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Validation of an isotope dilution gas chromatography–mass spectrometry method for combined analysis of oxysterols and oxyphytosterols in serum samples

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ABSTRACT

We describe the validation of a method for the analysis of oxysterols, i.e. oxycholesterols and oxyphytosterols, in human serum using gas chromatography–mass spectrometry selected ion monitoring (GC–MS-SIM). Concentrations of 7α- and 7β-hydroxy-, and 7oxo-cholesterol, -campesterol, and -sitosterol as well as 4β-hydroxycholesterol and side-chain oxygenated 24S-, 25-, and 27-hydroxycholesterol were determined by isotope dilution methodology.

After saponification at room temperature the oxysterols were extracted, separated from their substrates, cholesterol, campesterol, and sitosterol, by solid phase extraction, and subsequently derivatised to their corresponding trimethylsilyl-ethers prior to GC–MS-SIM. In order to prevent artificial autoxidation butylated hydroxytoluene and ethylenediaminetetraacetic acid were added. The validation of the method was performed according to the International Conference on Harmonisation guidance, including limits of detection and quantification, ranges, recovery and precision. Due to improved instrumental settings and work-up procedure, limits of detection and quantification ranged between 8.0–202.0 pg/mL and 28.0–674 pg/mL, respectively. Recovery data in five calibration points varied between 91.9% and 116.8% and in serum samples between 93.1% and 118.1%. The mean coefficient of variation (CV) for the recovery of all compounds was <10%. Well satisfying CVs for within-day precision (2.1–10.8%) and for between-day precision (2.3–12.1%) were obtained. More than 20 samples could be processed in a single routine day and test series of about 300 samples can be realised without impairment of the validation parameters during a sequence. Comparison of oxysterol and oxyphytosterol content in serum and plasma revealed no difference.

A fully validated isotope dilution methodology for the quantification of oxycholesterols and oxyphytosterols from human serum or plasma is presented.

1. Introduction

Within the last five decades immense progress was achieved in the field of cholesterol and oxysterol research. Investigations on oxycholesterols reveal their important and multilateral effect on physiological processes in humans [1].

Most importantly, oxycholesterols were identified as precursor molecules of bile acids. Formed by cytochrome P450 (CYP) enzymes 7α-hydroxy (OH)-cholesterol (CYP7A1) and 27-OH-cholesterol (CYP27A1) are immediate cholesterol oxidation metabolites and precursors within the neutral and acidic bile acid pathways, respectively [2–4]. In brain, where 25% of whole body free cholesterol is located a unique 24(S)-OH-cholesterol (cerebrosterol) is generated by CYP46A1. Though more polar than cholesterol, this oxysterol is able to pass the lipophilic blood–brain barrier and subsequently catabolised to bile acids in hepatocytes [5–9].

Furthermore, oxycholesterols (OCS) have different effects on physiological processes [10]. In human, several different oxysterols binding proteins do exist, which directly regulate cellular processes like cholesterol homeostasis [11–16]. Especially 25-OH-cholesterol, an oxysterol that occurs in low concentrations in plasma apparently seems to be involved in a plurality of different biological effects [17]. Furthermore 4β-OH-cholesterol is nowadays used as indicator for CYP3A4 activity [18–21]. Many researchers focus on the pro-atherogenic effect of 7β-OH-cholesterol as well as the

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negative influence of increased levels of 27-OH-cholesterol on the formation of β-amyloid plaque in Alzheimer pathology [22–26]. Within the last fifteen years the development of more sensitive and specific GC- or LC–mass spectrometric methods enabled to determine very low concentrations of oxyphytosterols in blood and tissue.

The phytosterols campesterol and sitosterol that naturally occur in a major amount in plant products as nuts, seeds and vegetable oils are described since a long time. They are structurally similar to cholesterol but differ only by an additional methyl or ethyl-group at C24 or an additional double bond in the side chain [27,28]. Intake of sitosterol in a dose of 2 g/day reveals a cholesterol lowering effect in human. This positive effect on cholesterol level is recently used in cholesterol lowering spreads that are enriched with plant sterols in esterified form (functional food) [29,30]. Due to the fact of a broad commercial use of plant sterols as support for serum cholesterol lowering therapy, the interest in oxyphytosterols (OPS) has increased. OPS can directly be formed from plants; to a high extend during food production by light and heat, during storage, and during food preparation [31]. Reactive oxygen species are able to form oxysterols *in vivo* and *ex vivo* during workup procedure of blood sample [32]. To date the accepted scientific data on physiological and biological effects of OPS are rare. A pro-atherosclerotic and carcinogenic effect, cytotoxicity, genotoxicity and mutagenesis are discussed as possible undesirable effects [33,34].

Since the middle of the 1990s a plurality of GC–MS methods for the determination of different OCS and OPS have been published. We describe here the development and validation of a method for the combined analysis of OCS and OPS in human serum using an isotope dilution gas chromatography–mass spectrometry selected ion-monitoring (GC–MS-SIM) method. The present method contains a broad spectrum of OCS (7α-, 7β-, 4β-, 24(S)-, 25- and 27-OH, as well as 7oxo-cholesterol) and OPS (7α-, 7β-, 7oxo- campesterol/-sitosterol). This procedure presents high reproducibility for all analytical parameters in 500 μL serum samples.

2. Experimental

2.1. Materials

All commonly used solvents were of highly pure analytical grade for liquid chromatography. Dried Pyridine, sodium chloride, sodium hydroxide pellets (for analyses), butylated hydroxytoluene (BHT), phosphoric acid 85% (for synthesis), 1,1,1,3,3,3-hexamethyldisilazane, trimethylchlorosilane and EDTA where purchased from Merck KgaA (Darmstadt, Germany). Dichloromethane, toluene, chloroform, and cyclohexane of high pure grade were obtained from VWR (Darmstadt, Germany). Silica cartridges (Chromabond, bonded phase SI 100 mg/ml) from Marchery-Nagel (Düren, Germany) where used for solid phase extraction. Authentic oxycholesterols used as reference and deuterated OCS used as internal standards were purchased from Steraloids (Newport, Richmond, USA) and Avanti Polar Lipids, Inc. (Alabaster, Alabama, USA). D6-27-hydroxycholesterol was purchased from Sugaris (Münster, Germany). Oxyphytosterols and deuterium labelled OPS used as reference and deuterated internal standards, respectively, are currently not available from industrial sources. Lacking commercial availability, these compounds were synthesised according to a previous described method by Plat et al. [35]. All oxysterols were tested for interfering impurities by gas chromatography equipped with mass selective detector (GC–MS) and quantified by gas chromatography equipped with flame ionisation detector (GC-FID) with 5α cholestane as internal standard before preparation of standard solutions and deuterated internal standard mixtures.

2.2. Sample preparation for simultaneous analysis of OCS and OPS

One hundred μL of methanolic deuterium-labelled internal standard (ISTD) containing: d_4-7α-OH-Campesterol (Camp) (22.7 ng/mL); d_4-7β-OH-Camp (95.1 ng/mL); d_5-7-keto-Camp (200.6 ng/mL); d_4-7α-OH-Sitosterol (Sit) (41.4 ng/mL); d_4-7β-OH-Sit (124.6 ng/mL); d_5-7-keto-Sit (270.5 ng/mL); d_6-7α-OH-Cholesterol (CH) (319.2 ng/mL); d_6-7β-OH-CH (307.3 ng/mL); d_6-7-keto-CH (374.0 ng/mL); d_4-24-OH-CH (570.3 ng/mL); d_6-25-OH-CH (109.0 ng/mL); d_6-27-OH-CH (1159.8 ng/mL); d_6-4β-OH-CH (464.1 ng/mL) were added to 500 μL serum. To minimize artefacts of cholesterol and plant sterol oxides during saponification one hundred μL of an ethanolic butylated hydroxytoluene solution (150 mg in 1 mL EtOH) and one hundred μL of an alkaline ethanolic ethylenediaminetetraacetic acid (EDTA) (300 mg EDTA dissolved in 2.0 mL deionised water and 3.0 mL 1 N NaOH in 90% ethanol) were added to the sample prior to saponification at room temperature. Two mL of 1.5 N NaOH in 90% ethanol were added to perform saponification at room temperature. Subsequently the samples were evaporated under a gentle stream of nitrogen for 5 min to minimize the content of oxygen in the test tube. Tubes where sealed with a Teflon® inlet equipped cap and the saponification was performed for 2 h at room temperature in darkness on a NeoLaB orbital shaker at 283 rpm.

After saponification, 1 mL of deionised water was added and the solution was adjusted to pH 7.0 with about 275 μL phosphoric acid in water (1:1, v/v) and 2 mL sodium chloride solution 0.9% (9 g NaCl in 1 l deionised water). Oxysterols were extracted twice with 3.0 mL dichloromethane as follows: vortex for 30 s followed by centrifugation at 2000 rpm for 10 min (Heraeus multitude OKRA centrifuge, Osterode, Germany) to accelerate separation. Organic layer was separated from the upper aqueous phase and kept in darkness at 4–8 °C. Both organic phases were combined and evaporated under a gentle stream of nitrogen at 60 °C and the residue was dissolved in 1 mL toluene.

OCS and OPS were separated from cholesterol, neutral sterols and plant sterols by solid-phase extraction. Silicate cartridges were equilibrated with 3 × 1.0 mL n-hexane. The toluene dissolved samples were loaded onto a silica cartridge and allowed to pass through the column by gravity force at room temperature. Cholesterol, and non-cholesterol neutral sterols, including plant sterols, were eluted from the column with 8 × 1.0 mL 0.5% 2-propanol in n-hexane (v/v) followed by elution of the OCS and OPS with 3 × 1.0 mL 30.0% 2-propanol in n-hexane (v/v) as described by Dzeletovic et al. and Husche et al. [36,37]. To decrease workup duration and to avoid autoxidation vacuum equipment (Marchery-Nagel, Düren, Germany) was employed to accelerate the washing procedure.

Prior to analysis, the OCS and OPS containing phases were carefully dried under a stream of nitrogen at 60 °C and all oxysterols were derivatised to their trimethylsilyl-esters with 500 μL silylation-reagent (pyridine: hexamethyldisilazane: trimethylchlorosilane; 9:3:1, v/v/v). The silylation reaction was performed by heating at 90 °C for 1.5 h. The silylation-reagent was evaporated under a gentle stream of nitrogen and the residue was dissolved in 30 μL n-decane and transferred to an injection vial for GC–MS-SIM analysis. The authenticity of the compound was proven by the comparison of the spectra from the literature and quantification was performed by GC-flame ionisation detection (FID) as published previously [37].

2.3. Determination of oxysterols by gas chromatography-mass selective detection (GC-MSD)

The TMS-ether derivates of the oxysterols were analysed by GC–MS using a 6890 N gas chromatograph coupled to a 5973 mass