Sensitive UHPLC-MS/MS quantification method for 4β- and 4α-hydroxycholesterol in plasma for accurate CYP3A phenotyping

Yosuke Suzuki1,* Ayako Oda1 Jun Negami1 Daiki Toyama1 Ryota Tanaka2 Hiroyuki Ono2 Tadasuke Ando3 Toshitaka Shin1 Hiromitsu Mimata4 Hiroki Itoh2 and Keiko Ohno1

1Department of Medication Use Analysis and Clinical Research, Meiji Pharmaceutical University, Kiyose, Tokyo, Japan; 2Department of Clinical Pharmacy, Oita University Hospital, Yufu, Oita, Japan; and 3Department of Urology, Oita University Faculty of Medicine, Yufu, Oita, Japan

Abstract 4β-Hydroxycholesterol (4β-OHC) is formed by Cytochrome P450 (CYP)3A and has drawn attention as an endogenous phenotyping probe for CYP3A activity. However, 4β-OHC is also increased by cholesterol autooxidation occurring in vitro due to dysregulated storage and in vivo by oxidative stress or inflammation, independent of CYP3A activity. 4α-hydroxycholesterol (4α-OHC), a stereoisomer of 4β-OHC, is also formed via autooxidation of cholesterol, not by CYP3A, and thus may have clinical potential in reflecting the state of cholesterol autooxidation. In this study, we establish a sensitive method for simultaneous quantification of 4β-OHC and 4α-OHC in human plasma using ultra-high performance liquid chromatography coupled to tandem mass spectrometry. Plasma samples were prepared by saponification, two-step liquid-liquid extraction, and derivatization using picolinic acid. Intense [M+H]+ signals for 4β-OHC and 4α-OHC di-picolinyl esters were monitored using electrospray ionization. The assay fulfilled the requirements of the US Food and Drug Administration guidance for bioanalytical method validation, with a lower limit of quantification of 0.5 ng/ml for both 4β-OHC and 4α-OHC. Apparent recovery rates from human plasma ranged from 88.2% to 101.5% for 4β-OHC, and 91.8% to 114.9% for 4α-OHC. Additionally, matrix effects varied between 86.2% and 117.6% for 4β-OHC and between 89.5% and 116.9% for 4α-OHC. Plasma 4β-OHC and 4α-OHC concentrations in healthy volunteers, stage 3–5 chronic kidney disease (CKD) patients, and stage 5D CKD patients as measured by the validated assay were within the calibration ranges in all samples. We propose this novel quantification method may contribute to accurate evaluation of in vivo CYP3A activity.

Supplementary key words cholesterol • cytochrome P450 • kidney • kinetics • pharmacokinetics • 4β-hydroxycholesterol • 4α-hydroxycholesterol • cytochrome P450 3A • mass spectrometry • plasma

*For correspondence: Yosuke Suzuki, y-suzuki@my-pharm.ac.jp.

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elderly people, and patients with kidney failure or liver diseases including cirrhosis (16–21).

Several quantification methods have been reported for the measurement of plasma 4β-OHC concentrations using gas chromatography coupled to mass spectrometry (11) and high-performance liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) (22–26). Recently, Hautajärvi et al. (27) reported an ultra-high performance liquid chromatography coupled to high resolution mass spectrometry method for quantification of plasma 4β-OHC and 4α-hydroxycholesterol (4α-OHC) concentrations. 4α-OHC, a stereoisomer of 4β-OHC, is formed via autooxidation of cholesterol, and not by CYP3A. Therefore, plasma 4α-OHC concentration reflects plasma sample stability, because plasma 4α-OHC concentration increases in uncontrolled storage condition (28). Furthermore, oxysterols including 4β-OHC and 4α-OHC have been reported to be elevated by cholesterol autoxidation due to oxidative stress or inflammation in the liver, regardless of CYP3A activity (29). Thus, simultaneous quantification of 4β-OHC and 4α-OHC is preferred for phenotyping of CYP3A activity using clinical plasma samples.

In this study, we established a sensitive method for simultaneous quantification of 4β-OHC and 4α-OHC in human plasma using ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS). The method was applied to measure plasma 4β-OHC and 4α-OHC concentrations in healthy volunteers and patients with chronic kidney disease (CKD).

MATERIAL AND METHODS

Chemicals

The standards of 4β-OHC and 4β-OHC-D7 (4β-hydroxycholesterol-25, 26, 27, 27, 27-D7, isotopically labelled internal standard of 4β-OHC) were purchased from Avanti Polar Lipids, Inc (Alabama) and 4α-OHC standard from Toronto Research Chemicals (Ontario, Canada). The standards of 7α-OHC, 7β-OHC, 22(R)-OHC, 22(S)-OHC, 24(S)-OHC, 25-OHC, and 27-OHC, isomers of 4β-OHC and 4α-OHC, were purchased from Sigma-Aldrich (St. Louis). Human serum albumin (HSA) was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Other reagents (2-methyl-6-nitrobenzoic anhydride, 4-dimethylamino-pyridine, and picolinic acid) were purchased from Tokyo Chemical Industry Co, Ltd (Tokyo, Japan). Other solvents (water, methanol, 2-propanol, acetonitrile, n-hexane, formic acid, pyridine, triethylamine, and 28% sodium methoxide methanol solution) were of the highest analytical quality and were purchased from FUJIFILM Wako Pure Chemical Corporation.

Healthy volunteers

Nine healthy volunteers who fasted for at least 8 h before blood sampling were recruited in this study. Blood was collected in tubes containing EDTA anticoagulant, centrifuged, and plasma samples were frozen at −80°C until measurement. Total cholesterol level was measured by enzymatic assay. This study was approved by the Ethics Committee of Meiji Pharmaceutical University (approval number: 202001). Prior explanations were provided to all volunteers to inform the scientific purpose of the study, and all volunteers gave written informed consent for participation in this study. Plasma obtained from healthy volunteers was used as unadulterated plasma for preparation of quality control (QC) samples and for validation purposes.

CKD patients

Blood samples were collected from 15 patients with stage 3–5 CKD and from 14 patients with stage 5D CKD undergoing hemodialysis in Oita University Hospital. The patients fasted for at least 8 h before blood sampling. CKD stages were classified by estimated glomerular filtration rate according to the evidence-based clinical practice guideline for CKD published by Japanese Society of Nephrology (https://link.springer.com/content/pdf/10.1007%2Fs10157-014-0949-2.pdf, accessed 16 July 2020). Blood samples collected in tubes containing EDTA anticoagulant were centrifuged, and plasma samples were frozen at −80°C until assay. Total cholesterol level was measured by enzymatic assay. This study was approved by the Ethics Committees of Oita University Hospital (approval number: 1815) and the Ethics Committee of Meiji Pharmaceutical University (approval number: 202016). Prior explanations were provided to all patients to inform the scientific purpose of the study, and all patients gave written informed consent for participation in this study.

Stock and working solutions

Independent 4β-OHC and 4α-OHC stock solutions were made separately for the preparation of calibration and QC samples. 4β-OHC and 4α-OHC were weighed in volumetric flasks and dissolved in 2-propanol (5 mg/50 ml and 1 mg/50 ml, respectively). The internal standard 4β-OHC-D7 was also weighed in volumetric flasks and dissolved in 2-propanol (20 μg/ml). The stock solutions were stored at −80°C. All calibrating and QC solutions contained a mixture of 4β-OHC and 4α-OHC. Eight different calibrating solutions containing equal concentrations of 4β-OHC and 4α-OHC at 10, 20, 40, 100, 400, 1000, 4000, and 10,000 ng/ml (final concentrations in calibration samples: 0.5, 1, 2, 5, 20, 50, 200, and 500 ng/ml) were prepared by diluting the respective calibrating stock solutions with 2-propanol. QC solutions were prepared at lower limit of quantification (LLOQ), low (LQA), medium (QMB), and high (QCC) concentrations containing equal concentrations of 4β-OHC and 4α-OHC at 10, 50, 200, and 8000 ng/ml (final concentrations in QC samples: 0.5, 15, 25, and 400 ng/ml), respectively, by diluting the respective QC stock solutions with 2-propanol.

Derivatization solution

A derivatization solution was prepared by mixing the following reagents: 250 mg of 2-methyl-6-nitrobenzoic anhydride, 75 mg of 4-dimethylamino-pyridine, 200 mg of picolinic acid, 7.5 ml of pyridine, and 1 ml of triethylamine. The derivatization solution was freshly prepared just before plasma sample preparation.

Plasma sample preparation

For measurement of 4β-OHC and 4α-OHC concentrations, plasma samples were pretreated as follows. In each 20-mL
safe-lock tube, 50 μl of subject’s plasma sample was mixed with 50 μl of internal standards solution (20 ng/ml of 4β-OHC-D2) and 25 μl of 2-propanol for volume adjustment. After adding 200 μl of 28% sodium methoxide methanol solution, the mixture was vortex-mixed for 30 s and left at room temperature (15−25°C) for 20 min. Then, 250 μl of water and 1 ml of n-hexane were added, vortex-mixed for 5 min, and centrifuged (10 min at 1,500 × g, 25°C). The supernatant (700 μl) was transferred to a clean 1.5-ml safe-lock tube and evaporated to dryness under a N2 gas stream at 40°C. The residue was reconstituted with 100 μl of derivatization solution, vortex-mixed, and transferred to an autosampler vial.

QC sample was prepared by spiking 50 μl of unadulterated plasma with 2.5 μl of QC solution containing 4β-OHC and 4α-OHC. Calibration sample was prepared by spiking 50 μl of 2% HSA solution (blank matrix) with 2.5 μl of calibration solution containing 4β-OHC and 4α-OHC. Each calibration or QC sample was mixed with 50 μl of internal standards solution. Then, these samples were processed as for subjects’ plasma samples.

Instrumental analysis parameters

The UHPLC-MS/MS system (Shimadzu, Kyoto, Japan) consisted of a Nexera X2 LC system and a quadrupole mass spectrometer (LCMS-8040). For chromatographic separation, a Waters Acquity BEH C18 column (1.7 μm, 2.1 × 150 mm) and a Waters Acquity BEH C8 VanGuard precolumn (1.7 μm, 2.1 × 5 mm) were used at 55°C. 0.1% aqueous formic acid (A) and acetonitrile with 0.1% formic acid (B) were used as mobile phase. The gradient started at 80% B (0.5 min). The ratio was changed linearly to 95% B within 5.5 min and maintained for another 4 min. Then, the ratio was returned to 80% B and maintained for another 2.5 min. The flow rate was 0.4 ml/min, and the injection volume was 2 μl.

The ionization parameters were as follows: desolvation line temperature 250°C, heat block temperature 400°C, nebulizer gas flow 3 L/min, drying gas flow 15 L/min, and collision induced dissociation gas pressure 230 kPa. The mass spectrometer was tuned automatically to 4β-OHC and 4α-OHC and the internal standard using LabSolutions LCMS software (Shimadzu). Multiple reaction monitoring analysis was performed using argon as collision induced dissociation gas. The MS/MS transitions for the di-picolinyl esters and the electrode voltage of Q1 prebias, collision cell Q2, and Q3 prebias MS/MS transitions for the di-picolinyl esters and the internal standard using LabSolutions LCMS software. The ionization parameters were as follows: desolvation line temperature 250°C, heat block temperature 400°C, nebulizer gas flow 3 L/min, drying gas flow 15 L/min, and collision induced dissociation gas pressure 230 kPa. The mass spectrometer was tuned automatically to 4β-OHC and 4α-OHC and the internal standard using LabSolutions LCMS software (Shimadzu). Multiple reaction monitoring analysis was performed using argon as collision induced dissociation gas. The MS/MS transitions for the di-picolinyl esters and the electrode voltage of Q1 prebias, collision cell Q2, and Q3 prebias were as follows: m/z 613.3 → 497.6, −24 V, −13 V, and −16 V, respectively, for 4β-OHC and 4α-OHC and m/z 620.3 → m/z 497.6, −24 V, −15 V, and −16 V for 4β-OHC-D2 (supplemental Fig. S1). The dwell time for each transition was 0.1 s.

Full validation of the analytical method

Analytical full validation was conducted according to the recommendations published by the US Food and Drug Administration (https://www.fda.gov/media/70858/download, accessed 21 April 2020) as described in our previous studies (30, 31). Each validation batch contained eight calibration samples and 30 QC samples at different concentrations [blank (endogenous level), LLOQ, QCA, QC, and QCC in plasma; in sextuplicate each], and three validation batches were analyzed. Accuracy (%), a percentage of the measured concentration to the nominal concentration, and precision (% coefficient of variation (CV)) a percentage of the observed SD to the mean measured concentration, were calculated for individual analytical batch (within-batch) and for three validation batches (batch-to-batch). Extraction apparent recovery rates from plasma were calculated by comparing the peak areas from QCA, QCB, and QCC obtained from validation analysis with the respective peak areas obtained from unadulterated plasma (from 6 different healthy volunteers) spiked at these QC levels after extraction (representing 100% of the analyte amount in an identical matrix), in sextuplicate determination. Matrix effects were evaluated by comparing peak areas of unadulterated plasma samples (from 6 different healthy volunteers) spiked at QC levels A-C after extraction with the respective peak areas of matrix-free LC eluent containing 100% of standards and internal standards, in sextuplicate determination. Stability of the analytes was evaluated using QCB and QCC samples subjected to three freeze-and-thaw cycles, and the respective accuracies were calculated. Stability in the autosampler was evaluated by measuring QCB and QCC samples after standing in the autosampler at 10°C for 24 h, and the respective accuracies were calculated. Long-term stability of 4β-OHC and 4α-OHC has been reported (22).

Stability of 4β-OHC and 4α-OHC over time in plasma under various conditions

Changes in plasma 4β-OHC and 4α-OHC concentrations over time were assessed under various conditions in vitro. Fresh unadulterated plasma samples from six healthy volunteers were dispensed in open or safe-lock tubes and stored at room temperature or 4°C for 1, 2, 7, 14, and 30 days, each tested in sextuplicate. After storage, 4β-OHC and 4α-OHC concentrations were determined by the UHPLC-MS/MS method developed. Measured concentrations of 4β-OHC and 4α-OHC in each sample were compared with the concentrations in freshly prepared samples without storage and expressed as percentage. 4β-OHC and 4α-OHC were considered stable when the residual percentage was within 85%−115%.

Data analysis and statistics

Calibration curves for 4β-OHC and 4α-OHC were constructed using the calibration samples and analyte-specific multiple reaction monitoring quantifier transitions. Peak area ratios of each analyte to internal standard were calculated, and weighted linear regression (1/x) was performed for each analytical batch using the LabSolutions LCMS software (Shimadzu). Data are expressed as mean ± SD. Differences between healthy volunteers, stage 3–5 CKD patients, and stage 5D CKD patients were analyzed using one-way ANOVA with Dunnett’s posthoc test or Kruskal-Wallis test with Dunn’s posthoc test. A P value less than 0.05 was considered statistically significant. Statistical analyses were performed using Graph Pad Prism 7 (GraphPad Software, La Jolla, CA).

RESULTS

Mass spectrometric and chromatographic characteristics

Intense [M+H]+ signals for 4β-OHC and 4α-OHC di-picolinyl esters were monitored using ESI. The same
mass transition (m/z 613.3→m/z 490.5) was monitored for 4β-OHC and 4α-OHC because they are the stereoisomers. The mass transition of internal standard 4β-OHC-D7 (m/z 620.3→m/z 497.6) differed from that of the two analytes by only the mass shift due to isotopic labeling.

Figure 1 shows the chromatograms of 4β-OHC, 4α-OHC, and 4β-OHC-D7 for 2% HSA solution (surrogate matrix), LLOQ sample, QC sample, plasma sample of a healthy volunteer, and plasma sample of a CKD patient. Retention times were approximately 8.3 and 8.8 min after injection for 4β-OHC and 4α-OHC, respectively. In the chromatogram, 4β-OHC and 4α-OHC were separated from the isomers comprising 7α-OHC, 7β-OHC, 22(R)-OHC, 22(S)-OHC, 24(S)-OHC, 25-OHC, and 27-OHC (supplemental Fig. S2).

Validation results

For 4β-OHC, the correlation coefficient (r²) of the calibration curve for the calibration range of 0.5–500 ng/ml was ≥0.9986. For 4α-OHC, r² for the same calibration range was 0.9989.

Within-batch and batch-to-bath accuracy and precision for 4β-OHC are shown in Table 1. Within-batch
accuracy and precision for QCA, QCB, and QCC ranged from 98.6% to 103.1% and 1.3% to 4.7% CV, respectively. Batch-to-batch accuracy and precision for the three QCs ranged from 100.2% to 101.7% and 3.6% to 4.8% CV, respectively. Within-batch accuracy and precision for LLOQ ranged from 96.5% to 102.3% and 2.1% to 4.0% CV, and batch-to-batch accuracy and precision were 99.4% and 3.9% CV, respectively.

Within-batch accuracy and precision for 4\(\beta\)-OHC are shown in Table 1. Within-batch accuracy and precision for QCA, QCB, and QCC ranged from 95.5% to 103.8% and 1.2% to 3.9% CV, and batch-to-batch accuracy and precision were 101.5% and 6.3% CV, respectively.

The apparent recovery rates and matrix effects of 4\(\beta\)-OHC for QCA, QCB, and QCC ranged from 88.2% to 101.5% and 86.2% to 117.6%, respectively (Table 1). The apparent recovery rates and matrix effects of 4\(\alpha\)-OHC for QCA, QCB, and QCC ranged from 91.8% to 114.9% and 89.5% to 116.9%, respectively (Table 2).

The three-cycle freeze-and-thaw stability test for QCB and QCC revealed no significant changes in measured concentrations, with accuracy ranging from 99.4% to 104.2% for 4\(\beta\)-OHC and 103.4% to 112.5% for 4\(\alpha\)-OHC. Stability test in the autosampler was also acceptable for QCB and QCC, with accuracy ranging from 95.5% to 103.8% and 1.2% to 3.9% CV, and batch-to-batch accuracy and precision were 101.5% and 6.3% CV, respectively.

The apparent recovery rates and matrix effects of 4\(\alpha\)-OHC for QCA, QCB, and QCC ranged from 88.2% to 101.5% and 86.2% to 117.6%, respectively (Table 1). The apparent recovery rates and matrix effects of 4\(\alpha\)-OHC for QCA, QCB, and QCC ranged from 91.8% to 114.9% and 89.5% to 116.9%, respectively (Table 2).

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### Table 1. Summary of validation results for 4\(\beta\)-OHC concentrations in human plasma

| Endogenous | LLOQ | QCA | QCB | QCC |
|------------|------|-----|-----|-----|
| -          | 0.5 + Endo | 1.5 + Endo | 25 + Endo | 400 + Endo |
| **Within-batch** | | | | |
| 1 Mean (ng/ml) | 40.1 | 39.4 | 42.9 | 67.0 | 441.7 |
| Accuracy (%) | 96.9 | 103.2 | 102.9 | 98.5 |
| Precision (%CV) | 7.5 | 4.5 | 5.1 | 8.0 | 8.3 |
| 2 Mean (ng/ml) | 40.7 | 40.8 | 42.5 | 70.0 | 462.0 |
| Accuracy (%) | 99.0 | 100.8 | 106.6 | 104.3 |
| Precision (%CV) | 3.7 | 3.4 | 3.5 | 6.5 | 6.1 |
| 3 Mean (ng/ml) | 37.1 | 37.3 | 39.2 | 61.9 | 402.4 |
| Accuracy (%) | 99.2 | 101.7 | 99.7 | 92.1 |
| Precision (%CV) | 5.4 | 18 | 19 | 6.7 | 6.4 |
| **Batch-to-batch** | | | | |
| Accuracy (%) | - | - | 920 (894-937) | 936 (882-1002) | 980 (94.9-1015) |
| Precision (%CV) | - | - | 4.7 | 8.1 | 6.7 |

### Table 2. Summary of validation results for 4\(\alpha\)-OHC concentrations in human plasma

| Endogenous | LLOQ | QCA | QCB | QCC |
|------------|------|-----|-----|-----|
| -          | 0.5 + Endo | 1.5 + Endo | 25 + Endo | 400 + Endo |
| **Within-batch** | | | | |
| 1 Mean (ng/ml) | 6.9 | 6.9 | 8.9 | 33.8 | 409.1 |
| Accuracy (%) | 93.4 | 105.7 | 106.0 | 100.5 |
| Precision (%CV) | 5.9 | 29 | 29 | 6.4 | 6.3 |
| 2 Mean (ng/ml) | 6.8 | 6.9 | 8.6 | 34.1 | 387.3 |
| Accuracy (%) | 95.1 | 103.6 | 107.2 | 95.2 |
| Precision (%CV) | 7.7 | 6.7 | 4.0 | 5.2 | 5.1 |
| 3 Mean (ng/ml) | 6.9 | 7.2 | 8.2 | 31.1 | 365.9 |
| Accuracy (%) | 97.5 | 96.9 | 97.3 | 89.9 |
| Precision (%CV) | 10.4 | 5.6 | 3.2 | 9.9 | 4.8 |
| **Batch-to-batch** | | | | |
| Accuracy (%) | - | - | 1011 (97.7-106.9) | 1036 (918-112.3) | 1054 (928-114.9) |
| Precision (%CV) | - | - | 4.7 | 8.1 | 6.7 |
| Apparent recovery rate [% (range)] | - | - | 4.7 | 8.1 | 6.7 |
| Matrix effect [% (range)] | - | - | 1015 (97.2-108.2) | 100.4 (89.5-116.9) | 1018 (902-1135) |

4\(\beta\)-OHC, 4\(\beta\)-hydroxycholesterol; CV, coefficient of variation; Endo, endogenous level; LLOQ, lower limit of quantitation; QC, quality control; QCA, low; QCB, medium; QCC, high.
Changes in $4\beta$-OHC and $4\alpha$-OHC concentrations in human plasma under various conditions

Figure 2 shows the changes in plasma $4\beta$-OHC and $4\alpha$-OHC concentrations in plasma samples stored in various conditions. Plasma $4\beta$-OHC concentration was stable for 30 days in safe-lock tubes stored at room temperature and 4°C. On the other hand, plasma $4\beta$-OHC concentration decreased on day 2 followed by an increase from day 7 in open tubes at room temperature and decreased on day 7 followed by an increase from day 14 in open tubes at 4°C. Similarly, when stored in open tubes, plasma $4\alpha$-OHC concentration decreased on day 2 followed by a sharp increase from day 7 at room temperature and decreased on day 7 followed by a sharp increase from day 14 at 4°C. Plasma $4\alpha$-OHC concentration was stable for 14 days in safe-lock tubes at room temperature and 4°C, followed by an increase on day 30.

Application of the method to measure $4\beta$-OHC and $4\alpha$-OHC in healthy volunteers and CKD patients

The validated UHPLC-MS/MS method was used to simultaneously measure plasma concentrations of $4\beta$-OHC and $4\alpha$-OHC in healthy volunteers and CKD patients (Table 3). Serum creatinine was elevated in stage 3–5 CKD patients and stage 5D CKD patients. As shown in Fig. 3A, plasma $4\beta$-OHC concentrations were 23.6 ± 7.4 in healthy volunteers, 31.2 ± 10.3 in stage 3–5 CKD patients, and 22.7 ± 6.7 ng/ml in stage 5D CKD patients, with a significant difference among three groups by one-way ANOVA ($P = 0.022$), whereas no significant difference was detected by posthoc test. There was no significant difference in the ratio of $4\beta$-OHC to total cholesterol among three groups ($P = 0.65$) (Fig. 3B).

Plasma $4\alpha$-OHC concentrations were 3.6 ± 0.9 in healthy volunteers, 5.9 ± 1.4 in stage 3–5 CKD patients, and 4.8 ± 0.9 ng/ml in stage 5D CKD patients; a significant difference was observed among three groups by one-way ANOVA ($P < 0.0001$), and significant differences were also detected by posthoc test (Fig. 3C).

Similarly, a significant difference in the ratio of $4\alpha$-OHC to total cholesterol was observed among three groups by Kruskal-Wallis test ($P = 0.0045$), and significant differences were also detected by posthoc test (Fig. 3D).

The ratios of $4\beta$-OHC to $4\alpha$-OHC were 6.6 ± 1.6 in healthy volunteers, 5.4 ± 1.8 in stage 3–5 CKD patients, and 4.8 ± 1.1 in stage 5D CKD patients, with a significant difference among three groups by one-way ANOVA ($P = 0.021$) (Fig. 4). Posthoc test revealed a significantly smaller $4\beta$-OHC to $4\alpha$-OHC ratio in stage 5D CKD patients compared to healthy volunteers. All measured concentrations of $4\beta$-OHC and $4\alpha$-OHC were within the calibration ranges.

DISCUSSION

In 2001, Bodin et al. (11) reported for the first time that plasma $4\beta$-OHC concentration was useful for phenotyping CYP3A activity in vivo. Since then, many studies have evaluated the usefulness of plasma $4\beta$-OHC concentration as an endogenous marker for phenotyping CYP3A activity. Interestingly, these studies reported the pros and cons of the use of plasma $4\beta$-OHC concentration. For example, Gravel et al. (32) recently reported that $4\beta$-OHC was a valid and convenient marker for phenotyping CYP3A activity with high correlation with midazolam pharmacokinetics. On the other hand, a recent review by Penzak et al. (33) did not support the use of $4\beta$-OHC as an endogenous biomarker for phenotyping CYP3A activity, because of the mild correlation with midazolam pharmacokinetics. The inconsistency may be partially explained by autooxidation of cholesterol. $4\beta$-OHC is produced by in vivo hydroxylation of cholesterol at the $4\beta$-position by CYP3A, followed by in vitro autooxidation of cholesterol after blood sampling (28). Thus, improper control of blood samples, such as prolonged storage at room temperature in open tubes, may increase plasma $4\beta$-OHC concentration after blood sampling, causing inaccurate CYP3A phenotyping. Furthermore, $4\beta$-OHC was reported to be produced in vivo by autooxidation of cholesterol due to oxidative stress or inflammation in the liver (29). Like $4\beta$-OHC, $4\alpha$-OHC is produced by autooxidation of cholesterol, but $4\alpha$-OHC is not formed by CYP3A in vivo (28). Therefore, plasma $4\alpha$-OHC concentration has clinical potential in providing information on the status of cholesterol autooxidation, i.e., plasma $4\beta$-OHC concentration may not accurately indicate CYP3A activity if plasma $4\alpha$-OHC concentration is high. Thus, simultaneous quantification of $4\beta$-OHC and $4\alpha$-OHC may enhance the accuracy of $4\beta$-OHC for phenotyping CYP3A.

Three studies have already reported methods of simultaneous measurement of plasma $4\beta$-OHC and $4\alpha$-OHC concentrations. Bodin et al. (11) reported a simultaneous quantification method using gas chromatography coupled to mass spectrometry, but the method was not validated. Goodenough et al. (22) reported an LC-MS/MS method, which was validated for $4\beta$-OHC but not for $4\alpha$-OHC. Recently, Hautaanjärvi et al. (27) reported a validated method for simultaneous quantification of $4\beta$-OHC and $4\alpha$-OHC in plasma using ultra-high performance liquid chromatography coupled to high resolution mass spectrometry. Their method allowed accurate measurements of $4\beta$-OHC and $4\alpha$-OHC, with LLOQ of 0.5 and 2 ng/ml for $4\beta$-OHC and $4\alpha$-OHC, respectively, using 100 μl of plasma sample. However, the LLOQ for $4\alpha$-OHC may not be low enough, because $4\alpha$-OHC concentrations in human plasma were reported to be 2.12–5.65 ng/ml in 24 subjects (27). The method of Hautaanjärvi et al. avoided the derivatization process in plasma sample preparation.
and monitored sodium adducted ions, causing inadequate sensitivity. Our novel method adopted a short derivatization process using picolinic acid and monitored the proton adducted ions, which increased ionization efficiency and achieved better LLOQ of 0.5 ng/ml for 4α-OHC. Our newly developed method thus has the advantage of having sufficient sensitivity to evaluate plasma 4α-OHC concentrations in different clinical settings, and it seems to be superior to previous reported methods for simultaneous quantification of 4β-OHC and 4α-OHC in human plasma.

We prepared plasma samples by saponification using sodium methoxide, two-step liquid-liquid extraction using n-hexane, and derivatization using picolinic acid. Sodium methoxide was used for saponification of 4β-OHC and 4α-OHC comprised in lipoproteins and was utilized in previous reports (23, 26, 27). Derivatization is an option to enhance ionization efficiency.

**Fig. 2.** Changes 4β-OHC (A) and 4α-OHC (B) concentrations over time in unadulterated plasma samples from six healthy volunteers stored under various conditions for 30 days. 4α-OHC, 4α-hydroxycholesterol; 4β-OHC, 4β-hydroxycholesterol.
because $\beta$-OHC and $\alpha$-OHC has poor ionization efficiency, but the process is not essential for LC-MS/MS method. Van de Merbel et al. (23) and Hasan et al. (26) quantified $\beta$-OHC without derivatization using LC-MS/MS equipped with atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI) probes, respectively. APCI and APPI provide efficient ionization for relatively nonpolar
compounds such as steroids and are suggested to be feasible methods for quantification of 4β-OHC and 4α-OHC. However, APCI and APPI seemed to be inferior to ESI with derivatization process in sensitivity for 4β-OHC (23, 26). Thus, APCI and APPI may not be suitable for sensitive quantification, especially for 4α-OHC, and ESI with derivatization using picolinic acid achieves sensitive quantification of 4β-OHC and 4α-OHC in plasma.

Validation results for 4β-OHC and 4α-OHC are within the acceptable ranges according to the recommendations published by the US Food and Drug Administration. High apparent recovery rates and matrix effects suggest effective sample preparation and minimal ion suppression or enhancement. When samples exposed to air were stored under various in vitro conditions, plasma 4β-OHC and 4α-OHC concentrations increased after a transient decrease. However, 4β-OHC and 4α-OHC were stable when stored in safe-lock tubes, suggesting increased stability by avoiding contact with air. The elevation is speculated to be due to autooxidation of cholesterol. However, detailed mechanism of the transient decrease in plasma 4β-OHC and 4α-OHC concentrations is unknown, but other pathways such as additional autooxidation of 4β-OHC and 4α-OHC may be involved. These findings suggest that plasma samples should be stored in rocked tubes to avoid autooxidation of cholesterol to 4β-OHC and 4α-OHC.

The validated UHPLC-MS/MS method was used to measure plasma 4β-OHC and 4α-OHC concentrations in healthy volunteers and CKD patients. Plasma 4β-OHC concentrations in this study were similar to previously reported values in healthy volunteers (11, 22, 23, 27), stage 3–5 CKD patients (18, 21), and stage 5D CKD patients (17, 19). Plasma 4α-OHC concentrations in healthy volunteers in this study were also similar to previous reports (11, 22, 27). To the best of our knowledge, this is the first report of plasma 4α-OHC concentrations in CKD patients. All the measured concentrations were within the calibration ranges. Especially, our novel method with lower LLOQ for 4α-OHC achieved to measure plasma 4α-OHC concentrations well in advance in healthy volunteers. These suggest that the established method is clinically useful for measuring 4β-OHC and 4α-OHC concentrations in human plasma samples from healthy volunteers as well as CKD patients and would contribute to accurate evaluation of in vivo CYP3A activity.

The 4β-OHC to total cholesterol ratio has been reported to be superior to 4β-OHC alone for CYP3A phenotyping (34). However, there was no significant difference in 4β-OHC to total cholesterol ratio among three groups (Fig. 3B), although CKD was reported to decrease CYP3A activity in patients (35). On the other hand, a significant difference in 4α-OHC to total cholesterol ratio was observed among three groups, with significant increases in stage 3–5 CKD patients (0.030 ± 0.0079) and stage 5D CKD patients (0.051 ± 0.0088) compared to healthy volunteers (0.023 ± 0.0037) (Fig. 3D). Studies have shown that increases in 4β-OHC and 4α-OHC by cholesterol autooxidation were caused not only by in vitro uncontrolled storage condition but also by in vivo oxidative stress or inflammation (28, 29). Moreover, elevated oxidative stress and inflammation are observed in CKD patients (36, 37). In this study, collection, storage, and pretreatment of blood samples from healthy volunteers, stage 3–5 CKD patients, and stage 5D CKD patients were conducted under the same conditions; therefore, the degree of in vitro cholesterol autooxidation of all samples probably did not vary greatly. Therefore, the significant elevations of 4α-OHC concentration and 4α-OHC to cholesterol ratio in stage 3–5 CKD and stage 5D CKD patients compared to healthy subjects likely reflect the elevated oxidative stress and inflammation in these patients. Similarly, elevated oxidative stress and inflammation in stage 3–5 CKD patients and stage 5D CKD patients are probably involved in the elevation of 4β-OHC concentration in these patients and in apparently no change in plasma 4β-OHC concentration among three groups. Thus, correction by plasma 4α-OHC concentration may control the fluctuation of in vitro and in vivo cholesterol autooxidation among patients and improve the accuracy of 4β-OHC as an endogenous biomarker for CYP3A phenotyping. Indeed, as shown in Fig. 4, a significant difference in 4β-OHC to 4α-OHC ratio was observed among three groups, with a significant difference between healthy volunteers and stage 5D CKD patients.

![Graph showing the ratio of 4β-OHC to 4α-OHC in healthy volunteers, stage 3–5 CKD patients, and stage 5D CKD patients.](image-url)
To the best of our knowledge, this is the first report of the potential use of the 4β-OHC to 4α-OHC ratio in CYP3A phenotyping. Further large-scale study is needed to validate this finding.

Our method has some limitations. First, because the retention times of 4α-OHC and 4β-OHC-D₇ were different, the matrix effect of the 4α-OHC assay was not corrected by internal standard. However, the variation due to matrix effect was not large in the 4α-OHC assay and thus appeared to have little effect on the results. Second, the rationale for the use of 4β-OHC to 4α-OHC ratio in CYP3A phenotyping is not well established. We showed the potential of 4β-OHC to 4α-OHC ratio for cross-sectional CYP3A phenotyping in healthy volunteers and CKD patients in this study, but the interindividual variability in 4β-OHC to 4α-OHC ratio was large within each group, although a significant difference was observed among three groups (Fig. 4). To establish a better clinical approach for CYP3A phenotyping, further clinical study by approaches such as model-based analysis using 4β-OHC and 4α-OHC concentrations is needed.

In conclusion, simultaneous quantification method for plasma 4β-OHC and 4α-OHC concentrations using UHPLC-MS/MS was developed and validated and was applied to measurement of plasma concentrations in healthy volunteers and CKD patients. Our novel method may contribute to accurate evaluation of in vivo CYP3A activity.

Data availability
The data supporting this study are available in the article, the supplemental data, or available from the corresponding author upon reasonable request.

Supplemental data
This article contains supplemental data.

Author contributions
Y. S. and K. O. conceptualization; Y. S. methodology; Y. S. and A. O. formal analysis; Y. S. writing-original draft; A. O., J. N., and D. T. validation; A. O., J. N., D. T., R. T., H. O., T. A., and T. S. investigation; A. O., J. N., D. T., R. T., H. O., T. A., H. M., H. I., K. O., and T. S. writing-review & editing; H. M., H. I., and K. O. supervision.

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Conflict of interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Abbreviations
4α-OHC, 4α-hydroxycholesterol; 4β-OHC, 4β-hydroxycholesterol; APCI, atmospheric pressure chemical ionization; APPI, atmospheric pressure photoionization; CKD, chronic kidney disease; CV, coefficient of variation; CYP, cytochrome P450; HSA, human serum albumin; LC-MS/MS, high-performance liquid chromatography coupled to tandem mass spectrometry; LLOQ, lower limit of quantification; QC, quality control; UHPLC-MS/MS, ultra-high performance liquid chromatography coupled to tandem mass spectrometry.

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