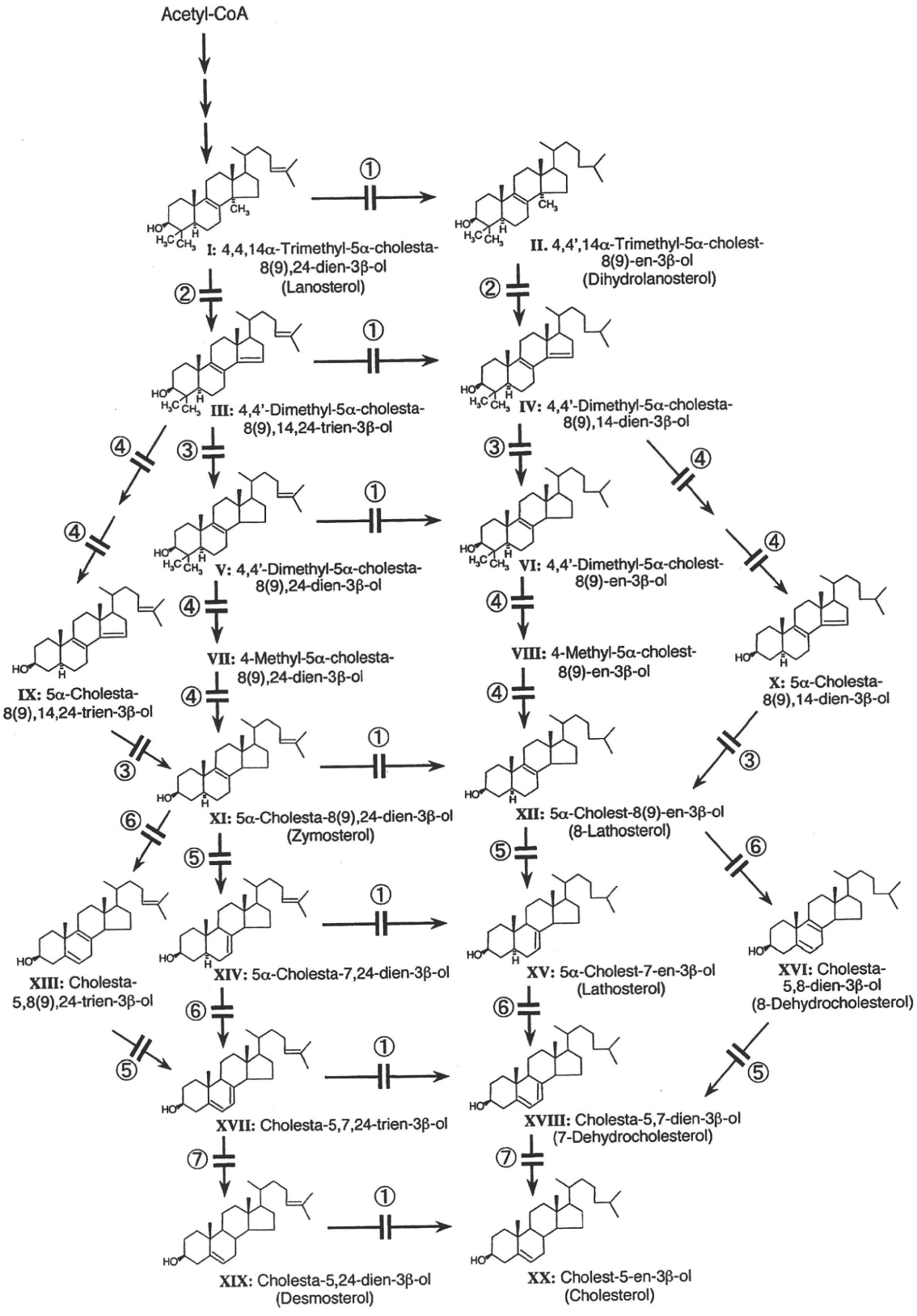

Fig. 1. Enzymatic steps and sterol intermediates in the cholesterol biosynthetic pathway. Biochemical blocks are indicated by the dashed lines. 1, desmosterolosis (3β-hydroxysteroid Δ^{24}-reductase deficiency); 2, Antley-Bixler syndrome (defective lanosterol 14α-demethylase activity due to cytochrome 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]			HPLC Data[c] (RRT[e])
							Collision Energy	Precursor to Product	S/N[d]	
		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]		HPLC Data[c]	
		Collision Energy	Precursor to Product	RRT[d]	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.

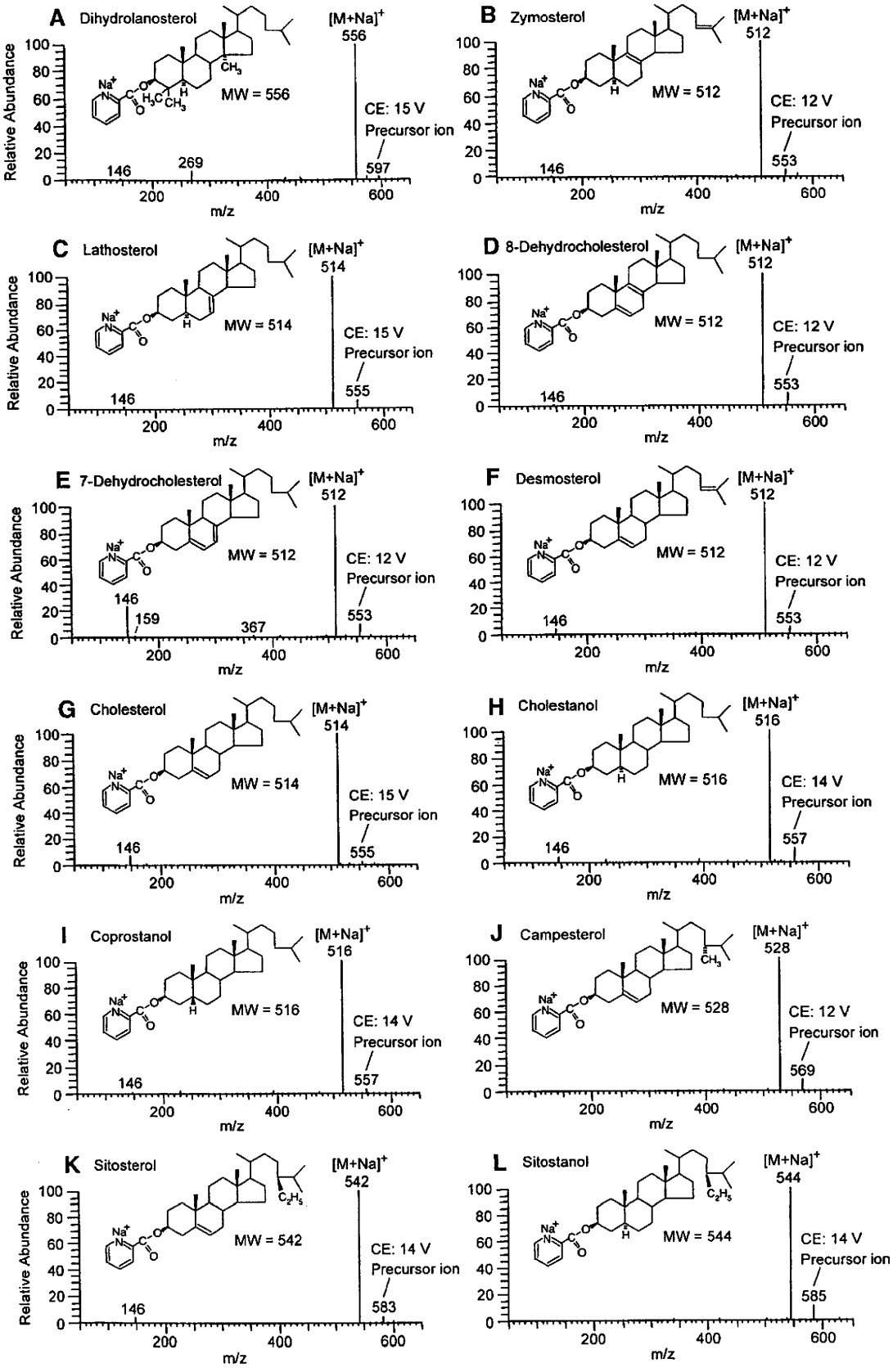

Fig. 2. Positive ESI product ion mass spectra of authentic 3β-picolinates of (A) dihydrolanosterol, (B) zymosterol, (C) lathosterol, (D) 8-dehydrocholesterol, (E) 7-dehydrocholesterol, (F) desmosterol, (G) cholesterol, (H) cholestanol, (I) coprostanol, (J) campesterol, (K) sitosterol, and (L) sitostanol. 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).

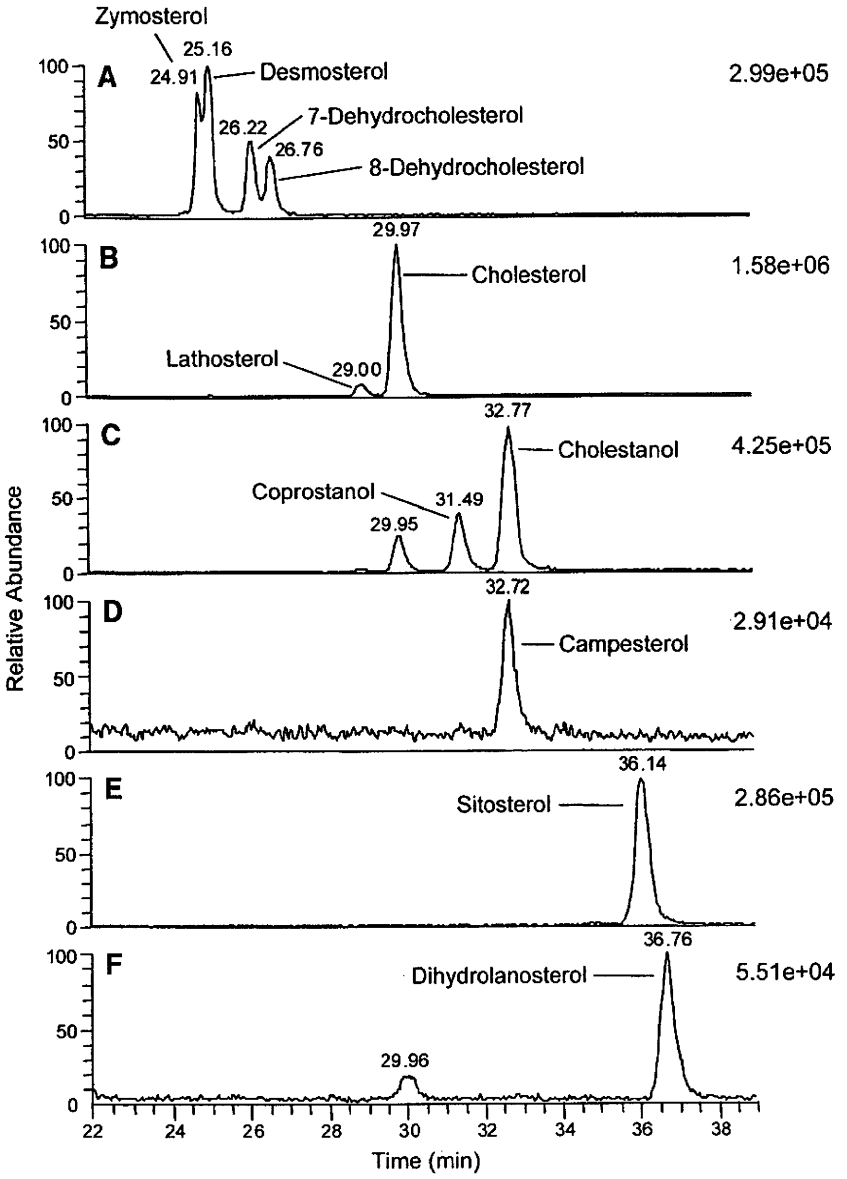

Fig. 3. Representative selected reaction monitoring chromatograms of authentic sterol-3β-picolinates. A: *m/z* 553 → 512 (collision energy: 12 V) for 3β-picolinate of cholestadien. B: *m/z* 555 → 514 (15 V) for 3β-picolinate of cholesten. C: *m/z* 557 → 516 (14 V) for 3β-picolinate of cholestan. D: *m/z* 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

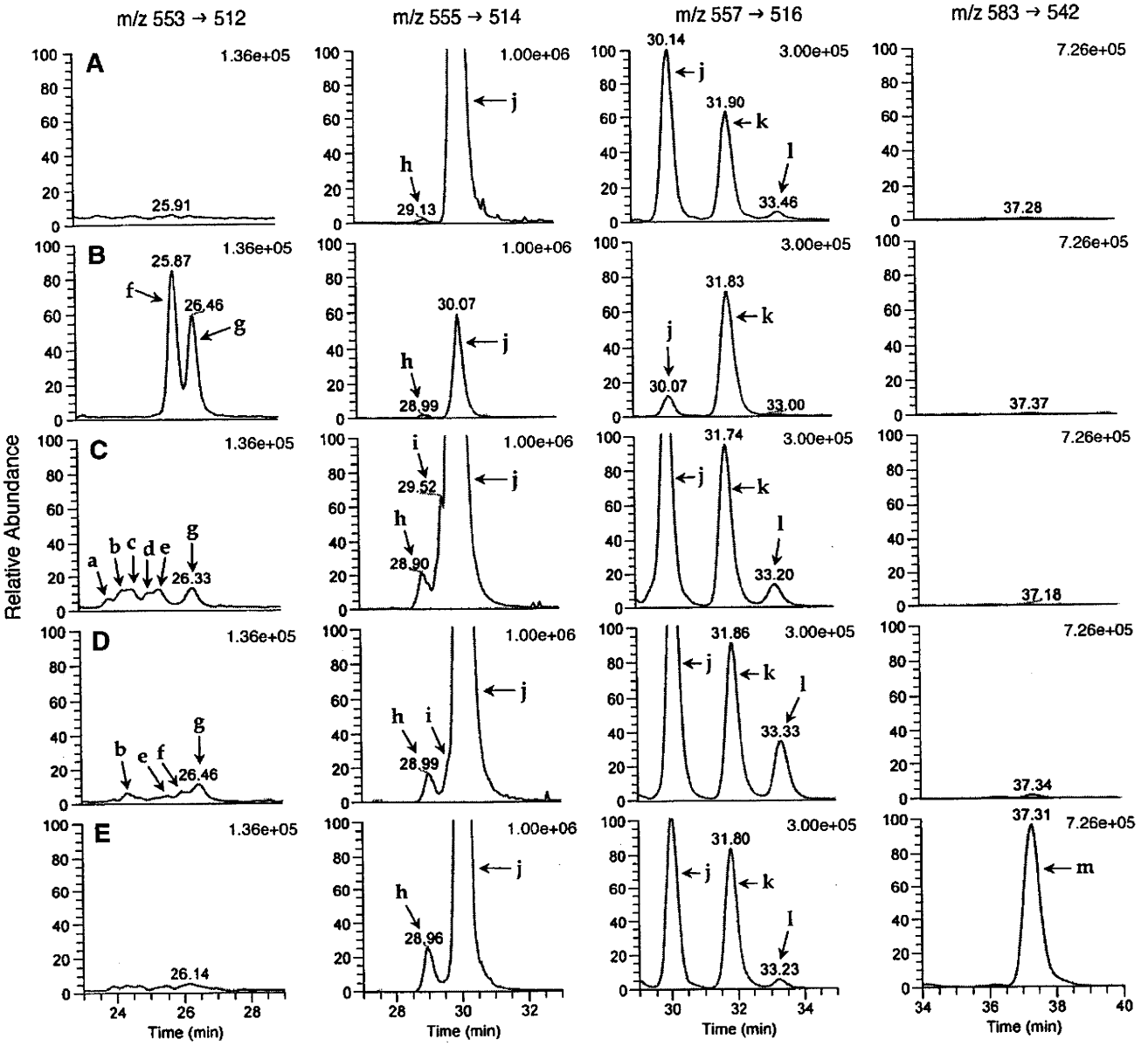

Fig. 4. Comparison of SRM chromatograms obtained from 1 μl of serum from a normal volunteer (A), and patients with SLOS (B), CDPX2 (C), CTX (D), and sitosterolemia (E). The numbers on the right side of each panel represent the full scale of the chromatogram, and were adjusted to the same number at every 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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REVIEW

Determination of Key Intermediates in Cholesterol and Bile Acid Biosynthesis by Stable Isotope Dilution Mass Spectrometry

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Abstract: For more than a decade, we have developed stable isotope dilution mass spectrometry methods to quantify key intermediates in cholesterol and bile acid biosynthesis, mevalonate and oxysterols, respectively. The methods are more sensitive and reproducible than conventional radioisotope (RI), gas-chromatography (GC) or high-performance liquid chromatography (HPLC) methods, so that they are applicable not only to samples from experimental animals but also to small amounts of human specimens. In this paper, we review the development of stable isotope dilution mass spectrometry for quantifying mevalonate and oxysterols in biological materials, and demonstrate the usefulness of this technique.

Keywords: isotope dilution mass spectrometry, biomarker, cholesterol synthesis, bile acid synthesis, mevalonate, oxysterol

Pathways for Cholesterol and Bile Acid Biosynthesis

Cholesterol homeostasis in human is maintained by two input pathways, comprised of dietary absorption and *de novo* synthesis, and two output pathways, comprised of direct secretion from liver to bile and conversion into bile acids (Everson, 1992). The rate-limiting step in the *de novo* cholesterol synthesis is the conversion of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) into mevalonic acid (MVA) by HMG-CoA reductase (HMGCR) (Dietschy and Brown, 1974). In contrast, the bile acid biosynthetic pathway is initiated by either hepatic 7α-hydroxylation or hepatic and extrahepatic 27-hydroxylation of cholesterol. The former is catalyzed by microsomal cholesterol 7α-hydroxylase (CYP7A1), the first and rate-limiting enzyme in the classic pathway, while the latter is catalyzed by mitochondrial sterol 27-hydroxylase (CYP27A1), a key enzyme in the alternative pathway (Vlahcevic et al. 1992). Bile acid synthesis by the classic pathway accounts for more than 90% of total bile acids in humans (Duane and Javitt, 1999) while less than 50% of total bile acids is produced by this pathway in rats (Vlahcevic et al. 1997) and mice (Schwarz et al. 1996). Therefore, the measurement of CYP7A1 activity is more important than that of CYP27A1 activity for the evaluation of bile acid biosynthesis in humans.

Direct and Indirect Assays of HMGCR and CYP7A1 Activities

Since HMGCR and CYP7A1 are crucial enzymes in understanding whole body cholesterol metabolism, a great deal of effort has been made to develop suitable assay methods for these enzyme activities. The primary methods have the great disadvantage that an invasive tissue biopsy is necessary for direct determination of these enzyme activities in humans. To overcome this problem, plasma biomarkers for evaluation of these enzyme activities has been explored.

Plasma levels of MVA, the immediate product of HMGCR, were positively correlated with HMGCR activities in rat liver (Popjak et al. 1979). In humans, the plasma MVA concentrations reflected (i) increased rates of whole-body cholesterol synthesis by treatment with cholestyramine resin, and (ii) decreased rates of whole-body sterol synthesis after consumption of a cholesterol-rich diet (Parker et al. 1982 and 1984). In addition, plasma concentration of lathosterol, an intermediate in the late cholesterol biosynthetic pathway, was reported to reflect hepatic HMGCR activity (Björkhem et al. 1987a) as well as whole body cholesterol synthesis (Kempen et al. 1988) in humans.

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As for bile acid biosynthesis, Björkhem et al. (Björkhem et al. 1987b) demonstrated that serum levels of 7α-hydroxycholesterol (7A) correlated well with the activities of CYP7A1 in patients with gallstones treated with cholestyramine. In addition, Axelson et al. (Axelson et al. 1988) measured serum concentrations of 7α-hydroxy-4-cholesten-3-one (C4), the product of the next reaction following 7α-hydroxylation of cholesterol, and showed that it was a good marker for CYP7A1 activity in humans (Axelson et al. 1991). It was subsequently reported that serum concentrations of 7A (Hahn et al. 1995) and C4 (Sauter et al. 1996) reflected not only CYP7A1 activities but also bile acid synthesis in humans.

The Methods for the Quantification of MVA

Table 1 summarizes the previously described methods for the quantification of MVA in the liver (enzyme assay), plasma or urine. The primary methods for assaying HMGCR activity have utilized a RI technique that measures the radioactivity in [^{14}C]MVA produced from [^{14}C]HMG-CoA (Shapiro et al. 1969; Goldfarb and Pitot, 1971; Shefer et al. 1972). The methods have been used for the direct determination of enzyme activity but they are not applicable to the quantification of plasma or urinary MVA. In contrast, the following methods, i.e. radioenzymatic assay, enzyme immunoassay, gas chromatography-mass spectrometry (GC-MS), high-performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS) and liquid chromatography-tandem mass spectrometry (LC-MS/MS), can measure not only enzyme activity but also MVA concentrations in plasma and urine.

Radioenzymatic assay

Radioenzymatic assay of the plasma MVA concentration was reported by Popjak et al. (Popjak et al. 1979). The method depends on the phosphorylation of MVA with [γ-^{32}P]ATP and MVA kinase to 5-[^{32}P]phospho-MVA, and the subsequent isolation of the 5-[^{32}P]phospho-MVA together with known amounts of added 5-phospho[^{14}C]MVA by ion-exchange chromatography. The detection limit of their radioenzymatic assay was 1–2 pmol (148–296 pg) indicating that it was not adequate for determining MVA in small amounts of plasma. In addition, there was a safety concern due to the handling of radioactive materials.

Enzyme immunoassay

In 1998, Hiramatsu et al. developed an enzyme immunoassay for urinary MVA using a specific monoclonal antibody against MVA (Hiramatsu et al. 1998). This method is not only simpler than the previously described radioenzymatic assay but also completely avoids the risk of radiation hazards. However, the limit of detection was not better than that of the radioenzymatic assay.

GC-MS

In 1972, Hagenfeldt and Hellström attempted to determine MVA concentration in rat blood by using GC-MS (Hagenfeldt and Hellström, 1972). In this procedure, the MVA was extracted from the acid aqueous phase as the lactone. The lactonization increased the hydrophobicity of MVA, so that they could extract it into organic phase. The resulting extract was treated with diazomethane to convert the coexisting fatty acids into their methyl esters. The unchanged mevalonolactone (MVL) with diazomethane in the extract was quantified by GC-MS in electron ionization mode (GC-EI-MS). The peak corresponding to the retention time of MVL appeared large due to interfering materials, such as fatty acids. However, the MVL could be quantified selectively because MVL exhibited an intensive peak at m/z 71 in the spectrum, whereas all fatty acid methyl esters gave rise to the inherent peak at m/z 74 produced by the McLafferty rearrangement ion of the methyl ester. Since then, urinary MVA has been successfully quantified by a similar GC-EI-MS method described above (Woollen et al. 2001).

In the 1970s, the RI technique was the standard method for assaying HMGCR activity, but the handling of radiolabeled materials was a great disadvantage of this method. In 1978, Miyazaki et al. developed a new non-RI method for assaying HMGCR activity in rat liver microsomes or liver slices using [$^{2}H_3$]HMG-CoA as a substrate and GC-MS in chemical ionization mode (GC-CI-MS) (Miyazaki et al. 1978). In this method, the resulting [$^{2}H_3$]MVL was derivatized to the corresponding n-propylamide-n-butylboronate, and deuterium labeled [$^{2}H_7$]MVL was first used as an internal standard.

Table 1. Methods for quantification of MVA in biological samples.

Author	Year	Method (ionization mode)	Derivatization	Lower limit of detection	Intra-assay variation	Inter-assay variation	Recovery	Application
Hagenfeldt et al.	1972	GC-MS (P-EI)	MVL	NA	NA	6.2%	87% ± 4%	blood
Miyazaki et al.	1978	GC-MS (P-CI)	MVL- PABB	NA	NA	NA	NA	liver
Popjak et al.	1979	radioenzymatic assay	5-[^{32}P]phospho-MVA	150–300 pg	NA	NA	100%	plasma
Cighetti et al.	1981	GC-MS (P-EI)	MVL-TMS	NA	1.5%	6.1%	NA	liver
Del Puppo et al.	1989	GC-HR-MS (P-EI)	MVL-TMS	NA	6.5%	NA	101% ± 4%	plasma urine
Honda et al.	1991	GC-HR-MS (P-EI)	MVL-B-DMES	800 fg	4.9%	7.8%	96%–100%	liver
Scoppola et al.	1991	GC-MS (N-CI)	MVA-TFB-TMS	10 pg	5.1%	7.7%	NA	plasma
Yoshida et al.	1993	GC-HR-MS (P-EI)	MVL-B-DMES	NA	2.8%	5.6%	91%–96%	plasma
Ishihama et al.	1994	GC-MS (P-CI)	MVL	NA	2.2%	4.5%	101%–103%	plasma
Siavoshian et al.	1995	GC-MS (P-CI)	MVL-TMS	NA	4.0%	8.0%	70% ± 2%	urine
Saisho et al.	1997	GC-MS (N-CI)	MVA-PFB-CB	NA	2.0%	7.5%	100%–107%	plasma
Hiramatsu et al.	1998	enzyme immunoassay	MVA	195 pg	3.4%	5.2%	102% ± 7%	urine
Woollen et al.	2001	GC-MS (P-EI)	MVL	NA	<13.7%	<9.8%	82%–110%	urine
Park et al.	2001	LC-MS (P-ESI)	MVL	6.5 pg	4.1%	9.4%	95% ± 4%	liver
Ndong-Akoume et al.	2002	LC-MS/MS (P-ESI)	MVL	NA	<1.0%	NA	98%–99%	liver
Abrar et al.	2002	LC-MS/MS (P-ESI)	MVL	NA	4.1%–15%	13%–16%	89%–114%	plasma
Jemal et al.	2003	LC-MS/MS (N-ESI)	MVA	NA	<4.5%	<3.3%	98%–103%	plasma urine
Buffalini et al.	2005	HPLC-UV	MVL	741 ng	NA	<3.0%	97%–103%	liver
Saini et al.	2006	LC-MS/MS (N-ESI)	MVA	NA	1%–17%	3%–12%	99%–108%	plasma
Honda et al.	2007	LC-MS/MS (P-ESI)	MV-PLEA	31 fg	1.8%	3.2%	93%–96%	liver

Abbreviations: P-EI: positive electron ionization; NA: not available; P-CI: positive chemical ionization; N-CI: negative chemical ionization; MVL-PABB: MVL n-propylamide-n-butylboronate; MVL-TMS: trimethylsilyl ether of MVL; GC-HR-MS: high-resolution GC-MS; MVL-B-DMES: dimethylethylsilyl ether of mevalonylbenzylamide; MVA-TFB-TMS: trimethylsilyl ether of bis(trifluoromethyl)benzyl ester of MVA; MVA-PFB-CB: cyclic boronate-pentafluorobenzyl ester of MVA; P-ESI: positive electrospray ionization; N-ESI: negative electrospray ionization; HPLC-UV: high-performance liquid chromatography equipped with an ultraviolet detector; MV-PLEA: MV-(2-pyrrolidin-1-yl-ethyl)-amide.

In 1991, Scoppola et al. (Scoppola et al. 1991) extended this approach, and quantified plasma MVA concentrations. The MVA was lactonized, extracted with [$^2H_3$]MVL and reconverted to the free acid. The resulting MVA was then converted to 3,5-bis(trifluoromethyl)benzyl ester followed by its trimethylsilyl (TMS) ether derivative. The quantification method was based on GC-CI-MS using ammonia as a reagent gas and the detection limit of MVA in plasma was 100 pg/mL. The GC-CI-MS method for the quantification of plasma MVA was subsequently improved by Ishihama et al. (Ishihama et al. 1994) and Saisho et al. (Saisho et al. 1997), and the method for the determination of urinary MVA was developed by Siavoshian et al. (Siavoshian et al. 1995). However, the GC-CI-MS methods have one disadvantage in that they required frequent cleaning of the CI ion source to maintain the high sensitivity.

To eliminate the aforementioned tedious operations in GC-CI-MS, another approach by gas chromatography-electron ionization-mass spectrometry (GC-EI-MS) was also developed. Cighetti et al. (Cighetti et al. 1981; Galli Kienle, 1984) assayed HMGCR activity by GC-EI-MS after conversion of MVL into the corresponding trimethylsilyl (TMS) ether. They used the ions at m/z 187 (M–15) for MVL-TMS and m/z 150 (M–15–CH_2CO) for [$^2H_5$]MVL-TMS because these ions were not influenced by interfering peaks in extracts from liver microsomes. In 1989, the same group improved their original method by using GC-high-resolution (HR)-EI-MS (Del Puppo et al. 1989). This group lactonized plasma and urinary MVA into MVL using a cation exchange resin, and extracted with organic solvent after the addition of [$^2H_5$]MVL as an internal standard. The extracted MVL was then converted into the TMS ether derivative, and quantified by GC-HR-EI-MS with a mass spectral resolution of 5,000. The ions at m/z 145.0685 for MVL-TMS and m/z 150.0965 for [$^2H_5$]MVL-TMS were used for selected ion monitoring (SIM).

We also developed new assay methods to measure hepatic HMGCR activity (Honda et al. 1991) and plasma MVA concentration (Yoshida et al. 1993) by GC-HR-EI-MS. These methods made it possible to simultaneously quantify not only MVA but also 7A. Other features of these methods are described below.

(i) A purification procedure was developed by the serial use of commercially available solid-phase extraction cartridges, which provided high recovery and reproducibility. In brief, plasma MVA was extracted by an anion exchange Bond Elut SAX cartridge, and then eluted as MVL with 0.6 M HCl. The MVL was further purified by a reversed phase Bond Elut C18 and a normal phase Bond Elut CN cartridges. In addition, an excess benzylamine was removed by another Bond Elut CN cartridge after derivatization into mevalonylbenzylamide. The recovery of spiked MVA through the purification procedures using these cartridges was 94.1%, and the relative standard deviations between sample preparations and between measurements by this method were 5.6% and 2.8%, respectively (Yoshida et al. 1993).

(ii) [$^2H_7$]MVL was used as an internal standard. This hepta-deuterated variant of MVL provided both good linearity of the calibration curve and easiness to distinguish between MVL peak and interfering peaks even if the MVL peak was small.

(iii) MVL was easily converted into mevalonylbenzylamide without any catalyst under mild conditions followed by its dimethylethylsilyl (DMES) ether derivative. This amidation via MVL from MVA is a characteristic reaction for γ-hydroxyfatty acids, such as MVA, however, the free fatty acids also present in the extract did not react without catalysts. The resulting derivative gave a [M-C_2H_5]$^+$ ion at *m/z* 380.2077 with a prominent intensity in the high mass region, which was a great advantage in the elimination of interfering peaks originating from endogenous substances in the extract by GC-EI-MS.

(iv) The DMES ether derivative was much more stable than the TMS ether derivative.

(v) The MVL derivative was quantified by GC-HR-EI-MS with a mass spectral resolution of 10,000, which was also useful to eliminate peaks of unknown substances that could interfere with the monitoring.

(vi) Trace amounts, less than 1 pg, of MVA could be detected by this method, and the lower limit of quantification in plasma sample was 180 pg/mL.

(vii) Using these methods, it was validated that there was a highly significant correlation between the hepatic HMGCR activities and plasma concentrations of MVA in ten patients ($r = 0.83$, $P < 0.01$) (Yoshida et al. 1993).

(viii) The GC-EI-MS method did not require frequent cleaning. This indicated that the GC-EI-MS

method was suitable for clinical applications, in which it is necessary to assay a large number of samples at once.

LC-MS and LC-MS/MS

Since the early 2000s, LC-MS or LC-MS/MS have been used more extensively than GC-MS to analyze relatively polar compounds, such as MVA or MVL, because LC-MS and LC-MS/MS do not generally require a derivatization step.

Park et al. (Park et al. 2001) and Ndong-Akoume et al. (Ndong-Akoume et al. 2002) assessed HMGCR activity by measuring MVL with LC-MS and LC-MS/MS using the positive electrospray ionization (P-ESI) mode. Plasma and urinary MVA concentrations were quantified by LC-P-ESI-MS/MS after conversion into MVL (Abrar and Martin, 2002), as well as directly by LC-negative (N)-ESI-MS/MS without lactonization (Jemal et al. 2003; Saini et al. 2006). The detection limit of MVL by the LC-MS method was 6.5 pg (Park et al. 2001), and the lower limit of quantification of plasma MVA by the LC-MS/MS methods were 200–500 pg/mL, which were similar to those obtained using GC-MS methods.

Recently, we developed a highly-sensitive method to assess HMGCR activity by LC-MS/MS (Honda et al. 2007a). In this method, MVA was extracted as MVL and its detection sensitivity was enhanced through derivatization (Fig. 1). The features of this method are described below.

(i) The P-ESI mode was selected to quantify MVA because the positive mode provides more abundant ions than the negative mode (Hiraoka and Kudaka, 1992).

(ii) To select the most suitable derivative of MVA for P-ESI mode, the amidation reaction from MVA via MVL, a characteristic reaction for γ-hydroxy fatty acids such as MVA, was conducted using seven types of primary alkylamines with a tertiary amine moiety to promote protonation. Of these amide derivatives, mevaonyl-2-pyrrolidin-1-yl-ethyl)-amide (MV-PLEA) was the best derivative for the LC-P-ESI-MS/MS method.

(iii) The detection limit of this MV-PLEA was about 30 fg (signal-to-noise ratio (S/N) = 3), indicating that this is the most sensitive method at present for the detection of MVL.

(iv) [$^2H_7$]MVL was used as an internal standard. The recovery of spiked MVA was 94.6%, and the relative standard deviations between sample preparations and between measurements by this method were 3.2% and 1.8%, respectively.

(v) MV-PLEA was determined by selected reaction monitoring (SRM) using m/z 245 (M+H) as a precursor ion and m/z 227 (M+H–H_2O) as a product ion, which almost completely eliminated the interfering peaks on the SRM chromatogram.

(vi) Hepatic HMGCR activities in 11 normal rats were measured by both the RI and LC-P-ESI-MS/MS methods. The HMGCR activities obtained by the present method correlated well with those obtained by the conventional RI method ($r = 0.93$, $P < 0.0001$). In the RI method, [^{14}C]HMG-CoA is usually used as 30 dpm/pmol = 33.3 fmol/dpm. When the standard deviation of background noise is 2 dpm, the signal would be 6 dpm when the S/N = 3. Therefore, the detection limit of the conventional RI method is calculated to be 200 fmol (S/N = 3). In comparison, the detection limit of the LC-P-ESI-MS/MS method is 240 amol (S/N = 3), ~800 times more sensitive than that of the conventional RI method.

HPLC

In 2005, Buffalini et al. reported a new method for the determination of HMGCR activity by HPLC (Buffalini et al. 2005). In this method, MVL produced from unlabeled HMG-CoA was extracted and quantified by HPLC with a fixed ultraviolet (UV) detector (200 nm). This method does not require very expensive equipment, such as a mass spectrometer, but the detection limit of MVL is at least 100,000 times less than that by mass spectrometry.

The methods for the quantification of 7A

CYP7A1 activity has previously been assayed by measuring the radioactivity of 7A produced from exogenously added [^{14}C]cholesterol by incubation with liver microsomes (Shefer et al. 1968). However, the extent of equilibration of exogenous labeled cholesterol with the endogenous cholesterol pool under different conditions still remains to be elucidated. To overcome this problem, several methods, i.e. a radioisotope derivative method, and GC-MS and HPLC methods, have been developed. These methods are able to measure the net amount of 7A produced from endogenous and exogenous

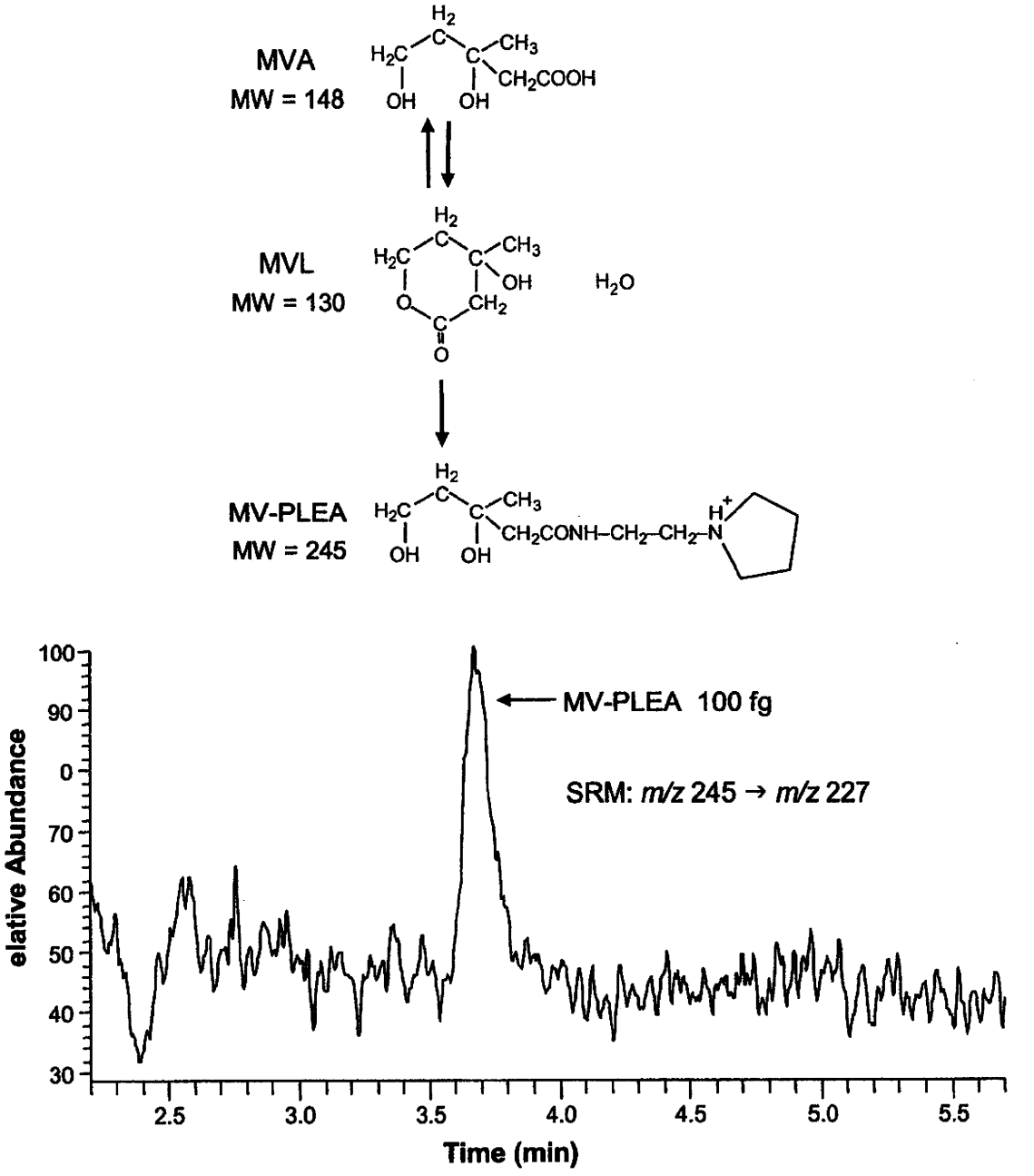

Figure 1. Representative chromatogram of mevalonyl-(2-pyrrolidin-1-yl-ethyl)-amide (MV-PLEA) by positive ESI-SRM at *m/z* 245 → *m/z* 227. Authentic standard of MV-PLEA (100 fg) was injected into the HPLC. LC-MS/MS conditions have been described previously (Honda et al. 2007a).

cholesterol. Table 2 summarizes the previously reported methods for the direct determination of the mass of 7A in the liver (enzyme assay) or plasma.

Radioisotope derivative method

This technique can measure the net amount of 7A produced from exogenous [^{14}C]cholesterol and endogenous unlabeled cholesterol (Mitropoulos et al. 1972; Shefer et al. 1975). The resultant 7A was extracted, acetylated with [^{3}H]acetic anhydride and purified by thin layer chromatography (TLC). The mass of 7A was calculated from the amount of radioactivity in the acetylated product based upon the specific radioactivity of the reagent.

Table 2. Methods for quantification of 7A in biological samples.

Author	Year	Method (ionization mode)	Derivatization	Lower limit of detection	Intra-assay variation	Inter-assay variation	Recovery	Application
Mitropoulos et al.	1972	radioisotope derivative method	acetylated 7A	NA	NA	NA	NA	liver
Björkhem et al.	1974	GC-MS (P-EI)	7A-TMS	NA	NA	2.2%	95%	liver
Sanghvi et al.	1981	GC-MS (P-EI)	7A-TMS	50 pg	3.5%	2.3%–4.7%	NA	liver
Noshiro et al.	1985	NP-HPLC-UV	7A	NA	NA	NA	>85%	liver
Ogishima et al.	1986	NP-HPLC-UV	C4	NA	NA	NA	NA	liver
Björkhem et al.	1987	GC-MS (P-EI)	7A-TMS	1–2 ng/mL	NA	4%–8%	105%	serum
Hylemon et al.	1989	RP-HPLC-UV	C4	8 ng	NA	NA	NA	liver
Yamashita et al.	1989	GC-MS (P-EI)	7A-TMS	NA	3.8%	4.6%	92%–99%	liver
Oda et al.	1990	GC-MS (P-EI)	7A-TMS	NA	NA	3%	97%–109%	serum
Honda et al.	1991	GC-HR-MS (P-EI)	7A-DMES	1.6 pg	7.9%	7.0%	94%–102%	liver
Yoshida et al.	1993	GC-HR-MS (P-EI)	7A-DMES	NA	4.2%	2.6%	93%–95%	serum

Abbreviations: NA: not available; P-EI: positive electron ionization; 7A-TMS: trimethylsilyl ether of 7α-hydroxycholesterol; HPLC-UV: high-performance liquid chromatography equipped with an ultraviolet detector; NP: normal-phase; C4: 7α-hydroxy-4-cholesten-3-one; RP: reversed-phase; GC-HR-MS: high-resolution GC-MS; 7A-DMES: dimethylethylsilyl ether of 7α-hydroxycholesterol.

GC-MS

In 1974, Björkhem and Danielsson developed a method for the assay of hepatic CYP7A1 activity by GC-MS (Björkhem and Danielsson, 1974). Their method was based on stable isotope dilution-mass spectrometry using [$^2H_3$]7A as an internal standard. In this method, 7A produced from endogenous microsomal cholesterol was extracted in organic solvent, purified by TLC, converted to the TMS ether derivative, and analyzed by GC-MS. In 1981, Sanghvi et al. reported an alternative method by GC-MS in which 7A produced from microsomal cholesterol was extracted with 5α-cholestane as an internal standard by organic solvent, converted to the TMS ether derivative, and quantified by SIM (Sanghvi et al. 1981). Meanwhile, Yamashita et al. measured hepatic CYP7A1 activity by GC-SIM using 5α-cholestane-3β,7β-diol as an internal standard (Yamashita et al. 1989).

We also developed a new assay method for hepatic CYP7A1 activity by GC-HR-SIM (Honda et al. 1991). As mentioned in the previous MVA section, this method made it possible to quantify simultaneously not only 7A but also MVA. [$^2H_7$]7A was used as an internal standard and 7A was converted into its DMES ether derivative before analysis by GC-HR-MS. This DMES ether derivative was not only more stable but also much advantageous compared with the TMS ether derivative for the separation of 7A from contaminated cholesterol on GC chromatograms.

The concentration of 7A in human serum was first quantified by Björkhem et al. using GC-SIM (Björkhem et al. 1987b). They also showed that serum free (unesterified) 7A reflected hepatic CYP7A1 activities in humans. In contrast, Oda et al. quantified human serum free and esterified 7A concentrations by GC-SIM and reported that the hepatic CYP7A1 activities correlated better with the serum esterified 7A than with the free 7A (Oda et al. 1990).

In 1993, we applied our GC-HR-SIM method to the determination of human serum 7A concentrations

and confirmed that there was a significant correlation ($r = 0.76$, $p < 0.05$) between serum free 7A concentrations and hepatic CYP7A1 activities in humans (Yoshida et al. 1993). However, neither the esterified 7A ($r = 0.45$, $p > 0.05$) nor the total (free + esterified) 7A concentrations ($r = 0.51$, $p > 0.05$) correlated significantly with CYP7A activities.

HPLC

The assay method for hepatic CYP7A1 activity by HPLC was first reported by Noshiro et al. (Noshiro et al. 1985). The 7A produced from microsomal cholesterol was extracted and separated by normal phase HPLC. Although the absorption maximum of 7A was lower than 200 nm, they monitored 7A at 214 nm because there was an interference due to absorption of oxygen and/or solvent impurities at lower wavelengths.

In 1986, the same group improved the assay method by converting the produced 7A into C4 by incubating with cholesterol oxidase (Ogishima et al. 1986). Because C4 exhibits an intense absorption at 240 nm and there are fewer interfering peaks at this wavelength than at 214 nm, this improved method exhibited a more than 10-fold increase in the sensitivity compared with the previous one (Noshiro et al. 1985). In 1989, Hylemon et al. modified Ogishima's method by using reverse-phase HPLC and adding 7β-hydroxycholesterol as an internal standard (Hylemon et al. 1989).

The Methods for the Quantification of C4

Another plasma or serum marker for the evaluation of hepatic CYP7A1 activities is C4, which is a product of the next oxidative enzymatic reaction after CYP7A1. In fact, CYP7A1 activities correlated better with serum C4 levels compared with those of 7A irrespective of the esterification (Yoshida et al. 1994). Table 3 shows the previously described methods for the quantification of serum C4 concentrations by HPLC, GC-MS, and LC-MS/MS.

HPLC

In 1988, Axelson et al. (Axelson et al. 1988) reported a method for the quantification of plasma C4 using normal-phase HPLC with UV detection, and demonstrated that plasma C4 concentration reflected bile acid biosynthesis in humans. In addition, they reported that there was a strong positive correlation between the plasma levels of C4 and the activities of CYP7A1 in patients treated with cholestyramine, chenodeoxycholic acid, or ursodeoxycholic acid (Axelson et al. 1991). However, their method required the addition of ^{3}H-labeled 25-hydroxyvitamin D_3 as an internal standard. On the other hand, Pettersson et al. (Pettersson and Eriksson, 1994) and Gälman et al. (Gälman et al. 2003) used unlabeled 7β-hydorxy-4-cholesten-3-one as an internal standard and analyzed C4 levels using HPLC with a reversed-phase column. The detection limits of C4 by these HPLC-UV methods were nearly 1 ng, so that at least 1 mL of plasma was required for each assay.

GC-MS

In 1994, we developed a more sensitive method for the quantification of plasma C4 by GC-HR-MS using $[^{2}H_7]$C4 as an internal standard (Yoshida

Table 3. Methods for quantification of serum C4 concentration.

Author	Year	Method (ionization mode)	Derivatization	Lower limit of detection	Intra-assay variation	Inter-assay variation	Recovery	Application
Axelson et al.	1988	NP-HPLC-UV	C4	0.5–1.5 ng/mL	NA	5%	82%–106%	plasma
Pettersson et al.	1994	RP-HPLC-UV	C4	3 ng	3.2%	3.8%	96%–105%	serum
Yoshida et al.	1994	GC-HR-MS (P-EI)	C4-MO-DMES	1 pg	2.54%	5.16%	94%–100%	plasma
Gälman et al.	2003	RP-HPLC-UV	C4	500 pg	4.4%	5.6%	NA	blood
Honda et al.	2007	LC-MS/MS (P-ESI)	C4-picolinate	30 fg	3.9%	5.7%	92%–94%	serum

Abbreviations: HPLC-UV: high-performance liquid chromatography equipped with an ultraviolet detector; NA: not available; NP: normal-phase; RP: reversed-phase; GC-HR-MS: high-resolution GC-MS; P-EI: positive electron ionization; C4-MO-DMES: methyloxime dimethylethylsilyl ether of C4; P-ESI: positive electrospray ionization.

et al. 1994). C4 was extracted from 200 μL of plasma by a salting-out extraction, and then purified by serial solid-phase extractions. The extract was treated with O-methylhydroxylamine hydrochloride and then dimethylethylsilylated. The resulting methyloxime-DMES ether derivative was quantified by GC-HR-SIM. This method was very sensitive as well as specific, and a lower limit of detection of 1 pg was achieved.

We compared the relationships between hepatic CYP7A1 activity and plasma concentrations of C4 and free 7A in humans using our GC-HR-SIM methods (Yoshida et al. 1994). Both biomarkers correlated significantly with hepatic CYP7A1 activity (C4: $r = 0.84$, $p < 0.001$; free 7A: $r = 0.73$, $p < 0.01$), and C4 correlated better with CYP7A1 activity compared with free 7A. However, perhaps these plasma markers do not precisely reflect hepatic CYP7A1 activities in some patients with markedly changed concentrations of plasma lipoproteins. Because plasma oxysterols including C4 and 7A are transported in lipoproteins, the concentrations of oxysterols can be affected by the half-life of the lipoproteins. This hypothesis was supported by another study by ourselves (Honda et al. 2004), in which plasma C4 concentrations and hepatic CYP7A1 activities were compared in New Zealand white rabbits that were fed a high cholesterol diet and/or a bile fistula was constructed. Feeding cholesterol markedly increased and bile drainage reduced plasma cholesterol concentrations. Initially, in these models there was no correlation between plasma C4 concentrations and hepatic CYP7A1 activities ($r = -0.24$, $p > 0.05$). Cholesterol feeding was associated with downregulated CYP7A1 activities, while plasma C4 concentrations were elevated in the presence of increased plasma cholesterol levels. However, this discrepancy was overcome and a significant correlation was observed ($r = 0.73$, $p < 0.05$) by expressing C4 levels relative to cholesterol. These results suggested that plasma C4 relative to cholesterol was a better marker for hepatic CYP7A1 activity than the absolute concentration when plasma cholesterol concentrations were changed markedly.

LC-MS/MS

HPLC with UV detection is a more convenient method than GC-MS for the measurement of plasma C4 concentrations. However, the sensitivity is not sufficient to quantify C4 in limited amounts of human serum. Therefore, we recently developed a highly-sensitive new method by LC-MS/MS (Honda et al. 2007b). After the addition of $[^2H_7]$C4 as an internal standard, C4 was extracted from human serum (2–50 μL) by a salting-out procedure, derivatized into the picolinoyl ester (C4-7α-picolinate), and then purified using a disposable C_{18} cartridge. The resulting picolinoyl ester derivative of C4 was quantified by LC-P-ESI-MS/MS (Fig. 2). LC-MS/MS method do not always require a derivatization step. However, it is also true that the introduction of charged moieties enhances the ionization efficiency of neutral steroids in ESI and atmospheric pressure chemical ionization processes. In P-ESI mode, the picolinoyl ester of C4 exhibited an $[M+H]^+$ ion at *m/z* 506 as the base peak. In the MS/MS spectrum, the $[M-C_6H_5O_2N]^+$ ion was observed at *m/z* 383 as the most prominent peak. The SRM was conducted using *m/z* 506 → *m/z* 383 for the C4-7α-picolinate and *m/z* 513 → *m/z* 390 for the $[^2H_7]$ variant. The detection limit of the C4-7α-picolinate was 30 fg (S/N = 3), which was more than 1,000 times more sensitive than that of C4 with a conventional HPLC-UV method. The recovery of spiked C4 was 93.4%, and the relative standard deviations between sample preparations and between measurements by this method were 5.7% and 3.9%, respectively. Thus, this LC-MS/MS method is not only the most sensitive method at present for the detection of C4 but it is also highly reliable and reproducible.

Applications to Clinical Studies

The quantification of MVA, 7A or C4 in human blood has made it possible to monitor *in vivo* cholesterol and bile acid synthesis without the need for an invasive liver biopsy. Therefore, these methods are very useful for basic or clinical time-course studies of cholesterol metabolism (Table 4).

Diurnal cycle

In 1982, Parker et al. observed the diurnal cycle of the MVA concentrations in human plasma (Parker et al. 1982). At the peak of the cycle (between midnight and 3 a.m.), the MVA concentrations were 3–5 times greater than those at the nadir (between 9 a.m. and noon). Pappu et al. also reported that the plasma concentrations of MVA exhibited a diurnal cycle in normal subjects and patients with abetalipoproteinemia, and the highest levels were observed between midnight and 4 a.m. (Pappu and Illingworth, 1994).

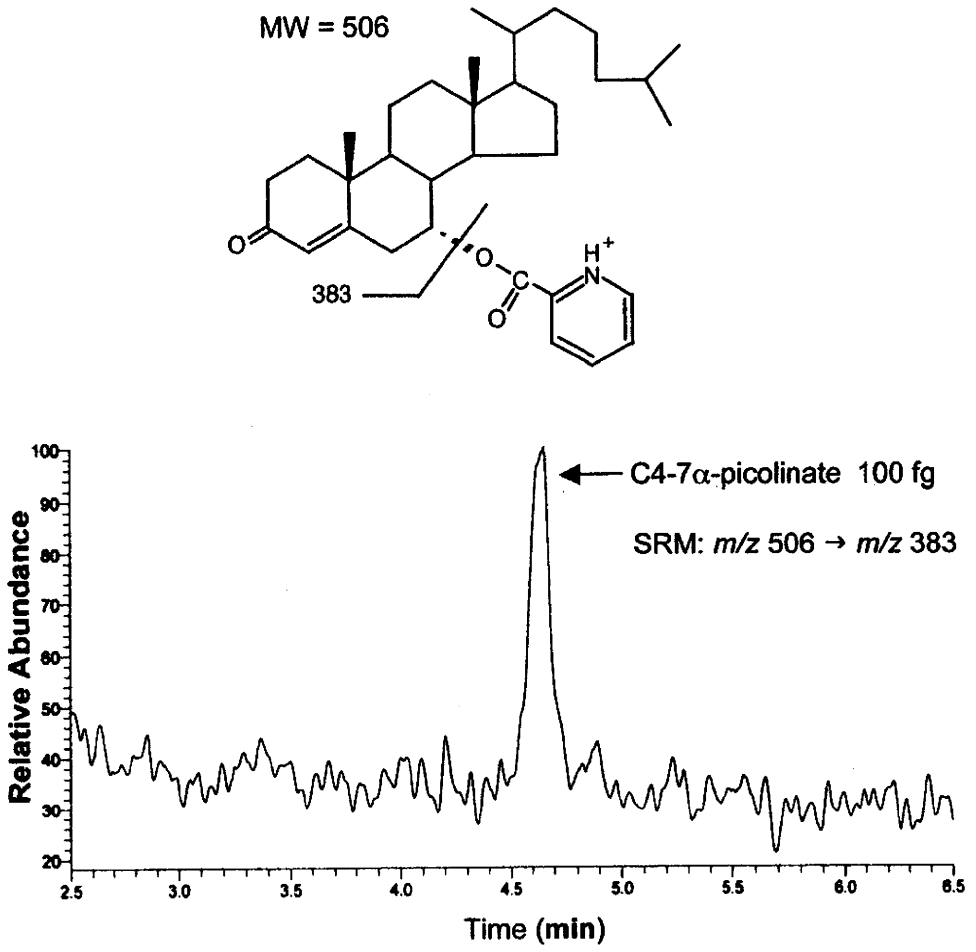

Figure 2. Representative chromatogram of C4-7α-picolinate by positive ESI-SRM at *m/z* 506 → *m/z* 383. Authentic standard of C4-7α-picolinate (100 fg) was injected into HPLC. LC-MS/MS conditions have been described previously (Honda et al. 2007b).

On the other hand, the diurnal cycle of bile acid biosynthesis in the human liver was reported (deletion) by Duane et al. (Duane et al. 1983). They used a radioisotope technique and demonstrated for the first time that humans with an intact enterohepatic circulation exhibited a diurnal cycle of bile acid synthesis with an amplitude of ± 35%–55% around mean synthesis, and an acrophase at about 9 a.m. The same group also reported in 1988 that neither chenodeoxycholic acid nor ursodeoxycholic acid administration significantly altered the circadian rhythm of bile acid synthesis in humans (Pooler and Duane, 1988).

We investigated the diurnal cycle of bile acid biosynthesis by using plasma C4 and 7A concentrations (Yoshida et al. 1994). Plasma was obtained every 2 hours from three normal volunteers and the C4 and 7A concentrations were determined using our GC-HR-MS method (Fig. 3). These levels were fitted to a cosine curve as reported in the previous studies using the isotope kinetic method (Duane et al. 1983; Pooler and Duane, 1988). The amplitudes of C4 and free 7A averaged 45% and 32%, respectively, and the acrophases of C4 and free 7A averaged 5:35 a.m. and 5:39 a.m., respectively, which was compatible with the previous results obtained using the radioisotope technique (Fig. 4). In contrast, total 7A and esterified 7A did not exhibit any significant diurnal cycle.

In 2005, Gälman et al. also reported the diurnal cycle of C4 and lathosterol, another biomarker of cholesterol biosynthesis (Gälman et al. 2005). They concluded that bile acid synthesis in humans exhibits a diurnal cycle with 2 peaks during the daytime, which is opposite from the circadian