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Andrea E. DeBarber
Oregon Health & Science University

Yana Sandler
Kennedy Krieger Institute, y.sandlers@csuohio.edu

Anuradha S. Pappu
Oregon Health & Science University

Louise S. Merkens
Oregon Health & Science University

P. Barton Duell
Oregon Health & Science University

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DeBarber, Andrea E.; Sandler, Yana; Pappu, Anuradha S.; Merkens, Louise S.; Duell, P. Barton; Lear, Steven R.; Erickson, Sandra K.; and Steiner, Robert D., "Profiling Sterols in Cerebrotendinous Xanthomatosis: Utility of Girard Derivatization and High Resolution Exact Mass LC-ESI-MSn Analysis" (2011). *Chemistry Faculty Publications*. 551.

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Authors

Andrea E. DeBarber, Yana Sandler, Anuradha S. Pappu, Louise S. Merkens, P. Barton Duell, Steven R. Lear, Sandra K. Erickson, and Robert D. Steiner

“Profiling Sterols in Cerebrotendinous Xanthomatosis: Utility of Girard Derivatization and High Resolution Exact Mass LC-ESI-MSⁿ Analysis”

Andrea E. DeBarber Yana Sandlers Anuradha S. Pappu Louise S. Merkens Barton Duell Steven R. Lear Sandra K. Erickson and Robert D. Steiner

Abstract

In this study we profile free 3-oxo sterols present in plasma from patients affected with the neurodegenerative disorder of sterol and bile acid metabolism cerebrotendinous xanthomatosis (CTX), utilizing a combination of charge-tagging and LC-ESI-MSⁿ performed with an LTQ-Orbitrap Discovery instrument. In addition, we profile sterols in plasma from 24 month old cyp27A1 gene knockout mice lacking the enzyme defective in CTX. Charge-tagging was accomplished by reaction with cationic Girard's P (GP) reagent 1-(carboxymethyl) pyridinium chloride hydrazide, an approach uniquely suited to studying the 3-oxo sterols that accumulate in CTX, as Girard's reagent reacts with the sterol oxo moiety to form charged hydrazone derivatives. The ability to selectively generate GP-tagged 3-oxo-4-ene and 3-oxo-5(H) saturated plasma sterols enabled ESI-MSⁿ analysis of these sterols in the presence of a large excess (3 orders of magnitude) of cholesterol. Often cholesterol detected in biological samples makes it challenging to quantify minor sterols, with cholesterol frequently removed prior to analysis. We derivatized plasma (10 μ l) without SPE removal of cholesterol to ensure detection of all sterols - present in plasma. We were able to measure 4-cholesten-3-one in plasma from untreated CTX patients (1207 \pm 302 ng/ml, mean \pm SD, n=4), as well as other intermediates in a proposed pathway to 5 α -cholestanol. In addition, a number of bile acid precursors were identified in plasma using this technique. GP-tagged sterols were identified utilizing high resolution exact mass spectra (\pm 5 ppm), as well as MS² ([M]⁺ \rightarrow) spectra that possessed characteristic neutral loss of 79 Da

(pyridine) fragment ions, and MS³ ([M]⁺→[M-79]⁺→) spectra that provided additional structurally informative fragment ions.

1. Introduction

Cerebrotendinous xanthomatosis (CTX; OMIM#213700) is a rare genetic disorder associated with deficient sterol 27-hydroxylase (CYP27A1); an enzyme important in the conversion of cholesterol to cholic and chenodeoxycholic acid (CDCA) (Figure 1 neutral pathway to primary bile acids). A damaging consequence of CYP27A1 deficiency is the accumulation of 5 α -cholestanol (a 5 α -dihydro derivative of cholesterol) in the tissues of affected patients. Patients often develop 5 α -cholestanol and cholesterol containing xanthomas. Extensive deposition of 5 α -cholestanol in the brain [1], is associated with the development of severe neurological dysfunction. In addition to 5 α -cholestanol, tissues and blood from untreated CTX patients contain high concentrations of bile acid precursors, in particular 7 α -hydroxy-4-cholesten-3-one (C4-7 α -ol-3-one, see Figure 1) [2–8].

The data reported thus far suggest that a major synthetic pathway to 5 α -cholestanol in CTX patients originates from C4-7 α -ol-3-one [9], in contrast to a normal synthetic pathway that originates from cholesterol. Both pathways for 5 α -cholestanol synthesis appear to converge through 4-cholesten-3-one (C4-3-one, Figure 1). *In vivo* studies in CTX patients demonstrated the formation of biliary 5 α -cholestanol from intravenous dosed [¹⁴C]-labeled C4-3-one [10]. Studies in rabbits [11] and in rats [12] demonstrated that 5 α -cholestanol was rapidly found in circulation after oral dosing with [¹⁴C]-labeled C4-3-one. Interestingly, prolonged feeding of C4-3-one (0.5%) in birds resulted in severe aortic arteriosclerosis and the accumulation of large amounts of 5 α -cholestanol in blood and tissues [12,13]. The synthesis of 5 α -cholestanol from C4-3-one was demonstrated to occur *via* 5 α -cholestan-3-one under *in vitro* conditions utilizing animal liver tissue preparations [14,15].

Although *in vitro* experiments initially suggested C4-3-one was formed from cholesterol in CTX [10], more recent studies indicate a major pathway to C4-3-one in CTX from 4,6-cholestadiene-3-one produced from C4-7 α -ol-3-one (Figure 1) [2,7]. High concentrations of 4,6-cholestadiene-3-one (500–850ng/ml) were shown to be present in hydrolyzed serum from untreated CTX patients [2], and the turnover of 4,6-cholestadiene-3-one to C4-3-one and 5 α -cholestanol was demonstrated in liver and brain tissue studies [16]. Although C4-7 α -ol-3-one can undergo non-enzyme catalyzed dehydration to form 4,6-cholestadiene-3-one, *in vitro* studies with liver tissue have characterized enzymatic conversion of C4-7 α -ol-3-one to 4,6-cholestadiene-3-one and subsequent saturation of the Δ -6 double bond to form C4-3-one [3,17].

To provide insight into alternate pathways that may be accentuated when bile acid synthesis is perturbed in CTX, we hypothesized a recently described approach utilizing the hybrid Thermo LTQ-Orbitrap instrument coupled with sterol derivatization [18] could be utilized for highly sensitive, selective ESI-MSⁿ analysis of 3-oxo-4-ene sterols present in blood and tissues of CTX patients. The approach involves sterol derivatization with cationic Girard's P (GP) reagent to enhance ESI ionization, as well as to provide MS² ([M]⁺→) spectra fragment ions possessing characteristic GP derivative neutral losses of 79 and 107 Da [18,19] and MS³ ([M]⁺→[M-79]⁺→) spectra fragment ions that provide further structural information. The Girard derivatives are resolved using HPLC and are identified by exact mass analysis (\pm 5 ppm), with MS² and MS³ spectra obtained to confirm sterol identity. Exact mass data is generated in the Orbitrap mass analyzer and MSⁿ spectra are generated in the LTQ linear ion trap (LIT) mass analyzer. We set out to detect sterols possessing a 3-oxo-4-ene or 3-oxo-5(H) saturated structure proposed to be *in vivo* intermediates in CTX

biochemical pathways (Figure 1) in a targeted manner utilizing derivatization with GP reagent and LC-ESI-MSⁿ analysis with an LTQ-Orbitrap Discovery instrument. We undertook this effort as part of a larger project designed to investigate altered metabolic pathways in CTX, in order to develop improved methods of screening, diagnosis, and treatment for this disorder. We describe here profiling of the free 3-oxo sterols present in plasma from affected patients and from 24 month old *cyp27A1* gene knock out (*cyp27A1* $-/-$) mice deficient in sterol 27-hydroxylase, the enzyme defective in CTX [5,20,21]. Bile acid precursors are elevated in these mice, which display a 3–5 fold increase in cholesterol 7 α -hydroxylase activity [22]. Although large amounts of C4-7 α -ol-3-one are present in liver tissue and blood from the mice [5,20], they do not develop xanthomas, or accumulate 5 α -cholestanol to the extent observed in human disease [5,5,22,23]. Recently significant cerebral accumulation of 5 α -cholestanol was noted to occur in 12 month old *cyp27A1* $-/-$ mice, especially in female mice [20], although corresponding to only 3% of the sterol pool versus 20–40% in patients with CTX [24]. The *cyp27A1* $-/-$ mice were used to demonstrate formation of cerebral 5 α -cholestanol from intravenously dosed [²H]-labeled C4-7 α -ol-3-one [20].

To summarize, in this study we report on the utility of GP derivatization and high resolution exact mass LC-ESI-MSⁿ analysis for profiling the free 3-oxo sterols present in plasma from untreated CTX patients and *cyp27A1* $-/-$ mice. We previously examined the utility of GP derivatization for quantitative LC-ESI-MS² analysis of a specific 3-oxo-4-ene sterol elevated in plasma from CTX patients [2–8]; C4-7 α -ol-3-one. We found C4-7 α -ol-3-one demonstrated improved utility as a diagnostic marker of disease and to monitor treatment compared to 5 α -cholestanol [8]. We anticipate GP-tagged sterols identified with high resolution exact mass LCESI-MSⁿ analysis will be amenable to LC-ESI-MS² analysis performed with unit resolution instrumentation as previously described for C4-7 α -ol-3-one [8], and we describe here preliminary LC-ESI-MS² experiments to measure 5 α -cholestanol precursors. We discuss our results in relation to the alternate pathways utilized when bile acid synthesis is perturbed in CTX and in the *cyp27A1* $-/-$ mouse.

2. Experimental

2.1 Chemicals and reagents

4,6-Cholestadiene-3-one, 5 α -cholestan-3 β -ol (5 α -cholestanol), 5 α -cholestan-3-one, 4-cholesten-3-one (C4-3-one), cholesterol (5-cholesten-3 β -ol), 7 α -hydroxy-4-cholesten-3-one (C4-7 α -ol-3-one) and 3 α ,7 α ,12 α -trihydroxy-5 β -cholestane were from Steraloids (Newport, RI). 5 β -cholestan-3 α -ol (5 β -cholestanol) was obtained from Sigma-Aldrich (St Louis, MO). 7 α ,12 α -dihydroxy-5 β -cholestan-3-one was synthesized from 3 α ,7 α ,12 α -trihydroxy-5 β -cholestane using 3 α -hydroxysteroid dehydrogenase from Sigma-Aldrich in the presence of β -NAD [25]. C4-7 α -ol-3-one-d₇ and C4-3-one-d₇ internal standards were synthesized from their respective 3 β -hydroxy- Δ 5 analogues using *streptomyces* sp. cholesterol oxidase obtained from Sigma-Aldrich as described previously [8,26,27]. 7 α -Hydroxycholesterol-d₇ and cholesterol-d₇ were obtained from CDN Isotopes (Pointe-Claire, Quebec, Canada). Methanol and water (GC-MS grade) were from Burdick and Jackson (Muskegon, MI). Formic acid (90%) was J.T. Baker brand and glacial acetic acid (99.99%) was from Aldrich. 'Girard's reagent P' (1-(carboxymethyl)pyridinium chloride hydrazide) was obtained from TCI America (Portland, OR). Volume 0.5ml Ultrafree-MC centrifugal filters (0.45 mm) were from Millipore (Bedford, MA). Human pooled plasma (K₂EDTA) was purchased from Biological Specialty Corporation (Colmar, PA).

2.2 Human and animal studies

Informed consent was obtained from the CTX patients and patient study protocols were approved by the Oregon Health & Science University Institutional Review Board. Blood samples were collected from untreated patients aged >16 years (n=4). Blood was collected from both male and female *cyp27A1* gene knock out mice and their wild type littermates -at 24 months of age. Plasma (K₂EDTA) was separated from the blood samples and stored at -80°C. The *cyp27A1* -/- and *cyp27A1* wild type (+/+) mice were from a heterozygote breeding colony at the Department of Veterans Affairs Medical Center San Francisco (VAMC), which was transferred onto the C57BL/6J background by backcrossing more than ten generations [22]. Mouse study protocols were approved by the Animal Studies Subcommittee, VAMC San Francisco. The animals were maintained on rodent chow and fed ad libitum. The Animal Research Facility at VAMC San Francisco is specific pathogen free and is 'Association for Assessment and Accreditation of Laboratory Animal Care International' accredited.

2.3 Preparation of calibrators and samples

Calibrators for the GC-FID and GC-MS methods were generated using dilutions of cholesterol and 5 α -cholestanol authentic standard in n-propyl alcohol or chloroform. To measure cholesterol and 5 α -cholestanol, plasma samples (25 to 100 μ l) were spiked with internal standard (5 β -cholestanol), hydrolyzed, extracted and derivatized to form trimethylsilyl ethers. GC was performed using a Phenomenex ZB1701 column (30m, ID=0.25mm, 0.25 μ m) with the method temperature held at 170°C for 2 min, the temperature increased at 18°C/min to 260°C, the temperature held for 12min and increased at 3°C/min to 280°C. Cholesterol was analyzed by FID and cholestanol by SIM MS; with detection of an m/z =355.4 target ion for internal standard and m/z = 306.3 ion for 5 α -cholestanol. Calibrators for the LC-ESI-MS methods were generated using dilutions of C4-7 α -ol-3-one, C4-3-one and 5 α -cholestan-3-one authentic standard in methanol or by spiking commercially available plasma with authentic standard solution. After addition of 20 μ l of methanol containing C4-7 α -ol-3-one-d₇ (10 ng) and C4-3-one-d₇ (10 ng) internal standards, sterols present in calibrators or 10 μ l plasma samples were derivatized with 175 μ l of 10 mM GP reagent in methanol at 1% acetic acid. The samples were shaken (200 rpm) for 120 min at room temperature and for plasma samples the precipitate was removed using centrifugal filters (0.45 μ m) prior to LC-ESI-MSⁿ analysis.

2.4 LC-ESI-MSⁿ

GP-tagged sterols were resolved using an Accela UPLC system (Thermo Fisher, San Jose, CA) with sample injection volume of 10 μ l. Samples were injected onto a Michrom BioResources (Auburn, CA) trap C₁₈ cartridge that was subsequently washed at 0.2 ml/min to remove excess GP reagent with solvent consisting of 33% methanol, 17% acetonitrile and 50% water. After 2 min the GP-tagged sterols were back-flushed onto a 50 \times 2.1 mm, 1.9 μ m, Hypersil Gold C₁₈-HPLC column from Thermo Hypersil (Waltham, MA, USA). The gradient mobile phase for HPLC separation (modified from Ogundare *et al* [28]) was delivered at 0.2 ml/min and consisted of two solvents (both containing 0.1% formic acid); **A** consisting of 33% methanol, 17% acetonitrile and 50% water and **B** consisting of 63% methanol, 32% acetonitrile, 5% water. Solvent **B** was increased from 25% to 100% over 3 min. The column was kept at 100% **B** for 6 min prior to increasing the flow to 0.25 ml/min for 2 min. The column was then equilibrated at 0% **B** for 2 min at 0.25 ml/min, giving a total run time of 15 min. The HPLC eluent was directed to a LTQ-Orbitrap XL Discovery instrument (San Jose, CA, USA), equipped with an ESI ion max source. The ionization interface was operated in the positive mode using the following settings: source voltage, 4 kV; sheath and aux gas flow rates, 50 and 5 units respectively; tube lens voltage, 90 V; capillary voltage, 49 V; and capillary temperature, 300°C. The Orbitrap mass analyzer was

externally calibrated prior to analysis to obtain mass accuracy within ± 5 ppm. An LC-ESI-MSⁿ method was created to perform three scan events in a data dependent manner as described by Griffiths *et al* [18]; a high resolution (30,000) full scan event over the m/z range 600–800 utilizing the Orbitrap analyzer, followed by data-dependent MS² and MS³ events performed in the LIT. For the MS² and MS³ events the normalized collision energies were 30 % and 35 %, respectively. A precursor ion inclusion list was generated based on the m/z of [M]⁺ ions for GP derivatives of potential sterols present, and MS² spectra were collected if precursor ions were present in the full scan spectra above a threshold of 500 counts. If a [M-79]⁺ fragment ion was observed in MS² spectra above a threshold of 200 counts, MS³ spectra were collected for the [M-79]⁺ ion.

Plasma GP-tagged C4-7 α -ol-3-one, C4-3-one and 5 α -cholestan-3-one were quantified using calibrators and high resolution data generated with the Orbitrap analyzer or LC-ESI-MS² data generated with an Applied Biosystems/SCIEX 4000 QTRAP instrument (Foster City, CA) scanning in triple quadrupole mode. The TurboIonSpray® ESI interface was operated in the positive mode with settings similar to those previously described for detection of GP-tagged C4-7 α -ol-3-one [8]. The MRM transitions monitored for GP-tagged C4-7 α -ol-3-one, C4-3-one and 5 α -cholestan-3-one were m/z 534.4→455.4, m/z 518.4→439.4 and m/z 520.4→441.4, respectively. Collision energies were 40 V, dwell times were 75 ms, and Q1 and Q3 were operated at unit resolution. The UPLC-ESI-MS² system was composed of an in-line Shimadzu (Columbia, MD) SIL-20AC XR auto-sampler and 2 LC-20AD XR LC pumps. GP-tagged sterols were resolved with a gradient mobile phase (consisting of water and methanol at 0.1% formic acid) as previously described [8], using a 30×2.1 mm, 1.9 μ m Hypersil Gold C₁₈-HPLC column with a 10×2.1 mm, 3 μ m Betabasic C₁₈-guard from Thermo Hypersil. LC-APCI-MS² analysis of underivatized C4-7 α -ol-3-one was performed with the 4000 QTRAP instrument to assess the signal enhancement factor for GP derivatization. The ionization interface was operated in the positive mode using the following settings: source temp (TEM), 450°C, ion spray needle voltage (IS), 5.0 kV; curtain (CUR), heater (GS1) and nebulizer (GS2) nitrogen gas flow rates, 15, 55 and 20 psi respectively; declustering potential (DP), 91 V; entrance potential (EP), 10 V; and collision cell exit potential (CXP), 24 V. The MRM transition monitored for was m/z 401.3→383.3. The collision energy (CE) was 25 V, dwell time was 100 ms and Q1 and Q3 were operated at unit resolution. The LC-MS² system was equipped with a 100×2.1 (i.d.) mm, 5 μ m Betabasic C₁₈-HPLC column with a 10×2.1 mm, 5 μ m Betabasic C₁₈-guard from ThermoHypersil. The gradient mobile phase was delivered at a flow rate of 0.4 ml/minute. The mobile phase consisted of two solvents: A, water and 0.1% formic acid, and B, methanol, 5mM ammonium acetate and 0.1% formic acid. Solvent B was increased from 60–98% over 2 min, the column was washed at 98% B for 3 min and re-equilibrated at 60% B for 3 min. The LC column temperature was kept at 40°C using a Shimadzu CTO-20AC column oven.

3. Results

3.1 Derivatization and LC-ESI-MSⁿ methodology

Derivatization of plasma sterols with 10 mM GP reagent in methanol containing 1% acetic acid was performed for 120 min [8], after which time the reaction mixture was injected for automated C₁₈-solid phase extraction (SPE) to remove excess derivatization reagent, with subsequent back-flushing of GP-tagged sterols onto a reversed phase C₁₈-HPLC column for chromatographic separation. Although the removal of plasma cholesterol prior to derivatization with an additional SPE step minimizes generation of cholesterol auto-oxidation products [29], we chose to perform our analysis without this step to ensure detection of sterols that otherwise may have been removed alongside the cholesterol. Detection of GP-tagged C4-7 α -ol-3-one in plasma from a representative untreated CTX

patient and *cyp27A1* $-/-$ mouse is shown with reconstructed ion chromatograms (RICs \pm 5 ppm) derived from data generated in the Orbitrap mass analyzer (Figure 2, upper RIC for CTX plasma, *Panel C*, and upper RIC for female *cyp27A1* $-/-$ mouse plasma, *Panel D*). In addition to expected presence of C4-7 α -ol-3-one, we detected C4-3-one at elevated concentrations, particularly in the CTX plasma (Figure 2, upper RIC for CTX plasma, *Panel G*, and upper RIC for *cyp27A1* $-/-$ mouse plasma, *Panel H*). Fragmentation routes for GP-tagged sterols are illustrated by the MS² and MS³ spectra obtained for C4-3-one (Figure 3; a major [M-79]⁺ fragment ion is observed in MS² ([M]⁺ \rightarrow) spectra, structurally informative fragment ions are observed in MS³ ([M]⁺ \rightarrow [M-79]⁺ \rightarrow) spectra). GP-tagged sterols were identified by comparison to authentic standard where available (for example with 5 α -cholestan-3-one, Figure 4) and by exact mass and MSⁿ data where no standard was available (for example with 7 α ,12 α -dihydroxy-4-cholesten-3-one, Figure 5).

3.2 Quantification

For the profiling experiments performed with the LTQ-Orbitrap Discovery a semi-quantitative determination of concentration was made for most compounds listed in Table 1, by isotope dilution mass spectrometry against known amounts of [²H]7-labeled C4-7 α -ol-3-one and C4-3-one internal standards. Peak areas obtained from RICs (\pm 5 ppm) generated in the Orbitrap were used to calculate the concentration for GP-tagged 3-oxo-4-ene sterols according to equation: analyte peak area/IS peak area= analyte concentration/IS concentration. This equation is valid as GP-tagged 3-oxo-4-ene sterols have been shown to have an identical response factor [30]. For GP-tagged 3-oxo-5(H) saturated sterols the calculated concentrations were normalized to the response factor for 5 α -cholestan-3-one according to the equation: (analyte peak area/IS peak area)/(5 α -cholestan-3-one peak area/IS peak area) = analyte concentration/IS concentration. Concentrations were determined for C4-7 α -ol-3-one, C4-3-one and 5 α -cholestan-3-one by generating calibration curves. Acceptable linearity (up to 10,000 ng/ml for C4-7 α -ol-3-one and 2,000 ng/ml for C4-3-one and 5 α -cholestan-3-one) was demonstrated for each calibration curve with characteristic correlation coefficients (r^2) >0.95. The within-run precision (RSD) for calculated GP derivative concentration from replicate analyses of a CTX sample is shown in Table 1.

We previously fully validated an LC-ESI-MS² method for quantification of GP-tagged C4-7 α -ol-3-one in CTX plasma with a 4000 QTRAP operating in triple quadrupole mode [8]. We modified this method to include quantification of GP-tagged C4-3-one and 5 α -cholestan-3-one in CTX patient and *cyp27A1* $-/-$ mouse plasma. Calibration curves were generated by performing a least-squares linear regression for calibrant peak area ratios obtained (sterol peak area/C4-3-one-d₇ peak area) versus concentration in ng/ml plasma. Acceptable linearity was demonstrated for each calibration curve (up to 2,000 ng/ml) with characteristic correlation coefficients (r^2) >0.95. Plasma calibrators were included with each sample set and monitored over a month (RSD for all calibrators apart from LLOQ was <15% and accuracy was between 85–115%; data not shown). The LLOQ (with RSD <20%) for C4-3-one was 50 ng/ml and for 5 α -cholestan-3-one was 100 ng/ml. To determine the effect of plasma matrix on detection of GP-tagged sterols, authentic standards were derivatized in the presence of commercially available plasma, and the ion abundance detected was compared to the ion abundance detected for authentic standard derivatized without plasma. Signal recovery was >80%, similar to results we previously observed for C4-7 α -ol-3-one [8].

3.3 Applications of the methodology

We hypothesized derivatization with GP reagent would provide a window with which to examine the 3-oxo sterols that accumulate in CTX (Figure 1) as Girard's reagent reacts with the sterol oxo moiety to enable selective ESI-MSⁿ analysis of these sterols. We profiled the

3-oxo-4-ene and 3-oxo-5(H) saturated sterols present in plasma from a representative untreated CTX patient and *cyp27A1*^{-/-} mice using GP derivatization and LC-ESI-MSⁿ analysis and were able to obtain semi-quantitative concentration estimates from data generated in the Orbitrap (data for *cyp27A1*^{-/-} mice shown in Table 1). The most noticeable gender difference was a higher concentration of bile acid precursors C4-7 α -ol-3-one and 7 α -hydroxy-5 β -cholestan-3-one in plasma from female *cyp27A1*^{-/-} mice. We found concentrations of C4-7 α -ol-3-one and 7 α -hydroxy-5 β -cholestan-3-one to be fairly comparable in *cyp27A1*^{-/-} mice and in the CTX patient (see also Figure 2).

Concentrations of 12 α -hydroxylated bile acid precursors were markedly lower in *cyp27A1*^{-/-} mouse plasma compared to those in the CTX patient, although they were elevated compared to *cyp27A1* wild type littermates (data not shown). Concentrations of plasma 4,6-cholestadiene-3-one, C4-3-one and 5 α -cholestan-3-one in the *cyp27A1*^{-/-} mice were between 9 and 15% of those in the CTX patient.

We further quantified plasma C4-7 α -ol-3-one, C4-3-one and 5 α -cholestan-3-one for a number of untreated CTX patients and female and male *cyp27A1*^{-/-} and *cyp27A1* wild type mice *via* LC-ESI-MS² analysis performed with a 4000 QTRAP instrument and, in addition, quantified 5 α -cholestanol and cholesterol in the plasma samples using GC-methodology (Table 2). The mean plasma C4-7 α -ol-3-one, C4-3-one and 5 α -cholestan-3-one concentrations obtained were similar to those determined from Orbitrap data, with the mean concentration of C4-7 α -ol-3-one in the *cyp27A1*^{-/-} mice being 52% of the concentration in CTX patients, but 14.9-fold increased compared to wild type mice (P=0.016). The concentrations of C4-3-one and 5 α -cholestan-3-one in the *cyp27A1*^{-/-} mice were <25% of the concentrations in CTX patients, with C4-3-one increased 4.7-fold (P=0.003) and 5 α -cholestan-3-one increased 1.7-fold (P=0.016) compared to wild type mice. The mean concentration of 5 α -cholestanol in the *cyp27A1*^{-/-} mice was 17% the concentration in CTX patients, but 1.6-fold increased compared to wild type mice (P=0.010).

4. Discussion

We present here an example of incorporating a derivatization step for analysis with ESI-MSⁿ, not only to improve analytical sensitivity, but also to tag a specific class of molecules present in a genetic disorder. The selective generation of GP-tagged 3-oxo-4-ene and 3-oxo-5(H) saturated plasma sterols enabled ESI-MSⁿ detection of proposed *in vivo* sterol intermediates in CTX biochemical pathways (shown in grey, Figure 1). Sterol detection was accomplished with good sensitivity using conventional HPLC-ESI methodology, despite the presence of a large excess of cholesterol. The signal enhancement factor for Girard derivatization of C4-7 α -ol-3-one and analysis with ESI-MS/MS relative to analysis of underivatized C4-7 α -ol-3-one with APCI-MS/MS was >30 fold. Some sterols were identified by comparison with authentic standards and others by information provided from LC-ESI-MSⁿ experiments performed with the LTQ-Orbitrap Discovery instrument.

To our knowledge measurement of elevated C4-3-one or 5 α -cholest-3-one has not been previously described in blood or tissues from CTX patients, although these sterols are known to be immediate precursors of 5 α -cholestanol based on [¹⁴C]4-labeled C4-3-one dosing experiments [10,11] and *in vitro* studies [14]. It is of interest to note that despite comparable plasma cholesterol concentrations between CTX patients and an unaffected subject, there is a large difference in C4-3-one concentrations (Table 1). Elevated C4-7 α -ol-3-one and 4,6-cholestadiene-3-one were previously detected in CTX serum [2] and an alternative pathway to C4-3-one was proposed from C4-7 α -ol-3-one through 4,6-cholestadiene-3-one [9].

In accordance with previous studies in *cyp27A1*^{-/-} mice [20], we determined the mean plasma concentration of C4-7 α -ol-3-one in 24 month old *cyp27A1*^{-/-} mice was 52% of the plasma concentration in CTX patients, compared with a plasma concentration of 5 α -cholestanol in *cyp27A1*^{-/-} mice that was only 17% of the concentration in CTX patients. Female 12 month old *cyp27A1*^{-/-} mice were reported to display marked cerebral accumulation of 5 α -cholestanol [20], although this was small in comparison to the accumulation in CTX patients. The difference in cerebral accumulation was hypothesized to be due to decreased 7 α -hydroxylation in the mice, with decreased transfer of C4-7 α -ol-3-one (and 7 α -hydroxycholesterol) across the blood brain barrier. As the concentration of C4-7 α -ol-3-one in *cyp27A1*^{-/-} mice is around half the concentration found in CTX patients, but the concentrations of 4,6-cholestadiene-3-one, C4-3-one and 5 α -cholest-3-one range from a quarter to a fifth, we propose differences between the *cyp27A1*^{-/-} mouse and human disease may reside in decreased enzyme-catalyzed conversion of C4-7 α -ol-3-one to 4,6-cholestadiene-3-one and C4-3-one or more efficient removal of these sterols from the alternative pathway to 5 α -cholestanol in *cyp27A1*^{-/-} mice.

GP derivatization and LC-ESI-MSⁿ analysis also enabled the detection in plasma of intermediates in the neutral bile acid pathway (Figure 1). The *cyp27A1*^{-/-} mice displayed quantitative differences in bile acid precursor profiles when compared with CTX patients, notably decreased 12 α -hydroxylated intermediates like 7 α ,12 α -dihydroxy-4-cholesten-3-one and 7 α ,12 α -dihydroxy-5 β -cholestan-3-one. Although 7 α ,12 α -dihydroxy-4-cholesten-3-one, 5 β -cholestan-3 α ,7 α ,12 α -triol and 5 β -cholestan-3 α ,7 α -diol were previously detected at high concentrations in plasma and liver tissue from CTX patients [4–6], to our knowledge elevated plasma 7 α ,12 α -dihydroxy-5 β -cholestan-3-one and 7 α -hydroxy-5 β -cholestan-3-one have not been previously reported.

A fragmentation route for GP-tagged 3-oxo-4-ene sterols has been proposed with the structure for [M-79]⁺ ion shown as an *inset* to Panel A (Figure 3) [29]. GP-tagged 3-oxo-5(H) saturated sterols fragment in an alternate manner with fragment ions observed in the MS² and MS³ spectra that are similar in nature. The MS² ([M]⁺→) spectra are dominated by [M-79]⁺ fragment ions (formation of a [M-79]⁺ fragment ion for GP-tagged 5 α -cholestan-3-one was previously noted [29]) and the MS³ ([M]⁺→[M-79]⁺→) spectra contain [M-107]⁺ ions. There is an absence of characteristic 3-oxo-4-ene b ions indicating fragmentation likely does not involve B ring cleavage. There is also little evidence of C and D ring cleavage, except in the case of GP-tagged 7 α ,12 α -dihydroxy-5 β -cholestan-3-one. Modification of B and C rings by introduction of hydroxyl groups at C7 and C12 (Figure 5) leads to formation of characteristic [M-79-(n18)]⁺ and [M-107-(n18)]⁺ fragment ions [29]. Overall less structurally informative fragments are present the MS³ spectra than for the corresponding 3-oxo-4-enes.

A limitation of the experiments we describe was the focus on 3-oxo-4-ene and 3-oxo-5(H) saturated sterols and bile alcohols, however these experiments could be easily modified to also examine 3 α -hydroxy-5 β (H) bile alcohols present in CTX, for example 3 α ,7 α ,12 α -trihydroxy-5 β -cholestane and 3 α ,7 α -dihydroxy-5 β -cholestane (by conversion to 3-oxo-5 β (H) bile alcohols using 3 α -hydroxysteroid dehydrogenase as described for synthesis of 7 α ,12 α -dihydroxy-5 β -cholestan-3-one in methods section [25]). Such experiments are underway in our laboratory and also possess the advantage that cholesterol in the samples remains underivatized.

In conclusion, we report here that GP derivatization of plasma and high resolution exact mass LC-ESI-MSⁿ analysis can be utilized to selectively profile the free 3-oxo sterol signature present in the systemic circulation of untreated CTX patients and in *cyp27A1*^{-/-} mice, even without removal of cholesterol, which is present in great excess. The techniques

we describe facilitate the study of sterol and bile acid pathways in CTX, and should lead to improvements in screening, diagnosis, and treatment of this debilitating disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by an NIH grant awarded to S.K.E. (DK072187), a Department of Veterans Affairs Merit Award to S.K.E., and a RDCRN fellowship (U54HD061939) and United Leukodystrophy Foundation grant awarded to A.E.D. We are grateful to the CTX patients and their families for participation. The research was accomplished using instrumentation housed in the OHSU Department of Physiology and Pharmacology Bio-Analytical Shared Resource and Portland State University Chemistry Department MS Facility (where LTQ-Orbitrap instrument was purchased with NSF grant 0741993). A.E.D would like to dedicate this paper to the late Dr William E Connor for sharing his wealth of knowledge and CTX samples and providing enthusiasm and encouragement.

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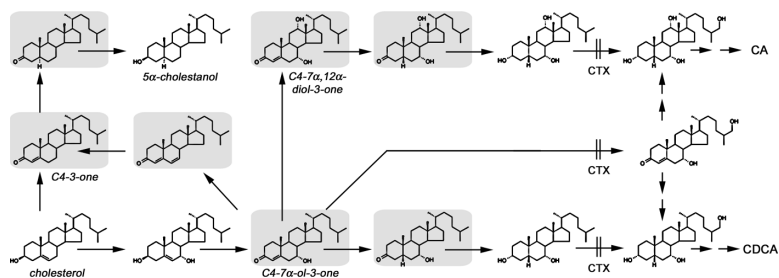


Figure 1. The neutral pathway to primary bile acids in cerebrotendinous xanthomatosis (CTX) Bile acid precursors (bile alcohols) are generated by increased cholesterol 7 α -hydroxylase activity, in addition to deficient 27-hydroxylation in CTX affected patients. With CDCA treatment of CTX the plasma concentration of 7 α -hydroxy-4-cholesten-3-one (C4-7 α -ol-3-one) rapidly declines [8,9]; reflecting feedback inhibition by CDCA to decrease cholesterol 7 α -hydroxylation, and limit bile acid synthesis [9]. In the normal sequence of events C4-7 α -ol-3-one can be further hydroxylated to form 7 α ,12 α -dihydroxy-4-cholesten-3-one (C4-7 α ,12 α -diol-3-one). Both C4-7 α ,12 α -diol-3-one and C4-7 α -ol-3-one are reduced by a soluble 3-oxosteroid Δ^4 -steroid 5 β -reductase to form 7 α ,12 α -dihydroxy-5 β -cholestan-3-one and 7 α -hydroxy-5 β -cholestan-3-one, respectively [9]. Prior to 27-hydroxylation the 3-oxo moiety in each bile alcohol is further reduced by a 3 α -hydroxysteroid dehydrogenase to form 5 β -cholestane-3 α ,7 α ,12 α -triol and 5 β -cholestane-3 α ,7 α -diol respectively [9].

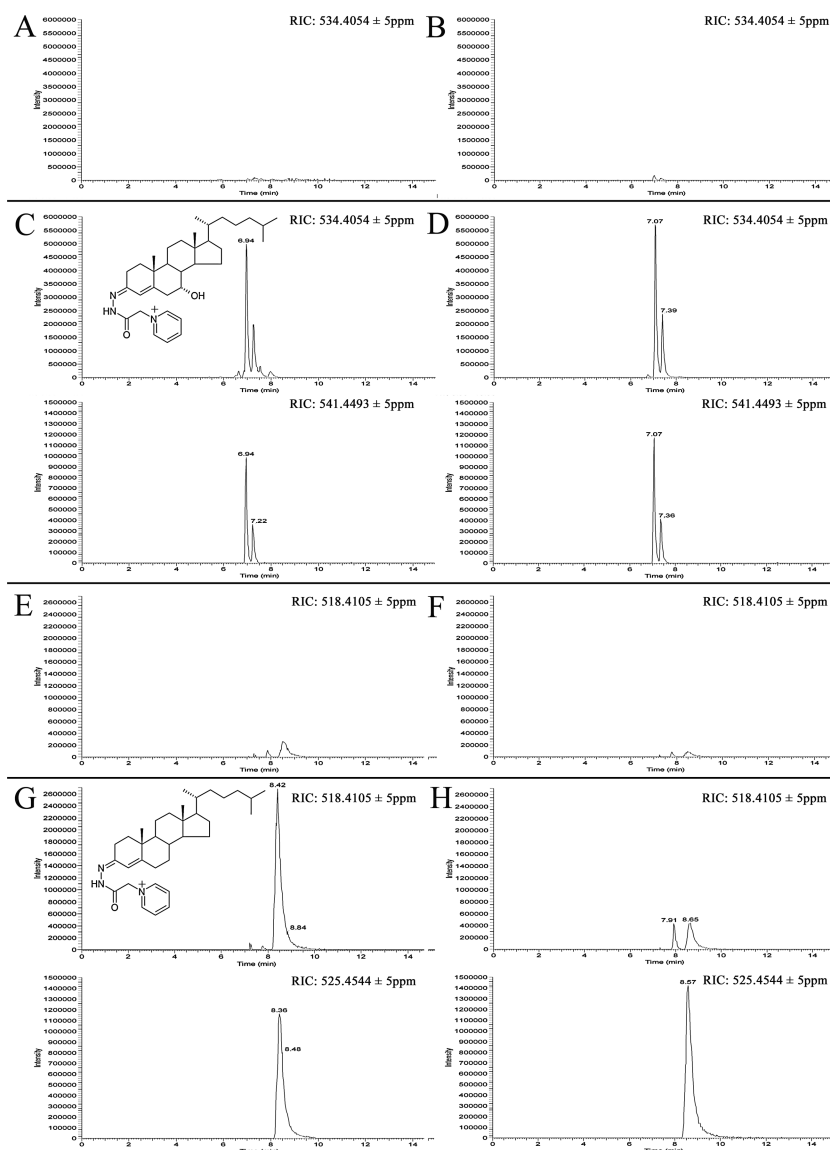


Figure 2. 7α -Hydroxy-4-cholesten-3-one and 4-cholesten-3-one present in plasma from untreated CTX patient and *cyp27A1*^{-/-} mice
Panel A & B and E & F are RICS from data generated in the Orbitrap analyzer for plasma from representative unaffected human and 24 month old female wild-type mouse, respectively. Panel C & D and G & H (upper chromatograms) are RICS for plasma from representative CTX affected patient and 24 month old female *cyp27A1*^{-/-} mouse, respectively. GP-tagged C4- 7α -ol-3-one (exact mass 534.4054) appears in chromatograms as *syn* and *anti* conformers eluting at 7 and 7.3 min. GP-tagged C4-3-one (exact mass 518.4105) appears in chromatograms as broad peak eluting at 8.4 min. Panel C & D and G & H (lower chromatograms) are RICS for [2 H]7-labeled C4- 7α -ol-3-one (exact mass 541.4493) and [2 H]7-labeled C4-3-one (exact mass 525.4544) internal standards respectively spiked into plasma samples.

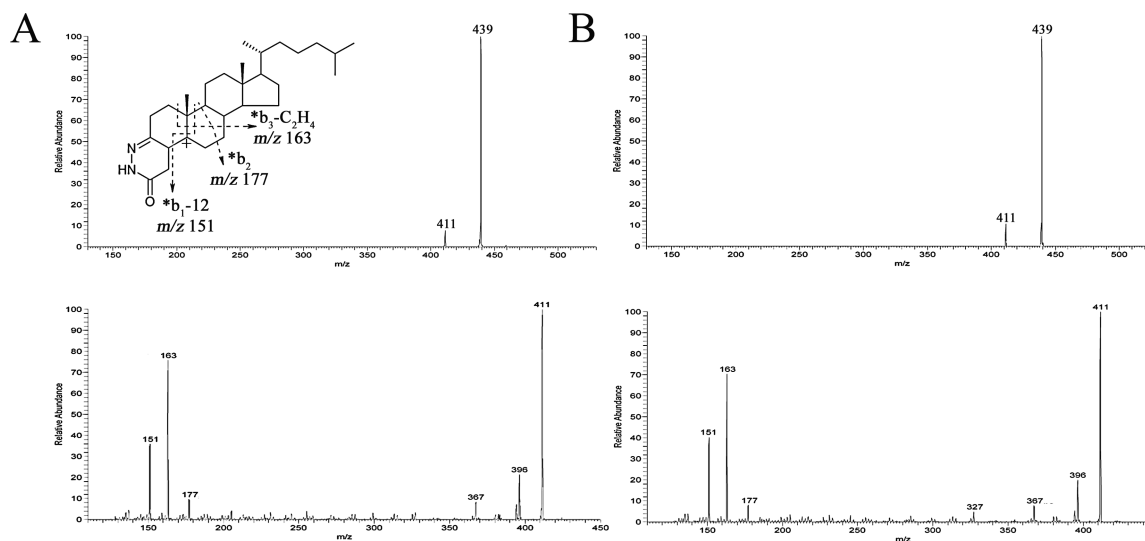


Figure 3. Fragmentation routes for GP-tagged 3-oxo-4-ene sterols illustrated by 4-cholesten-3-one

Panel A is MS² (518→; upper panel) and MS³ (518→439→; lower panel) spectra for GP-tagged C4-3-one from CTX plasma eluting at 8.4 min. Panel B is MS² and MS³ spectra for GP-tagged authentic standard at same retention time. Major [M-79]⁺ and [M-107]⁺ fragment ions formed by the neutral loss of pyridine, and pyridine plus CO, respectively, are observed in MS² ([M]⁺→) spectra; structure for [M-79]⁺ ion is shown as *inset* to Panel A [29]. Formation of structurally informative fragment ions observed in MS³ ([M]⁺→[M-79]⁺→) spectra are indicated according to Griffiths *et al* [29]. MSⁿ spectra were generated in LIT analyzer.

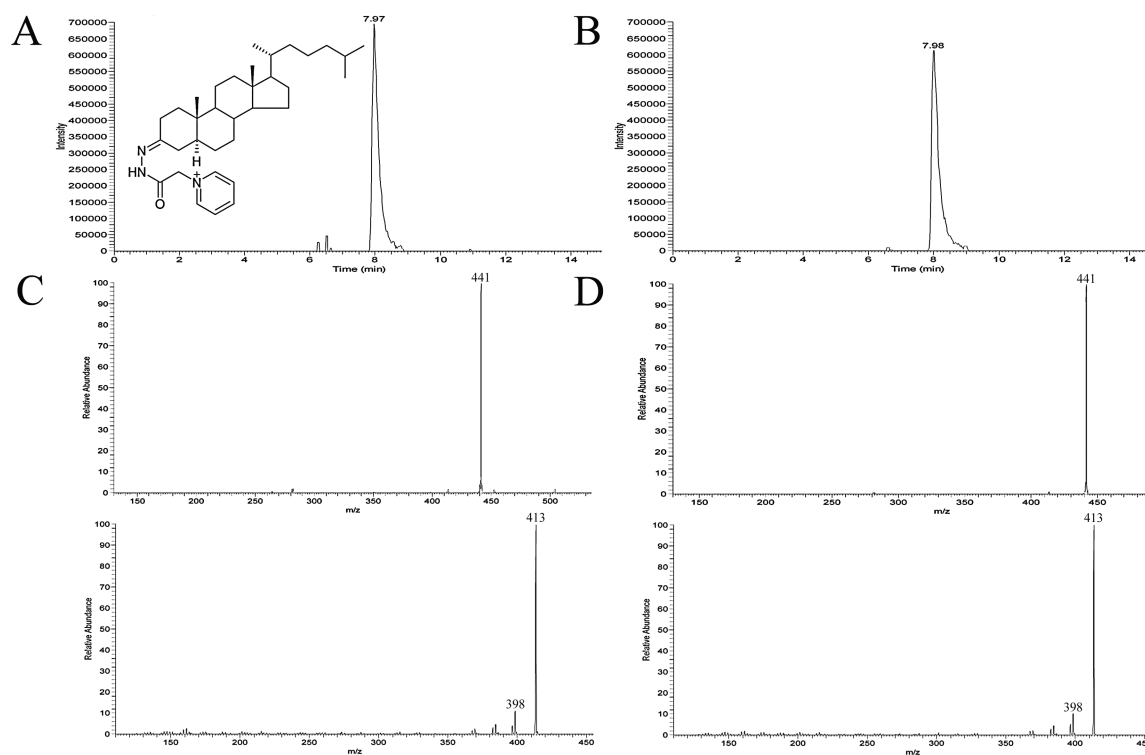


Figure 4. 5α -Cholestan-3-one present in plasma from untreated CTX patient

Panels A and B are RICs from data generated in the Orbitrap analyzer for representative CTX plasma sample and authentic standard, respectively. GP-tagged 5α -cholestan-3-one appears in chromatogram as broad peak eluting at 8 min. Panels C are MS^2 ($520 \rightarrow$; upper panel) and MS^3 ($520 \rightarrow 441 \rightarrow$; lower panel) spectra for GP-tagged 5α -cholestan-3-one from CTX plasma. Panels D are MS^2 and MS^3 spectra for GP-tagged authentic 5α -cholestan-3-one. Collision induced dissociation of $[M-79]^+$ ion observed in MS^2 spectra leads to formation of two ions in MS^3 spectra; m/z 413 corresponding to loss of pyridine plus CO $[M-107]^+$ and m/z 398, likely arising from loss of pyridine plus isocyanic acid $[M-79-HNCO]^+$.

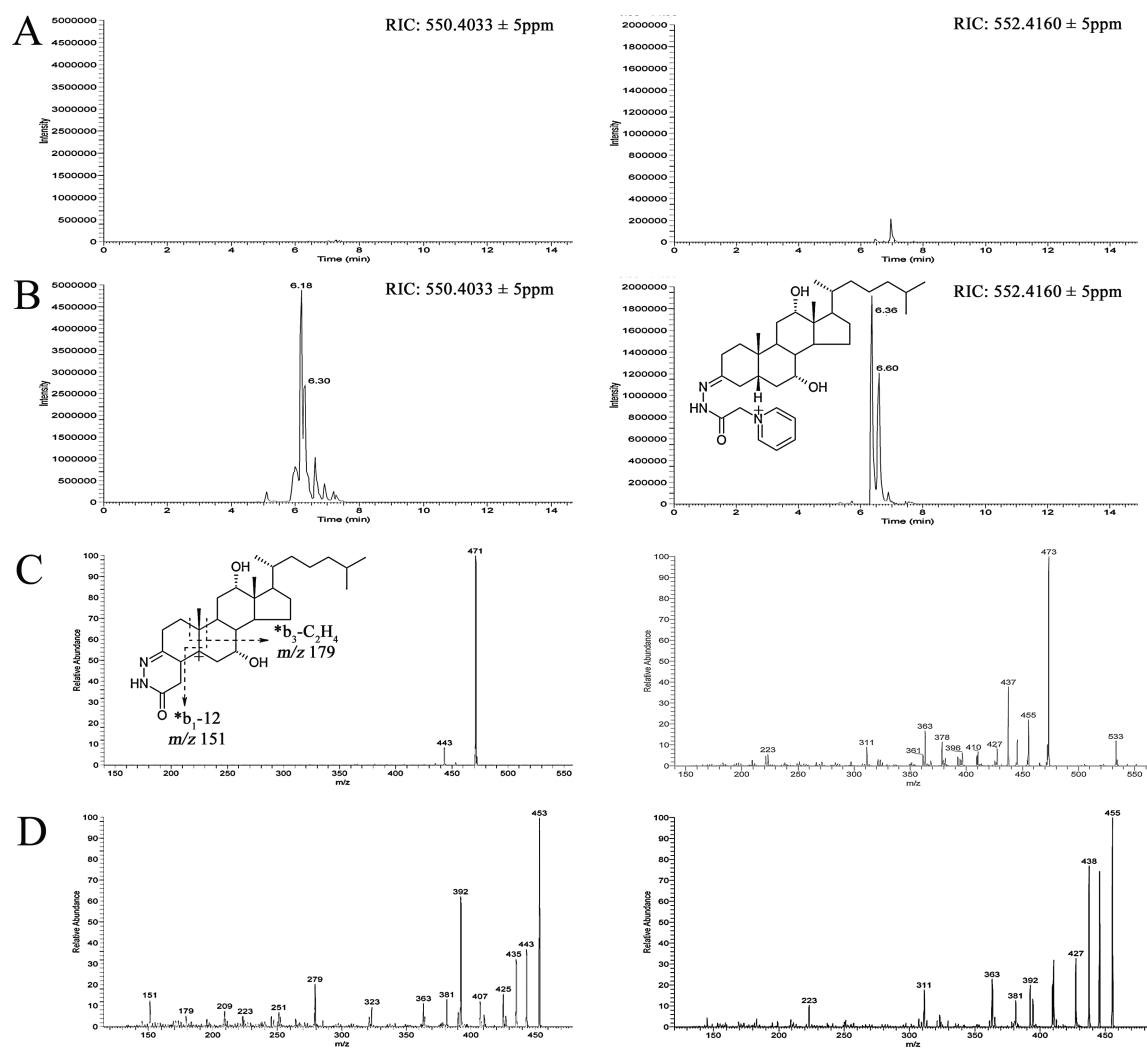


Figure 5. $7\alpha,12\alpha$ -Dihydroxy-4-cholesten-3-one and $7\alpha,12\alpha$ -dihydroxy-5 β -cholestan-3-one present in plasma from untreated CTX patient

Panels A and B are RICs from data generated in the Orbitrap analyzer for plasma from representative un-affected and CTX affected subject, respectively. Left panels; putative GP tagged $7\alpha,12\alpha$ -dihydroxy-4-cholesten-3-one appears in chromatogram as *syn* and *anti* conformer split peak eluting at 6.2–6.3 min. Right panels; GP-tagged $7\alpha,12\alpha$ -dihydroxy-5 β -cholestan-3-one appears in chromatogram as peak eluting at 6.6 min (peak at 6.4 min is unidentified putative dihydroxy-5 β -cholestan-3-one). Panels C are respective MS² spectra (550→left panel, 552→right panel) and Panels D are respective MS³ spectra (550→471→left panel; 552→473→left panel). Formation of structurally informative fragment ions observed in MS³ spectra for $7\alpha,12\alpha$ -dihydroxy-4-cholesten-3-one [M-79]⁺ ion are indicated (*inset* to Panel C); including *b₃-C₂H₄ fragment observed at *m/z* 179 indicating presence of 7α -hydroxyl group [29]. There is no *c₂+2H-H₂O fragment observed at *m/z* 231 (from cleavage of C-ring) [29], indicating C-ring is modified, likely with 12α -hydroxyl group. The presence of two hydroxyl groups is consistent with observation of characteristic [M-79-(nH₂O)]⁺ and [M-107-(nH₂O)]⁺ ions [29]. GP-tagged $7\alpha,12\alpha$ -dihydroxy-5 β -cholestan-3-one from CTX plasma was identified by comparison to GP-tagged authentic standard. Fragment ions observed in the MS³ spectrum include *m/z* [M-79-(nH₂O)]⁺ and [M-107-(nH₂O)]⁺ ions indicative of two hydroxyl groups. Low abundance

ions at m/z 392 and 394 most likely arise from neutral loss of pyridine, NH_3 , CO and two molecules of water $[\text{M}-79-\text{NH}_3-\text{CO}-2\text{H}_2\text{O}]^+$ and loss of pyridine, isocyanic acid and water $[\text{M}-79-\text{HNCO}-2\text{H}_2\text{O}]^+$, respectively.

Table 1

Sterols identified in plasma from untreated CTX patient and 24 month old cyp27A1 $-/-$ mice utilizing data generated with LTQ-Orbitrap instrument.

GP derivative	Formula	Exact mass	RT (min)	Conc. for representative CTX patient (ng/ml)	Method RSD* (%)	Mean conc. (n=3) male cyp27A1 $-/-$ mice (ng/ml)	Mean conc. (n=3) female cyp27A1 $-/-$ mice (ng/ml)
4,6-cholestadiene-3-one 3GP ^{1,4,5,8}	C ₃₄ H ₅₀ N ₃ O ₁ ⁺	516.3948	7.6	1215	8.5	150	187
4-cholesten-3-one 3GP ^{1,4}	C ₃₄ H ₅₂ N ₃ O ₁ ⁺	518.4105	8.0	4662	2.2	725	539
5 α -cholestan-3-one 3GP ^{1,4,7,8}	C ₃₄ H ₅₄ N ₃ O ₁ ⁺	520.4261	8.2	407	2.7	77	35
7 α -hydroxy-4-cholesten-3-one 3GP ^{2,4,6}	C ₃₄ H ₅₂ N ₃ O ₂ ⁺	534.4054	7.2	8842	8.9	2431	4344
7 α -hydroxy-5 β -cholestan-3-one 3GP ^{2,3,7,8}	C ₃₄ H ₅₄ N ₃ O ₂ ⁺	536.4211	7.3	3231	9.0	2740	5031
7 α ,12 α -dihydroxy-4-cholesten-3-one 3GP ^{2,3,6,7,8}	C ₃₄ H ₅₂ N ₃ O ₃ ⁺	550.4033	6.2	2098	8.8	198	291
Putative dihydroxy 5 β -cholestan-3-one 3GP ^{2,3,7,8,9}	C ₃₄ H ₅₄ N ₃ O ₃ ⁺	552.4160	6.2	848	3.9	106	99
7 α ,12 α -dihydroxy 5 β -cholestan-3-one 3GP ^{2,4,7,8}	C ₃₄ H ₅₄ N ₃ O ₃ ⁺	552.4160	6.4	1282	6.7	ND	ND

ND, not detected

* RSD is for representative CTX sample calculated concentration (within-run relative standard deviation for three replicates).

¹ Quantitative estimate was based on area ratio of analyte to 4-cholesten-3-one-d7 internal standard (formula C₃₄D₇H₄₅N₃O₁⁺, exact mass 525.4544).

² Quantitative estimate was based on area ratio of analyte to 7 α -hydroxy-4-cholesten-3-one-d7 internal standard (formula C₃₄D₇H₄₅N₃O₂⁺, exact mass 541.4493).

³ Identification based on exact mass and MSⁿ spectra.

⁴ Identification based on comparison with authentic standards.

⁵ 4,6-cholestadien-3-one levels augmented by significant dehydration of C₄-7 α -ol-3-one under our derivatization conditions (<20% of 4,6-cholestadien-3-one response by comparison with dehydration of C₄-7 α -ol-3-one authentic standard, data not shown). Dehydration of C₄-7 α -ol-3-one is negligible in plasma stored at -20 °C for up to 10 months [26].

⁶ Resolved *syn* and *anti* conformers.

⁷ Not detected in plasma from unaffected subjects.

⁸ Not detected in plasma from cyp27A1 $+/+$ mice.

⁹ [M-79-(n18)]⁺ and [M-107-(n18)]⁺ fragment ions observed in MS² ([M]⁺→[M-79]⁺→) spectra consistent with two hydroxyl groups.

Table 2
Concentration of 5 α -cholestanol precursors in plasma from untreated CTX patients and 24-month old cyp27A1 $-/-$ mice.

	7 α -hydroxy-4-cholesten-3-one ^{a,b} (ng/ml)	4-cholesten-3-one ^{a,c,d} (ng/ml)	5 α -cholestan-3-one ^{a,c,d} (ng/ml)	5 α -cholestanol ^e (μ g/dl)	Cholesterol ^f (mg/dl)
<i>Untreated CTX patients (n=4)</i>	5675 \pm 1975 (3720–7860)	1207 \pm 302 (918–1580)	342 \pm 90 (267–461)	3040 \pm 910 (2510–4400)	148 \pm 11 (132–156)
<i>Representative unaffected subject</i>	148	94	ND	220 ^e	151
<i>Female cyp27A1 $-/-$ mice (n=3)</i>	3897 \pm 1952 (2740–6150)	170 \pm 49 (113–201)	71 \pm 10 (48–83)	329 \pm 108 (224–440)	36 \pm 11 (26–48)
<i>Female cyp27A1^{+/+} mice (n=3)</i>	258 \pm 124 (138–385)	42 \pm 16 (25–55)	37 \pm 10 (27–46)	228 \pm 96 (171–339)	86 \pm 42 (56–134)
<i>Male cyp27A1 $-/-$ mice (n=3)</i>	2018 \pm 1555 (694–3730)	115 \pm 42 (83–162)	97 \pm 14 (85–112)	706 \pm 198 (518–913)	70 \pm 8 (65–79)
<i>Male cyp27A1^{+/+} mice (n=3)</i>	140 \pm 115 (65–272)	17 \pm 20 (6–41)	63 \pm 15 (48–78)	405 \pm 58 (344–460)	64 \pm 15 (50–79)

Mean \pm S.D. and (range of results) are given; ND, not detected.

^a Analysis performed with 4000 QTRAP instrument scanning in triple-quad mode.

^b Free sterol; quantification based on area ratio of 7 α -hydroxy-4-cholesten-3-one to 7 α -hydroxy-4-cholesten-3-one-d7 internal standard synthesized as described previously [8].

^c Free sterols; quantification was based on area ratio of sterol to 4-cholesten-3-one-d7 internal standard.

^d LLOQ for C4-3-one 50ng/ml, for 5 α -cholestan-3-one 100ng/ml.

^e Hydrolyzed sterols; quantification was by GC-FID and GS-MS.

^f Normally <600 μ g/dl and for CTX between 1,300–15,000 μ g/dl [9].