methods

Determination of 24S- and 27-hydroxycholesterol in plasma by high-performance liquid chromatography-mass spectrometry

Ines Burkard, Katharina M. Rentsch,[1] and Arnold von Eckardstein

Institute of Clinical Chemistry, University Hospital Zurich, CH-8091 Zurich, Switzerland

Abstract **24S-Hydroxycholesterol (24S-OH-Chol) and 27-hydroxycholesterol (27-OH-Chol) are oxidized derivatives of cholesterol and of potential diagnostic interest because their circulating levels may reflect the cholesterol metabolism of the brain and macrophages, respectively. We developed a sensitive and specific HPLC-MS method for the quantification of 24S-OH-Chol and 27-OH-Chol in human plasma. In contrast to currently available procedures based on gas chromatography-mass spectrometry, this methodology offers the advantage that no time-consuming derivatization is needed. After saponification, solid-phase extraction, and HPLC separation under reversed-phase column conditions, detection by MS was performed using atmospheric pressure chemical ionization and selected ion monitoring mode. The standard curves were linear throughout the calibration range for both oxysterols. Within-day and between-day coefficients of variation were less than 9%, and the recoveries ranged between 98% and 103%. The quantification limits were 40 and 25 μg/l for 24S-OH-Chol and 27-OH-Chol, respectively. Mean values for both oxysterols were determined in plasma from 22 healthy volunteers. The sensitive and selective HPLC-MS method described here combined with the appropriate workup procedure allow the quantification of 24S-OH-Chol and 27-OH-Chol in plasma samples, for example in clinical studies to elaborate the clinical usefulness of these two oxysterols.**—Burkard, I., K. M. Rentsch, and A. von Eckardstein. **Determination of 24S- and 27-hydroxycholesterol in plasma by high-performance liquid chromatography-mass spectrometry.** ***J. Lipid Res.*** **2004.** 45: **776–781.**

Supplementary key words atmospheric pressure chemical ionization • selected ion monitoring • oxysterols

Both 24S-hydroxycholesterol (24S-OH-Chol) and 27-hydroxycholesterol (27-OH-Chol) are side chain-hydroxylated derivatives of cholesterol. Together with other mono-oxygenated metabolites of cholesterol, they are often referred to as oxysterols. In addition to being obligatory intermediates in bile acid synthesis, oxysterols activate the nuclear liver X receptors α and β and thereby modulate the transcription of several pivotal genes involved in lipid metabolism (1). Moreover, some of them are cytotoxic in cultured cells (2).

24S-OH-Chol is enzymatically formed by cholesterol 24-hydroxylase (CYP46) (3), a member of the cytochrome P450 family. In accordance with the predominant expression of CYP46 in the brain (4), 24S-OH-Chol present in the human circulation originates almost exclusively from the brain (5). Combined with a constant flux across the blood-brain barrier and subsequent hepatic metabolism into bile acids (6), conversion of cholesterol into the polar metabolite 24S-OH-Chol is an important mechanism for the elimination of excess cholesterol from the brain (7, 8). Because 24S-OH-Chol is particularly located in myelin, demyelination caused by neuronal degeneration results in an increased flux of 24S-OH-Chol across the blood-brain barrier (9). As a clinical consequence, plasma levels of 24S-OH-Chol were increased slightly in patients with early stages of Alzheimer's disease (10). However, the diagnostic value of plasma 24S-OH-Chol concentrations in Alzheimer's disease may be limited by the fact that the circulating levels are increased in vascular dementia as well (11). Therefore, further studies are needed to unravel the diagnostic relevance of 24S-OH-Chol in neurodegenerative and other cerebral diseases.

27-OH-Chol is the quantitatively most prevailing oxysterol in the human circulation (1) and is formed by sterol 27-hydroxylase (CYP27) (3). Cell culture experiments demonstrated that, among other mammalian cell types, human macrophages have the highest capacity to convert cholesterol to 27-OH-Chol (12). After release into the

Manuscript received 11 November 2003 and in revised form 6 January 2004.

Published, JLR Papers in Press, January 16, 2004.
DOI 10.1194/jlr.D300036-JLR200

Abbreviations: CTX, cerebrotendinous xanthomatosis; CYP27, sterol 27-hydroxylase; CYP46, cholesterol 24-hydroxylase; 27-OH-Chol, 27-hydroxycholesterol; 24S-OH-Chol, 24S-hydroxycholesterol.

[1] To whom correspondence should be addressed.
e-mail: rentsch@ikc.unizh.ch

blood and transport to the liver, 27-OH-Chol is converted to bile acids (4). In addition to HDL-dependent reverse cholesterol transport, the formation and excretion of 27-OH-Chol were suggested to serve as an alternative pathway for reducing cholesterol accumulation in macrophages (13). The importance of CYP27 and 27-OH-Chol for the regulation of cholesterol homeostasis in macrophages is illustrated by the clinical presentation of patients with cerebrotendinous xanthomatosis (CTX) (4, 14), which is caused by mutations in the gene coding for CYP27. Patients with CTX accumulate cholesterol and its metabolite cholestanol in xanthomas and suffer from premature atherosclerosis despite normal circulating levels of cholesterol (15).

Several methods for the analysis of cholesterol oxides in biological materials have been described. HPLC combined with UV detection is an accessible and low-cost technique, particularly for the determination of cholesterol auto-oxidation products in animal tissues (16) and cholesterol-containing foods (17). However, it has limitations attributable to the lack of specificity of the detection system. In contrast, MS represents a sensitive and selective detection technique that is especially effective for the analysis of biological samples. During the last decade, GC-MS has been applied for the determination of cholesterol oxides not only in foodstuffs (18) but also in human plasma (19, 20). A drawback of this analytical technique is the time-consuming derivatization step, which is required for the subsequent analysis of oxysterols by GC-MS. To date, HPLC-MS has been used to a very limited extent for the determination of cholesterol oxides. The published methods are restricted to the analysis of cholesterol auto-oxidation products in lyophilized beef (21) and processed food (22) and do not include enzymatically formed oxygenated derivatives of cholesterol such as 24S-OH-Chol and 27-OH-Chol. Therefore, we developed a method for the determination of 24S-OH-Chol and 27-OH-Chol in plasma by HPLC-MS.

To correct for variations in sample preparation and analysis, the 7-fold deuterated internal standard 24-OH-Chol-25,26,26,26,27,27,27-d_7 was added before sample processing. This deuterated analog of 24S-OH-Chol behaves almost identical to 24S-OH-Chol and 27-OH-Chol through all chemical and ionization processes and differs only in its mass-to-charge ratio (m/z), which enables quantification.

Oxysterols in human plasma are present not only as free sterols but also as fatty acid and sulfate esters. Approximately 70% of 24S-OH-Chol and 90% of 27-OH-Chol in plasma are esterified with fatty acids (20). Sulfate esters have been reported to account for ~10% of the total content of 24S-OH-Chol in human plasma (5). Because alkaline hydrolysis is included in the present method, the sum of free sterols and fatty acid esters is recorded.

The sensitive method presented here allows the quantification of 24S-OH-Chol and 27-OH-Chol in plasma and can be used in large studies to elucidate the clinical usefulness of these two oxysterols.

MATERIALS AND METHODS

Chemicals and reagents

24S-OH-Chol and 27-OH-Chol were purchased from Steraloids (Newport, RI), and 24-OH-Chol-25,26,26,26,27,27,27-d_7 was from Medical Isotopes (Pelham, NH). Solvents were of HPLC grade and obtained from Scharlau (Barcelona, Spain). Butylated hydroxytoluene was obtained from Fluka (Buchs, Switzerland), and all other chemicals and reagents were obtained from Merck ABS (Dietikon, Switzerland) in the highest purity available.

Sample collection

Blood was collected in evacuated blood collection tubes containing EDTA (3 ml Vacutainer, 0.184 M K_3EDTA; Becton Dickinson). After centrifugation at 2,000 *g* for 10 min, 50 μg of butylated hydroxytoluene was added per milliliter of plasma. The samples were stored at −70°C until analysis.

Sample preparation

For the hydrolysis of oxysterol esters, the plasma samples were first subjected to saponification. Plasma (0.5 ml) was transferred to a screw-capped vial, and 40 μl of deuterium-labeled internal standard (40 ng of 24-OH-Chol-25,26,26,26,27,27,27-d_7) was added. After the addition of 1.5 ml of freshly prepared 1 M ethanolic sodium hydroxide, alkaline hydrolysis was performed for 2 h at 50°C in a water bath. The samples were neutralized to pH 7 with 50% phosphoric acid (v/v) and 1 ml of 100 mM phosphate buffer, pH 7.0. After centrifugation for 5 min at 1,000 *g*, the supernatant (hydrolyzed plasma sample) was taken for the subsequent clean-up procedure performed by solid-phase extraction. The C_{18} cartridges (200 mg, Bond Elut; Varian, Zug, Switzerland) were preconditioned with 1 ml of *n*-heptane/2-propanol (50:50, v/v), 1 ml of methanol, and 2 ml of water. The hydrolyzed plasma sample was then applied to the cartridge using only gravity. Afterward, the cartridge was washed with 4 ml of methanol-water (75:25, v/v) and briefly dried under vacuum. The oxysterols were desorbed with 2 ml of *n*-heptane/2-propanol (50:50, v/v) using only gravity. The eluted substances were dried at 30°C by evaporation (Rotavapor; Büchi, Flawil, Switzerland), the residue was dissolved in 100 μl of methanol, and 40 μl of the residue was injected into the HPLC-MS system.

HPLC-MS

The HPLC system consisted of a Rheos 2000 pump (Flux Instruments, Basel, Switzerland), an HTC PAL autosampler (CTC Analytics, Zwingen, Switzerland), and a TSQ 7000 triple quadrupole mass spectrometer (Thermo-Quest Finnigan, San Jose, CA). The ionization mode was positive atmospheric pressure chemical ionization. The vaporizer temperature and the capillary temperature were kept at 450°C and 190°C, respectively, the discharge current was fixed at 6 μA, and the capillary voltage was set at 40 V. The auxiliary gas pressure was held at 5 U, and the sheath gas pressure was kept at 35 pounds per square inch.

Quantification of oxysterols was performed using the single ion monitoring mode. The mass spectra were characterized by fragment ions because of the secession of one and two molecules of water. No molecule ions have been observed. 24S-OH-Chol and 27-OH-Chol were finally detected by the fragment ion at m/z 385.4 $[M+H-H_2O]^+$, and 24-OH-Chol-25,26,26,26,27,27,27-d_7 was detected by the fragment ion at m/z 374.4 $[M+H-2H_2O]^+$.

The oxysterols were separated using a Nucleosil C_{18} HD, 5 μm particle size column (125 mm × 2 mm; Macherey-Nagel, Oensingen, Switzerland) protected with a guard column (8 × 4 mm; Macherey-Nagel).

For the first 14 min, the mobile phase consisted of methanol-acetonitrile-10 mM ammonium acetate buffer (pH 4.5) (45:40:15,

v/v/v) with a corresponding flow rate of 250 μl/min. The eluents were then linearly changed in a gradient system to methanol-acetonitrile (50:50, v/v) within a few seconds and maintained for 10 min to elute lipophilic substances. Afterwards, the eluents were changed to the original ratio and maintained for 11 min to enable equilibration of the column.

Determination of 24S-OH-Chol and 27-OH-Chol normal levels

Twenty-two healthy volunteers (11 men and 11 women) from the staff of our institute, between 19 and 57 years of age (mean age, 35 years) and with serum cholesterol concentration of 4.5 ± 1.0 mmol/l (mean ± SD), were studied for the determination of normal values. The blood samples were taken in the morning after an overnight fast.

RESULTS

Chromatographic separation

Representative mass chromatograms of plasma standards for 24S-OH-Chol and 27-OH-Chol are shown in **Fig. 1**. All peaks were symmetric and sufficiently resolved.

The retention times were 11.4 min for 24S-OH-Chol, 12.8 min for 27-OH-Chol, and 11.3 min for the internal standard.

Linearity

Defined amounts of 24S-OH-Chol and 27-OH-Chol dissolved in methanol were added to human plasma for the preparation of standards; their concentrations ranged from 40 to 400 μg/l for both oxysterols. These standard samples were extracted as described above. The standard curves were plotted as the peak area ratio of the respective compound to the internal standard versus the concentration and then corrected for endogenous oxysterols in the human plasma by subtracting blank peak area ratios.

The standard curves were linear in the calibration range for both oxysterols (**Fig. 2**). Least-squares regression data afforded correlation coefficients (n = 6, mean ± SD) of 0.9983 ± 0.0011 for 24S-OH-Chol and 0.9941 ± 0.0025 for 27-OH-Chol.

Sample material and stability

To investigate the importance of the sample matrix and sample storage on the measurement of 24S-OH-Chol and 27-OH-Chol, blood of a healthy volunteer was collected in different evacuated blood collection tubes containing EDTA, heparin, or no additives. Whole blood samples were stored at 25°C for 8 h and at 8°C for 24 h until centrifugation and analysis. Plasma or serum samples were either analyzed directly or stored at 25°C for a maximum of 24 h and at 8°C for a maximum of 48 h. After each centrifugation, 50 μg of butylated hydroxytoluene was added per milliliter of plasma or serum. Oxysterol concentrations determined in EDTA plasma, heparin plasma, or serum did not reveal any significant differences. Moreover, oxysterol levels of the immediately quantified blood samples did not differ significantly from any blood sample stored under the above-mentioned conditions before analysis. Because EDTA is able to quench potential metal ions that might contribute to auto-oxidation, EDTA plasma was chosen as the matrix for all subsequent experiments.

Recovery

To determine the efficiency of the extraction procedure, 24S-OH-Chol and 27-OH-Chol were analyzed without the addition of the internal standard. For this pur-

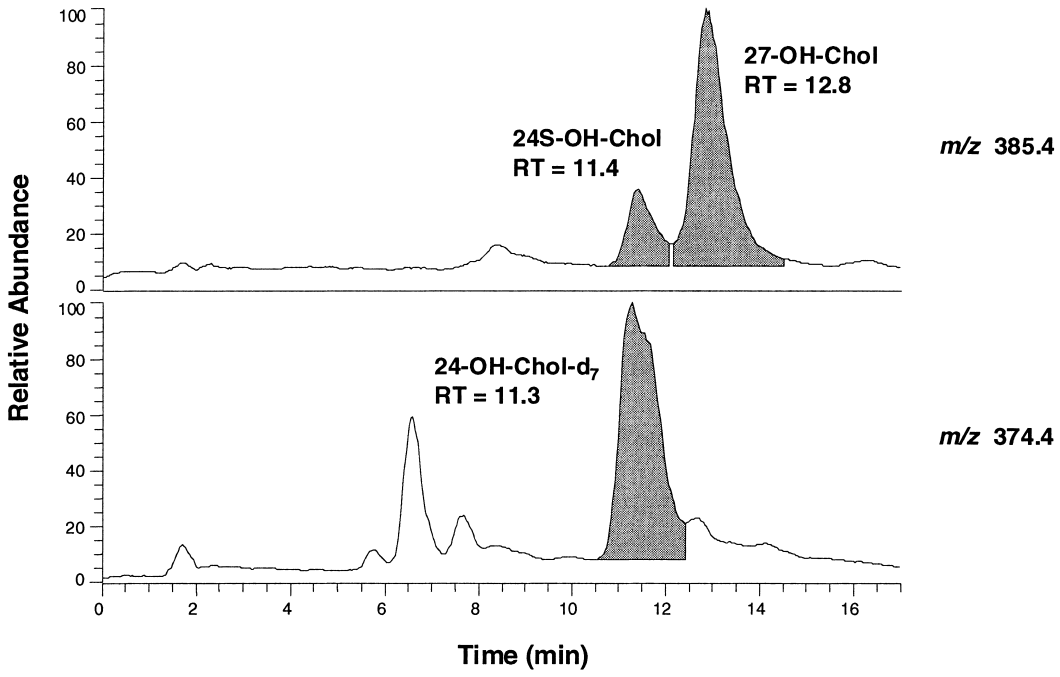

Fig. 1. Mass chromatograms of an extracted plasma standard spiked with 80.5 μg/l 24S-hydroxycholesterol (24S-OH-Chol) and 27-hydroxycholesterol (27-OH-Chol). RT, retention time (measured in minutes).

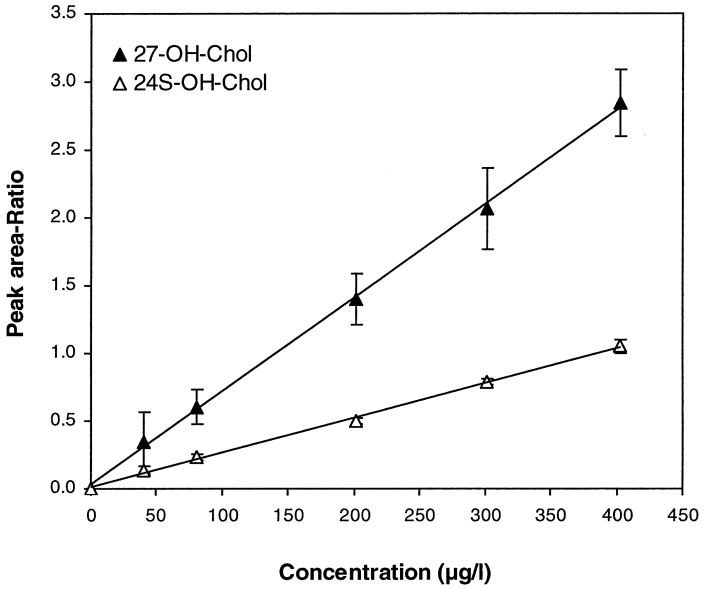

Fig. 2. Means and standard deviations of six separately recorded standard curves of 24S-OH-Chol and 27-OH-Chol. Plasma standards were spiked with defined amounts of 24S-OH-Chol and 27-OH-Chol, extracted, and analyzed by HPLC-MS (see Materials and Methods). To assess linearity, the line of best fit was determined by least-squares regression.

pose, defined amounts of both analytes were added to 0.5 ml of plasma (sample) or in a clean vial (standard). The samples were extracted six times as described above, whereas the standards were only evaporated; afterward, samples and standards were dissolved in methanol. The average peak areas of both oxysterols of the sample were compared with the corresponding peak areas of the standard.

The recoveries for 24S-OH-Chol were 102% (81 µg/l) and 99.1% (302 µg/l), and those for 27-OH-Chol were 103% (81 µg/l) and 98.6% (302 µg/l).

Precision and accuracy

For the determination of between-day and within-day precision, calibration curves for both oxysterols as well as for two samples of different oxysterol concentrations were analyzed once on six different days and six times on the same day, respectively. The accuracy of the method was assessed by expressing the mean of the assayed concentration as a percentage of the weight-in concentration.

The results of the precision and accuracy experiments are summarized in **Table 1**. All within-day and between-day coefficients of variation were less than 9%. The accuracy amounted to 100% to 108%.

Quantification limit

The quantification limit of the method was calculated using a signal-to-noise ratio of 3. For this purpose, the noise signal was obtained as the amplitude of the peaks from a segment of the chromatogram that preceded both peaks.

The quantification limits for plasma samples of 0.5 ml were 40 µg/l for 24S-OH-Chol and 25 µg/l for 27-OH-Chol.

Determination of 24S-OH-Chol and 27-OH-Chol normal levels

Oxysterols were determined in plasma from 22 healthy volunteers. The concentrations (mean ± SD) of 24S-OH-Chol and 27-OH-Chol were 64 ± 14 ng/ml (range, 39–91 ng/ml) and 120 ± 30 ng/ml (range, 67–199 ng/ml), respectively.

DISCUSSION

We describe a sensitive and selective HPLC-MS method together with the appropriate workup procedure for the

TABLE 1. Precision and accuracy of 24S-hydroxycholesterol and 27-hydroxycholesterol determination in plasma

Oxysterol	Concentration	No.	Mean	SD	Coefficient of Variation	Accuracy
	µg/l		*µg/l*	*µg/l*	%	%
24S-Hydroxycholesterol						
Within-day	80.5	6	83.8	4.6	5.5	104
	302	6	318	6.0	1.9	105
Between-day	80.5	6	87.1	5.4	6.2	108
	302	6	305	10.0	3.3	101
27-Hydroxycholesterol						
Within-day	80.5	6	80.4	3.1	3.8	99.8
	302	6	302	12.5	4.1	100
Between-day	80.5	6	81.8	6.7	8.2	102
	302	6	315	16.9	5.4	104

determination of 24S-OH-Chol and 27-OH-Chol, two oxysterols that are of potential diagnostic interest because their circulating concentrations may reflect the cholesterol metabolism of different organs or cells.

A crucial point in method development turned out to be the chromatographic separation of 24S-OH-Chol and 27-OH-Chol. Because both analytes have the same molecular mass and also behave identically through the ionization process, a sufficient peak resolution was a prerequisite for further analyses.

Validation data showed good within-day and between-day reproducibility for both oxysterols tested, with coefficients of variation of less than 9% and accuracy between 100% and 108%. These results ensure precise and accurate analysis within the calibration range. The recoveries for two different concentrations of 24S-OH-Chol and 27-OH-Chol ranged between 98% and 103%, which demonstrate no analyte losses during the workup procedure. These results indirectly support the general view that 24S-OH-Chol and 27-OH-Chol are enzymatically formed products rather than artifactual products of the auto-oxidation of cholesterol. In contrast to other oxysterols (e.g., 25-, 7α-, or 7β-OH-Chol) that are believed to be products of cholesterol auto-oxidation, 24S-OH-Chol and 27-OH-Chol can be determined without rigorous preanalytical adherence to antioxidative arrangements. The sole action implemented in this context involved the addition of butylated hydroxytoluene as the antioxidant to plasma, which ensured unaltered plasma composition until analysis. Even though sample storage under various conditions did not reveal significant differences in oxysterol concentrations, it is advisable to centrifugate the blood samples and to add a potential stabilizing reagent as soon as possible. Subsequent storage in the refrigerator or, for future analysis, at −70°C would certainly be an optimal prerequisite for an accurate analysis.

To account for any analyte losses throughout sample preparation and injection, quantification of oxysterols was accomplished by the use of a deuterium-labeled internal standard, 24-OH-Chol-25,26,26,26,27,27,27-d_7. Unfortunately, a deuterated analog of 27-OH-Chol is not commercially available at present. Even though this lack did not result in imprecise 27-OH-Chol quantification, a deuterated internal standard would probably decrease the standard deviations of 27-OH-Chol concentration measurements.

The current procedures for oxysterol analysis, usually based on GC-MS (19, 20), require a time-consuming derivatization step to convert the low volatile substances into more vaporable substances. The methodology described here offers the advantage that reproducible fragment ions can be observed without further derivatization. Nevertheless, if derivatization would lead to fragment ions that would undergo tandem mass spectrometry, this would probably increase the sensitivity and the specificity of the developed method.

Using our novel HPLC-MS technique, we found plasma concentrations of 24S-OH-Chol that were in the same range as those reported by authors who used GC-MS [64 ± 14 ng/ml versus 64 ± 24 ng/ml (20), 83 ng/ml (23), and 60 ± 21 ng/ml (11)]. However, we found 27-OH-Chol levels that were lower than those reported previously [120 ± 30 ng/ml versus 154 ± 43 ng/ml (20) and 159 ng/ml (23)]. Yet, it is important to note that all studies, including our own, analyzed a very limited number of subjects, which decreases the reliability and significance of the reported mean levels.

In conclusion, we present a HPLC-MS method for the accurate quantification of 24S-OH-Chol and 27-OH-Chol in plasma.

This work was supported by a grant from the University of Zurich, Forschungskommission (to K.M.R.).

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