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Detection of dihydroxycholesterols in human plasma using HPLC–ESI-MS/MS

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ABSTRACT

We report a straightforward sample preparation procedure and a direct liquid chromatography electrospray ionization tandem mass spectrometry (LC–ESI-MS/MS) method for the analysis of 7alpha,25-dihydroxycholesterol (7α25-OHC) and 7alpha,27-dihydroxycholesterol or (7α27-OHC). By applying a slow protein precipitation approach using cold ethanol, we were able to detect and quantify 7α25-OHC and 7α27-OHC in a fast and reliable manner. The average concentrations from 20 healthy individuals were determined to be 0.21 ± 0.05 nM for 7α25-OHC and 3.4 ± 0.1 nM for 7α27-OHC. In addition, we are the first to report the average degrees of esterification ($n = 8$) to be 73.8% and 82% for 7α25-OHC and 7α27-OHC, respectively. Using the established method, we achieved the sensitivity sufficient for detecting low abundant dihydroxylated oxysterols in healthy individuals. This result should enable extension of these studies towards a comprehensive analysis of oxysterol levels under disease conditions.

1. Introduction

The oxidized forms of cholesterol are important precursors of steroid hormones and bile acids. In addition to their roles as metabolic intermediates, oxysterols possess key functions in cholesterol homeostasis, sterol transport, apoptotic cell death, transcriptional control of metabolism, inflammation, as well as cell signaling during development [1,2]. A recent review article summarized the finding on the oxysterols' contribution to the pathogenesis of various chronic disease processes, such as atherosclerosis, neurodegenerative diseases, retina degeneration, and inflammatory bowel disease [3].

Among all oxysterols, the monohydroxylated cholesterols, such as 25-, 27-, or 24S-hydroxycholesterols, have been intensively studied since the 1960s. In a recent discovery, 7α,25-dihydroxycholesterol (7α25-OHC) has been identified as a natural ligand for a G protein-coupled receptor (GPCR) called Epstein-Barr virus-induced gene 2 (EBI2, also known as GPR183) [4,5]. Since then, the important physiological roles of EBI2 receptor and its ligand have been studied by different groups [6,7]. It was discovered that both 7α25-OHC and its precursor, 25-hydroxycholesterol (25-OHC), have wide-ranging influences on innate and adaptive immunity [7–17]. In addition to 7α25-OHC, its structural isomer 7α,27-dihydroxycholesterol (7α27-OHC) was also shown to be a potent ligand for EBI2 [4]. Furthermore Soroosh et al. have demonstrated that both 7β27-OHC and 7α27-OHC enhance the differentiation of murine and human IL-17 producing Th17 cells in a RORγt-dependent manner and the most potent and selective activator for RORγt is 7β27-OHC [11]. Since dihydroxylated cholesterols play important roles in the physiology and pathophysiology of the immune system, determining circulating levels is critical for a comprehensive understanding of their biology. Furthermore, inter-individual variability might be helpful to define a possible role of these oxysterols in the pathophysiology of autoimmune and inflammatory diseases.

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7α25-OHC and 7α27-OHC are oxysterol isomers formed by hydroxylation of the precursors, 25-OHC and 27-OHC. Their structures and biosynthetic pathways are shown in Fig. 1 (as described in KEGG pathway database [18] and in [19]). The systematic names for 7α25- and 7α27-OHC are cholest-5-ene-3β,7α,25-triol and cholest-5-ene-3β,7α,(25R)26-triol, respectively [20]. Following the systematic nomenclature, 7α27-OHC is also called 7α,(25R)26-dihydroxycholesterol. But the name 7α,27-dihydroxycholesterol is more widely accepted. Unlike their biosynthetic precursors, research on the biological functions of dihydroxylated cholesterol derivatives has only been started since their discovery as the ligands of EBI2 [4,5]. Methods for oxysterol analyses such as gas chromatography–mass spectrometry (GC–MS) and liquid chromatography–mass spectrometry (LC–MS) have been described for the monohydroxylated cholesterols in human circulation [21–32]. In contrast, data on the dihydroxycholesterols has been limited. The endogenous levels of dihydroxylated cholesterols vary in different biological matrices and the reported levels in human plasma were low [25,33].

LC–MS is widely used as the identification and quantification tool of choice in conventional bio-analytical investigations. Its success is mainly due to the high sensitivity, specificity as well as precision and accuracy of detection. It can be applied without chemical derivatization for the analyses of broad range of molecules allowing for a fast method development process. A sensitive detection of low abundant molecules in biological matrices is dependent on key factors such as ionization efficiencies, gas-phase fragmentation patterns, chromatographic separations, and extraction recoveries during the sample work up.

Oxysterols are neutral molecules and are generally not readily ionized by the commonly used ionization techniques, such as electrospray ionization (ESI). Introduction of a charged or readily ionized functional group has been applied to improve the ionization efficiency and thus the sensitivity of oxysterol analysis. Various derivatization reagents have been reported such as N,N-dimethylglycine, picolinyl esters, or Girard P reagents [25–27]. Among these approaches, the most sensitive method reported by Griffiths and co-workers has been applied to analyze both dihydroxycholesterols in plasma. This approach is called 'enzyme assisted derivatization for sterol analysis' (EADSA) [24,25]. Without any chemical derivatization, the poor ionization efficiency of oxysterols molecules can be aggravated by the gas-phase formation of multiple ions: protonated $[M+H]^+$, cationic adducts (e.g., Na^+, NH_4^+), and many in-source fragmentation product ions, e.g., $[M+H-H_2O]^+$. It has been reported that atmospheric pressure chemical ionization (APCI) and dopant-assisted atmospheric pressure photoionization (DA-APPI) are more sensitive compared to ESI for the analyses of sterols and oxysterols in general [22,29,30,32,34,35]. We therefore investigated how these ionization techniques influence the detection limit of dihydroxycholesterols.

Many oxysterol isomers are isobaric and have similar gas-phase fragmentation patterns, meaning that some of them share identical multiple reaction monitoring (MRM) transitions (same Q1 and Q3 masses). This makes the differentiation of oxysterol isomers based on MS/MS detection difficult [31]. Therefore, the LC separation of oxysterol isomers is crucial for a selective detection. With the introduction of sub-2 μm particle size as the column packing material in ultra-high pressure liquid chromatography (UHPLC) and the new generation of core–shell column technology, obtaining high chromatographic resolution with LC has become routine in many analytical laboratories. Hence, the analyses of sterols and steroids, including oxysterols, have been done more and more using UHPLC or HPLC with core–shell columns [31,36–38].

The detection of low abundant endogenous molecules such as 7α25-OHC by LC–MS is strongly affected by baseline problems, i.e., high noise or elevated baseline due to closely eluted interferences. For a sensitive detection, the removal of the "background materials" that are present in the biological matrix and co-extracted during the sample workup is essential. The co-eluting materials not only cause the high noise or elevated baseline, they also can compete for the charge with the target analyte during the ionization process and cause the so-called ion suppression effect. The total extraction of lipids is generally done using Folch and Bligh-and-Dyer protocols with chloroform–methanol [39,40]. Various groups have developed alternative procedures that use low toxic and non-halogenated solvents to replace chloroform. These protocols usually combine semi-polar solvents (methyl-tert-butyl-ether (MTBE), ethyl acetate, butanol) and water-miscible alcohols (methanol, ethanol). Sometimes non-polar solvents such as heptane and hexane are also applied for a two-phase extraction [41–45]. Furthermore, various solid-phase extraction (SPE) using a C18 or aminopropyl column to remove cholesterol and non-polar lipids have also been applied [24,31]. We thus compared different extraction protocols based on the detection sensitivity of the endogenous 7α25-OHC in human plasma samples.

In summary, we have investigated how these factors described above influence the detection sensitivity and proposed an analytical strategy for a sensitive and direct detection of 7α25- and 7α27-OHC in human plasma using LC–ESI-MS/MS.

2. Materials and methods

2.1. Materials

Oxysterol standards and internal standards, 7α,25-dihydroxycholesterol (7α25-OHC), 7α,27-dihydroxycholesterol (7α27-OHC), 7α,24(S)-dihydroxycholesterol (7α24S-OHC), 7β,25-dihydroxycholesterol (7β25-OHC), 7β,27-dihydroxycholestrol (7β27-OHC), 7α,25-dihydroxycholesterol-d6 (7α25-OHC-d6), 7α,27-dihydroxycholesterol-d6 (7α27-OHC-d6), 25-hydroxycholesterol (25-OHC), 27-hydroxycholesterol (27-OHC), 24(S)-hydroxycholesterol (24S-OHC), 25-hydroxycholesterol-d6 (25-OHC-d6), 27-hydroxycholesterol-d6 (27-OHC-d6), were purchased from Avanti Polar Lipids Inc. (Alabaster, Alabama, USA). Butylated hydroxytoluene (BHT) was purchased from SAFC Sigma–Aldrich® Chemie (St. Louis, MO, USA). Water (Biosolve BV, Valkenswaard, the Netherlands), methanol (MeOH) (Merck Co. Whitehouse Station, NJ, USA), formic acid (FA) (Sigma Aldrich, Buchs, Switzerland).

2.2. Oxysterol extraction methods

Different extraction protocols were applied directly to pooled naïve plasma and the endogenous 7α25-OHC was detected using the same LC–MS methods. For each protocol, two to five aliquots with 200 μl volume each are extracted and the endogenous 7α25-OHC was detected using the same LC–MS methods. There were 9 different protocols tested, i.e. fast and slow protein precipitation (PP) using ethanol and acetone (fPP-EtOH, fPP-Acetone, sPP-EtOH and sPP-Acetone), solid phase extraction (SPE) with a C18 column (SPE-C18), liquid–liquid extraction (LLE) using a mixture of Chloroform–MeOH, MTBE–MeOH, MTBE–butanol, and SPE-based phospholipid removal with Ostro™ plate (Ostro).

The protocol used for the sPP-EtOH or sPP-Acetone was as described in "LC–ESI-MS/MS analysis of human plasma" section. The protocols used for the SPE-C18 [25], the Chloroform–MeOH [39] and MTBE–MeOH [44] LLE, and the Ostro™ plate (Waters, Milford, Mass, USA) [46] were as described in the literature. For the fPP-EtOH or fPP-Acetone, a 10-times volume of solvent was added to 200 μl plasma. The mixture was shaken immediately and centrifuged. The supernatant was evaporated and reconstituted in 40 μl EtOH/water. An aliquot of the reconstituted sample was injected

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