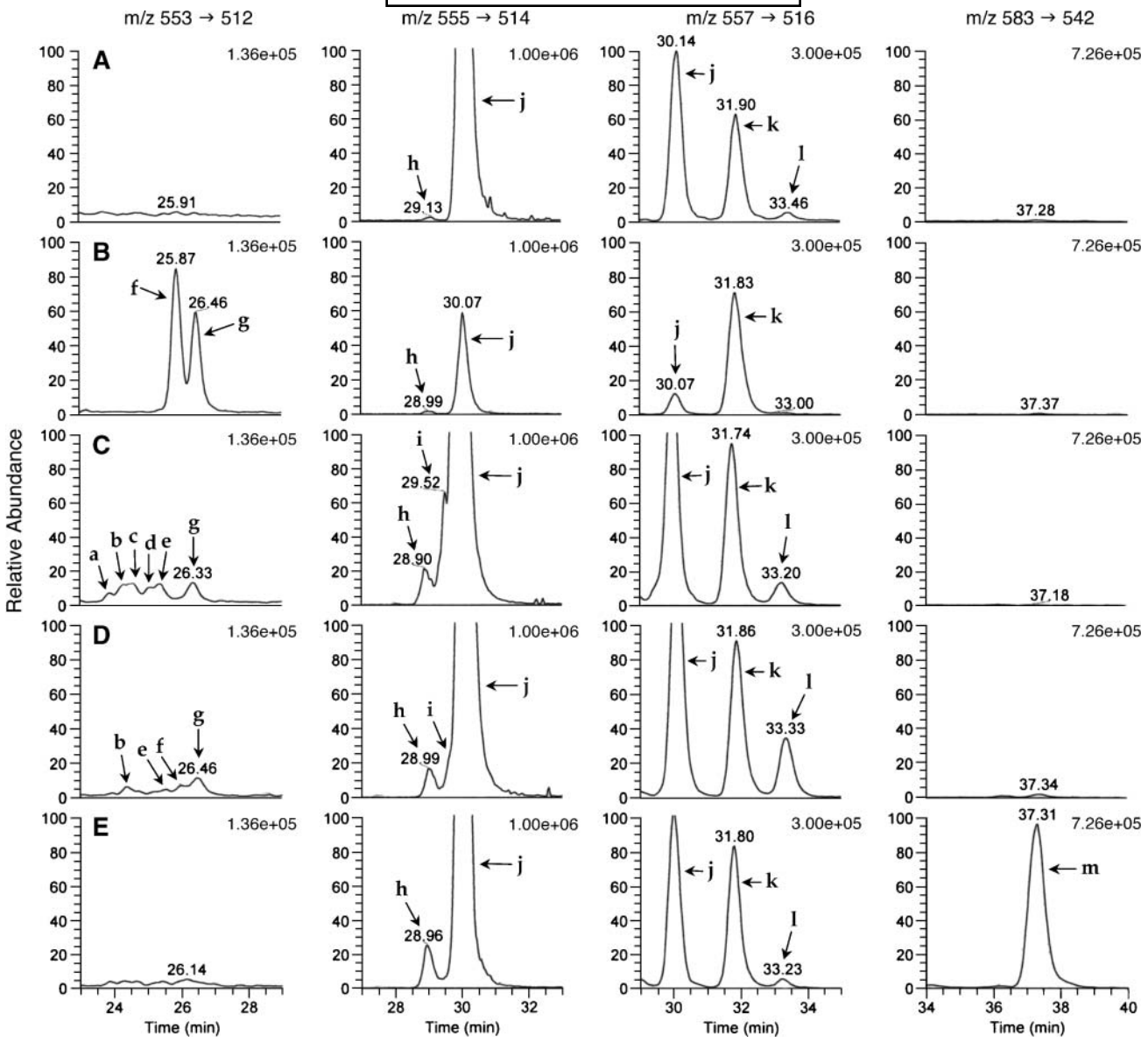

Fig. 4. Comparison of SRM chromatograms obtained from 1 μl of serum from a normal volunteer (A), and patients with SLOS (B), CDPX2 (C), CTX (D), and sitosterolemia (E). The numbers on the right side of each panel represent the full scale of the chromatogram, and were adjusted to the same number at every monitoring ion. a, 5α-cholesta-7,24-dien-3β-ol; b, zymosterol; c, desmosterol; d, 5α-cholesta-8(9),14-dien-3β-ol; f, 7-dehydrocholesterol; g, 8-dehydrocholesterol; h, lathosterol; i, 8-lathosterol; j, cholesterol; k, coprostanol (internal standard); l, cholestanol; m, sitosterol. The peaks a, d, and i were tentatively identified. Peak e was suspected to be 5α-cholesta-6,8(9)-dien-3β-ol reported by Axelson (41).

N-methylpyridyl ether (29), a ferrocenecarbamate ester (30), a sulfate (31), a mono-(dimethylaminoethyl) succinyl ester (32), Girard P hydrazone (33), and a dimethylglycine ester (34). These derivatizations enhanced the ionization efficiency of the sterols in the ESI process and significantly increased the sensitivity.

We have also successfully introduced picolinyl moiety into the hydroxyl group of various steroids and demonstrated that the picolinyl ester derivatization is a simple and versatile method suitable for sensitive and specific quantification by positive LC-ESI-MS/MS (26, 35, 36). The limit of detection [signal-to-noise ratio (S/N) = 3] of cholesterol picolinate by our LC-ESI-MS/MS analysis was about 100 fg on-column, which was ~3,860 times more sensitive than that of native cholesterol by LC-ESI-MS/MS analysis (1 pmol = 386 pg on-column) (24). We also determined the detection limit of native cholesterol by LC-APCI-MS/MS analysis, and it was found to be 40 pg on-column (data not shown). Thus, although an additional half-hour is necessary, the derivatization step is very useful for the highly sensitive analysis of sterols by LC-MS/MS. In addition, these picolinyl ester derivatives were stable for at least 6 months in acetonitrile solution.

In this new LC-ESI-MS/MS method, the picolinyl ester derivatization and thorough chromatographic separation were important for the highly sensitive and specific analysis,

TABLE 4. Reproducibility of the quantification of each sterol in human serum

Sterol	Mean ± SD (n = 12)	Relative SD	
		Sample Preparation	Error (SRM)
	ng	*%*	
Dihydrolanosterol	0.042 ± 0.006	8.2	16.5
Zymosterol	0.27 ± 0.01	3.7	5.1
Lathosterol	2.51 ± 0.14	1.6	6.5
8-Dehydrocholesterol	2.14 ± 0.06	1.9	2.8
7-Dehydrocholesterol	1.63 ± 0.04	2.8	2.5
Desmosterol	0.24 ± 0.01	2.1	4.5
Cholestanol	1.01 ± 0.08	6.1	8.2
Sitosterol	1.62 ± 0.07	3.9	4.4

Each sterol was quantified in 1 μl of normal human serum. Four samples were prepared and quantified in triplicate by LC-MS/MS. The results were analyzed by a one-way layout, in which the analytical errors were divided into two sources: sample preparation and SRM measurement.

because we needed to discriminate between different sterols that have the same molecular weight and a virtually identical MS/MS spectrum. A previous study by Ruan et al. (20) showed that reverse-phase and normal-phase HPLC had very limited capabilities for the separation of C_{27} sterols differing in the number and location of double bonds, whereas silver ion HPLC provided remarkable separation of the same compounds. However, silver ion HPLC requires a special column and hydrophobic mobile phase (acetone-hexane 3:97), which is not suitable for conventional LC-ESI-MS/MS analysis. In addition, it takes about 2 h for the best separation of important sterols for the diagnosis of inherited disorders involved in cholesterol metabolism. Therefore, we developed a new reverse-phase HPLC method for the separation of key sterols within 40 min. In our method, some sterol isomers were not completely separated from each other on the chromatograms. For example, zymosterol and desmosterol were separated with a resolution factor (Rs) of 0.68, and Rs between 7- and 8-dehydrocholesterol was 0.98, which was not complete but acceptable at least for the detection of abnormal, altered sterol levels in patients with inherited disorders.

Although most of the inherited disorders of cholesterol metabolism can be diagnosed through the analysis of serum sterols, a few affected patients may exhibit minimal or no sterol abnormalities in their serum. For biochemical diagnosis of such atypical cases, sterol analysis of cultured fibroblasts (37) or lymphoblasts (9) grown in delipidated medium are very useful. As shown in the sterol analysis of Caco-2 cells, our analytical method can be applied to the determination of sterol profiles in cultured cells. In addition, this method is applicable to the quantification of serum lathosterol and plant sterol concentrations as markers for whole-body cholesterol biosynthesis and cholesterol absorption, respectively. In these cases, lipids in the cells and serum are usually hydrolyzed and extracted before derivatization and LC-ESI-MS/MS analysis, whereas these steps could be omitted for the screening of the inherited disorders by serum sterol analysis. Serum total (free + esterified) sterol concentrations in 19 normal volunteers, measured by our LC-ESI-MS/MS method, were almost in the same range as those reported by other authors using different methods (**Table 6**). However, 7-dehydrocholesterol

TABLE 5. Recovery of each sterol from human serum

Sterol	Amount Added	Average Recovery[a] (Mean ± SD) (n = 6)
	ng	*%*
Dihydrolanosterol	1.00	100.1 ± 0.6
	2.00	97.3 ± 6.8
	3.00	98.0 ± 5.0
Zymosterol	1.00	100.9 ± 3.0
	2.00	99.0 ± 1.9
	3.00	98.2 ± 0.9
Lathosterol	1.00	88.1 ± 24.9
	2.00	101.2 ± 7.7
	3.00	94.5 ± 8.9
8-Dehydrocholesterol	1.00	97.1 ± 6.5
	2.00	98.5 ± 2.8
	3.00	98.1 ± 3.4
7-Dehydrocholesterol	1.00	99.7 ± 7.4
	2.00	97.2 ± 4.3
	3.00	97.0 ± 3.9
Desmosterol	1.00	100.3 ± 3.2
	2.00	99.3 ± 6.4
	3.00	98.4 ± 4.7
Cholestanol	1.00	98.7 ± 5.6
	2.00	97.1 ± 6.7
	3.00	99.5 ± 1.9
Sitosterol	2.05	95.5 ± 16.8
	4.10	102.5 ± 3.9
	6.15	97.3 ± 11.0

Known amounts of each sterol were spiked into 1 μl of normal human serum before sample preparation.

[a] Recovery (%) = (amount found $-\overline{X}_0$) / amount added × 100; $\overline{X}_0$ value was obtained from TABLE 4.

TABLE 6. Concentrations of total (free + esterified) sterols in normal human serum: comparison with previous methods

Sterol	Present Method[a]	Previous Methods	
	Mean ± SD (n = 19)	Mean ± SD (n)	Method (Reference)
	μg/ml	*μg/ml*	
Dihydrolanosterol	0.21 ± 0.07	0.20 ± 0.12 (4)[b]	GC (38)
Zymosterol	0.63 ± 0.36	NA[c]	
Lathosterol	6.12 ± 4.87	3.2 ± 1.5 (148)	GC (39)
		2.40 ± 1.21 (161)	GC-MS (18)
8-Dehydrocholesterol	2.49 ± 1.44	<3.8 (14)	GC (40)
7-Dehydrocholesterol	3.81 ± 1.48	0.13 ± 0.04 (11)	GC-MS (17)
		5.61 ± 2.23 (50)[d]	ESI-MS/MS (32)
Desmosterol	0.69 ± 0.27	2.0 ± 0.7 (148)	GC (39)
		0.91 ± 0.40 (161)	GC-MS (18)
Cholestanol	3.12 ± 1.00	4.2 ± 1.1 (148)	GC (39)
		1.84 ± 1.00 (161)	GC-MS (18)
Campesterol	4.11 ± 1.66	3.3 ± 1.4 (148)	GC (39)
		1.47 ± 0.78 (161)	GC-MS (18)
Sitosterol	3.46 ± 1.33	2.5 ± 1.0 (148)	GC (39)
		2.45 ± 1.50 (161)	GC-MS (18)
Sitostanol	0.05 ± 0.02	NA[c]	

[a] Coprostanol (10 ng) as an internal standard was added to 1 μl of serum, and saponification was carried out in 0.5 ml of 1 N ethanolic KOH at 37°C for 1 h. After addition of 0.25 ml of distilled water, sterols were extracted twice with 1 ml of *n*-hexane. The following derivatization and LC-ESI-MS/MS analysis were performed as described in Materials and Methods.

[b] Serum from patients with prostate cancer.

[c] Not available.

[d] Concentration of the sum of 7- and 8-dehydrocholesterol.

level determined by GC-MS (17) was significantly low compared with that by our method. In the GC-MS method, high temperature during GC separation may have caused degradation of this relatively unstable steroid.

In summary, we have developed a very sensitive and specific method for the analysis of sterol profiles in human biological samples. Derivatization of neutral sterols into the picolinyl ester allowed them to be quantified by LC-ESI-MS/MS with excellent sensitivity and reliability. This method is useful for the diagnosis of inherited disorders in cholesterol metabolism as well as the quantification of serum biomarkers for the synthesis and absorption of cholesterol in the human body.

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