Quantitative analysis of 4β- and 4α-hydroxycholesterol in human plasma and serum by UHPLC/ESI-HR-MS

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Abstract

Cholesterol oxidation product 4β-hydroxycholesterol (4β-OHC) may possibly be used as an endogenous biomarker of CYP3A enzyme activity and as CYP3A4 is involved in the metabolism of approximately 50% of the drugs in clinical use, the monitoring of CYP3A activity by 4β-OHC plasma or serum levels, may be of clinical significance. The plasma and serum concentrations of 4α-hydroxycholesterol (4α-OHC), an isomer of 4β-OHC, increase during uncontrolled storage conditions and therefore serve as an indicator of proper handling of samples.
A sensitive and simple high-throughput method for the simultaneous quantification of both 4α-OHC and 4β-OHC in human plasma and serum was developed utilizing ultrahigh performance liquid chromatography coupled with high resolution mass spectrometry (UHPLC/ESI-HR-MS). The chromatographic analysis was carried out on a Waters HSS T3 C18 reversed phase column with a mobile phase composed of 0,1% formic acid with 200 mg/L sodium acetate, and methanol. 4β-OHC and 4α-OHC and also internal standard d7-4β-OHC were monitored using HR-MS as sodium adducts, which could not be used as a precursor ions in conventional tandem mass spectrometry methods due to their extensive stability in collision for MS/MS. The use of HR-MS detection enabled avoiding laborious sample derivatization, which is required with triple quadrupole mass spectrometer-based methods to achieve adequate analytical sensitivity for 4β-OHC, as the underivatized molecule is otherwise poorly ionized to other molecular ions than sodium adduct. Chromatographic separation of 4α-OHC and 4β-OHC was obtained and confirmed with standard samples prepared in blank surrogate matrix. The lower limits of quantitation in the assay were 0.5 ng/ml for 4β-OHC, and 2 ng/ml for 4α-OHC. Endogenous levels of 4β-OHC can vary between 10 - 100 ng/ml depending on the possible induction or inhibition of CYP3A4, whereas the levels of 4α-OHC can vary between 5 - 100 ng/ml, depending on the storage conditions of the samples. Thus, the sensitivity of the assay developed allows for the simultaneous measurement of endogenous levels of 4α-OHC and 4β-OHC cost-effectively and with high troughput.The method was successfully used for the determination of 4β-OHC and 4α-OHC concentrations in clinical plasma and serum samples collected before and after treatment with a known CYP3A4 inducer rifampicin. The endogenous levels in clinical human samples before treatment varied between 13.4 – 31.9 ng/ml for 4β-OHC, and between 3.53 – 5.65 ng/ml for 4α-OHC, and a three-fold increase in 4β-OHC plasma levels was observed after the rifampicin treatment, while 4α-OHC levels remained unaffected.

Keywords: LC/ESI-HR-MS, 4β-hydroxycholesterol, plasma, serum.

1. Introduction

A cell membrane element and a substrate for biosynthesis of bile acids and steroid hormones, cholesterol, undergoes oxidation via multiple mechanisms and may be transformed into different oxidation products known as oxysterols. One of the quantitatively important oxysterols in human circulation is 4β-hydroxycholesterol (4β-OHC) (Figure 1), which is predominantly formed via CYP3A4 enzyme [1], though also CYP3A5 has a minor role in oxidation of cholesterol into 4β-OHC [2]. In contrast to the enzyme-mediated formation of 4β-OHC, its isomer 4α-hydroxycholesterol (4α-OHC) (Figure 1) is formed via auto-oxidation of cholesterol, and its levels can be used as a marker for clinical sample stability. The plasma and serum concentrations of 4α-OHC increase during uncontrolled storage conditions and therefore work as an indicator of proper handling of samples. [1,3].

Pregnane X receptor (PXR) and constitutively active receptor (CAR) are the most important regulators of CYP3A expression although many other ligand-activated receptors such as glucocorticoid receptor and farnesoid X receptor have also been demonstrated to affect the expression of CYP3A4 [4]. PXR is activated by a multitude of agonists such as pharmaceuticals including dexamethasone, rifampicin and enzyme-inducing antiepileptics, as well as many environmental contaminants and constituents of herbal remedies [5, 6]. Also CAR is activated by several environmental chemicals and pharmaceuticals [7]. As suggested in several publications, 4β-OHC could possibly be used as an endogenous biomarker of CYP3A enzyme activity [3, 8, 9]. As CYP3A4 is involved in the metabolism of approximately 50% of the drugs in clinical use [10], the monitoring of CYP3A4 activity by 4β-OHC may be of clinical significance. However, the use of 4β-OHC as a CYP3A4 biomarker has been criticized, since the correlations of 4β-OHC concentrations with dose requirements and clearances of CYP3A-metabolized medications are usually quite poor [11, 12].

Observation of 4β-OHC requires sensitive and selective analytical methods, including optimized sample treatment to avoid analytical interference by other oxysterols. In addition, cholesterol itself may add interference as it has up to about 100-fold higher concentration in plasma than any oxysterol [13]. Sensitivity is required also in situations where inhibition of CYP3A4 activity is monitored via decrease of plasma 4β-OHC levels [14]. Oxysterols, including 4β-OHC and 4α-OHC, exist in plasma both as esterified to fatty esters and as free forms, hence it is necessary to hydrolyze the cholesterol esters to free sterols for the analysis of clinical samples. Commonly, a saponification method, e.g, incubation with alkaline is used for this [15]. The natural levels of 4β-OHC in human plasma in previous studies have been 13 - 44 ng/ml [1, 15, 16, 17, 18]. Drugs known to induce cytochrome P450 enzymes elevate the level of 4β-OHC in plasma up to about eight times higher concentration, whereas 4α-OHC levels stay the same, about 5 - 7 ng/ml [1].

The reported sample treatment methods of quantitative analysis of oxysterols in human plasma have been laborious, and chromatographic separation of 4α-OHC and 4β-OHC is not always obtained [17, 19, 20, 21]. Methods for determination of 4β-OHC in plasma using isotope-dilution GC-MS method have been reported [8, 12, 23,], as well as an LC/MS method using atmospheric pressure photoionization (APPI) that does not require derivatization [24, 25]. Several LC-ESI-MS/MS methods using derivatization have been reported [15, 16, 26], a dual stable-label LC-APCI-MS/MS method without derivatization [27] and a LC-ESI-MS/MS method using dispersive liquid-liquid microextraction [28], as well as a stable isotope dilution technique by LC-MS/MS [29]. All previously reported methods require either derivatization of the samples or relatively large sample volume to enable sufficient analytical sensitivity. With these previously reported methods, the routinely reported quantitation limits for 4β-OHC vary in the range of 2-10 ng/ml in plasma, and the detection limits vary in the range 1-5 ng/ml, whereas for 4α-OHC, detection or quantitation limits are not often reported [19]. In this study we describe a robust, sensitive and high-throughput method for the simultaneous quantification of both 4α-OHC and 4β-OHC in human plasma and serum without derivatization, utilizing ultrahigh performance liquid chromatography coupled with high resolution mass spectrometry.

2. Materials and methods

2.1 Materials, matrices & reagents

HPLC-grade acetonitrile, Potassium chloride (CAS 7447-40-7), Sodium chloride (CAS 7647-14-5), Monopotassium phosphate (CAS 7778-77-0), Disodium phosphate (CAS 7558-79-4) and Sodium acetate trihydrate (CAS 6131-90-4) were purchased from Merck (Darmstadt, Germany). Laboratory water was distilled and purified with a Direct-Q water purifier (Millipore, Molsheim, France). 4α-hydroxycholesterol (CAS 34310-86-6) was purchased from Toronto Research Chemicals, Toronto, Canada. 4β-hydroxycholesterol (CAS 17320-10-4) and D7-4β-hydroxycholesterol (Figure 1.) (CAS 1246302-80-6) were purchased from Avanti Polar Lipids. Inc. Alabama, USA. Sodium methoxide (CAS 124-41-4) was purchased from Sigma-Aldrich, Missouri, USA. Bovine serum albumin (CAS 9048-46-8) was purchased from Sigma life sciences Merck (Darmstadt, Germany). Technical ethanol Etax AaS was purchased from Altia Indrustial, Rajamäki, Finland. Human K_2EDTA plasma and human serum were purchased from Seralab, West Sussex, United Kingdom. Human citrate plasma was purchased from Finnish Red Cross Blood Service, Oulu, Finland.

2.2 Instrumentation

The LC/ESI-HR-MS system consisted of a Thermo Vanquish Horizon UHPLC with an autosampler, vacuum degasser, photodiode-array (PDA) detector, and column oven coupled to a Q-Exactive Orbitrap Focus mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The analytical column used was a Waters Acquity HSS T3 2.1x30 mm with 1.8 µm particle size (Waters Corp, Milford, MA, USA). The temperature of the column oven was 50°C, and the injection volume was 6 µl. The aqueous eluent (A) was 0.1% formic acid + 200 mg/L sodium acetate in ultrapure water, and the organic eluent (B) was methanol. A gradient elution with 80-80-95-95% (B) in 0-0.5-4-5 min was applied, followed by 1 min equilibration time. The eluent flow rate was 0.5 ml/min and the flow was directed to the MS through a PDA detector. The data acquisition was performed using positive ionization polarity with a spray voltage of 4000 V. Capillary temperature was 400^{0}C and auxiliary gas temperature was 550^{0}C. Scan was performed with a resolution of 35 000 (full width at half maximum at m/z 200), while an Automated Gain Control target of a million ions, maximum injection time of 100 ms, and a scan range of 400−450 m/z were used. Nitrogen was used as a sheath gas with 55 units, auxiliary gas with 5 units and as a sweep gas with 3 units. Ion chromatograms were extracted from the total ion chromatograms using calculated monoisotopic accurate masses with 7 mDa window. Calibration curves were generated using a weighted (1/x) quadratic regression of peak area ratios (PAR) of the analytes and the deuterium-labeled internal standard internal. The data were processed with Thermo Xcalibur 4.1.31.9 software.

2.3 Stock solutions, calibration standard and quality control sample preparation

2 mg/mL stock solutions of 4β-OHC, 4α-OHC and stable-labeled internal standard d7-4b-hydroxycholesterol were prepared in borosilicate vials by dissolving accurately weighed amounts of the analytes in acetonitrile. Quality control (QC) stock solution was prepared from an independently weighted volume of the analytes. Surrogate matrix used in preparation of the calibration standards was 40 mg/ml bovine serum albumin in 150 mM phosphate buffered saline (pH 7.4).

4β-OHC and 4α-OHC working solutions were prepared by dilution with acetonitrile to create 12 calibration standard spiking solutions (2, 5, 10, 20, 50, 100, 200, 500, 1000, 5000 and 10 000 ng/mL) and four quality control spiking solutions (500, 1000, 1500, 3000 ng/mL). Internal standard working solution (ISWS) was prepared by dilution with acetonitrile to 400 ng/mL. The stock and working solutions were prepared to a volume of 1 mL and were stored at -20 °C when not in use.

The calibration standard samples were prepared by spiking the surrogate matrix into concentrations 0.2 - 1000 ng/mL of the analytes. As the human blank plasma endogenously contains the analytes, the quality control samples were prepared by spiking the surrogate matrix and blank human plasma into added concentrations of 10, 30, 100 and 300 ng/ml. Therefore, the actual concentrations of quality control samples in human plasma were set based on the concentrations quantified from the blank plasma against the calibration curve.

2.4 Sample preparation

Standards, QCs, and clinical samples were analyzed in 96-well plates. 100 µl of plasma containing dipotassium ethylenediaminetetra-acetic acid (K_2EDTA) as an anticoagulant was spiked with 10 µl of ISWS, mixed throughout and

saponified with 200 µl of 2 mM sodium methoxide in ethanol. The samples were then mixed 1 x g 30 minutes at 20^{0}C (Eppendorf Thermomixer, Eppendorf, Hamburg, Germany). Following saponification, the samples were protein precipitated with 600 µl of 1.5% formic acid in acetonitrile, mixed 2 x g for 5 minutes (Eppendorf Thermomixer, Eppendorf, Hamburg, Germany), ultrasonicated at 45kHz for 15 min (Ultrasonic cleaning bath, VWR, Radnor, Pennsylvania. USA), followed by centrifugation for 20 min at 2200 x g (Fisher Scientific SL 16 centrifuge, Thermo Fisher Scientific, Waltham, MA, USA). 500 µl of the supernatants were transferred to Waters Ostro 96-well plate to purify the samples from excess protein and remove phospholipids (25 mg 1/Pkg, Waters Corp, Milford, MA, USA.) The samples were drawn through the plate by applying 15 inHg vacuum for 10 min using Thermo 96-well plate as a collection plate, and submitted to LC/ESI-HR-MS analysis.

3. Analytical method validation

The method for detecting 4β-OHC and 4α-OHC in plasma was developed and validated based on current guidance and laboratory standard operating procedures [30]. The outline of the key validation experiments conducted is described in the following sections.

During analytical method development all mass spectrometric parameters were optimized by infusing a mixture of 4β-OHC and 4α-OHC in acetonitrile into the mass spectrometer. Both analytes and also internal standard were detected as a sodium adducts $[M+Na]^+$ and therefore the amount of sodium in aqueous eluent (A) was optimized to achieve the best ionization of the analytes. The most abundant ionization was achieved with 200 mg/L sodium acetate added to 0.1% formic acid in ultrapure water, this corresponds 33.8 mg/L sodium in the eluent.

3.1 Accuracy and precision

The lower limit of quantification (LLOQ) is the lowest concentration of analytes in a sample which can be quantified reliably, with an acceptable accuracy and precision. Also, the analyte signal at the lowest limit of quantification should be at least 5 times the signal of the blank surrogate sample. For a calibration curve to be acceptable, the back-calculated calibration concentrations needed to be within ± 15% of the nominal (± 20% at the LLOQ), and within 15% relative standard deviation (RSD) and at least 75% of the calibration standards must fulfil the criterion. The accuracy and precision of the method were evaluated using six replicates of QC samples at four concentrations (10, 30, 100 and 300 ng/ml) in both, surrogate matrix and blank human plasma, over three days with calibration curve samples generated on each day. Acceptance criteria for the accuracy and precision determinations of the QC samples were specified to be ± 15% of the nominal value in blank surrogate matrix whereas the actual concentrations of quality control samples in human plasma were set based on the concentrations quantified from the blank plasma against the calibration curve.

3.2 Selectivity and Matrix effects

The selectivity of the assay should be assessed using both blank surrogate matrix and blank human plasma. 4β-OHC and 4α-OHC being endogenous compounds with basal level in human plasma higher than LLOQ, selectivity in human plasma could not be tested. Conversely, blank surrogate matrix was analyzed and any responses in the chromatographic regions of interest were assessed. Similarly to selectivity, matrix effect in human plasma could not be assessed conventionally. The matrix effects were determined non-quantitatively by post-column infusion method [31] by introducing a constant flow of analyte solution into mobile phase while injecting blank solvent sample, blank surrogate matrix, blank human plasma and serum, and observing disruptions in the background signal.

3.3 Carry- over and stability

Carry-over was assessed by injection of two blank surrogate matrix samples after the highest calibration samples. Peak area responses in the blank surrogate matrix samples were compared with the analyte area responses of the LLOQ of the method, and values ≤20% of the corresponding analyte response of the LLOQ level were considered acceptable. Stability of 4β-OHC and 4α-OHC in plasma has been extensively covered [15, 16, 19, 24, 26, 27] and only freeze-thaw stability was evaluated in this study. For one freeze-thaw cycle a 150 µl of pooled human K_2EDTA

plasma or pooled human serum (n=6) was incubated in polypropylene tubes at room temperature for 1 hour, after which the samples were refrozen on dry ice for 30 minutes. Following three freeze-thaw cycles, samples were prepared for analysis as described earlier.

3.4 Clinical samples

To investigate rifampicin induced levels of 4β-OHC in plasma, the samples from a previous clinical study exploring the effect of PXR activation on incretin hormone excretion were utilized [32]. Twelve healthy volunteers, aged 18 – 45 years, with body mass index between 19 – 28 kg/m^2 were recruited for the study. Exclusion criteria included major medical or psychiatric conditions including any liver disease (as judged by the study physician on the basis of history, physical examination, and basic laboratory values); any continuous medication including oral contraceptives; insensitivity to rifampicin; pregnancy; breast feeding; continuous use of soft contact lenses; history of difficult venipuncture; drug or alcohol abuse; and participation in any other trial within 1 month. The study was approved (number 73/2010) by the Ethics Committee of the Northern Ostrobothnia Hospital District (Oulu, Finland) and the Finnish Medicines Agency. Written, informed consent was obtained from each subject. The study procedures performed were in accordance with the ethical standards of the Declaration of Helsinki and guidelines on Good Clinical Practice. The participants were financially compensated for participation. The trial was registered at ClinicalTrials.gov as NCT01293422.

The study had a one-phase, open-label design. Twelve subjects were given 600 mg of rifampicin (Rimapen; Orion Corporation, Espoo, Finland) a day for a week. The daily dose of 600 mg is the maximum clinically used dose as well as the most commonly used dose in experimental human studies. The participants were asked to abstain from the use of alcohol, over-the-counter-medications, and dietary and herbal supplements for 5 days before and during the study. The study was conducted on an outpatient basis, and each subject visited the Internal Medicine Research Laboratory of Oulu University Hospital two times. On the morning of the first day an i.v. catheter was inserted in the forearm for blood drawings and oral glucose tolerance test (OGTT) was performed. Blood samples were taken using K_2EDTA as an anticoagulant, after which the first rifampicin tablet was administered under the supervision of a study nurse. The subsequent daily doses were taken by the subjects at home between 4 – 8 p.m. at least 1 h before a meal or 2 h after a meal, at the subjects' convenience. To monitor the compliance to drug regimen, the volunteers wrote the date and time of each dose taken in a medication diary, and the participants were required to return the used medication containers. The subjects consumed their regular diets during the study. On the eighth day, the OGTT was performed as in the first day. A 3 ml blood sample was drawn using K_2EDTA as an anticoagulant followed by 30 min incubation in room temperature, 10 min centrifugation 2000 x g at +4°C, and storage at –70°C prior to sample analysis. On both the Day 1 and Day 8, only the fasting samples taken prior the oral glucose tolerance test were utilized to analyze 4β-OHC. As 4β-OHC has a half-life of about 17 days [3], the analysis of the samples from other OGTT time points was deemed redundant.

4. Results and discussion

The analytical methods with triple quadrupole mass spectrometer require laborious sample derivatization to achieve adequate analytical sensitivity for 4β-OHC due to poor ionization of the underivatized molecule. Both 4β-OHC and 4α-OHC and also internal standard d7-4β-OHC were detected with high-resolution mass spectrometry as sodium adducts $[M+Na]^+$, which could not be used as a precursor in LC/MS/MS methods due to extensive stability. With LC/ESI-HR-MS the use of sodium adduct as a main quantifier ion instead of protonated molecule is possible, thus increasing the analytical sensitivity compared to LC/MS/MS methods and also making sample derivatization unnecessary.

4.1 Accuracy and precision

Quantitation was based on PAR of the analytes and the deuterium-labeled internal standard. Calibration curves were generated using a weighted (1/x) quadratic regression of PAR of the analytes and the deuterium-labeled internal standard internal. The lower limit of quantitation in the assay was 0.5 ng/ml for 4β-OHC, and 2 ng/ml for 4α-OHC. The calibration ranges were 0.5 - 1000 ng/ml for 4β-OHC, and 2 - 1000 ng/ml for 4α-OHC prepared in blank

surrogate matrix. The back-calculated calibration concentrations were within 90-116% of the theoretical value for 4β-OHC and 82-114% for 4α-OHC. The relative standard deviations in all four calibration curves above lowest limit of quantification were less than 15% for both analytes and less than 35% at the lowest limit of quantification. The intra- and interday accuracy and precision of the method were determined by analyzing four QC samples (10, 30, 100 and 300 ng/ml) prepared in surrogate matrix and in blank human plasma on four different days. A total of six replicates were evaluated for each QC concentration on each of the four different days. A summary of the intra- and interday accuracy and precision measurements in surrogate matrix is shown in Table 1. The interday accuracy in surrogate matrix was ±8% for both 4β-OHC and 4α-OHC for all four QC concentrations, and the intraday accuracies were all within ±13% of the nominal value for both analytes. The interday precisions in surrogate matrix were ≤9% RSD for 4β-OHC and 4α-OHC for all four QC concentrations, while the intraday RSD were less than 13% for both analytes.

4.2 Selectivity and matrix factor evaluation

In the analytical method development a chromatographic separation of 4β-OHC and 4α-OHC was obtained, as well as separation from other oxysterol isomers. Separation of especially 4β-OHC and 4α-OHC from another is crucial in cases where evaluation of sample stability is of interest. Even though stability would not be assessed, the auto-oxidation of cholesterol into 4α-OHC could cause bias in determination of levels of 4β-OHC in plasma, if the chromatographic separation is not confirmed. In most of the studies reported earlier it has not been clear if the chromatographic separation of 4β-OHC and 4α-OHC has been evaluated and thus taken into consideration. In our experience, the only way to confirm the separation is to use standard samples of both 4β-OHC and 4α-OHC in solvent blank and compare situation in blank plasma samples, confirming the identification of endogenous plasma oxysterols.

Different batches of blank human plasma may contain various and unknown levels of the endogenous compounds such as 4β-OHC and 4α-OHC, and therefore it is not reasonable to use human blank plasma to prepare calibration standards. We used surrogate matrix for the preparation of calibration standards and the QC samples, but also made another set of the QC samples using authentic matrix (pooled blank human K_2 EDTA plasma) in order to demonstrate that there are no stability issues or matrix effect in the analysis. In Table 2 a summary of the intra- and interday accuracy and precision assay performance is shown for QC samples prepared in blank human plasma. The interday and intraday accuracies of blank plasma were all within the same values as in blank surrogate matrix (Table 1). The interday accuracies (n=24 from 4 separate days) in human plasma were ±7% for both 4β-OHC and 4α-OHC for all four QC concentrations, and the intraday (n=6) accuracies were all within ±13% of the nominal value for 4β-OHC and within ±24% for 4α-OHC. Also, interday precision (n=24 from 4 separate days) were ≤10% RSD for all four QC concentrations for 4β-OHC and ≤17% RSD for 4α-OHC, while the intraday RSD (n=6) were less than 15% for both analytes in blank plasma, showing no evident differences in neither interday nor intraday assay performance between plasma and surrogate matrix, indicating that there is no matrix effect in the analysis.

The matrix effects of the assay were also determined non-quantitatively in human K_2 EDTA plasma, human citrate plasma, human serum and surrogate matrix by post-column infusion method where no disruptions in the background signal were observed near or at the retention times of 4β-OHC and 4α-OHC (Figure 2).

The selectivity in human plasma could not be reported due to endogenous nature of 4β-OHC and 4α-OHC with basal level higher than LLOQ, therefore selectivity in blank surrogate matrix was evaluated. As can be noted in the Figure 3, no interfering components near or at the retention times of 4β-OHC or 4α-OHC were found in the blank surrogate matrix, suggesting good selectivity. Also the endogenous levels of 4β-OHC or 4α-OHC in blank human K_2EDTA plasma, blank human citrate plasma and blank human serum are apparent in Figure 3. The levels of 4β-OHC in blank human citrate plasma were observed to differ from human K_2EDTA plasma and human serum in the selectivity tests and were therefore investigated further. Three replicate samples were prepared from blank human K_2EDTA and citrate plasma and human serum, and the average (n=3) LC/ESI-HR-MS peak area responses of 4β-OHC were compared using average of K_2EDTA plasma as 100%. The values obtained were 97.5% (10.6 % RSD) for human blank serum and 189.5% (7.8 % RSD) for human blank citrate plasma (data not shown). In the literature, anticoagulants used in 4β-OHC analysis have been either K_3EDTA or K_2EDTA, or the anticoagulant has not been reported, although it is common to obtain plasma samples using citrate or EDTA as an anticoagulant. These observations strongly suggest that the effect of anticoagulant on analysis of 4β-OHC in clinical samples should be investigated further with broader selection of anticoagulants, including heparin, and with more replicates to provide reliable insight on the phenomenon.

4.3 Carry –over and Stability

Carry-over was assessed by injection of two blank surrogate matrix samples without internal standard after the highest calibration samples. No peaks in the blank surrogate matrix samples were observed, indicating no carry-over in the analysis (Figure 4). Due the endogenous nature of 4β-OHC and 4α-OHC with basal level higher than LLOQ, instead of blank plasma, blank surrogate matrix samples were injected as a precaution after samples with expected high concentrations, including high QC samples, before the analysis of the next study samples to avoid possible interference by carry-over.

The known concentration levels of 4α-OHC in human plasma and serum varies between 5- 7 ng/ml and its levels can be used as a marker for clinical sample stability. The plasma or serum concentrations of 4α-OHC can increase during storage up to 100 ng/mL due to non-enzymatic formation and auto-oxidation of cholesterol [3] and hence the long-term stability of clinical samples can be monitored by measuring 4α-OHC levels. The levels of 4α-OHC in our blank K_2EDTA plasma pool was on average 5.24 ng/mL, and in clinical samples the levels varied between 2.12 – 5.65 ng/mL indicating good sample stability.
Freeze-thaw stability of 4β-OHC and 4α-OHC was evaluated by measuring the endogenous levels of the analytes in pooled human plasma and serum samples (n=6) after multiple freeze-thaw cycles. The samples were incubated at room temperature for 1 hour and refrozen on dry ice for 30 minutes totally 3 times before analysis. This freeze-thaw stability test demonstrates the worst-case scenario of handling samples and the differences between various freeze-thaw cycles are therefore not evaluated. The results for freeze-thaw stability (in Table 3) show that the levels of 4α-OHC and 4β-OHC stayed stable during three freeze-thaw cycles.

4.4 Assay performance in clinical studies

The developed LC/ESI-HR-MS method was used to evaluate rifampicin-induced levels of 4β-OHC in human plasma. Six women and six men participated in the study. The mean age was 23 years (SD ± 3.5; range 19 – 31), the mean weight 65 kg (SD ± 7.6; range 55 – 78) and the BMI 22.5 (SD ± 2.2; range 19.5 – 26.3). All the subjects were Caucasian. The volunteers were administered orally 600 mg rifampicin daily for a week, and the plasma samples were collected on the first day before the first administration of rifampicin, and on the eight study day. The observed levels of 4α-OHC and 4β-OHC in plasma are presented in Table 4. The average value in predose samples for 4β-OHC was 21.6 ng/mL, and for 4α-OHC 4.2 ng/mL. The average increase in 4β-OHC levels was 338 % compared to predose samples, mean concentration being 70.2 ng/mL, with precision of 20.6 % RSD. Concurrently, the levels of 4α-OHC stayed stable, decreasing on average 7 % compared to predose samples, with precision of 26.3 % RSD.

5. Conclusions

LC/ESI-HR-MS assay was developed for the quantitative analysis of cholesterol oxidation products 4β-OHC and 4α-OHC in preclinical and clinical plasma and serum samples. The assay was partially validated and the performance was found to be within typical bioanalytical acceptance criteria. The lower limits of quantitation in the assay were 0.5 ng/ml for 4β-OHC, and 2 ng/ml for 4α-OHC. Endogenous levels of 4β-OHC can vary between 10 - 100 ng/ml depending on the possible induction or inhibition of CYP3A4, whereas the levels of 4α-OHC can vary between 5- 100 ng/ml depending on the storage conditions of the clinical samples, making the assay developed well suitable for clinical use. Fast turnaround time and sample preparation in 96-well plate format enable robust and cost effective high throughput screening of vast amounts of clinical samples. The method has successfully been used for determination of 4β-OHC and 4α-OHC concentrations in clinical plasma and serum samples before and after rifampicin treatment. The availability of sensitive bioanalytical method to simultaneously quantify 4β-OHC and 4α-OHC in human plasma and serum samples enables the evaluation of CYP3A enzyme activity in patients. The method provides a possibility to understand in vivo induction or inhibition potential of certain medical substances.
The use of detection selectivity of high-resolution MS and lack of need for MS/MS-detection reaction enables use of sodium adduct as a main quantifier ion instead of protonated molecule, on the contrary to triple quadrupole mass spectrometers. Due to this, the laborious sample derivatization is not needed for electrospray MS analysis, and analytical sensitivity is increased.

The observations made in this study suggest that the effect of anticoagulants on analysis of 4β-OHC in clinical samples should be investigated further, and with broader selection of anticoagulants, including heparin.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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Figure 1. Structures of 4α-hydroxycholesterol (4α-OHC) (A) and 4β-hydroxycholesterol (4β-OHC) (B)

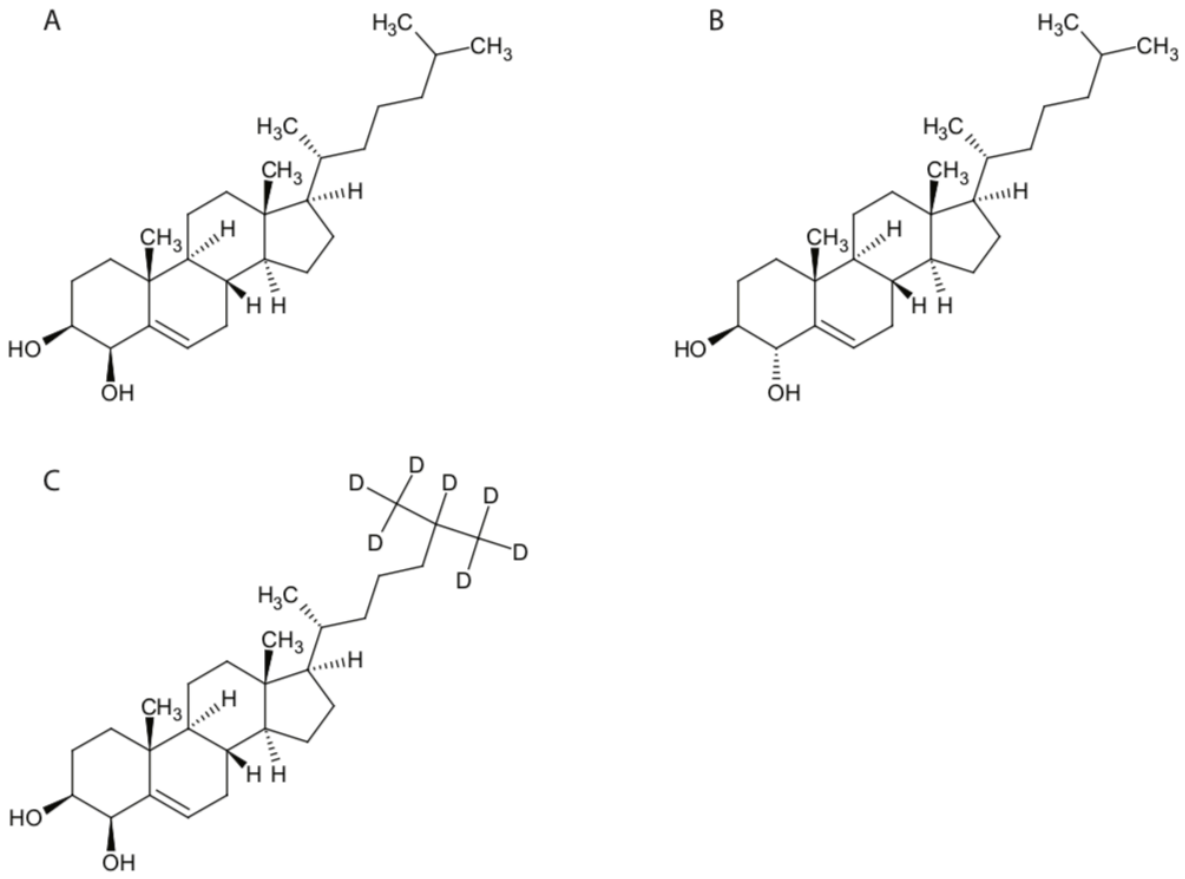

Figure 2. LC/ESI-HR-MS post-column infusion chromatograms of blank solvent, blank human serum, blank human citrate plasma, blank human K_2 EDTA plasma, blank surrogate matrix, and a LC/ESI-HR-MS chromatogram of 200 ng/ml standard sample of 4β-hydroxycholesterol (4b-OHC) and 4α-hydroxycholesterol (4a-OHC) in blank surrogate matrix. The infusion concentration (2 µg/ml) is about 100-fold to endogenous levels of 4b-OHC and 4a-OHC and therefore no change in baseline is observed at their retention times.

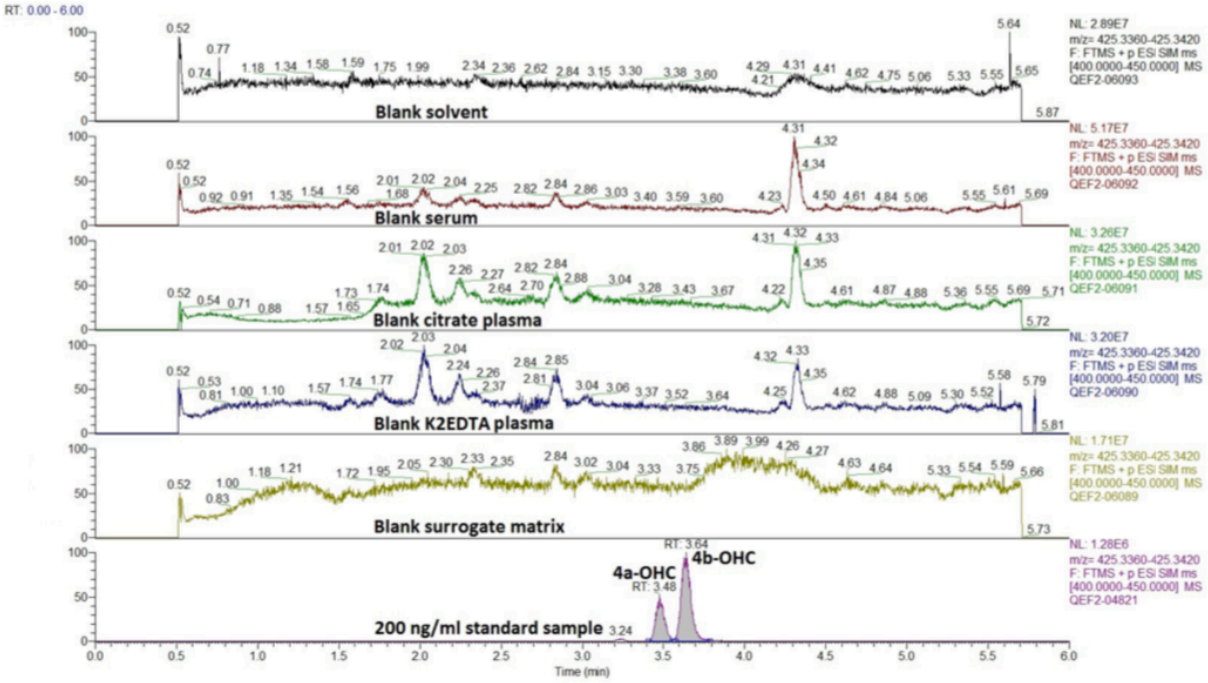

Figure 3. LC/ESI-HR-MS chromatograms of blank human serum, blank human citrate plasma, blank human K_2 EDTA plasma, blank surrogate matrix, showing endogenous 4α-hydroxycholesterol (4a-OHC,retention time 3.48 min) and 4β-hydroxycholesterol (4b-OHC, retention time 3.64 min).

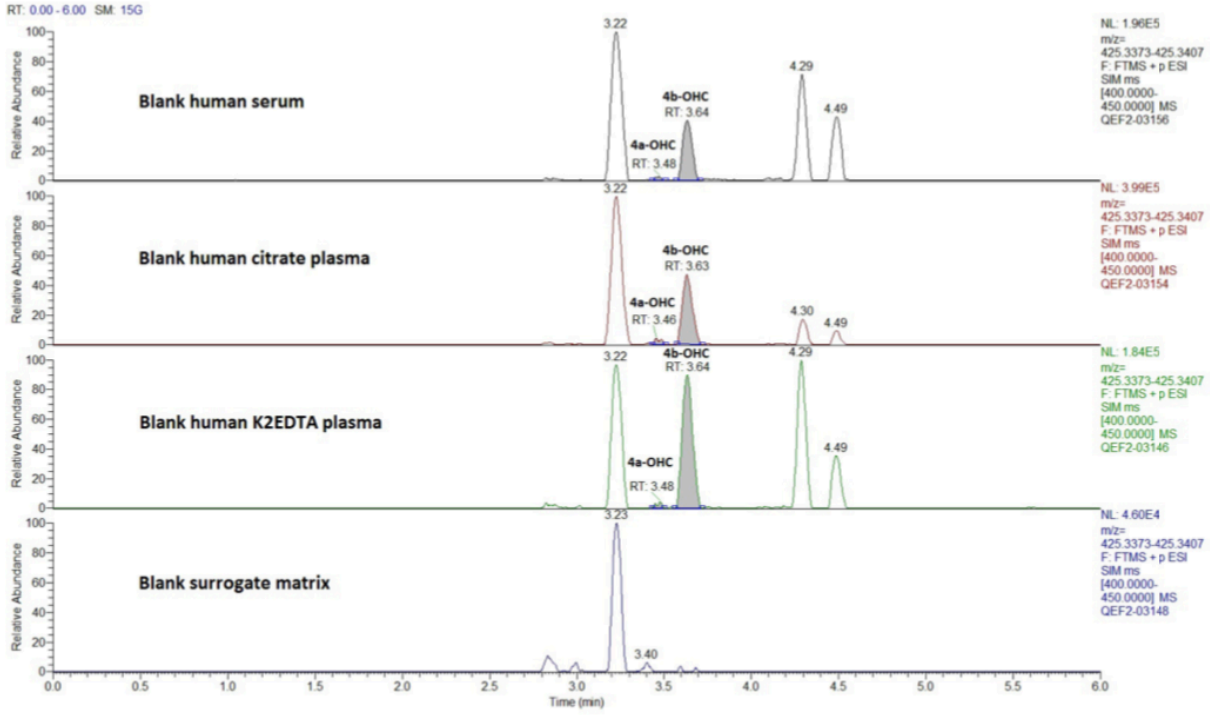

Figure 4. LC/ESI-HR-MS chromatograms of 1000 ng/ml standard sample in blank surrogate matrix, blank surrogate matrix sample injected after 1000 ng/ml standard sample for carry-over estimation, and 0.5 ng/ml standard sample in blank surrogate matrix showing 4α-hydroxycholesterol (4a-OHC) and 4β-hydroxycholesterol (4b-OHC).

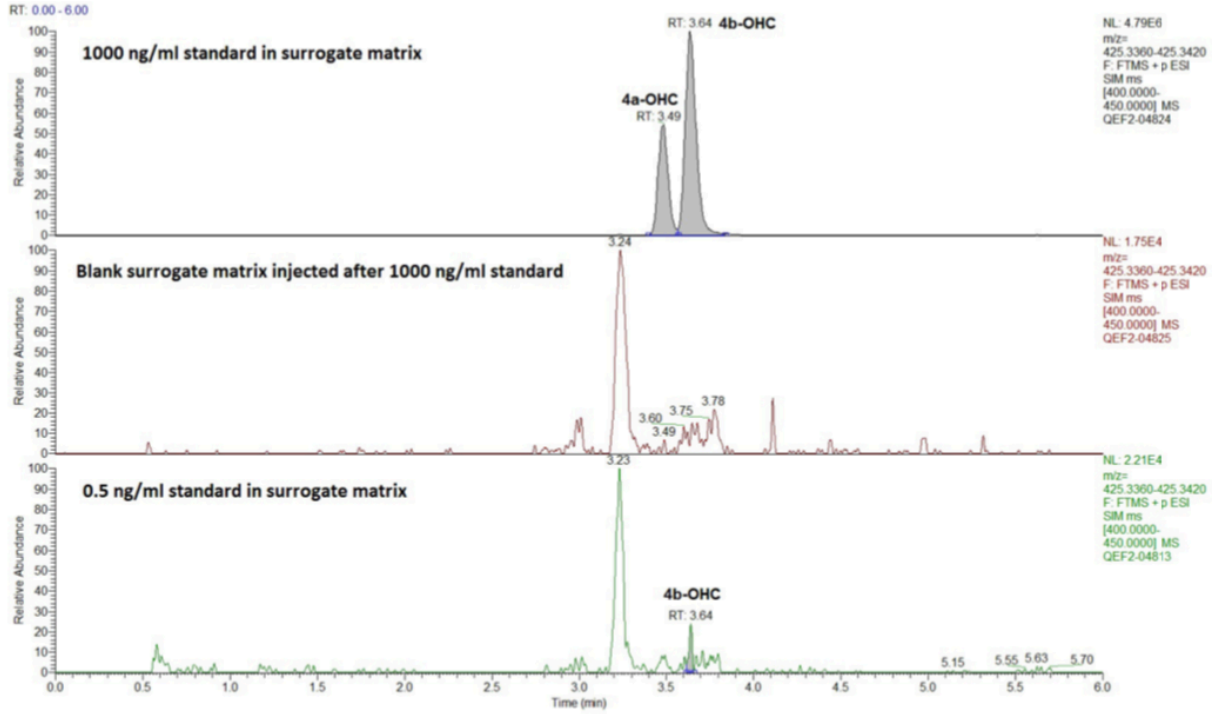

Table 1. Intra- and interday accuracies and precision for quality control samples in blank surrogate matrix on each of the four study days. SD = standard deviation in ng/ml, Δ % = percentual deviation of mean value from theoritical value, % RSD = 100*SD/mean value.

	4α-OHC	**10 ng/ml**	**30 ng/ml**	**100 ng/ml**	**300 ng/ml**	**4β-OHC**	**10 ng/ml**	**30 ng/ml**	**100 ng/ml**	**300 ng/ml**
	mean,					mean,				
day 1	ng/mL	10.7	30.6	109.9	330.2	ng/mL	10.0	31.3	105.5	318.1
	SD,					SD,				
(n=6)	ng/mL	0.6	1.3	4.2	7.1	ng/mL	0.3	0.7	3.2	6.1
	Δ%	6.9	2.2	9.9	10.1	Δ%	0.0	4.3	5.5	6.0
	% RSD	5.2	4.3	3.9	2.1	% RSD	2.7	2.1	3.0	1.9
	mean,					mean,				
day 2	ng/mL	10.1	33.7	111.4	320.3	ng/mL	9.7	31.0	103.7	297.4
	SD,					SD,				
(n=6)	ng/mL	0.7	2.3	4.0	13.1	ng/mL	0.8	0.9	1.1	11.5
	Δ%	0.9	12.4	11.4	6.8	Δ%	-2.8	3.3	3.7	-0.9
	% RSD	7.1	6.8	3.6	4.1	% RSD	7.9	2.9	1.1	3.9
	mean,					mean,				
day 3	ng/mL	11.0	31.3	103.3	318.4	ng/mL	11.1	31.4	99.1	296.4
	SD,					SD,				
(n=6)	ng/mL	1.3	1.2	4.7	9.5	ng/mL	0.4	0.7	2.8	9.7
	Δ%	9.8	4.4	3.3	6.1	Δ%	10.9	4.7	-0.9	-1.2
	% RSD	12.2	3.8	4.6	3.0	% RSD	3.4	2.3	2.8	3.3
	mean,					mean,				
day 4	ng/mL	10.9	30.7	106.6	315.6	ng/mL	10.6	31.6	100.8	307.2
	SD,					SD,				
(n=6)	ng/mL	0.5	1.1	4.3	3.9	ng/mL	0.2	0.7	2.5	4.6
	Δ%	9.2	2.2	6.6	5.2	Δ%	5.6	5.4	0.8	2.4
	% RSD	5.0	3.5	4.0	1.2	% RSD	2.1	2.1	2.5	1.5
	mean,					mean,				
Interday	ng/mL	10.7	31.6	107.8	321.2	ng/mL	10.3	31.3	102.3	304.8
(n=24)	Δ%	6.7	5.3	7.8	7.1	Δ%	3.4	4.4	2.3	1.6
	% RSD	8.2	6.1	4.8	3.2	% RSD	6.7	2.3	3.4	3.9

Table 2. Intra- and interday accuracies and precision for quality control samples in blank human plasma on each of the four study days. SD = standard deviation in ng/ml, Δ % = percentual deviation of mean value from theoritical value, % RSD = 100*SD/mean value.

	4α-OHC	10 ng/ml	30 ng/ml	100 ng/ml	300 ng/ml	4β-OHC	10 ng/ml	30 ng/ml	100 ng/ml	300 ng/ml
	mean,					mean,				
day 1	ng/mL	9.6	27.3	97.8	291.7	ng/mL	10.4	27.9	99.7	286.3
	SD,					SD,				
(n=6)	ngg/mL	0.8	2.5	8.9	10.8	ng/mL	1.5	2.6	7.5	10.4
	Δ%	-4.4	-9.1	-2.2	-2.8	Δ%	4.5	-6.9	-0.3	-4.6
	% RSD	8.5	9.2	9.1	3.7	% RSD	14.3	9.2	7.6	3.6
	mean,					mean,				
day 2	ng/mL	12.4	34.5	110.1	334.2	ng/mL	10.7	30.1	99.6	306.7
	SD,					SD,				
(n=6)	ng/mL	0.9	1.3	2.8	14.7	ng/mL	1.3	2.3	3.5	10.4
	Δ%	23.8	15.1	10.1	11.4	Δ%	6.8	0.2	-0.4	2.2
	% RSD	7.5	3.6	2.5	4.4	% RSD	12.3	7.5	3.5	3.4
	mean,					mean,				
day 3	ng/mL	10.2	28.7	95.5	283.7	ng/mL	11.3	32.3	107.6	311.6
	SD,					SD,				
(n=6)	ng/mL	1.3	2.4	7.6	10.0	ng/mL	1.1	1.3	5.0	8.4
	Δ%	1.6	-4.2	-4.5	-5.4	Δ%	12.5	7.5	7.6	3.9
	% RSD	12.7	8.2	8.0	3.5	% RSD	9.7	4.1	4.7	2.7
	mean,					mean,				
day 4	ng/mL	8.5	29.7	104.2	310.9	ng/mL	10.4	31.4	102.5	311.0
	SD,					SD,				
(n=6)	ng/mL	0.6	2.0	5.5	4.6	ng/mL	0.4	1.6	3.0	4.9
	Δ%	-15.4	-1.0	4.2	3.6	Δ%	4.4	4.7	2.5	3.7
	% RSD	6.7	6.7	5.3	1.5	% RSD	3.8	5.2	3.0	1.6
	mean,					mean,				
Interday	ng/mL	10.1	30.1	101.9	305.1	ng/mL	10.7	30.4	102.4	303.9
(n=24)	Δ%	1.4	0.2	1.9	1.7	Δ%	7.0	1.4	2.4	1.3
	% RSD	16.8	11.3	8.3	7.3	% RSD	10.4	8.2	5.6	4.4

Table 3. Stability results for 4α-OHC and 4β-OHC (n=6) in human plasma and serum after three freeze-thaw cycles, compared to untreated blank human plasma and serum.

	Plasma		Serum	
	% of untreated sample	RSD%	% of untreated sample	RSD%
4α-OHC	92.9	12.5	104.7	8.1
4β-OHC	103.4	3.49	108.6	3.94

466 **Table 4. Results for 4α-OHC and 4β-OHC in human plasma before and after rifampicin administration.**

patient	Before 4α-OHC ng/ml	After 4α-OHC ng/ml	% of initial	Before 4β-OHC ng/ml	After 4β-OHC ng/ml	% of initial
1	4.07	2.74	67.3	13.4	59.2	443
2	3.53	2.93	83.0	19.1	58.6	307
3	3.87	2.33	60.3	16.0	68.6	428
4	4.35	2.70	62.0	14.9	51.3	345
5	3.82	4.00	104.5	17.3	76.4	442
6	4.23	2.12	50.3	20.0	56.4	281
7	4.58	3.72	81.1	31.9	76.8	241
8	3.54	2.75	77.7	24.5	64.2	262
9	4.21	4.57	108.4	21.8	75.2	345
10	3.75	4.67	124.4	19.7	71.5	363
11	5.65	4.73	83.8	30.3	101	333
12	4.93	4.57	92.6	30.0	83.1	277
Average	4.2	3.5	83.0	21.6	70.2	339
SD	0.6	1.0	21.8	6.3	13.7	69.9
% RSD	14.6	28.5	26.3	29.2	19.5	20.6

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