

## methods

# Highly sensitive quantification of 7 $\alpha$ -hydroxy-4-cholesten-3-one in human serum by LC-ESI-MS/MS

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**Abstract** We describe a highly sensitive and specific method for the quantification of serum 7 $\alpha$ -hydroxy-4-cholesten-3-one (C4), which has been used as a biomarker for bile acid biosynthesis. This method is based upon a stable isotope dilution technique by liquid chromatography-tandem mass spectrometry (LC-MS/MS). C4 was extracted from human serum (2–50  $\mu$ l) by a salting-out procedure, derivatized into the picolinoyl ester (C4-7 $\alpha$ -picolinate), and then purified using a disposable C<sub>18</sub> cartridge. The resulting picolinoyl ester derivative of C4 was quantified by LC-MS/MS using the electrospray ionization mode. The detection limit of the C4 picolinoyl ester was found to be 100 fg (signal-to-noise ratio = 10), which was  $\sim$ 1,000 times more sensitive than the detection limit of C4 with a conventional HPLC-ultraviolet method. The relative standard deviations between sample preparations and between measurements by our method were calculated to be 5.7% and 3.9%, respectively, by one-way layout analysis. The recovery experiments were performed using serum spiked with 20.0–60.0 ng/ml C4 and were validated by a polynomial equation. The results showed that the estimated concentration with 95% confidence limit was  $23.1 \pm 2.8$  ng/ml, which coincided completely with the observed  $\bar{X}_0 \pm SD = 23.3 \pm 1.0$  ng/ml with a mean recovery of 93.4%. This method provides highly reliable and reproducible results for the quantification of C4, especially in small volumes of blood samples.—Honda, A., K. Yamashita, M. Numazawa, T. Ikegami, M. Doy, Y. Matsuzaki, and H. Miyazaki. Highly sensitive quantification of 7 $\alpha$ -hydroxy-4-cholesten-3-one in human serum by LC-ESI-MS/MS. *J. Lipid Res.* 2007; 48: 458–464.

**Supplementary key words** bile acid synthesis • cholesterol 7 $\alpha$ -hydroxylase • picolinic acid • liquid chromatography electrospray ionization-tandem mass spectrometry

The conversion of cholesterol to bile acids is a major reaction for the catabolism of cholesterol in the body (1).

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Cholesterol 7 $\alpha$ -hydroxylase (CYP7A1) is the rate-limiting enzyme in one of the pathways of bile acid biosynthesis (2). Bile acid synthesis by this classic pathway accounts for <50% of total bile acid production in rats (3) and mice (4), whereas it represents a major route synthesizing >90% of total bile acids in humans (5). Therefore, the measurement of CYP7A1 activity is clinically useful for exploring the mechanisms of hypercholesterolemia (6) and evaluating the effects of hypcholesterolemic treatments (7, 8).

Because CYP7A1 is expressed solely in the liver, an invasive liver biopsy is necessary to measure the activity of this enzyme. However, a breakthrough of this problem was achieved in 1987 by Björkhem et al. (9). They quantified the plasma concentrations of 7 $\alpha$ -hydroxycholesterol, the immediate product of CYP7A1, by gas chromatography-mass spectrometry with selected ion monitoring (GC-SIM) and reported that this measurement reflected hepatic CYP7A1 activities in humans.

In 1988, Axelson, Aly, and Sjövall (10) determined the plasma concentrations of 7 $\alpha$ -hydroxy-4-cholesten-3-one (C4), a product of the next oxidative enzymatic reaction after CYP7A1, using HPLC with ultraviolet (UV) detection and found out that C4 is also a good marker for CYP7A1 activity in humans (11). It was subsequently reported that serum concentrations of 7 $\alpha$ -hydroxycholesterol (12) and C4 (13) reflected not only CYP7A1 activities but also bile acid synthesis in humans.

We also developed new high-resolution GC-SIM methods for the quantification of these sterols in plasma. We found that there was a significant correlation between

Abbreviations: C4, 7 $\alpha$ -hydroxy-4-cholesten-3-one; CYP7A1, cholesterol 7 $\alpha$ -hydroxylase; ESI, electrospray ionization; GC-SIM, gas chromatography-mass spectrometry with selected ion monitoring; LC-MS/MS, liquid chromatography-tandem mass spectrometry; SRM, selected reaction monitoring; UV, ultraviolet.

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hepatic CYP7A1 activities and plasma levels of free but not esterified or total 7 $\alpha$ -hydroxycholesterol (14) and that CYP7A1 activities correlated better with plasma levels of C4 than those of free 7 $\alpha$ -hydroxycholesterol (15). In addition, we applied these methods to monitor the circadian rhythms of these sterol levels in human plasma. A zero-amplitude test (15) revealed that C4 was the most reliable plasma biomarker for hepatic CYP7A1 activity. Furthermore, our recent study demonstrated that plasma C4 relative to cholesterol is a better marker for hepatic CYP7A1 activity than the absolute concentration when hypercholesterolemia is present (16).

The aim of this study was to develop a simple, more sensitive and reliable method to quantify C4 in a small volume of human serum. For this purpose, we derivatized C4 into the picolinoyl ester (C4-7 $\alpha$ -picolinate) and determined the concentrations using liquid chromatography-tandem mass spectrometry (LC-MS/MS) coupled in electrospray ionization (ESI) mode.

## MATERIALS AND METHODS

### Chemicals

C4 and 7 $\beta$ -hydroxycholesterol were purchased from Steraloids (Wilton, NH). [25,26,26,26,27,27,27- $^2$ H<sub>7</sub>]7 $\alpha$ -hydroxycholesterol was prepared as described previously (17). [ $^2$ H<sub>7</sub>]C4 and 7 $\beta$ -hydroxy-4-cholesten-3-one were synthesized from [ $^2$ H<sub>7</sub>]7 $\alpha$ -hydroxycholesterol and 7 $\beta$ -hydroxycholesterol, respectively, with cholesterol oxidase (18). 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. 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

C4 was extracted from serum by the salting-out method described previously (15). Fifty microliters of serum was diluted with 200  $\mu$ l of distilled water in a microcentrifuge tube (1.5 ml; Eppendorf, Hamburg, Germany), and 1 ng of [ $^2$ H<sub>7</sub>]C4 in 500  $\mu$ l of acetonitrile was added as an internal standard. After the addition of ~100 mg of ammonium sulfate, the sample tube was vortexed for 1 min and centrifuged at 2,000 g for 5 min. The supernatant acetonitrile phase was collected and evaporated to dryness under nitrogen.

Derivatization to the picolinoyl ester was performed according to the method of Yamashita et al. (19) 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  $\mu$ l) and triethylamine (20  $\mu$ l) were added to the extract, and the reaction mixture was allowed to stand at room temperature for 30 min. After the ad-

dition of 0.5 ml of 5% sodium bicarbonate, the mixture was applied to a Bond Elut C<sub>18</sub> cartridge (100 mg; Varian, Harbor City, CA) preconditioned with 1 ml of tetrahydrofuran, 1 ml of methanol, and 2 ml of distilled water. The cartridge was washed with 1 ml of 5% sodium bicarbonate, 1 ml of distilled water, 2 ml of 5% HCl, 1 ml of distilled water again, and 2 ml of acetonitrile-water (1:1, v/v). The picolinoyl ester derivative of C4 was then eluted with 4 ml of acetonitrile-water (95:5, v/v) containing 0.1% acetic acid. After evaporation, the residue was redissolved in 50  $\mu$ l of the same solvent, and an aliquot (1  $\mu$ l) was injected into the 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  $\times$  2.1 mm, 3  $\mu$ m; Thermo Fisher Scientific) at 40°C. The mobile phase consisted of acetonitrile-water (95:5, v/v) containing 0.1% acetic acid and was used at a flow rate of 200  $\mu$ l/min. The general LC-MS/MS conditions were as follows: spray voltage, 1,000 V; vaporizer temperature, 350°C; sheath gas (nitrogen) pressure, 35 p.s.i.; auxiliary gas (nitrogen) flow, 45 arbitrary units; ion transfer capillary temperature, 350°C; collision gas (argon) pressure, 1.5 mTorr; collision energy, 16 V; and ion polarity, positive.

### Evaluation of the solid-phase purification process

The absolute recovery of C4-7 $\alpha$ -picolinate from the Bond Elut C<sub>18</sub> cartridge was determined by the addition of [ $^2$ H<sub>7</sub>]C4-7 $\alpha$ -picolinate (1 ng) to the cartridge with a derivatizing mixture, which was prepared from 50  $\mu$ l of serum without adding [ $^2$ H<sub>7</sub>]C4. After the purification steps, the picolinoyl ester of 7 $\beta$ -hydroxy-4-cholesten-3-one (1 ng) was added to the eluate as an internal standard, and both [ $^2$ H<sub>7</sub>]C4-7 $\alpha$ -picolinate and 7 $\beta$ -hydroxy-4-cholesten-3-one-7 $\beta$ -picolinate were quantified by LC-selected reaction monitoring (SRM).

To calculate the elimination rate of cholesterol by the Bond Elut C<sub>18</sub> cartridge, unesterified cholesterol concentration in the first salting-out extraction was measured by colorimetric enzyme assay. The extract was then derivatized and purified by the cartridge, and the picolinoyl ester of [ $^2$ H<sub>7</sub>]cholesterol (100 ng) was added to the final eluate as an internal standard. Cholesterol-3-picolinate and its  $^2$ H<sub>7</sub> variant were quantified by the LC-SIM method to measure the absolute amount of cholesterol-3-picolinate contaminant in the final eluate.

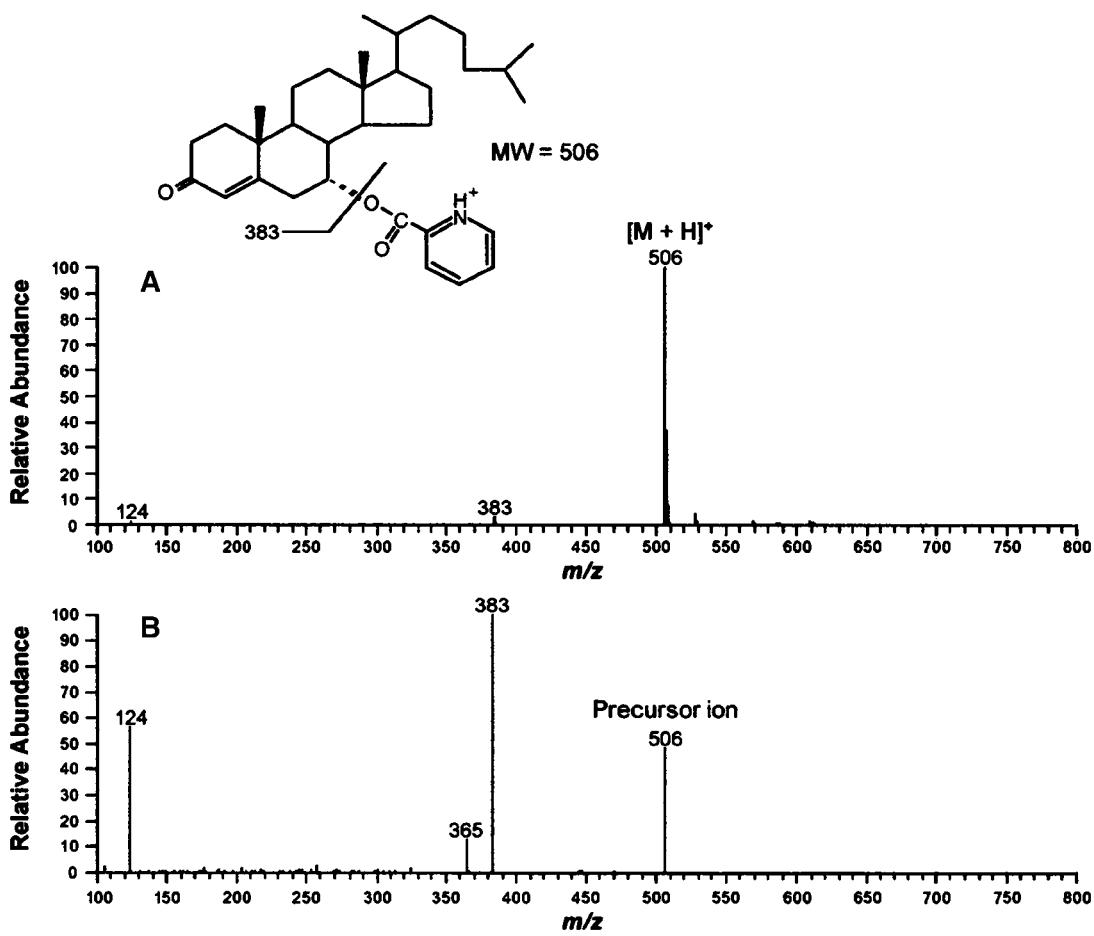
### Statistics

Data are reported as means  $\pm$  SD. Reproducibility was analyzed by one-way layout (JMP software; SAS Institute, Inc., Cary, NC). Recovery was analyzed using a polynomial equation (20). Linearity of the calibration curve and correlation between serum volume and the amount of C4 were analyzed by simple linear regression. The regression analysis was also used to calculate the estimated amount  $\pm$  95% confidence limit in the recovery study. For all analyses, significance was accepted at  $P < 0.05$ .

## RESULTS

### SRM

A typical ESI positive mass spectrum of the C4-7 $\alpha$ -picolinate is shown in Fig. 1A. This picolinoyl ester deriv-



**Fig. 1.** Typical electrospray ionization (ESI) positive mass spectrum (A) and product ion mass spectrum using  $m/z$  506 as a precursor ion (B) of the picolinoyl ester derivative of  $7\alpha$ -hydroxy-4-cholesten-3-one (C4). Mass spectrometric conditions were as follows: introducing solvent, acetonitrile-water (95:5, v/v) containing 0.1% acetic acid; flow rate, 200  $\mu$ l/min; spray voltage, 1,000 V; collision energy, 16 V.

ative 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, as shown in Fig. 1B. The SRM was conducted using  $m/z$  506  $\rightarrow$   $m/z$  383 for the C4- $7\alpha$ -picolinate and  $m/z$  513  $\rightarrow$   $m/z$  390 for the  $^2H_7$  variant.

#### Sensitivity of the present method

To determine the sensitivity of our LC-SRM method, the standard C4- $7\alpha$ -picolinate solution was diluted and injected into the LC-MS/MS system. As shown in Fig. 2A, the C4- $7\alpha$ -picolinate was easily detected to 100 fg, with a signal-to-noise ratio of 10, whereas conventional UV detection at 241 nm was barely able to detect 100 pg of C4 (Fig. 2B).

#### Absolute recovery of C4 and elimination rate of cholesterol in the solid-phase purification process

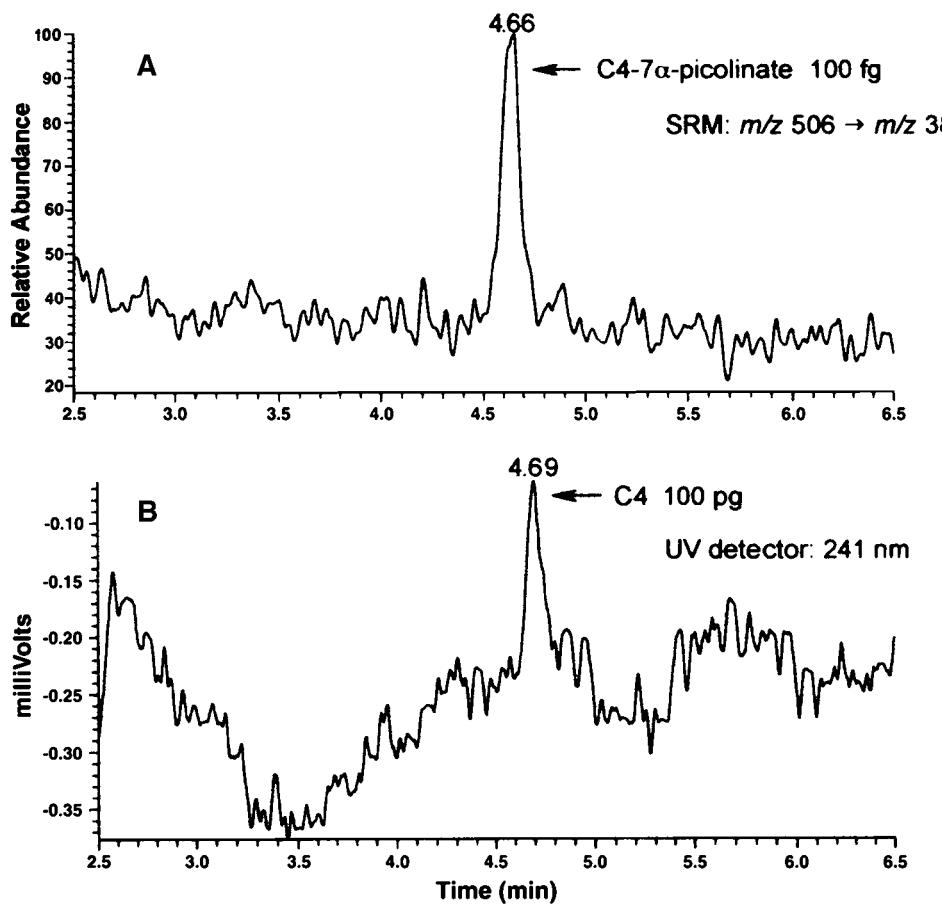
In this absolute recovery,  $[^2H_7]C4-7\alpha$ -picolinate was used instead of the corresponding C4 derivative and was quantified by SRM using the picolinoyl ester of  $7\beta$ -hydroxy-4-cholesten-3-one as an internal standard. Both picolinoyl esters of C4 and  $7\beta$ -hydroxy-4-cholesten-3-one

exhibited similar mass spectra, but the retention times were 4.66 and 5.00 min, respectively. Thus, the recovery of  $[^2H_7]C4-7\alpha$ -picolinate was  $104.0 \pm 2.3\%$  ( $n = 4$ ).

The elimination rate of cholesterol by the Bond Elut C<sub>18</sub> cartridge was calculated by quantifying cholesterol-3-picolinate contamination in the final eluate. The multiple-ion detector was focused on  $m/z$  492 for cholesterol-3-picinate and  $m/z$  499 for  $[^2H_7]$  cholesterol-3-picinate. Under the same HPLC conditions as for the determination of C4, the retention time of cholesterol-3-picinate was  $\sim$ 11 min. The elimination rate of cholesterol by the cartridge was calculated as  $98.6 \pm 0.7\%$  ( $n = 4$ ).

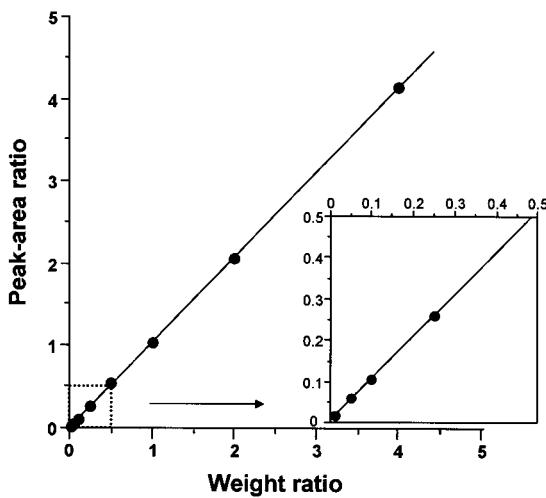
#### Calibration curve

A calibration curve was established for C4 (Fig. 3). Different amounts (0.01, 0.05, 0.1, 0.25, 0.5, 1, 2, and 4 ng) of authentic C4 were mixed with 1 ng of  $[^2H_7]C4$ , derivatized to the picolinoyl ester, and purified by the Bond Elut C<sub>18</sub> cartridge, as described in Materials and Methods. The weight ratio of C4, relative to the corresponding deuterated internal standard, was plotted on the abscissa, and the peak area ratio of the C4- $7\alpha$ -picolinate to the  $^2H_7$  variant, as measured by LC-SRM, was plotted



**Fig. 2.** Comparison of the detection limit of C4-7 $\alpha$ -picolinate by selected reaction monitoring (SRM) at  $m/z$  506 →  $m/z$  383 (A) with that of C4 by ultraviolet (UV) detection at 241 nm (B). Authentic standard of C4-7 $\alpha$ -picolinate (100 fg) or C4 (100 pg) was injected into the HPLC system.

on the ordinate. The linearity of the standard curve, as determined by simple linear regression, was excellent for weight ratios between 0.01 and 4.0 ( $n = 8$ ;  $r = 1.000$ ;  $P < 0.0001$ ).



**Fig. 3.** Calibration curve for the weight ratio of C4 to the corresponding deuterated internal standard. Linearity was checked by simple linear regression, and the equation for the line of best fit was  $y = 1.035x + 0.008$  ( $n = 8$ ;  $r = 1.000$ ;  $P < 0.0001$ ).

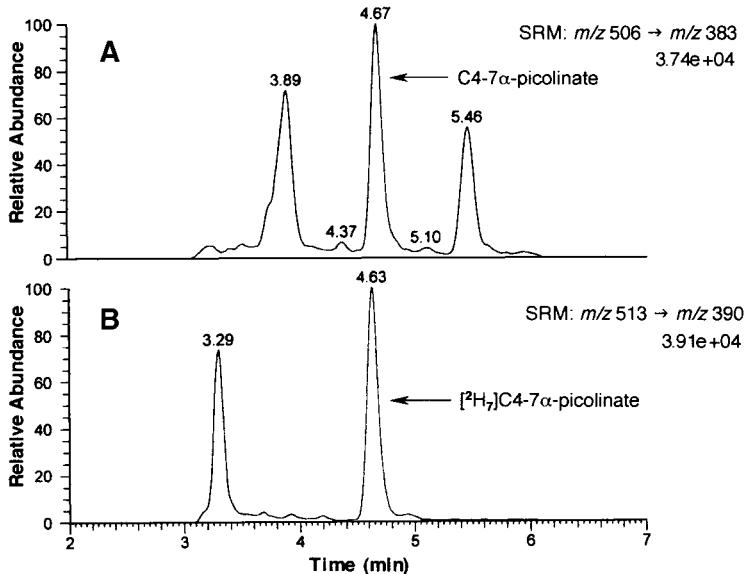
#### Representative LC-SRM

Figure 4 shows typical LC-SRM chromatograms of C4-7 $\alpha$ -picolinate and the  $^2\text{H}_7$  variant obtained from 50  $\mu\text{l}$  of normal human serum. Both peaks corresponded to  $\sim 20$  pg. The peak appearing at 5.46 min on this chromatogram (Fig. 4A) was identified as the picolinoyl ester derivative of 7-oxo-cholesterol by comparison with authentic 7-oxo-cholesterol-3-picolinate.

#### Precision and accuracy of the present method

The following studies were performed to determine the precision and accuracy of our method using the same serum obtained from a normal human subject. Reproducibility was investigated by analyzing four samples in triplicate by LC-SRM (Tables 1, 2). The results were analyzed by one-way layout, in which the analytical errors were divided into two sources of sample preparation and LC-SRM measurement. The variances were considered to be attributable to the measurement, because the errors during sample preparation were negligible. The interassay coefficients of variation for the between- and within-sample variations were 5.7% and 3.9%, respectively.

For the recovery experiment (Table 3), known amounts of C4 (1.0–3.0 ng, 20.0–60.0 ng/ml) were spiked into 50  $\mu\text{l}$  aliquots of the serum samples. After the clean-up



**Fig. 4.** Representative LC-SRM chromatograms of C4-7 $\alpha$ -picolinate (A) and [ $^2\text{H}_7$ ]C4-7 $\alpha$ -picolinate (B) obtained from 50  $\mu\text{l}$  of normal human serum. The peak of C4-7 $\alpha$ -picolinate corresponds to  $\sim$ 20 pg (20 ng/ml).

procedure, LC-SRM was carried out in triplicate for each sample. The recoveries of the known spiked amounts of C4 ranged from 91.9% to 94.3%, with a mean of 93.4%. In addition, the amount of endogenous C4 found in unspiked 50  $\mu\text{l}$  of serum was within the 95% confidence limit for the estimated amount of C4 calculated by linear regression analysis, which also constituted an index for the precision and accuracy of the method.

#### Application to a microscale assay

We studied the effect of reduced serum volume on the quantification of C4 amount. Different volumes (2, 5, 10, 25, and 50  $\mu\text{l}$ ) of normal human serum were diluted to 250  $\mu\text{l}$  with distilled water, and extraction and quantification of C4 were conducted, as described in Materials and Methods. As shown in Fig. 5, proportionality was observed when the volume of serum ranged from 2  $\mu\text{l}$  to at least 50  $\mu\text{l}$  for the amount of C4.

#### DISCUSSION

HPLC with UV detection has been the most commonly used method for the determination of serum C4 concentrations (10, 11, 13, 21, 22). In HPLC-UV methods, a great deal of effort has been made to find appropriate internal standards and to develop superior clean-up procedures. In

the latest method by Gälman et al. (22), 7 $\beta$ -hydroxy-4-cholesten-3-one was added as an internal standard, and a commercially available solid-phase extraction column ( $C_8$ ) was used at a temperature of 64°C. One weak point of this method is that the sensitivity was not sufficient to quantify C4 in limited amounts of human serum. At least 1 ml of serum was needed for each assay, which is a considerably large volume for clinical blood chemistry tests.

We previously developed a more sensitive and specific method based on a stable isotope dilution technique using high-resolution GC-SIM (15). In this method, C4 was extracted from 200  $\mu\text{l}$  of human plasma by a salting-out extraction, purified by a commercially available solid-phase extraction column ( $C_{18}$  and unbonded silica) at room temperature, and converted to the methyloxime-dimethylsilyl ether derivative before GC-SIM analysis. However, the sensitivity of this method was still not sufficient for the quantification of C4 in limited amounts of human serum, because the resulting derivatives exhibited two peaks of *syn* and *anti* isomers and their fragmentation patterns were not simple.

LC-MS or LC-MS/MS has come to be used more readily than GC-MS, because these methods do not always require

TABLE 2. Reproducibility in the quantification of C4 in human serum: ANOVA

Source	S	f	V	$F_0$	Relative SD	%
Sample preparation	5.23	3	1.74	2.08	5.7	
Error (selected reaction monitoring)	6.72	8	0.84		3.9	
Total	11.95	11				
				$F(3,8,0.05)$ = 4.07		

S, residual sum of squares; f, number of degrees of freedom;  $f_1$ , f<sub>sample preparation</sub>;  $f_2$ , f<sub>error</sub>; V, unbiased variance;  $F_0$ , observed value following Fdistribution variance ratio ( $V_{\text{sample preparation}}/V_{\text{error}}$ );  $F(f_1, f_2, \alpha)$ , density function of F distribution with  $f_1$  and  $f_2$  degrees of freedom.

C4, 7 $\alpha$ -hydroxy-4-cholesten-3-one. C4 was quantified in 50  $\mu\text{l}$  of normal human serum.

TABLE 3. Recovery of C4 from human serum

Sample ( $X_0 + na$ ) (n = 0,1,2,3)	Amount Added	Amount Found	Recovery <sup>a</sup> (mean $\pm$ SD)	Estimated Amount $\pm$ 95% Confidence Limit <sup>b</sup>
<i>ng/ml</i>				
$X_0$	0	$\bar{X}_0 \pm SD = 23.3 \pm 0.1^c$		
$X_0 + a$	20.0	43.1	44.2	43.4
$X_0 + a$	20.0	41.0	40.1	40.9
$X_0 + 2a$	40.0	60.6	59.1	62.6
$X_0 + 2a$	40.0	62.5	58.3	57.0
$X_0 + 3a$	60.0	82.7	78.1	76.6
$X_0 + 3a$	60.0	81.1	78.1	81.3
				<i>ng/ml</i>
				$23.1 \pm 2.8$

Known amounts of C4 were spiked into 50  $\mu$ l of normal human serum before sample preparation.

<sup>a</sup>Recovery (%) = (amount found -  $\bar{X}_0$ ) / amount added  $\times$  100.

<sup>b</sup>The estimated amount was calculated by linear regression analysis.

<sup>c</sup>This value was obtained from Table 1.

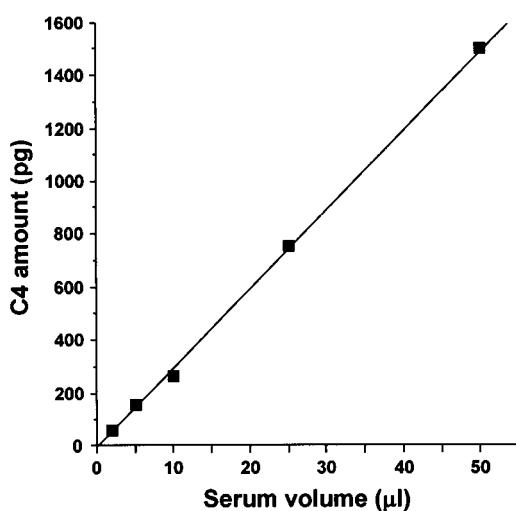
a derivatization step. However, it is also true that the introduction of charged moieties enhances the ionization efficiency of neutral steroids in ESI and atmospheric pressure chemical ionization processes. A permanently charged *N*-methylpyridyl group was introduced into the hydroxyl group of cholesterol (23) and 5 $\alpha$ -dihydrotestosterone (24, 25), whereas a permanently charged quaternary pyridinium moiety was introduced to the carbonyl group of oxosteroids with Girard P reagent (26–28) or 2-hydrazino-1-methylpyridine (29). Very recently, Yamashita et al. (19) developed a new picolinoyl derivatization that is a simple and versatile method suitable for the sensitive and specific quantification of hydroxysteroids by positive ESI-LC-MS/MS. Interestingly, because the picolinoyl group is not charged permanently, even in the case of estradiol with two hydroxyl groups, a single charged ion was observed in the positive ESI mass spectrum. Therefore, picolinoyl ester may be a suitable derivative not only for monohydroxy steroids but also for hydroxycholesterols with plural hydroxyl groups.

Then, we applied this derivatization to quantify C4 in a very small volume of human serum by LC-SRM. The picolinoyl ester of C4 exhibited [M+H]<sup>+</sup> as a single ion in ESI mode. The detection limit of C4 (as the C4 picolinoyl ester) in SRM mode was 100 fg, which was ~1,000 and 30 times more sensitive than that by the HPLC-UV and high-resolution GC-SIM methods, respectively. The detection limit of 100 fg corresponds to 0.1 ng/ml in 50  $\mu$ l of serum, whereas the range of serum C4 concentrations has been reported to be 3–40 ng/ml (median, 12 ng/ml) in healthy subjects (10). Consequently, even when a sufficient volume of serum was not available, C4 concentrations could be determined by our method using a few microliters of serum. However, we recommend that 50  $\mu$ l of serum be used for routine assay because some subjects treated with chenodeoxycholic acid (11) and patients with CYP7A1 deficiency (6) appear to exhibit unusually low serum concentrations of C4.

The derivatization and purification steps in this method are very simple. After the previously described salting-out extraction procedure (15), hydroxysteroids, including C4 and cholesterol, were derivatized to the picolinoyl esters within 30 min at room temperature. The C4-7 $\alpha$ -picolinate was completely recovered from a commercially available C<sub>18</sub> column with >98% elimination of cholesterol-3-picoline. However, the amount of cholesterol-3-picoline contaminant in the final eluate was still 300 times greater than that of C4-7 $\alpha$ -picolinate. Therefore, the next injection into the LC-MS/MS system was not performed until cholesterol-3-picoline had eluted completely from the column (~12 min), whereas the retention time of C4-7 $\alpha$ -picolinate was ~4.7 min.

[<sup>2</sup>H<sub>7</sub>]C4 was added to serum as an ideal internal standard for quantification by SRM. Although the object of our study was to quantify a very small amount of C4, the specificity and reproducibility of this method were highly satisfactory.

In summary, a very sensitive and specific method for the quantification of C4 in human serum was developed. Derivatization of C4 into the picolinoyl ester made it possible to be quantified by LC-ESI-MS/MS with excellent sensitivity. Recovery and reproducibility experiments verified that this method provided analytical results with high reliability and reproducibility. ■



**Fig. 5.** Effects of reduced serum volume on quantification of the amount of C4. Linearity was checked by simple linear regression, and the equation for the line of best fit was  $y = 30.23x - 7.83$  ( $n = 5$ ;  $r = 0.999$ ;  $P < 0.0001$ ).

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