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Quantification of oxysterols in human plasma and red blood cells by liquid chromatography high-resolution tandem mass spectrometry

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ABSTRACT

Oxysterols are important intermediates in numerous metabolic and catabolic pathways and their biological significance is also proven. The present paper describes a reliable and short liquid chromatography–high-resolution mass spectrometry method (LC–MS/HR-MS) for the quantification of 8 different oxysterols (24(S)-hydroxycholesterol, 25-hydroxycholesterol, 27-hydroxycholesterol, 4β-hydroxycholesterol, 7α-hydroxycholesterol, 7β-hydroxycholesterol, 7-ketocholesterol and cholestan-3β,5α,6β-triol) in human plasma and red blood cells. Oxysterols were extracted with iso-octane after saponification of esterified sterols. Due to the poor ionization efficiency of the target compounds in electrospray ionization (ESI) derivatization of the molecules has been performed with *N,N*-dimethylglycine (DMG). Within less than 8 min we were able to achieve baseline separation of the isobaric 24(S)-hydroxycholesterol, 25-hydroxycholesterol, 27-hydroxycholesterol, 4β-hydroxycholesterol, 7α-hydroxycholesterol and 7β-hydroxycholesterol. Moreover, high mass resolution was advantageously applied to resolve quasi-isobaric interferences. The method was validated based on the recommendations of US Food and Drug Administration and the European Medicines Agency guidelines. Oxysterol concentrations were determined in human plasma and red blood cells from healthy volunteers. Furthermore, the applicability for clinical use has been proven by the analysis of oxysterols as biomarkers in Niemann–Pick type C or cerebrotendinous xanthomatosis patients.

1. Introduction

Sterols are a biologically important class of organic molecules which contain a free OH group on their polycyclic steroid backbone. These compounds play essential roles as elements of cellular membranes, signaling, regulation and metabolism in almost all living systems [1,2]. From the clinical point of view, the most noteworthy classes of sterols in biological fluids are cholesterol and cholesteryl esters, cholesterol precursors, oxysterols, bile acids, and steroid hormones [3].

Oxysterols are oxygenated derivatives of cholesterol that are formed by enzymatic oxidation or by reactive oxygen species. Oxysterols are intermediates in the conversion of cholesterol to hormonal steroids and bile acids. They are involved in neurogenesis, protein prenylation and may activate the liver X receptor (LXR) [4,5]. Moreover, oxysterols are suspected to play a role in several diseases and pathological processes (e.g. neurodegeneration, atherosclerosis, apoptosis, necrosis, inflammation) [6–10].

For example, 7-ketocholesterol and cholestane-3β,5α,6β-triol have been shown to be significantly elevated in various tissues and in blood plasma of patients with Niemann–Pick type C disease (NPC) [11–14]. Oxysterols, like 24(S)-hydroxycholesterol in combination with total cholesterol and 27-hydroxycholesterol are discussed as potential biomarkers in plasma and liquor for neurodegenerative diseases [15]. In addition, these side chain oxysterols together with 25-hydroxycholesterol are associated to Huntington's and Alzheimer's diseases [16]. 7-Ketocholesterol, 7α- and 7β-hydroxycholesterol, and cholestane-3β,5α,6β-triol possess cytotoxic and inflammatory properties in vitro [6].

The analysis of oxysterols is challenging due to their low concentrations in biological fluids (lower ng/ml) accompanied by 10^3–10^6-fold excess of cholesterol. Secondly, some of the oxysterols may be generated by autoxidation of cholesterol during storage and sample preparation. This process might be partially reduced by adding antioxidants (e.g. butylated hydroxytoluene [BHT]). Several chromatographic methods coupled to MS for sterol analysis have been proposed and an extensive review about recent methodologies is given in [4]. GC-MS methods are most commonly used to detect sterols exhibiting good peak shape and high chromatographic resolution but often suffer from long run times and

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the necessity of large sample volumes. The separation and quantification of the target compounds are done usually after silylation [5,17–20]. However, in clinical laboratories instrumental equipment is often restricted to liquid chromatography (LC). LC coupled to tandem MS (LC–MS/MS) methods without [21,22] or with derivatization procedures have also been reported [13,23–27]. Chemical derivatization can enhance analytical sensitivity in mass spectrometry, e.g. derivatization into *N*,*N*-dimethylglycine (DMG) esters improves the mass spectrometric detection of oxysterols [28]. The derivatization with DMG is straightforward, adoptable for automatization and suitable for high throughput analysis. Successful applications after DMG derivatization have been shown only for the quantification of 7-ketocholesterol and cholestane-3β,5α,6β-triol [12,14]. Here, we used DMG derivatization to analyze 8 oxysterols, including more of the biochemically relevant species.

Essential requirements for application in large cohort studies are short LC run times. LC–MS approaches below 10 min run time that cover a panel of both side-chain and ring substituted oxysterols can be hardly found in literature. In LC–MS a chromatographic separation is essential since many of the target oxysterols are isobaric (with same transition masses in most cases) and separation by mass spectrometry is not possible. In the work described here, we present for the first time a baseline LC separation of the isobaric species 24(S)-, 25-, 27-, 4β-, 7α-, and 7β-hydroxycholesterol to ensure reliable quantitation. Furthermore, high resolution tandem mass spectrometry is applied to enhance specificity because co-eluting compounds undergoing a similar transition can distort quantification. We illustrate the use of our LC–MS/HR-MS method for the analysis of 8 oxysterols in human plasma and red blood cells (RBCs).

2. Experimental

2.1. Chemicals and reagents

Acetonitrile, ammonium acetate analytical grade, formic acid analytical grade, ethanol absolute EMSURE, and *N*,*N*-dimethylpyridin-4-amine (DMAP) were purchased from Merck (Darmstadt, Germany). Methanol (MeOH) LC–MS Chromasolv was from Fluka (Buchs, Switzerland). Chloroform ROTISOLV® was from Carl Roth GmbH (Karlsruhe, Germany). Standards and isotopically labeled standards as 24(S)-hydroxycholesterol, 4β-hydroxycholesterol-D7, 7-ketocholesterol-D7 and cholestan-3β,5α,6β-triol-D7 were obtained from Toronto research chemicals (Toronto, Canada) while 25-hydroxycholesterol, 27-hydroxycholesterol, 4β-hydroxycholesterol, 7α-hydroxycholesterol, 7β-hydroxycholesterol, 7-ketocholesterol, 25-hydroxycholesterol-D6 and 7β-hydroxycholesterol-D7 were purchased from Avanti Polar Lipids (Alabaster, Alabama, USA). 24(S)-Hydroxycholesterol-D10 and 27-hydroxycholesterol-D6 were from Sugaris GmbH (Munster, Germany). Cholestan-3β,5α,6β-triol, butylated hydroxytoluene (BHT), *N*,*N*-dimethylglycine (DMG), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC), and iso-octane ACS reagent were from Sigma–Aldrich (Munich, Germany).

2.2. Sample preparation

2.2.1. Calibration samples

Pooled plasma samples from healthy volunteers were diluted with 0.1 M sodium chloride in water in a ratio of 1:10. Aliquots of this diluted plasma were supplemented with a combined standard solution to obtain five calibrators in appropriate concentration ranges. The standard solution was prepared from stock solutions of oxysterol standards by dilution with MeOH. The concentration range of the calibration samples used in this method was estimated based on our previous oxysterol studies [5,17] to cover the endogenous levels of oxysterols in human plasma and red blood cells. The concentration ranges of the oxysterols in the calibration solutions are given in Suppl. Table 1.

2.2.2. Plasma samples

EDTA blood samples were collected from healthy volunteers followed by centrifugation at 4000 × *g* for 10 min and addition of BHT to the supernatant at a concentration of 50 μg/ml. The samples were stored in aliquots at −80 °C.

Sample preparation was based on the method described by Dzeletovic et al. [29] and the methods used in our research group in previous studies [5,17]. Briefly, 100 μl of human plasma or calibrator was transferred to a screw-capped vial. To this sample 10 μl of isotopically labeled internal standard mixture (24(S)-hydroxycholesterol-D10 (2 μg/ml), 25-hydroxycholesterol-D6 (1 μg/ml), 27-hydroxycholesterol-D6 (2 μg/ml), 4β-hydroxycholesterol-D7 (1 μg/ml), 7β-hydroxycholesterol-D7 (1 μg/ml), 7-Ketocholesterol-D7 (3 μg/ml) and Cholestan-3β,5α,6β-triol-D7 (1 μg/ml) in methanol) and 500 μl of freshly prepared 1 M potassium hydroxide in ethanol were added. Unless stated otherwise alkaline hydrolysis was conducted at 25 °C in a water bath for 60 min under Argon atmosphere. Afterwards neutralization was performed with phosphoric acid solution. To extract lipids, 250 μl of 2 M sodium chloride solution was added to the sample, vortex-mixed with 1 ml of iso-octane and then centrifuged for 5 min at 4000 × *g*. The supernatant (iso-octane layer) was transferred by a Tecan Genesis (Männedorf, Switzerland) liquid handler to a new tube. The extraction step with 1 ml iso-octane was repeated followed by evaporation to dryness. The residue was dissolved in a mixture of 25 μl DMG (0.5 M) and DMAP (2 M) in $CHCl_3$ and 25 μl EDC (1 M) in $CHCl_3$. This mixture was allowed to react for 1 h at 45 °C. The excess of the derivatizing agent was deactivated with the addition of 50 μl MeOH at 45 °C for 30 min. Evaporation was performed until dryness. The residue was redissolved in MeOH and transferred to vials for direct injection.

2.2.3. Red blood cells

EDTA blood was centrifuged for 5 min at 2000 × *g*. The RBCs were separated from plasma and buffy coat and washed three times with physiological NaCl solution. After washing the RBCs were dispersed in NaCl solution and the concentration of RBCs was measured by a hematology analyzer (Sysmex 5000). For oxysterol analysis 100 μl of this suspension underwent the same preparation steps as plasma samples (as described above).

2.3. Apparatus and conditions

The analysis of different oxysterols was performed on a liquid chromatography-high resolution tandem mass spectrometry (LC–MS/HR-MS) system consisted of an UltiMate 3000 XRS quaternary UHPLC pump, an UltiMate 3000 RS column oven and an UltiMate 3000 isocratic pump (Thermo Fisher Scientific Waltham, MA USA). The system was equipped with a PAL HTS-xt autosampler (CTC Analytics, Zwingen, CH) and a hybrid quadrupole-orbitrap mass spectrometer QExactive (Thermo Fisher Scientific, Bremen, Germany) equipped with a heated electrospray ionization source. 5 μl of the resolved samples were injected and separated on a Kinetex™ 2.6 μm Biphenyl (50 × 2.1 mm, Phenomenex, Aschaffenburg, Germany) column at a column temperature of 30 °C. The separation was achieved under gradient elution conditions. Mobile phase A consisted of methanol/water (5/95; v/v), mobile phase B was methanol/acetonitrile (10/90; v/v), both containing 0.1% formic acid and 2 mM ammonium acetate. Gradient elution started at 42% B with a flow rate of 500 μl/min, a linear increase to 50% B in