Clinica Chimica Acta 411 (2010) 43–48

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

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

Andrea E. DeBarber [a,*], William E. Connor [b], Anuradha S. Pappu [b], Louise S. Merkens [c], Robert D. Steiner [d]

[a] Department of Physiology and Pharmacology, Oregon Health & Science University, Portland, OR 97239, USA
[b] Department of Medicine, Oregon Health & Science University, Portland, OR 97239, USA
[c] Department of Pediatrics, Oregon Health & Science University, Portland, OR 97239, USA
[d] Departments of Pediatrics and Molecular and Medical Genetics, Child Development and Rehabilitation Center and Doernbecher Children's Hospital, Oregon Health & Science University, Portland, OR 97239, USA

ARTICLE INFO

Article history:
Received 7 July 2009
Received in revised form 24 September 2009
Accepted 24 September 2009
Available online 3 October 2009

Keywords:
Derivatization
Girard reagent
7α-Hydroxy-4-cholestene-3-one
Plasma
Diagnostic
Cholestanol
Cataracts
Xanthoma
Bile acids
Chenodeoxycholic acid

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.

* Corresponding author. L334, OHSU, 3181, SW Sam Jackson Park Rd, Portland, OR 97239, USA. Tel.: +1 503 494 9292; fax: +1 503 494 9275.
E-mail address: debarber@ohsu.edu (A.E. DeBarber).

0009-8981/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.cca.2009.09.036

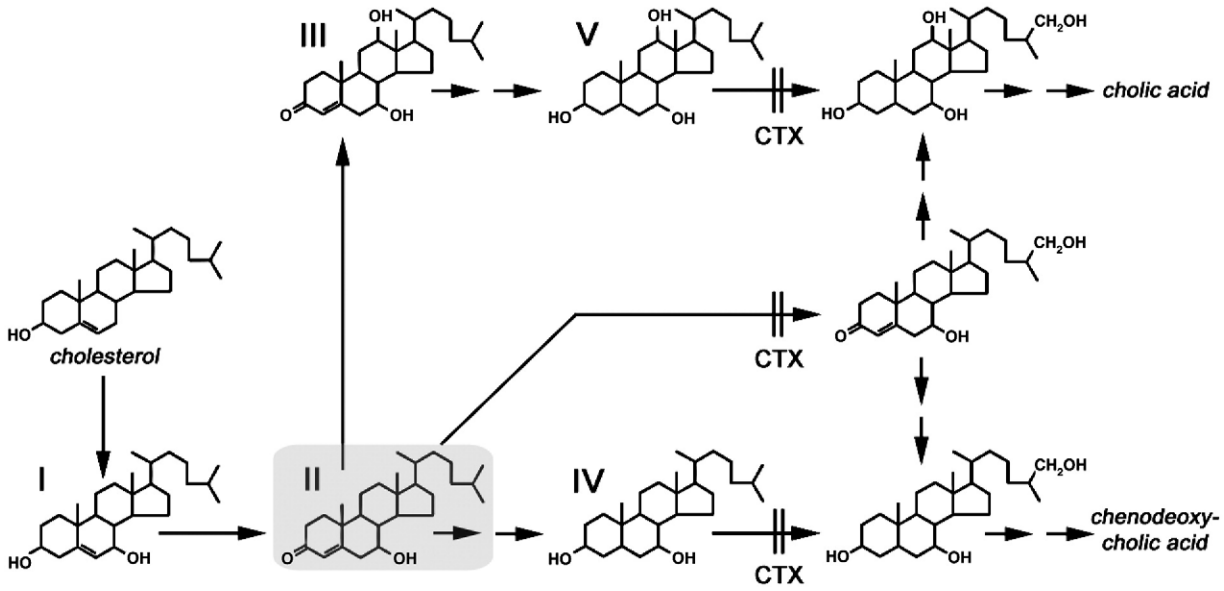

Fig. 1. "Neutral" pathway to CA and CDCA with nuclear transformations preceding those of the steroid side chain [1,2]. 7α-Hydroxy-4-cholestene-3-one (*3-oxo-Δ4* metabolite **II** indicated in grey) is synthesized from 7α-hydroxycholesterol (**I**), an oxysterol produced from cholesterol by CYP7A1, the 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