Short Communication

A useful multi-analyte blood test for cerebrotendinous xanthomatosis

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ARTICLE INFO

Article history:
Received 3 January 2014
Received in revised form 10 April 2014
Accepted 13 April 2014
Available online 21 April 2014

Keywords:
Cerebrotendinous xanthomatosis
CYP27A1
Bile acids
Ketosterols
Cholestanol

ABSTRACT

Objectives: Cerebrotendinous xanthomatosis (CTX) is a rare genetic disorder of bile acid (BA) synthesis that can cause progressive neurological damage and premature death. Blood (normally serum or plasma) testing for CTX is performed by a small number of specialized laboratories, routinely by gas chromatography–mass spectrometry (GC–MS) measurement of elevated 5α-cholestanol. We report here on a more sensitive biochemical approach to test for CTX particularly useful for confirmation of CTX in the case of a challenging diagnostic sample with 5α-cholestanol that, although elevated, was below the cut-off used for diagnosis of CTX (10 μg/mL or 1.0 mg/dL).

Design and methods: We have previously described liquid chromatography–electrospray ionization-tandem mass spectrometry (LC–ESI-MS/MS) methodology utilizing keto derivatization to enable the sensitive quantification of plasma ketosterol BA precursors that accumulate in CTX. We have expanded this methodology to perform isotope dilution LC–ESI-MS/MS quantification of a panel of plasma ketosterol BA precursors, with internal standards readily generated using isotopically-enriched derivatization reagent.

Results: Quantification of plasma ketosterol BA precursors (7α-hydroxy-4-cholesten-3-one, 7α,12α-dihydroxy-4-cholesten-3-one and 7α,12α-dihydroxy-5β-cholestan-3-one) in a single LC–ESI-MS/MS test provided better discrimination between a CTX-positive and negative samples analyzed (n = 20) than measurement of 5α-cholestanol alone.

Conclusions: Quantification of plasma ketosterol BA precursors provides a more sensitive biochemical approach to discriminate between CTX negative and positive samples. A multiplexed LC–ESI-MS/MS test quantifying a panel of plasma ketosterols, with simple sample preparation, rapid analysis time and readily available internal standards, can be performed by most clinical laboratories. Wider availability of testing will benefit those affected with CTX.

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Introduction

Cerebrotendinous xanthomatosis (CTX; OMIM#213700) is an autosomal recessive neurodegenerative disorder associated with deficient sterol 27-hydroxylase (CYP27A1), a mitochondrial enzyme important in conversion of cholesterol to bile acids (BAs). Childhood-onset symptoms can include diarrhea and juvenile cataracts. Adolescent- and adult-onset symptoms can include tendon and cerebral xanthomas associated with neurological symptoms. CTX is difficult to diagnose, often there are many years between the age of first symptom onset and the age at diagnosis, which occurs only after significant neurological involvement. As the disorder progresses, affected individuals can become incapacitated with motor dysfunction with premature death occurring due to advancing neurological deterioration. Although only around three hundred cases of CTX have been described worldwide [1], relatively large series of patients have been described by physicians with experience in recognizing the disorder.

An effective oral therapy for CTX is available in the form of chenodeoxycholic acid (CDCA), the main BA deficient in CTX. Treatment with CDCA has been shown to normalize the biochemical phenotype

Abbreviations: CTX, cerebrotendinous xanthomatosis; BA, bile acid; GC–MS, gas chromatography–mass spectrometry; LC–ESI-MS/MS, liquid chromatography–electrospray ionization-tandem mass spectrometry; CDCA, chenodeoxycholic acid; FAB, fast atom bombardment; 7α, C4, 7α-hydroxy-4-cholesten-3-one; IRB, Institutional Review Board; 7α,12α-C4, 7α,12α-dihydroxy-4-cholesten-3-one; 7α,12α-C5, 7α,12α-dihydroxy-5β-cholestan-3-one; DCS, double charcoal stripped; QAQ, quaternary ammonyx; MRM, multiple reaction monitoring; LLOQ, lower limit of quantification; S/N, signal-to-noise; RSD, relative standard deviation.

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and halt progression of disease [2,3]. In many cases treatment of patients with advanced neurological disease does not reverse the impairment [3], therefore it is essential to diagnose and treat CTX as early as possible.

Biochemical tests for CTX include screening blood samples using gas chromatography–mass spectrometry (GC–MS) measurement of elevated 5α-cholestanol [4,5]. The upper normal range for plasma 5α-cholestanol has been reported to range from 7 to 11 µg/mL [4,5]. Diagnostic confirmation is routinely performed using fast atom bombardment (FAB)–MS measurement of more specific markers for CTX, bile alcohol glucuronides in urine [6]. We have utilized keto-moieties derivatization to enable sensitive liquid chromatography–electrospray ionization–tandem MS (LC–ESI–MS/MS) measurement of ketosterol BA precursors that accumulate in CTX [7–9]. Free (non-hydrated) plasma 7α-hydroxy-4-cholesten-3-one (7αC4) possessed improved utility over 5α-cholestanol as a marker for CTX [7]. We describe here quantification of a panel of ketosterols in a plasma sample from a CTX affected individual with a plasma 5α-cholestanol concentration above the concentration range we determined for normal samples (n = 20) but below our cut-off for diagnosis of CTX (10 µg/mL).

Materials and methods

Human subject research considerations

Blood was obtained from participants enrolled in studies at OHSU under Institutional Review Board (IRB) approved protocols. Informed consent was obtained from all study participants. De-identified plasma samples submitted to the OHSU diagnostic laboratory for CTX testing using GC–MS measurement of elevated plasma 5α-cholestanol were used with IRB approval. For CTX positive samples diagnostic confirmation was by molecular genetic testing.

Chemicals and reagents

7αC4, 7α,12α-dihydroxy-4-cholesten-3-one (7αC4) and 7α,12α-dihydroxy-5β-cholestan-3-one (7α12αCSβ) were from Toronto Research Chemicals (Toronto, Ontario), 5α-Cholesterol was from Steraloids (Newport, RI) and epicoprostanol from Sigma-Aldrich (St. Louis, MO). BSTFA reagent was from Thermo Scientific (Bellefonte, PA). Human plasma and double charcoal stripped (DCS) plasma were from Golden West Bio (Temecula, CA). 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. Cholesterol was prepared with isotope dilution LC–MS/MS, and 7αC4 spiked into DCS plasma. QAO derivatization was complete. Previously prepared QAO-d3 tagged ketosterol internal standards (300 pg) in 10 µL methanol were added [9].

Preparation of calibrators and samples for LC–MS/MS measurement of ketosterols

We have previously described the approach used for LC–ESI–MS/MS measurement of elevated plasma ketosterols [9]. In brief, method calibrators were generated using dilutions of authentic standard in methanol spiked into DCS plasma. QAO reagent solution (210 µg in methanol plus 5% acetic acid, v/v) was added to calibrators or plasma samples (4 µL). After 2 h at RT QAO derivatization was complete. Previously prepared QAO-d3 tagged ketosterol internal standards (300 pg) in 10 µL methanol were added [9].

LC–ESI–MS/MS method

LC–ESI–MS/MS analyses were performed using a QTRAP® 5500 triple-quadrupole hybrid mass spectrometer with linear ion trap functionality (AB SCIEX, Framingham, MA), equipped with a TurbolonSpray® ESI source. The ionization interface was operated in the positive mode and multiple reaction monitoring (MRM) transitions monitored for quantification of ketosterols were as follows: QAO 7αC4 m/z 515.7 → 152.2, for QAO 7α12αC4 m/z 531.7 → 152.2, for QAO 7α12αCSβ m/z 533.7 → 145.0, for QAO-d3 7αC4 m/z 518.7 → 152.3, for QAO-d3 7α12αC4 m/z 534.7 → 152.1 and for QAO-d3 7α12αCSβ m/z 536.7 → 145.0. The QTRAP® 5500 was coupled to a Shimadzu UPLC system (Columbia, MD) composed of a SIL-20ACXR auto-sampler and two LC-20ADXR LC pumps. QAO derivatives were resolved using a 50 × 2.1 (i.d.) mm, 5.0 µm Luna C8–HPLC column with guard (Phenomenex; Torrance, CA). The gradient mobile phase was delivered at a flow rate of 0.8 mL/min and the water:acetonitrile:0.1% formic acid mobile phase [9]. The column temperature was kept at 35 °C using a Shimadzu CTO-20 AC column oven. The sample injection volume was 10 µL.

Data analysis and method performance

Calibration curves were generated by performing a least-square linear regression for peak area ratios (QAO-d3, ketosterol analyte/QAO-d3 ketosterol internal standard) plotted against specified calibrant concentration in plasma (ng/mL) [9]. The lower limit of quantification (LLOQ) was determined as the lowest spiked concentration in matrix for which the signal-to-noise (S/N) ratio was ≥5 and the within- and between-day reproducibility of the peak area was ≤20% relative standard deviation (RSD). Potential method interference was evaluated by examination of the peak shape, peak shoulder, and peak area ratio of two MRM transitions (quantifier and qualifier) acquired for each analyte and by analysis of possible method interferences. The matrix effect was determined by comparing the QAO ketosterol peak area for matrix-based against non-matrix calibrators.

Results

As we have previously described, DCS plasma calibration curves for QAO tagged 7αC4 and 7α12αC4, with QAO-d3 tagged 7αC4 and 7α12αC4 internal standards respectively, demonstrated acceptable linearity (correlation coefficients across the range 20–250 ng/mL possessed r² values >0.990) [9]. This was also the case for a novel ketosterol marker for CTX we quantified with isotope dilution LC–ESI–MS/MS, 7α12αCSβ. DCS plasma calibration curves for QAO tagged 7α12αCSβ with QAO-d3 tagged 7α12αCSβ internal standard demonstrated acceptable linearity (correlation coefficients across the range 20–250 ng/mL possessed r² values >0.990). Satisfactory between and within-run accuracy and precision data was also obtained for calculated concentrations of 7α12αCSβ (≤20% RSD at the LLOQ and <15% RSD between 50 and 250 ng/mL). The LLOQ for all ketosterols in plasma (including 7α12αCSβ) was 20 ng/mL.

The sensitivity of ketosterols as plasma markers for CTX compared to 5α-cholestanol was highlighted by analysis of a plasma sample from a
possible CTX-affected individual with a clinical history consistent with the disorder (see Fig. 1). GC–MS analysis revealed a plasma 5α-cholestanol concentration of 8.4 μg/mL, close to reported upper normal concentrations of 7–11 μg/mL [4,5]. The plasma ketosterol concentrations determined with LC–ESI-MS/MS were 795, 1548, and 1004 ng/mL for 7αC4, 7α12αC4 and 7α12αC5β, respectively (with upper normal concentrations of 22, 0.8, and 29 ng/mL determined; for reference ranges see Table 1).

Molecular genetic testing confirmed the individual possessed a homozygous R474Q CYP27A1 gene mutation, previously reported to be a causative mutation for CTX [10].

**Discussion**

5α-Cholesterol can be elevated in a number of liver diseases and concerns have been raised regarding the specificity of this disease marker for CTX [11]. Although not routinely used for diagnosis, other markers elevated in CTX include cholesterol precursors such as 7-dehydrocholesterol and 8-dehydrocholesterol [12]. Ketosterol BA precursors and bile alcohol glucuronides also accumulate in CTX [7–9,13–15]. We have previously described an approach using keto derivatization to incorporate a permanent charge and improve sensitivity [7]. We report here on expansion of this methodology to allow isotope dilution quantification of plasma ketosterols allowed for ready discrimination between the sample we describe here and CTX negative samples (n = 20), such that it serves as an improved blood test for CTX. The availability of LC–ESI-MS/MS methodology utilizing QAO derivatization with simple and rapid sample preparation amenable to automation, analysis times of 4–6 min using conventional LC instrumentation and buffers, and readily available stable-isotope labeled internal standard analogs, allows performance by most clinical laboratories. Wider availability of testing will benefit those affected with this disorder.

**Acknowledgments**

The authors would like to thank the Bioanalytical Shared Resource at OHSU for providing technical assistance and access to analytical instrumentation. AED has been supported as a KL2 awardee by the Oregon Clinical and Translational Research Institute (OCTRI), grant number (KL2TR000152) from the National Center for Advancing Translational Sciences (NCATS) at the National Institutes of Health (NIH) and also as a training grant awardee by the Sterol and Isoprenoid Diseases (STAIR) consortium. STAIR is part of the NIH Rare Diseases Clinical Research Network (RDCRN). Funding and/or programmatic support for this project has been provided by a grant (1U54HD061939) from the Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD) and the NIH Office of Rare Diseases Research (ORDR).

**Table 1**

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<th>7α-Hydroxy-4-cholesten-3-one&lt;sup&gt;a&lt;/sup&gt; (ng/mL)</th>
<th>7α12α-Dihydroxy-4-cholesten-3-one&lt;sup&gt;b&lt;/sup&gt; (ng/mL)</th>
<th>7α12α-Dihydroxy-5α-cholestan-3-one&lt;sup&gt;c&lt;/sup&gt; (ng/mL)</th>
<th>5α-cholestanol&lt;sup&gt;d&lt;/sup&gt; (μg/mL)</th>
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<tr>
<td>CTX-case described plasma</td>
<td>795</td>
<td>1548</td>
<td>1004</td>
<td>8.4</td>
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<td>Untreated CTX-affected adult plasma (n = 10)</td>
<td>1174 ± 711&lt;sup&gt;e&lt;/sup&gt; [204–1828]</td>
<td>1545 ± 1144&lt;sup&gt;e&lt;/sup&gt; [57–2420]</td>
<td>498 ± 310&lt;sup&gt;e&lt;/sup&gt; [100–1004]</td>
<td>31 ± 19&lt;sup&gt;d&lt;/sup&gt; [8.4–66]</td>
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<tr>
<td>Unaffected adult plasma (n = 20)</td>
<td>6.4 ± 5.3&lt;sup&gt;f&lt;/sup&gt; [1.6–22]</td>
<td>0.4 ± 0.3&lt;sup&gt;f&lt;/sup&gt; [0.1–0.8]</td>
<td>10 ± 5.5&lt;sup&gt;f&lt;/sup&gt; [0–29]</td>
<td>1.3</td>
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The mean concentration ± S.D. and [range of results] are given.

<sup>a</sup> Non-hydroxylated free sterol.

<sup>b</sup> This data has been reported previously [9].

<sup>c</sup> Hydrolyzed total sterol; quantification by GC–MS.

<sup>d</sup> CTX plasma samples with concentrations >250 ng/mL were diluted and re-analyzed.

<sup>e</sup> Includes values for CTX-case described plasma.

<sup>f</sup> Calculated outside the quantifiable range.

<sup>g</sup> Upper 5α-cholestanol normal cut-off reported as between 7 and 11 μg/mL [4,5].
References


