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ESI-MS/MS quantification of 7α-hydroxy-4-cholesten-3-one facilitates rapid, convenient diagnostic testing for cerebrotendinous xanthomatosis

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ABSTRACT

Background: The genetic disorder cerebrotendinous xanthomatosis (CTX) frequently remains undiagnosed for many years. Left untreated CTX is associated with the development of cataracts, xanthomas and severe neurological dysfunction. The method routinely used to screen for CTX is GC-based measurement of elevated 5α-cholestanol from hydrolyzed plasma. A plasma test for CTX utilizing ESI-MS/MS methodology would be beneficial.
Methods: Development of rapid, simple LC-ESI-MS/MS methodology to test plasma for CTX is described. Two hour Girard derivatization allowed for 7α-hydroxy-4-cholesten-3-one quantification by isotope dilution LC-ESI-MS/MS within 12 min from un-hydrolyzed affected patient plasma (5 μl).
Results: Adequate sensitivity and reproducibility were achieved for quantification of 7α-hydroxy-4-cholesten-3-one, which demonstrated improved utility as a diagnostic marker of disease and to monitor treatment compared to 5α-cholestanol. The mean plasma concentration of 7α-hydroxy-4-cholesten-3-one in untreated CTX-affected patients ($n=6$) was 107-fold that in unaffected subjects ($n=9$), with the lowest concentration in affected patients >10-fold the highest concentration in unaffected subjects.
Conclusion: Quantification of the bile acid precursor 7α-hydroxy-4-cholesten-3-one with LC-ESI-MS/MS is a novel approach to improved diagnostic testing of plasma for CTX, amenable to high-throughput analysis and automated sample handling. Development of ESI-MS/MS methodology should make CTX testing more widely available and facilitate easier diagnosis of CTX.

1. Introduction

Cerebrotendinous xanthomatosis (CTX, OMIM # 213700) is a rare genetic disorder associated with defective sterol 27-hydroxylation; an enzymatic step important in the conversion of cholesterol to the primary bile acid chenodeoxycholic acid (CDCA) (Fig. 1 hepatic neutral pathway to cholic acid (CA) and CDCA) [1,2]. CTX characteristically presents in the second or third decade; childhood signs and symptoms commonly include diarrhea, juvenile cataracts and failure to progress in school. Often the disorder remains undiagnosed for many years and the burden to those affected with CTX can become profound. The accumulation of 5α-cholestanol (a 5α-dihydro derivative of cholesterol) in the tissues of affected patients, especially in the brain [3], is associated with the development of severe neurological dysfunction. Although the progressive impairment of CTX can be effectively prevented and symptoms ameliorated by treatment with CDCA [4,5], treatment after many years of disease progression cannot completely reverse the neurological pathology of CTX [6], therefore the value of an early diagnosis cannot be stressed enough.

Increased 5α-cholestanol in the systemic circulation of CTX-affected patients was reported in 1971 [7], and since then measurement of elevated serum or plasma 5α-cholestanol by gas chromatography (GC)-FID or GC-mass spectrometry (MS) has been widely used for biochemical screening for CTX [8]. Measurement of plasma 5α-cholestanol has also been used as a tool to assess the efficacy of bile acid treatment in CTX patients [4]. 5α-Cholestanol is formed *in vivo* from cholesterol [9] and, in part, from the bile acid precursor 7α-hydroxy-4-cholesten-3-one (*3-oxo-Δ4* metabolite **II** in Fig. 1) [10,11], one of a number of CYP27A1 enzyme substrates that accumulates when

Abbreviations: CTX, cerebrotendinous xanthomatosis; CA, cholic acid; CDCA, chenodeoxycholic acid; ESI, electrospray ionization; MS/MS, tandem mass spectrometry; GirP, Girard P; CID, collision induced dissociation; LLOQ, lower limit of quantification; SPE, solid-phase extraction.

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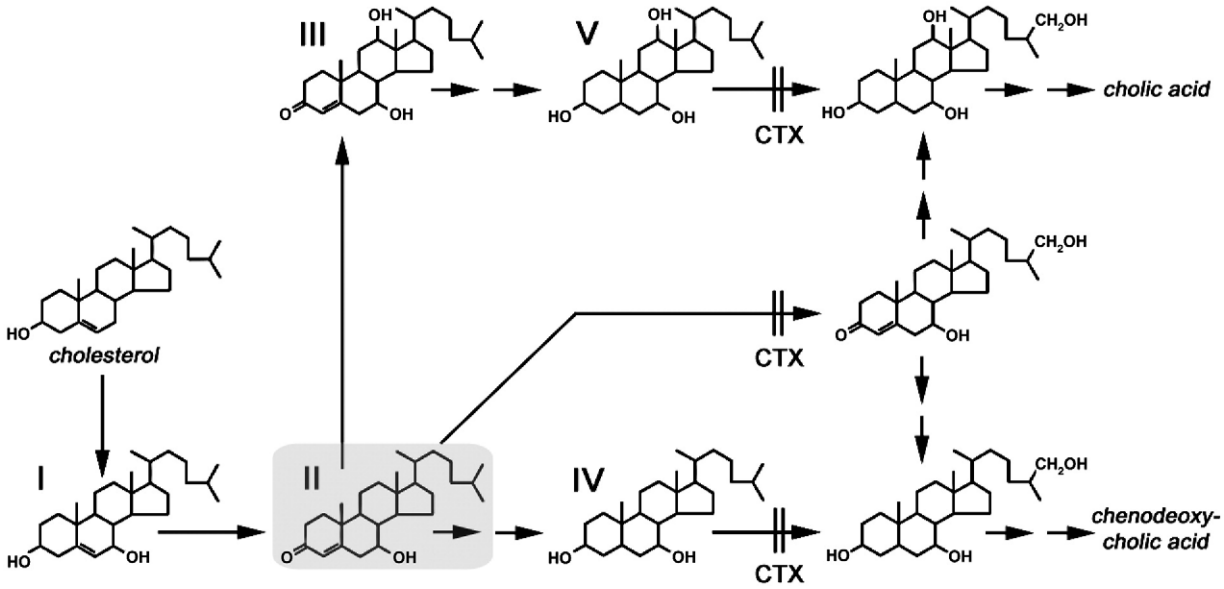

Fig. 1. "Neutral" pathway to CA and CDCA with nuclear transformations preceding those of the steroid side chain [1,2]. 7α-Hydroxy-4-cholestene-3-one (*3-oxo-Δ4* metabolite **II** indicated in grey) is synthesized from 7α-hydroxycholesterol (**I**), an oxysterol produced from cholesterol by CYP7A1, the rate-limiting enzyme that initiates the neutral pathway. 7α-Hydroxy-4-cholesten-3-one can undergo 12α-hydroxylation to form 7α,12α-dihydroxy-4-cholesten-3-one (*3-oxo-Δ4* metabolite **III**). Both *3-oxo-Δ4* metabolites **II** and **III** can be reduced to form the *bile alcohol* CYP27A1 substrates, 5β-cholestane-3α,7α-diol (**IV**) and 5β-cholestane-3α,7α,12α-triol (**V**) [12]. 27-Hydroxylation of the bile alcohols **IV** and **V,** in the normal pathway to CDCA and CA respectively, is blocked by CTX-causative mutations in the *CYP27A1* gene encoding the 27-hydroxylase enzyme CYP27A1 (EC = 1.14.13.15); although CA can still be synthesized by alternate hydroxylation of triol **V** [2].

sterol 27-hydroxylation is defective. 7α-Hydroxy-4-cholesten-3-one is highly elevated in liver tissue [12,13] and in serum or plasma [10,14,15] from CTX-affected patients.

Limitations of GC-based methodology for biochemical screening for CTX using measurement of elevated serum or plasma 5α-cholestanol include a lengthy analysis time (>30 min) and complex sample preparation. With the inexorable move from GC-MS to take advantage of the benefits offered by electrospray ionization (ESI)-tandem MS (MS/MS), development of ESI-MS/MS methodology able to detect diagnostic markers of CTX from plasma would be of great value. ESI-MS/MS offers the capability for rapid sample analysis with minimal sample work-up, and ESI-MS/MS instrumentation is now widely accessible in many clinical laboratories. Development of ESI-MS/MS-based diagnostic methodology should make more widespread adoption of diagnostic testing and facilitate easier diagnosis of CTX.

A sterol derivatization technique enabling ESI-MS/MS detection of *3-oxo-Δ4* metabolites (such as **II** in Fig. 1), known to be markedly elevated in the systemic circulation of patients affected with CTX [10,14,15], is Girard derivatization [16]. Derivatization of *3-oxo-Δ4* molecules with Girard's P- (GirP) or T-reagent forms charged hydrazone cations that are readily analyzed with ESI and fragment to give dominant CID (collision induced dissociation) product ions from the neutral loss of 79 Da (pyridine) or 59 Da (trimethylamine), respectively. We describe here one-step GirP derivatization with ESI-MS/MS detection of 7α-hydroxy-4-cholesten-3-one (*3-oxo-Δ4* metabolite **II**) from 5 µl of un-hydrolyzed plasma that accurately discriminates between samples from CTX patients and unaffected individuals and should prove useful as a diagnostic test for CTX, as well as to monitor CDCA treatment in CTX patients.

2. Materials and methods

2.1. Human research subjects

Blood for all studies was collected from volunteers or from CTX patients where informed consent was obtained according to the OHSU Institutional Review Board approved policies and procedures. The CTX blood samples were obtained from affected adults ($n = 5$) and a 16 year affected teenager. Treatment was with CDCA at a dose of 250 mg twice daily (12 mg/kg). The control blood samples were from healthy adult volunteers ($n = 6$) and children ranging from 3 to 19 months old ($n = 3$). Plasma (K_2EDTA) was separated from the blood samples and stored at −80 °C.

2.2. Chemicals and reagents

7α-Hydroxycholesterol, 5α-cholestanol, 5-cholesten-3β-ol (cholesterol) and 4-cholesten-3-one were from Steraloids (Newport, RI). 7α-Hydroxycholesterol-d_7 was from CDN Isotopes (Pointe-Claire, Quebec, Canada). 7α-Hydroxy-4-cholesten-3-one and 7α-hydroxy-4-cholesten-3-one-d_7 internal standard were synthesized from their 3β-hydroxy-Δ5 analogues using *streptomyces* sp. cholesterol oxidase obtained from Sigma-Aldrich (St Louis, MO) as described previously [17,18]. Their concentrations were determined from the absorbance at 241 nm using 4-cholesten-3-one as a standard [17,18]. 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 0.5 ml Ultrafree-MC centrifugal filters (0.45 µm) were from Millipore (Bedford, MA).

2.3. Preparation of calibrators and samples

Calibrators for the GC method were generated using dilutions of commercially available authentic standard in isopropanol or chloroform. 5α-Cholestanol was isolated from 50 µl of plasma following hydrolysis and was measured as trimethylsilyl ether with GC as previously described [19]. Calibrators for the LC-ESI-MS/MS method were generated using dilutions of authentic standard in methanol or by spiking 5 µl plasma aliquots from healthy subjects with authentic standard solution. After addition of 40 µl of methanol containing 7α-hydroxy-4-cholesten-3-one-d_7 (10 ng) internal standard, the 7α-hydroxy-4-cholesten-3-one present in calibrators or 5 µl plasma samples was derivatized with 160 µl of 10 mmol/l Girard's P reagent in methanol at 1% acetic acid. The samples were shaken (200 rpm) for

120 min at room temp and for plasma samples the precipitate was removed using centrifugal filters (0.45 µm) prior to LC-ESI-MS/MS analysis.

2.4. LC-ESI-MS/MS

Analysis of the derivatized calibrators and plasma samples required an LC step to remove excess derivatization reagent prior to detection with ESI-MS/MS. An LC-ESI-MS/MS method for quantification was created to monitor for GirP derivative transitions from precursor to $[M\text{-}79]^+$ product ion. Mass spectral experiments were performed and the LC-ESI-MS/MS quantification method was validated using an Applied Biosystems/MDS SCIEX 4000 QTRAP triple-quadrupole hybrid mass spectrometer with linear ion trap functionality (Foster City, CA), equipped with a TurboIonSpray® ESI source. The ionization interface was operated in the positive mode using the following settings: source temp (TEM), 500 °C, ion spray needle voltage (IS), 5.0 kV; curtain (CUR), heater (GS2) and nebulizer (GS1) nitrogen gas flow rates, 40, 40 and 30 psi respectively; declustering potential (DP), 100 V; entrance potential (EP), 10 V; and collision cell exit potential (CXP), 12 V. The MRM transitions monitored for were m/z 534.4 → 455.4 and 541.4 → 462.4. The collision energy (CE) was 40 V, dwell times were 75 ms and Q1 and Q3 were operated at unit resolution. For product ion scanning experiments the collision energy was 50 V. The UPLC-ESI-MS/MS system was composed of an in-line Shimadzu (Columbia, MD) SIL-20AC XR auto-sampler and 2 LC-20AD XR LC pumps. GirP derivatives were resolved using a 30 × 2.1 (i.d.) mm, 1.9 µm Hypersil Gold C_{18}-HPLC column with a 10 × 2.1 mm, 3 µm Betabasic C_{18}-guard from ThermoHypersil (Waltham, MA). The gradient mobile phase was delivered at a flow rate of 0.8 ml/min. The mobile phase consisted of 2 solvents: A, water and 0.1% formic acid, and B, methanol and 0.1% formic acid. Excess derivatization reagent was removed by diverting the LC flow to waste for the first 2 min of the 12 min LC-ESI-MS/MS method. Solvent B was increased from 65 to 77% over 6 min, then 77 to 85% over 2 min. The column was washed at 98% B for 2 min and re-equilibrated at 65% B for 2 min. The LC column temperature was kept at 40 °C using a Shimadzu CTO-20AC column oven. The sample injection volume was 10 µl.

3. Results

3.1. ESI-MS/MS optimization and validation of LC-ESI-MS/MS quantification method

The mass spectrometry method was optimized for $[M]^+$ ionization and to produce a dominant $[M\text{-}79]^+$ product ion using the GirP derivative of 7α-hydroxy-4-cholesten-3-one authentic standard. The MS/MS spectrum obtained for derivative $[M]^+$ ion of 7α-hydroxy-4-cholesten-3-one is shown in Fig. 2 Panel E. Under optimized method conditions the GirP derivative was readily detected with LC-ESI-MS/MS experiments monitoring for the transition from m/z 534 precursor $[M]^+$ ion to m/z 455 product $[M\text{-}79]^+$ ion (authentic standard at 500 pg on-column injection is shown in Fig. 2 Panel C). Although the GirP derivative was detected as *cis* and *trans* isomer peaks, method sensitivity enabled a lower limit of quantification (LLOQ) of 12.5 pg on-column injection (data not shown, signal-to-noise value 5:1). For analysis from 5 µ plasma aliquots this is equivalent to 50 ng/ml in plasma; close to the maximum circulating concentration of 7α-hydroxy-4-cholesten-3-one reported in healthy human subjects (range 2–50 ng/ml) [10,17,18,20]. We therefore expected the LC-ESI-MS/MS method to easily detect and quantify 7α-hydroxy-4-cholesten-3-one GirP derivative from 5 µl of CTX patient plasma. Indeed, the GirP derivative of 7α-hydroxy-4-cholesten-3-one was clearly detected in un-hydrolyzed plasma from a male patient affected with CTX (Fig. 2 Panel A; representative unaffected plasma profile shown in Panel B). Although LC separation is required for the analysis (to remove excess GirP reagent), no significant closely eluting endogenous interference was detected from plasma, potentially allowing for the LC method to be significantly shortened.

Calibration curves for quantification of 7α-hydroxy-4-cholesten-3-one were generated by performing a least-squares linear regression for calibrant peak area ratios obtained (7α-hydroxy-4-cholesten-3-one GirP derivative/7α-hydroxy-4-cholesten-3-one-d_7 GirP derivative) versus specified concentration in ng/ml plasma. Acceptable linearity was demonstrated for each calibration curve up to 20,000 ng/ml (characteristic correlation coefficients $r^2 > 0.999$). Internal standard-d_7 was spiked at 2000 ng/ml plasma. Calibrators generated using authentic standard were included with each sample set. These were monitored over 3 months to obtain an overview of the precision characteristics for detection with the LC-ESI-MS/MS method (Table 1). Acceptable reproducibility was obtained for analysis of 7α-hydroxy-4-cholesten-3-one across the range 12.5–5000 pg on-column injection (50–20,000 ng/ml plasma). Precision for the repeat analysis of this analyte from serial dilutions of CTX patient plasma was also determined. The within- and between-run precision determined for detection of 7α-hydroxy-4-cholesten-3-one from plasma was found to be satisfactory (Table 1).

We examined the effect of plasma matrix on the detection of the GirP derivative of 7α-hydroxy-4-cholesten-3-one. Plasma aliquots of 5 µl were added to analyte calibrants, which were derivatized with GirP reagent. The ion abundance detected for each analyte calibrant was compared to the ion abundance detected for analyte calibrants generated without plasma. Signal recovery for 7α-hydroxy-4-cholesten-3-one averaged 86.4% across the range 50–5000 pg on-column injection (at 50 pg was 104.8%, at 500 pg was 80.1%, at 2.5 ng was 79.3%, and at 5.0 ng was 81.2%). It was previously reported that 7α-hydroxy-4-cholesten-3-one was stable in plasma at −20 °C for up to 10 months [17]. We determined 7α-hydroxy-4-cholesten-3-one was stable in plasma at room temperature or at 4 °C for at least 4 h, although 18 h storage at 4 °C resulted in 60–70% degradation. We determined GirP derivative in derivatization solution kept at room temperature in the auto-sampler could be reproducibly quantified by analysis at time zero and after 12 h and that the derivative could be stored at −20 °C for up to 1 month. Methanol dilutions of authentic standard and internal standard were stable at −80 °C for at least 1 year.

3.2. Applications of LC-ESI-MS/MS methodology

We set out to examine if the ESI-MS/MS detectable 7α-hydroxy-4-cholesten-3-one from un-hydrolyzed plasma demonstrated utility as a diagnostic marker of CTX. Quantification of 7α-hydroxy-4-cholesten-3-one from CTX patient plasma with the LC-ESI-MS/MS methodology confirmed the utility of this molecule as a marker of disease (Table 2). The mean plasma concentration of 7α-hydroxy-4-cholesten-3-one measured in untreated patients affected with CTX (4049 ng/ml) was 107-fold that in healthy unaffected subjects, with the lowest concentration determined in untreated CTX patients >10-fold the highest concentration determined in healthy unaffected subjects.

We also examined whether 7α-hydroxy-4-cholesten-3-one was useful to monitor treatment efficacy or dosing with CDCA. To assess this we examined the correlation between plasma 7α-hydroxy-4-cholesten-3-one concentration and CDCA treatment in a patient affected with CTX (Fig. 3). Quantification of 7α-hydroxy-4-cholesten-3-one was with LC-ESI-MS/MS from plasma drawn at regular intervals over 360 days. The first post-treatment measurement was recorded 28 days after CDCA therapy started. Although the pre-treatment concentration of 7α-hydroxy-4-cholesten-3-one was lower than that of 5α-cholestanol, the LC-ESI-MS/MS detectable reduction in this marker appeared to demonstrate a stronger correlation with CDCA treatment than the reduction in 5α-cholestanol.

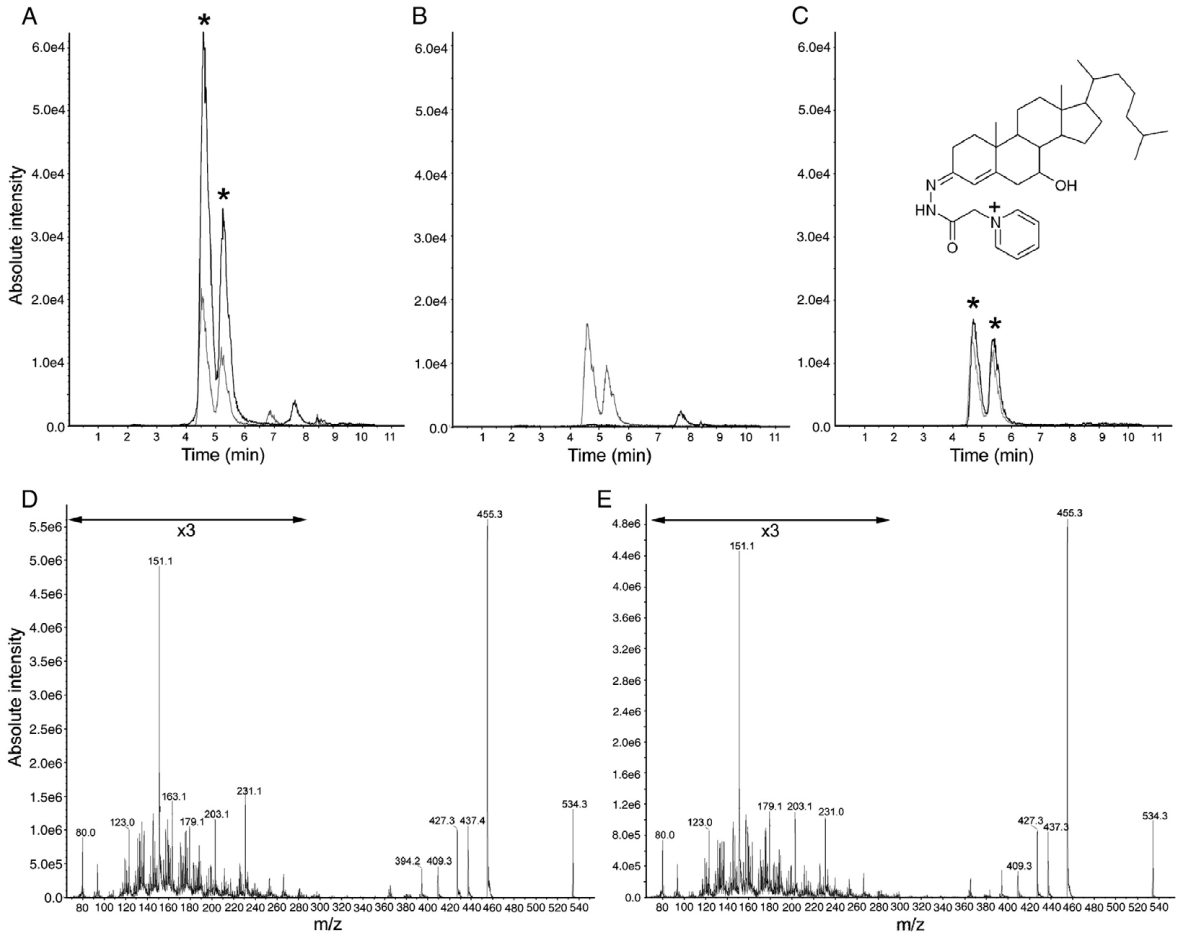

Fig. 2. Detection of 7α-hydroxy-4-cholesten-3-one in plasma from CTX patients. 7α-Hydroxy-4-cholesten-3-one was detected as GirP derivative isomers (designated by *) with LC-ESI-MS/MS experiments monitoring for the neutral loss of 79 (*Panel C* authentic standard at 500 pg on-column injection, RIC is for *m/z* 534→455). Endogenous 7α-hydroxy-4-cholesten-3-one was clearly detected at high levels in plasma from CTX patients (*Panel A* RIC is from representative untreated affected patient) compared to plasma from unaffected subjects (*Panel B* RIC is from representative healthy subject). Detection of the corresponding $[H^1]_7$-labeled 7α-hydroxy-4-cholesten-3-one standard derivative in each case is shown in grey. MS/MS spectra for 7α-hydroxy-4-cholesten-3-one derivative $[M]^+$ ions obtained at CE 50 V are shown (endogenous compound *Panel D*, authentic standard *Panel E*). In each case the characteristic GirP derivative product ions $[M\text{-}79]^+$ and $[M\text{-}107]^+$ are present [23]. The presence of a hydroxyl group is indicated by the formation of $[M\text{-}79\text{-}18]^+$ and $[M\text{-}107\text{-}18]^+$ product ions; the fact that it is a 7-hydroxy group is demonstrated by enhanced abundance of the product ions *m/z* 151 and 179 [23]. The α-positioning of the 7-hydroxyl group is indicated by heightened abundance of the product ion *m/z* 231 [23]. The *m/z* regions of 70–300 are magnified by a factor of 3.

Table 1
Precision characteristics of LC-ESI-MS/MS quantification for 7α-hydroxy-4-cholesten-3-one.

		RSD[a], %	
	ng/ml	Within-run	Between-run
7α-Hydroxy-4-cholesten-3-one	50	9.4	27.8
	200	–	9.7
	2000	–	8.3
	10,000	–	6.3
	20,000	–	11.9
7α-Hydroxy-4-cholesten-3-one from plasma[c]	1700	2.2	14.6[b]
	3400	1.9	15.0[b]
	6800	2.8	18.3[b]
	13,600[d]	1.5	15.3[b]

[a] Three replicates.
[b] Five replicates.
[c] Serial dilutions of CTX plasma.
[d] 10 µl CTX plasma.

Table 2
Plasma concentration of 7α-hydroxy-4-cholesten-3-one and 5α-cholestanol in patients affected with CTX.

	7α-hydroxy-4-cholesten-3-one (ng/ml)[a]	5α-cholestanol (µg/ml)[b]
Untreated CTX patients ($n=6$)	4149 ± 2370 (848–8034)	24.7 ± 13.2 (8.4–43.0)
Treated CTX patients ($n=2$)[c]	422 ± 244 (76–654)	8.4 ± 4.7 (3.5–17.8)
Unaffected subjects ($n=9$)	38 ± 18 (21–82)[d]	ND[e]

Mean ± S.D. and (range of results) are given.
[a] Determined from 5 µl plasma.
[b] Determined from 50 µl plasma.
[c] Seven measurements for time points 1–12 months after initiating CDCA treatment (12 mg/kg).
[d] LLOQ (50 ng/ml).
[e] Not determined, normally <6 µg/ml and for CTX between 13–150 µg/ml [1,2].

4. Discussion

We report the development and validation of novel diagnostic methodology useful to test for CTX and to monitor treatment with CDCA. Specifically, a convenient, rapid LC-ESI-MS/MS method to quantify increased 7α-hydroxy-4-cholesten-3-one (*3-oxo-Δ4* metabolite **II** in Fig. 1) from 5 µl of un-hydrolyzed plasma from CTX patients is described.

There were a number of challenges associated with sensitive MS/MS detection of neutral sterol markers from plasma including the lack of ionization efficiency for sterols and no generation of dominant CID product ions. Techniques for sterol derivatization have recently been described that greatly increase ionization efficiency. A charged moiety is incorporated, or a moiety that can readily accept a proton, to enhance ion formation with ESI, as well as to produce intense CID product ions. Honda et al. described simple derivatization methodology for the sensitive measurement of monohydroxy- and dihydroxy-sterols from small volumes of human serum using LC-ESI-MS/MS [21,22]. Picolinyl esterification allowed for detection of 5α-cholestanol 3β-picolinate with positive mode ESI, to permit diagnosis of CTX from 1 µl of un-hydrolyzed serum [21]. A drawback of the methodology was the >30 min LC separation required to remove cholesterol 3β-picolinate formed. With incorporation of an additional solid-phase extraction (SPE) step for removal of cholesterol 3β-picolinate, measurement of 7α-hydroxy-4-cholesten-3-one was possible in <12 min using picolinyl esterification [20].

Griffiths et al. identified brain dihydroxy-sterols by using cholesterol oxidase to convert oxysterols with a 3β-hydroxy-Δ5 structure into *3-oxo-Δ4* molecules, which lend themselves to derivatization at the keto-moiety to form GirP hydrazones detectable with LC-ESI-MS/MS [23]. The main drawbacks of this strategy are the enzyme-mediated sample work-up step required and the inability to differentiate endogenous *3-oxo-Δ4* molecules already present, for example 7α-hydroxy-4-cholesten-3-one. As 7α-hydroxy-4-cholesten-3-one is increased in the circulation of patients affected with CTX [10,14,15], we reasoned Girard derivatization, without the use of cholesterol oxidase, would allow for sensitive, convenient ESI-MS/MS detection of this metabolite from the plasma of CTX patients. We demonstrate here that 7α-hydroxy-4-cholesten-3-one present in un-hydrolyzed plasma from CTX patients can be detected using GirP derivatization ESI-MS/MS methodology. The method sensitivity enabled a LLOQ for 7α-hydroxy-4-cholesten-3-one GirP derivative of 12.5 pg on-column injection, with a limit of detection (LOD) comparable to the 5 pg on-column LOD obtained for analysis of GirP derivatives with conventional LC-ESI-MS/MS by Griffiths et al. [24]. An advantage of the direct analysis of endogenous *3-oxo-Δ4* metabolites, without the use of cholesterol oxidase, is the lack of enzyme-mediated conversion of cholesterol to 4-cholesten-3-one prior to derivatization. This abrogates the requirement for chromatographic isolation of excessive derivatized 4-cholesten-3-one (or SPE removal of cholesterol prior to treatment with cholesterol oxidase), as well as results in a simpler to perform one-step sample work-up.

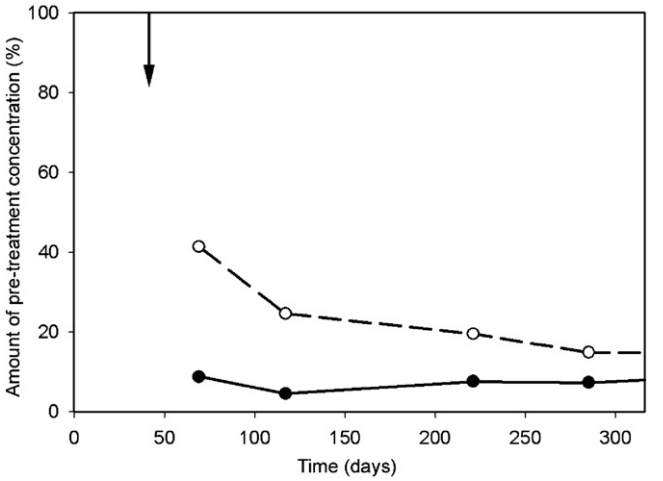

Fig. 3. Decline in plasma concentrations of 7α-hydroxy-4-cholesten-3-one and 5α-cholestanol during CDCA treatment. 7α-Hydroxy-4-cholesten-3-one (shaded circles) was quantified with the LC-ESI-MS/MS methodology in plasma from a 16 year old male patient affected with CTX. Levels of plasma 5α-cholestanol (open circles) were quantified using a GC-FID method [19]. The plasma concentrations of 7α-hydroxy-4-cholesten-3-one and 5α-cholestanol were 7.4 and 43 µg/ml, respectively, 40 days prior to the start of treatment with CDCA (500 mg/day; indicated by arrow).

7α-Hydroxy-4-cholesten-3-one appears to be a satisfactory marker of CTX that can be reproducibly quantified from un-hydrolyzed plasma with the LC-ESI-MS/MS methodology described to provide a diagnostic test for CTX. Measurement of 7α-hydroxy-4-cholesten-3-one allowed for excellent discrimination between plasma samples from healthy unaffected subjects ($n=9$) and untreated CTX patients ($n=6$) and the LC-ESI-MS/MS methodology provided a concentration range in untreated CTX patient plasma similar to that previously obtained in un-hydrolyzed serum (0.5 ml) from untreated CTX patients using GC-based methodology (2.3–8.0 µg/ml) [10]. The mean plasma concentration of 7α-hydroxy-4-cholesten-3-one determined with the LC-ESI-MS/MS method was 107-fold that in unaffected subjects, compared to 5α-cholestanol concentrations (currently used for diagnosis) that are generally only 3–15 fold the mean concentrations found in unaffected subjects [1,2]. As 5α-cholestanol can be increased with certain other diseases there are concerns regarding its specificity as a diagnostic marker of CTX [25]. 7α-Hydroxy-4-cholesten-3-one at concentrations >848 ng/ml appears to be relatively specific as a marker of CTX. Although increased plasma concentrations of 7α-hydroxy-4-cholesten-3-one can be observed

with bile acid malabsorption [26], concentrations >848 ng/ml were only measured with ileal resection ($n=20$, maximum 960 ng/ml, mean 170 ng/ml), unlikely to be confused clinically with CTX.5β-Cholestanetetrols (formed from 5β-cholestane-triol **V** in Fig. 1) were previously measured as their glucuronide conjugates in plasma (1 ml) from CTX-affected patients [27]. The predominate bile alcohol species, 5β-cholestane-3α,7α,12α,25-tetrol, was present at increased concentrations ranging from 1.6 to 7.7 μg/ml [27]. The amount of 5β-cholestane-3α,7α,12α,25-tetrol in plasma from affected patients was found to rapidly decrease during the first month of treatment with CDCA, on average by >90% [27]. Our measurements of 7α-hydroxy-4-cholesten-3-one precursor in plasma from an affected patient prior to and during CDCA treatment demonstrated a similar dramatic response during the first month of treatment. The reduction in amount of 7α-hydroxy-4-cholesten-3-one was 91%, compared to a characteristic slower 59% drop in 5α-cholestanol over the same time period [25].

In summary, a novel blood test for CTX is described. The test possesses suitable characteristics such that this technique can serve as an improved diagnostic assay, with the need for only minimal sample work-up and LC separation, and offering potential for automated sample handling and high-throughput sample analysis. It is hoped that development of this assay will facilitate easier and earlier diagnosis and treatment of patients with CTX.

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