methods

Highly sensitive quantification of key regulatory oxysterols in biological samples by LC-ESI-MS/MS[S]

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Abstract We describe a highly sensitive and specific method for the quantification of key regulatory oxysterols in biological samples. This method is based upon a stable isotope dilution technique by liquid chromatography-tandem mass spectrometry (LC-MS/MS). After alkaline hydrolysis of human serum (5 μl) or rat liver microsomes (1 mg protein), oxysterols were extracted, derivatized into picolinyl esters, and analyzed by LC-MS/MS using the electrospray ionization mode. The detection limits of the picolinyl esters of 4β-hydroxycholesterol, 7α-hydroxycholesterol, 22R-hydroxycholesterol, 24S-hydroxycholesterol, 25-hydroxycholesterol, 27-hydroxycholesterol, and 24S,25-epoxycholesterol were 2–10 fg (5–25 amol) on-column (signal-to-noise ratio = 3). Reproducibilities and recoveries of these oxysterols 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 1.8% to 12.7% and 2.9% to 11.9%, respectively. The recovery experiments were performed using rat liver microsomes spiked with 0.05 ng to 12 ng of oxysterols, and recoveries of the oxysterols ranged from 86.7% to 107.3%, with a mean recovery of 100.6%. This method provides reproducible and reliable results for the quantification of oxysterols in small amounts of biological samples.— Honda, A., K. Yamashita, T. Hara, T. Ikegami, T. Miyazaki, M. Shirai, G. Xu, M. Numazawa, and Y. Matsuzaki. **Highly sensitive quantification of key regulatory oxysterols in biological samples by LC-ESI-MS/MS. *J. Lipid Res.* 2009.** 50: **350–357.**

Supplementary key words liquid chromatography-tandem mass spectrometry • electrospray ionization • 24S,25-epoxycholesterol • 4β-hydroxycholesterol • 7α-hydroxycholesterol • 22R-hydroxycholesterol • 24S-hydroxycholesterol • 25-hydroxycholesterol • 27-hydroxycholesterol

Biological samples contain a large number of oxysterols (1), and most of them are formed from cholesterol by enzymatic oxidation (2–6) (**Fig. 1**) or autoxidation (7). By contrast, the oxysterol 24S,25-epoxycholesterol is not derived from cholesterol but is produced de novo from acetyl-CoA via a shunt in the mevalonate pathway (8).

These oxysterols are important molecules for preserving lipid homeostasis in the body. 7α-Hydroxycholesterol is a product of CYP7A1, which is the rate-limiting enzyme in the classic bile acid biosynthetic pathway. 27-Hydroxycholesterol, 24S-hydroxycholesterol, 4β-hydroxycholesterol, 22R-hydroxycholesterol, and 24S,25-epoxycholesterol are effective endogenous ligands of the nuclear receptors liver X receptor α (LXRα) and LXRβ (9–11). In addition, 27-hydroxycholesterol (12), 25-hydroxycholesterol (13), and 24S,25-epoxycholesterol (14) are known to downregulate the cholesterol biosynthetic pathway, presumably by blocking the processing of the sterol-regulatory element binding protein.

GC-MS has historically been used for the analyses of oxysterols in serum and tissues (1, 15) because the sensitivity and specificity of conventional GC with flame ionization detector is not sufficient to quantify oxysterols in biological samples. However, GC-MS is still not an ideal method, especially for the analysis of 24S,25-epoxycholesterol, because this epoxycholesterol does not survive the temperature required for GC analysis (16). Another approach to quantifying oxysterols in biological samples was HPLC with ultraviolet (UV) detection after derivatization to the Δ^4-3-ketones (16–19). This method made it possible to detect

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Abbreviations: CTX, cerebrotendinous xanthomathosis; ESI, electrospray ionization; LC-APCI-MS, liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LXRα, liver X receptor α; SRM, selected reaction monitoring; TMS, trimethylsilyl.

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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.

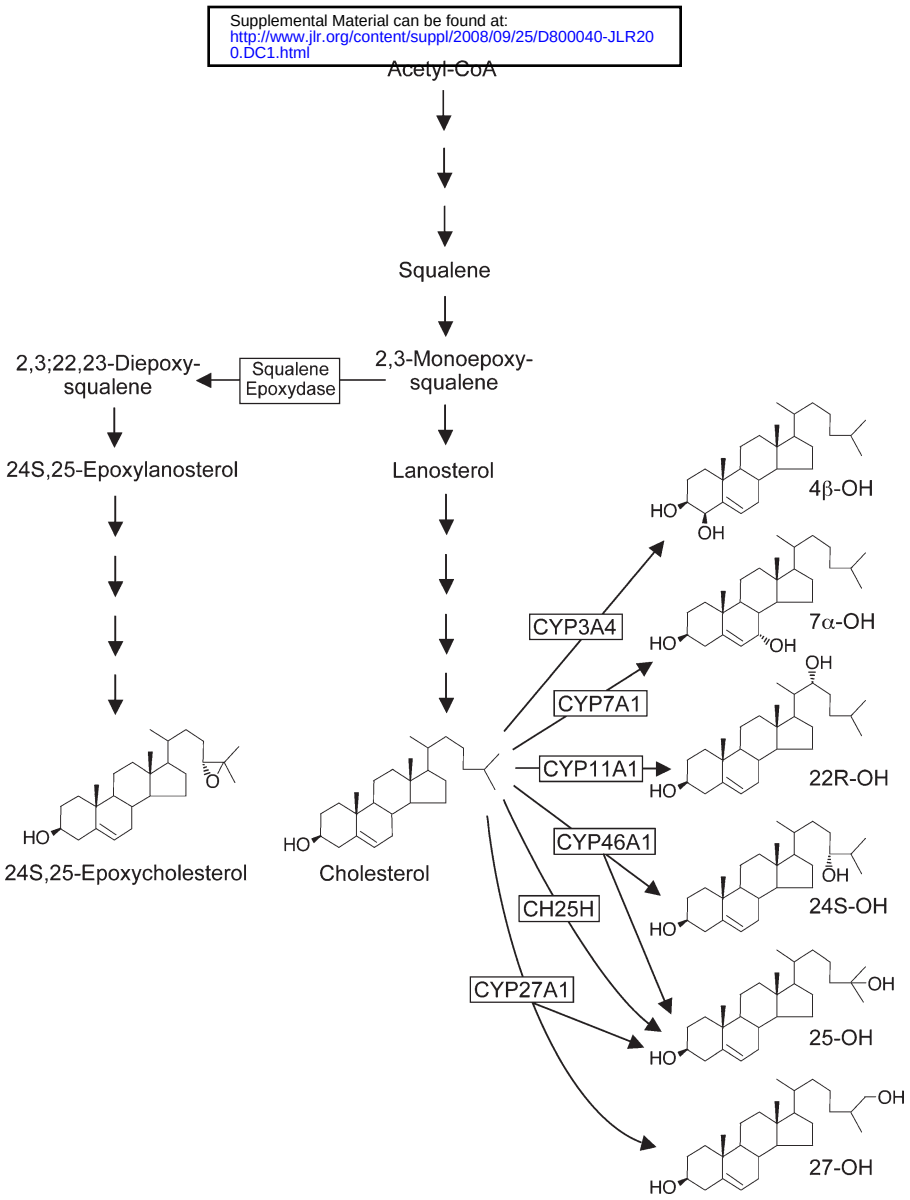

Fig. 1. Biosynthetic pathways for key regulatory oxysterols. Hydroxycholesterols are synthesized from cholesterol, whereas 24S,25-epoxycholesterol is derived from a shunt in the cholesterol biosynthetic pathway. CH25H, cholesterol 25-hydroxylase; 4β-OH, 4β-hydroxycholesterol; 7α-OH, 7α-hydroxycholesterol; 22R-OH, 22R-hydroxycholesterol; 24S-OH, 24S-hydroxycholesterol; 25-OH, 25-hydroxycholesterol; and 27-OH, 27-hydroxycholesterol.

the 24S,25-epoxycholesterol derivative as an intact form, but the lower limit of detection for the Δ^4-3-ketones of oxysterols was about 2 ng on-column (16), which was not sufficient for quantification of the oxysterols in a small amount of biological sample.

Recently, liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (LC-APCI-MS) was introduced as a sensitive, specific, and rapid method for the quantification of oxysterols (20, 21). In addition, LC-tandem mass spectrometry (LC-MS/MS) using electrospray ionization (ESI) has also been applied to the analysis of oxysterols (22). In general, ESI is not the best ionization method for neutral steroids because of its poor ionization efficiency. However, our recent study demonstrated that the derivatization of monohydroxysterols into picolinyl esters markedly enhanced the ionization efficiency in the ESI process, and the method was much more sensitive than the assay of native monohydroxysterols by LC-APCI-MS/MS (23). In this study, we have applied our derivatization method to dihydroxy- and epoxysterols. In each case, singly charged ions were observed as the base peaks in the positive ESI mass spectra and amol levels of these oxysterols were detectable.

MATERIALS AND METHODS

Chemicals

4β-Hydroxycholesterol (cholest-5-en-3β,4β-diol), 7α-hydroxycholesterol (cholest-5-en-3β,7α-diol), 22R-hydroxycholesterol (cholest-5-en-3β,22R-diol), 24S-hydroxycholesterol (cholest-5-en-3β,24S-diol), 25-hydroxycholesterol (cholest-5-en-3β,25-diol),

and 24S,25-epoxycholesterol (cholest-5-en-24S,25-epoxy-3β-ol) were purchased from Steraloids (Wilton, NH). [25,26,26,26,27,27,27-$^2H_7$]4β-hydroxycholesterol, [26,26,26,27,27,27-$^2H_6$]24-hydroxycholesterol, [27,27,27-$^2H_3$]25-hydroxycholesterol, and [26,26,26,27,27,27-$^2H_6$]24,25-epoxycholesterol were obtained from Avanti Polar Lipids (Alabaster, AL). 27-Hydroxycholesterol [(25R)-cholest-5-en-3β,26-diol], [25,26,26,26,27,27,27-$^2H_7$]27-hydroxycholesterol, and [25,26,26,26,27,27,27-$^2H_7$]7α-hydroxycholesterol were prepared as described previously (24).

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 from a patient with cerebrotendinous xanthomatosis (CTX). 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. Rat liver microsomes were prepared in our previous study (25) and had been stored at −70°C until they were used in the present experiments.

Sample preparation

[$^2H_7$]4β-hydroxycholesterol (5 ng), [$^2H_7$]7α-hydroxycholesterol (10 ng) [$^2H_6$]24-hydroxycholesterol (5 ng), [$^2H_3$]25-hydroxycholesterol (1 ng), [$^2H_7$]27-hydroxycholesterol (10 ng), and [$^2H_6$]24,25-epoxycholesterol (1 ng) as internal standards and 5 μg of butylated hydroxytoluene were added to serum (5 μl) or microsomes (1 mg protein), and saponification was carried out in 0.5 ml of 1 N ethanolic KOH at 37°C for 1 h. After the addition of 0.25 ml of distilled water, sterols were extracted twice with 1 ml of *n*-hexane, and the extract was evaporated to dryness under a stream of nitrogen. Derivatization to the picolinyl ester was performed according to our previous method (23) 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), pyridine (1.5 ml), and triethylamine (200 μl). The freshly prepared reagent mixture (170 μl) was added to the sterol extract, and the reaction mixture was incubated at 80°C for 60 min. After the addition of 1 ml of *n*-hexane, the mixture was vortexed for 30 s and centrifuged at 700 *g* for 3 min. The clear supernatant was collected and evaporated at 80°C under nitrogen. The residue was redissolved in 50 μl of acetonitrile, and an aliquot (1 μl) 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 was composed 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 an additional 20 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 optimized collision energy, as listed in **Table 1**.

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 (26). Linearity of the calibration curves was analyzed by simple linear regression. 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

Seven oxysterols were converted into the corresponding picolinyl ester derivatives and positive ESI-MS, MS/MS,

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

Oxysterols (Derivatives)	MS Data $[M+Na]^+$ (Relative Intensity)	MS/MS Data[a] (Collision Energy at Maximum Intensity)	SRM Data[b]			HPLC Data[b] (RRT[d])
			Collision Energy	Precursor to Product	S/N[c]	
	m/z (%)	*m/z* (*V*)	*V*	*m/z*		
4β-Hydroxycholesterol (cholest-5-en-3β,4β-dipicolinates)	635 (100)	146 (22) 512 (20)	22	635 → 146	200	0.77
7α-Hydroxycholesterol (cholest-5-en-3β,7α-dipicolinates)	635 (100)	146 (15) –[e]	15	635 → 146	200	0.62
22R-Hydroxycholesterol (cholest-5-en-3β,22R-dipicolinates)	635 (100)	146 (26) 512 (22)	22	635 → 512	40	0.45
24S-Hydroxycholesterol (cholest-5-en-3β,24S-dipicolinates)	635 (100)	512 (22) 146 (31)	22	635 → 512	80	0.48
25-Hydroxycholesterol (cholest-5-en-3β,25-dipicolinates)	635 (100)	512 (19) 146 (28)	22	635 → 512	40	0.51
27-Hydroxycholesterol (cholest-5-en-3β,27-dipicolinates)	635 (100)	512 (12) 146 (33)	22	635 → 512	80	0.56
24S,25-Epoxycholesterol (cholest-5-en-24S,25-epoxy-3β-picolinate)	528 (100)	146 (20) —[e]	20	528 → 146	80	0.41

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] $[M+Na]^+$ 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.

[b] The same HPLC column and flow rate described in Materials and Methods were employed.

[c] S/Ns were determined by injecting 100 fg of each derivative.

[d] RRTs are expressed relative to the retention time of cholesterol 3β-picolinate.

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

SRM, and HPLC data were obtained for each of them (Table 1). All picolinyl ester derivatives exhibited $[M+Na]^+$ ions as the base peaks. The fragmentation pattern of the base peak ion of each derivative was examined under various levels of collision energy, and [M+Na−picolinic acid $(C_6H_5NO_2)]^+$ (m/z = 512) or [picolinic acid $(C_6H_5NO_2)$+ Na]$^+$ (m/z = 146) ions were observed as the most-abundant product ions, so that they were selected as monitoring ions for authentic oxysterols by SRM. The monitoring ions and optimal collision energies for deuterated internal standards were m/z 642 → 146 (22 V) for 3β,4β-dipicolinates of [$^2H_7$] 4β-hydroxycholesterol, m/z 642 → 146 (15 V) for 3β,7α-dipicolinates of [$^2H_7$]7α-hydroxycholesterol, m/z 641 → 518 (22 V) for 3β,24-dipicolinates of [$^2H_6$]24-hydroxycholesterol, m/z 638 → 515 (22 V) for 3β,25-dipicolinates of [$^2H_3$]25-hydroxycholesterol, m/z 642 → 519 (22 V) for 3β,27-dipicolinates of [$^2H_7$]27-hydroxycholesterol, and m/z 534 → 146 (20 V) for 3β-picolinate of [$^2H_6$]24,25-epoxycholesterol.

Sensitivity of the present method

To determine the sensitivity of our SRM method, the standard mixture solution of the seven oxysterol derivatives was diluted and injected into the LC-MS/MS system. The limit of detection (signal-to-noise ratio of 3) of each steroid was 2 fg (5 amol) on-column for 4β-hydroxycholesterol and 7α-hydroxycholesterol, 5 fg (12.5 amol) on-column for 24S-hydroxycholesterol, 27-hydroxycholesterol, and 24S,25-epoxycholesterol, and 10 fg (25 amol) on-column for 22R-hydroxycholesterol and 25-hydroxycholesterol.

Calibration curves

A calibration plot was established for each oxysterol. Different amounts of authentic oxysterol were mixed with deuterated internal standard, derivatized to the picolinyl ester, and quantified as described in the Materials and Methods. The weight ratio of each oxysterol, relative to the corresponding deuterated internal standard, was plotted on the abscissa, and the peak area ratio of the picolinyl ester of the authentic oxysterol to the deuterated variant measured by SRM was plotted on the ordinate. Because deuterium-labeled 22R-hydroxycholesterol was not available, [$^2H_6$]24-hydroxycholesterol was used as an internal standard for 22R-hydroxycholesterol. The linearity of the standard curves, as determined by simple linear regression, was excellent, as shown in **Table 2**.

Representative SRM

The separation of various authentic oxysterol picolinates by SRM is shown in **Fig. 2A**. All oxysterol picolinates tested were successfully separated. 7β-Hydroxycholesterol, an autoxidation product of cholesterol, gave a peak just before 7α-hydroxycholesterol (not shown in the figure), and the retention times (relative to cholesterol) of these oxysterols (as picolinates) were 0.61 and 0.62, respectively. Figure 2B–D shows typical SRM chromatograms obtained from 1 mg of protein from rat liver microsomes (Fig. 2B) and 5 μl of sera from a control subject (Fig. 2C) and a CTX patient (Fig. 2D). In rat liver microsomes, a significant amount of 24S,25-epoxycholesterol was detected, whereas only a trace amount of 24S-hydroxycholesterol was observed. In contrast, human serum contained a very low concentration of 24S,25-epoxycholesterol, but a significant amount of 24S-hydroxycholesterol was present. When serum oxysterol profiles were compared between controls and CTX, markedly reduced serum 25- and 27-hydroxycholesterol concentrations were observed.

Precision and accuracy of the present method

The following studies were performed to determine the precision and accuracy of the present method using rat liver microsomes. Reproducibility was investigated by analyzing four samples in triplicate by LC-MS/MS (**Table 3**). 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 not considered to be attributable to the sample preparation, because the errors during sample preparation were not significantly larger than those between the measurements (see supplementary Tables I, II). The inter-assay coefficients of variation for the between- and within-sample variations were 1.8% to 12.7% and 2.9% to 11.9%, respectively.

For the recovery experiment, known amounts of oxysterols (a, 2a, 3a; a = 0.05–4.0 ng) were spiked into 1 mg of rat liver microsomal protein (n = 2). After alkaline hydrolysis and derivatization, LC-MS/MS was carried out in triplicate for each sample. The recoveries of the known spiked amounts of the oxysterols ranged from 86.7% to 107.3%, with a mean of 100.6% (**Table 4**). In addition, the amounts of each endogenous oxysterol found in 1 mg of unspiked microsomal protein were within the 95% confidence limit for the estimated amount of each

TABLE 2. Linearities of calibration plots for each oxysterol

Oxysterol	Range (n)	Linear Regression Equation[a]	Correlation Coefficient (r)
	ng		
4β-Hydroxycholesterol	0.05 – 10 (7)	$Y = 0.436X - 0.009$	0.999
7α-Hydroxycholesterol	0.1 – 20 (7)	$Y = 1.075X - 0.011$	1.000
22R-Hydroxycholesterol	0.05 – 5 (6)	$Y = 0.084X - 0.000$	0.993
24S-Hydroxycholesterol	0.05 – 5 (6)	$Y = 0.615X - 0.010$	0.996
25-Hydroxycholesterol	0.01 – 1 (6)	$Y = 0.935X - 0.007$	1.000
27-Hydroxycholesterol	0.1 – 10 (6)	$Y = 1.400X - 0.020$	0.998
24S,25-Epoxycholesterol	0.01 – 2 (7)	$Y = 0.444X - 0.004$	0.998

[a] X is the weight ratio of each oxysterol to the corresponding deuterated internal standard, and Y is the peak area ratio calculated as the peak area of the oxysterol-picolinate(s) divided by that of deuterated oxysterol-picolinate(s) (internal standard). [$^2H_6$]24-hydroxycholesterol was used as an internal standard for 22R-hydroxycholesterol.

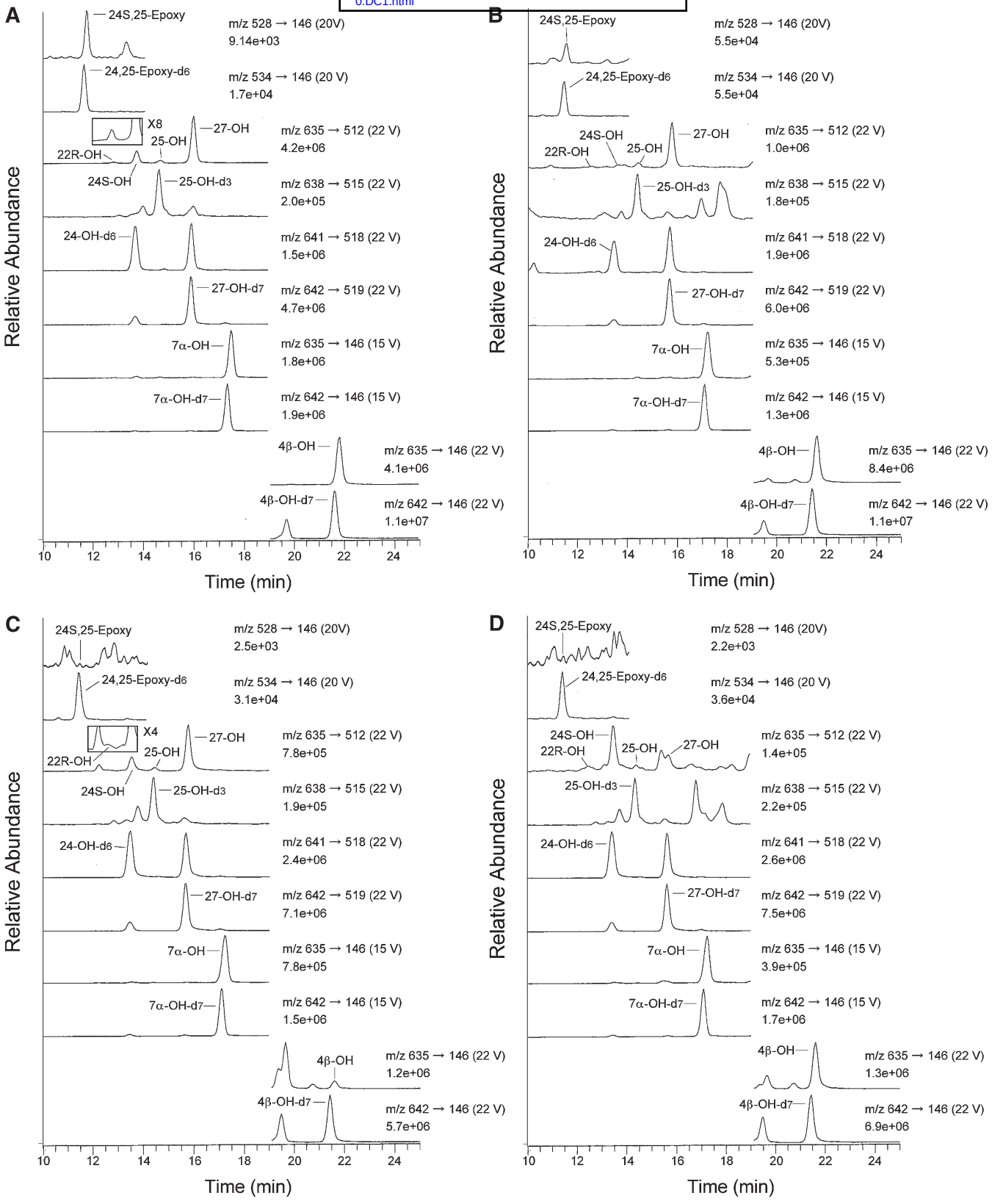

Fig. 2. Comparison of selected reaction monitoring chromatograms obtained from authentic oxysterols (A), 1 mg protein of the microsomal fraction from a normal rat liver (B), and 5 μl of sera from a normal volunteer (C) and a patient with CTX (D). The quantities of each peak (in A) of authentic oxysterol standards are: ~200 pg for 7α-hydroxycholesterol (7α-OH), [$^2H_7$]7α-OH (7α-OH-d_7), 27-hydroxycholesterol (27-OH), and [$^2H_7$]27-OH (27-OH-d_7), ~100 pg for 4β-hydroxycholesterol (4β-OH), [$^2H_7$]4β-OH (4β-OH-d_7), 24S-hydroxycholesterol (24S-OH), and [$^2H_6$]24-OH (24-OH-d_6), and ~20 pg for 25-hydroxycholesterol (25-OH), [$^2H_3$]25-OH (25-OH-d_3), 22R-hydroxycholesterol (22R-OH), 24S,25-epoxycholesterol (24S,25-Epoxy), and [$^2H_6$]24,25-Epoxy (24,25-Epoxy-d_6). The numbers on the right side of each chromatogram represent the full scale of the chromatogram.

TABLE 3. Reproducibility of the quantification of each oxysterol in rat liver microsomes

Oxysterol	Mean ± SD (n = 12)	Relative SD	
		Sample Preparation	Error (SRM)
	ng	%	
4β-Hydroxycholesterol	5.56 ± 0.28	3.3	5.6
7α-Hydroxycholesterol	4.22 ± 0.13	3.7	2.9
22R-Hydroxycholesterol	0.107 ± 0.013	12.7	11.9
24S-Hydroxycholesterol	0.104 ± 0.007	8.7	5.8
25-Hydroxycholesterol	0.64 ± 0.02	1.8	3.7
27-Hydroxycholesterol	3.16 ± 0.23	8.1	6.9
24S,25-Epoxycholesterol	1.11 ± 0.08	5.1	8.4

Each oxysterol was quantified in 1 mg protein from normal rat liver microsomes. Four samples were prepared and quantified in triplicate by liquid chromatography-tandem mass spectrometry. The results were analyzed by a one-way layout, in which the analytical errors were divided into two sources: sample preparation and SRM measurement.

oxysterol calculated by linear regression analysis; this also constituted an index for the precision and accuracy of the method (see supplementary Table III).

DISCUSSION

Neutral monohydroxysterols are poorly ionized by electrospray. To overcome this disadvantage, we have developed a new method for the enhancement of the ionization efficiency by derivatizing into picolinyl esters (23, 27). Dihydroxy- or epoxysterols are more efficiently ionized by electrospray, and their limit of detection (5–60 fmol on-column) was reported to be more than 10 times lower than that of monohydroxysterols (175–2,000 fmol on-column)

TABLE 4. Recovery of each oxysterol from rat liver microsomes

Oxysterol	Amount Added	Average Recovery[a] (Mean ± SD) (n = 6)
	ng	%
4β-Hydroxycholesterol	2.00	102.7 ± 8.7
	4.00	98.5 ± 9.9
	6.00	104.3 ± 11.7
7α-Hydroxycholesterol	4.00	89.5 ± 7.1
	8.00	86.7 ± 6.9
	12.00	90.8 ± 8.8
22R-Hydroxycholesterol	0.05	103.0 ± 15.5
	0.10	105.2 ± 6.9
	0.15	99.8 ± 5.6
24S-Hydroxycholesterol	0.05	107.3 ± 14.0
	0.10	100.3 ± 8.4
	0.15	102.0 ± 9.0
25-Hydroxycholesterol	0.20	106.6 ± 12.7
	0.40	100.1 ± 6.8
	0.60	103.1 ± 5.3
27-Hydroxycholesterol	1.00	98.2 ± 15.0
	2.00	102.6 ± 4.8
	3.00	103.7 ± 2.2
24S,25-Epoxycholesterol	0.40	97.5 ± 15.2
	0.80	107.2 ± 18.5
	1.20	104.2 ± 7.5

Known amounts of each oxysterol were spiked into 1 mg protein from normal rat liver microsomes before sample preparation.

[a] Recovery (%) = (amount found $-X0$)/amount added × 100. *X0* value was obtained from TABLE 3. (See Table 5 in ref. 35.)

(22). In this paper, we have studied the usefulness of our derivatization method on dihydroxy- and epoxysterols that are key regulatory oxysterols in biological samples. The detection limits of oxysterol dipicolinates and epoxycholesterol picolinate were 5–25 amol on-column, which was about 1,000-fold more sensitive than those with the underivatized ESI method (22). We also determined the detection limits of native dihydroxy- and epoxysterols by LC-APCI-MS/MS analysis, and they were about 10 fmol on-column (data not shown). Thus, highly sensitive LC-MS/MS analysis after picolinyl ester derivatization can be used not only for monohydroxysterols but also for dihydroxy- and epoxysterols.

A few derivatization methods that are suitable for LC-ESI-MS/MS analysis of dihydroxysterols have been reported. Griffiths et al. (28) converted oxysterols with a 3β-hydroxy-Δ^5 structure into 3-oxo-Δ^4 steroids by using cholesterol oxidase, and then derivatized with the Girard P reagent to Girard P hydrazone. This method improved the sensitivity by enhancing ionization and was successfully applied to the identification of oxysterols in the brain (29). However, this method has several disadvantages for simple and highly sensitive quantification of oxysterols in biological samples. First, two steps are needed to convert 3β-hydroxysterols into Girard P hydrazone derivatives. Second, the derivatization gives *syn* and *anti* forms with different retention times. Third, 3β-hydroxysterols with an oxo group are converted to the mono- and bis-Girard P hydrazone derivatives. Finally, this method produces the same derivative from 7α-hydroxycholesterol and 7α-hydroxy-4-cholesten-3-one, which are important intermediates in the hepatic bile acid biosynthetic pathway.

Recently, Jiang, Ory, and Han (30) reported another derivatizing method that converted oxysterols into dimethylglycine esters. This method appears to have overcome the weaknesses of the above Girard P hydrazone derivatives. However, overnight incubation at 50°C was necessary to make the dimethylglycine esters, and the formed dimethylglycine diesters provided a doubly protonated ion. MS/MS spectra of doubly protonated ions are more complicated than those of singly protonated ions. Therefore, singly charged ions are preferable as precursor ions for simple and highly sensitive MS/MS analysis.

In our picolinyl ester derivatization, Yamashita et al. (31) reported in a recent study that estradiol dipicolinates gave singly charged ions in the positive ESI mass spectrum. In the present study, oxysterols with two hydroxyl groups were also derivatized to picolinyl diesters showing singly charged ions in the positive ESI mass spectra, which appears to be a general characteristic of the picolinyl ester derivatization of steroids with two hydroxyl groups. Because of the better ionizing efficiency due to the double picolinyl moieties and a simple MS/MS spectra, the detection limits of dihydroxysterols (5–25 amol on-column) were about 100 times lower than those of monohydroxysterols (260–2,600 amol on-column) (23).

In addition, our method made it possible to quantify 24S,25-epoxycholesterol in biological samples with high sensitivity (12.5 amol on-column) and specificity. Although

this epoxycholesterol appears to be one of the most important regulatory oxysterols for cholesterol homeostasis (10, 14), the concentrations in biological samples have not been determined widely because of instability during GC-MS analysis and insufficient sensitivity by HPLC with UV detection (16). In fact, we have measured this epoxycholesterol concentration in hepatic tissues by high-resolution GC-MS after trimethylsilyl (TMS) ether derivatization (32). However, the derivative became decomposed during GC separation, giving several peaks with similar mass spectra, and 100 fmol of 24S,25-epoxycholesterol was barely detectable on-column. Although this sensitivity exceeded that obtained by the HPLC-UV method (16), it was still not sufficient to quantify this epoxycholesterol in small amounts of biological samples.

Another merit of highly sensitive quantification is that the loading amount on the HPLC column can be minimized, so that the solid-phase extraction/purification step was omitted in our assay. In human serum analysis, less than 20 pg of oxysterol picolinates was injected on the column with approximately 200 ng of cholesterol picolinate. Under our HPLC conditions, this amount of cholesterol picolinate was easily trapped in the column and eluted around 29 min, which was well separated from oxysterols and did not affect the separation or elution of each oxysterol picolinate. HPLC column separation was very important in the present method because many oxysterols have the same molecular weight and MS spectrum. By changing the collision energies, the specific MS/MS spectrum of each oxysterol was observed to some extent, but we selected less-specific SRM ion pairs rather than more-specific ones because the former showed higher sensitivities and better signal-to-noise ratios compared with the latter.

The procedure for picolinyl ester derivatization was essentially the same as that in our previous report (23), but a few modifications were made. First, the reagent mixture was prepared by using pyridine instead of tetrahydrofuran, and the incubation was performed at 80°C for 60 min. Usually, this esterification progresses easily at room temperature, but the only hydroxyl at the C-25 position of 25-hydroxycholesterol was resistant to picolinyl ester formation. However, complete esterification of this C-25 position was achieved by heating at 80°C for 60 min. After the derivatization step, excess reagents were precipitated by the addition of *n*-hexane, and picolinyl ester derivatives were recovered in the supernatant.

Serum total (free + esterified) oxysterol concentrations in 19 normal volunteers were measured by our LC-ESI-MS/MS method (**Table 5**), and the concentrations of 4β-hydroxycholesterol, 7α-hydroxycholesterol, 22R-hydroxycholesterol, 25-hydroxycholesterol, and 24S,25-epoxycholesterol looked higher than those determined by previous methods. However, 7α-hydroxycholesterol levels determined by our method did not differ significantly ($P > 0.05$) from those by the GC-MS method (33), and 22R-hydroxycholesterol and 24S,25-epoxycholesterol levels appeared to be less than the detection limits by the HPLC method (34). We cannot exclude the possibility that some 25-hydroxycholesterol was produced by cholesterol autoxidation, but it is also possible that the concentration was not quantified accurately by the low-resolution GC-MS method. This is because the TMS ether derivative of 25-hydroxycholesterol did not give an ideal mass spectrum in the high mass region and *m/z* 131 was used for the quantification by selected ion monitoring. In general, high background noise is expected when a low mass number is selected as a monitoring ion for GC-MS analysis of biological samples. We have measured 25-hydroxycholesterol and 4β-hydroxycholesterol concentrations by using different SRM ion pairs [*m/z* 635 → 146 (22 V) and *m/z* 635 → 512 (20 V), respectively], and virtually the same results have been obtained.

A recent study using Cyp27a1 knockout mice demonstrated that 25-hydroxycholesterol was also synthesized by CYP27A1 (6). Our results showed that not only 27-hydroxycholesterol but also 25-hydroxycholesterol concentrations were markedly lower in serum from a patient with CTX, CYP27A1 deficiency, compared with that from a control subject (Fig. 2C, D), which lends support to the idea that a portion of the 25-hydroxycholesterol circulating in human serum is derived from CYP27A1.

In summary, we have developed a very sensitive and specific method for the quantification of key regulatory oxysterols in biological samples. Derivatization of dihydroxy- and epoxysterols into the picolinyl esters allowed them to be quantified by LC-ESI-MS/MS with excellent sensitivity and reliability. This method is useful for the study of

TABLE 5. Concentrations of total (free + esterified) oxysterols in normal human serum: comparison with previous methods

	Present Method	Previous Methods	
Oxysterol	Mean ± SD (n = 19)	Mean ± SD (n)	Method (Reference)
	ng/ml	*ng/ml*	
4β-Hydroxycholesterol	77 ± 40	29 ± 10 (125)	GC-MS (2)
7α-Hydroxycholesterol	145 ± 82	99 ± 43 (12) 43 ± 48 (31)	GC-MS (33) GC-MS (1)
22R-Hydroxycholesterol	10 ± 18	ND (2)	HPLC (34)
24S-Hydroxycholesterol	51 ± 12	64 ± 24 (31) 64 ± 14 (22)	GC-MS (1) LC-APCI-MS (20)
25-Hydroxycholesterol	31 ± 11	2 ± 3 (22)	GC-MS (1)
27-Hydroxycholesterol	117 ± 35	154 ± 43 (31) 120 ± 30 (22)	GC-MS (1) LC-APCI-MS (20)
24S,25-Epoxycholesterol	2 ± 2	ND (2)	HPLC (34)

LC-APCI-MS, liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry; ND, not detectable.

lipid metabolism controlled by oxysterols as well as the screening and diagnosis of metabolic disorders in oxysterols.

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