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Preanalytical and analytical challenges in gas chromatographic determination of cholesterol synthesis and absorption markers☆

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ABSTRACT

Introduction: Cholesterol homeostasis disruption contributes to the development of different pathologies. Non-cholesterol sterols (NCSs) serve as cholesterol synthesis markers (desmosterol and lathosterol), and cholesterol absorption surrogate markers (campesterol, stigmasterol and β-sitosterol). The study aimed to resolve certain new pre-analytical and analytical problems and ensure a reliable and validated method.

Materials and methods: Method optimization, validation and stability studies were executed in human serum and plasma. Freeze-thaw cycles were done with and without antioxidant. Gas chromatography-mass spectrometer (GC-MS) was used for NCSs confirmation and plasticizer identification, while GC-flame ionization detector (GC-FID) was used for NCSs quantitation.

Results: Intra- and inter-assay variabilities for all NCSs were 2.75–9.55% and 5.80–7.75% for plasma and 3.10–5.72% and 3.05–10.92% for serum, respectively. Recovery studies showed satisfactory percentage errors for all NCSs: 93.4–105.7% in plasma and 87.5–106.9 in serum. Derivatized samples were stable up to 7 days at − 20 °C and derivatization yield was affected by presence of plasticizers. Fatty acid amids were identified as interfering plastic leachates. Statistically different NCSs concentrations were observed after the 1st freeze-thaw cycle, in antioxidant-free samples, and after the 4th cycle in antioxidant-enriched samples.

Conclusions: All of the in-house procedures proved to be useful for minimizing the preanalytical and analytical variations, as proven by the validation results.

1. Introduction

Although the overall cholesterol metabolism is still not entirely clarified, it is known that the cholesterol homeostasis is maintained through the balance between cholesterol synthesis and absorption [1]. Non-cholesterol sterols (NCSs) can provide an important insight into the cholesterol synthesis and absorption pathways. Plasma concentrations of endogenous sterols, which basically represent cholesterol precursors, are indicators of cholesterol endogenous production, while exogenous phytosterols serve as surrogate markers of cholesterol gastrointestinal absorption [2]. Disturbance of cholesterol homeostasis, which occurs at synthesis or absorption level, can be associated with the development of hypercholesterolemia, atherosclerosis, obesity, metabolic syndrome, diabetes mellitus and cardiovascular, cerebrovascular as well as certain neurodegenerative diseases [1,3–6]. Results of recent studies indicate the importance of estimating the efficiency of cholesterol absorption and synthesis for better identification of individual differences in cholesterol metabolism, and the selection of the most appropriate therapeutic approach (lifestyle changes or drug therapy with statins or ezetimibe) [2,7].

Abbreviations: ANOVA, Analysis of variance; AUC, area under the curve; BBP, benzylbutylphthalate; CS, calibration standard; CV, coefficient of variation; DBP, di-n-butyl phthalate; DEHP, di(2-ethylhexyl) phthalate; DMSO, dimethyl sulphoxide; Dv, derivatization yield; EDTA, Ethylenediaminetetraacetic acid; Ex, extraction yield; FDA, Food and Drug Administration; FID, flame ionization detector; GC, Gas chromatography; HMDS, 1,1,3,3,3-Hexamethyldisilazane; HPLC, high-performance liquid chromatography; IS, internal standard; KOH, Potassium hydroxide; LLE, liquid-liquid extraction; LOD, limit of detection; LOQ, limit of quantitation; MS, mass spectrometry; NSC, Non-cholesterol sterol; PTFE, polytetrafluoroethylene; RI, retention index; Rs, resolution factor; S/N, signal-to-noise ratio; THF, tetrahydrofurane; TMCS, Trimethylchlorosilane

☆ NCSs method validation and preanalytical problems

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Despite of its acknowledged clinical and diagnostic potential, sterol bioanalysis still lacks measurement harmonization and methodology standardization. So far, a considerable number of studies examined the clinical significance of sterols as cholesterol synthesis and absorption markers [1–7]. Nevertheless, a limited number of studies dealt with method validation and even fewer studies examined preanalytical factors which may influence the NCSs quantitation. Over the past decade, a positive trend towards reduction of laboratory errors is observed, but preanalytical phase still represents the most critical area to target [8].

Gas chromatography-flame ionization detection (GC-FID) and gas chromatography-mass spectrometry (GC-MS) methods for separation and quantification of NCSs represent an analytical challenge because NCSs concentrations in plasma are 200–1000 times lower compared to cholesterol levels [8,9]. Various authors have pointed out the importance of proper sample type selection and sample storage conditions [10]. However, hardly there is a study examining freeze-thaw cycle and analyte stability which can also represent potential sources of errors leading to unsatisfactory validation results. Sample preparation prior the NCSs quantitation requires multiple steps. Along with saponification and extraction, many authors state the necessity for the derivatization, while others try to avoid this procedure because of its complexity [8,11]. If one chooses to encompass the derivatization into the sample preparation procedure, an additional optimization of the whole process is needed. According to our best knowledge, there are no papers on this subject.

A recent study by Mackay et al. showed that humidity content and temperature which are present during the sample preparation greatly influence NCSs quantitation [8]. However, a small number of studies comprehensively examined the influence of labware on NCSs quantitation. Nevertheless, it often represents the overlooked preanalytical factor. Plastic labware and consumables are widely used in the laboratory setting, although plastics may spontaneously release its contents and interfere with the results [12,13]. Even if there is a growing concern regarding this issue, it isn't backed up with considerable literature data, especially regarding compound leakage due to the usage of organic solvents with plastic consumables during extraction and derivatization of steroid compounds.

Steroid structures are susceptible to oxidative changes. Therefore long-term preparation may cause the structural changes and represent a great preanalytical issue. It is acknowledged that this process can be accelerated by the presence of metal ions originating from the various labware components, such as tube seals [14,15]. During the employment of contemporary analytical techniques such as chromatographic methods with high-sensitive detection, every structural change of the analyte may influence the quantitation.

The aim of this study was to examine the most common variability causes, in particular those deriving from pre-analytical sample preparation, as well as gas chromatographic conditions, through comprehensive optimization process in order to ensure a reliable, validated method for further employment into the clinical studies based on NCSs quantitation.

2. Materials and methods

2.1. Reagents, samples and instrumentation

Peaks of desmosterol, lathosterol, campesterol, stigmasterol and β-sitosterol were identified by comparison with authentic standards (Supelco, Bellefonte, PA, USA). Internal standard (IS) 5α-cholestane (GC grade), 0.5 M methanolic potassium hydroxide (KOH) and tetrahydrofuran (HPLC grade) from Sigma-Aldrich (St. Louis, MO, USA) were used. KOH was purchased from POCH (Center Valley, PA, USA), and ethanol, chloroform, petroleum ether, n-hexane and acetonitrile (HPLC grade) from Fisher (Pittsburgh, PA, USA). Trolox® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) from Acros Organics (Geel, Belgium) was used as an antioxidant and Supelco's Sylon™ HTP (1,1,1,3,3,3–hexamethyldisilane + trimethylchlorosilane + pyridine, 3:1:9) was used as derivatization reagent. The C8-C40 Alkanes Mixture was used as a calibration standard for calculation of retention indices (Supelco, Bellefonte, PA, USA). Helium (5.0. purity), synthetic air free of hydrocarbons (5.0), hydrogen (5.0), and nitrogen (3.5, and 5.0.) were obtained from Messer Group (Bad Soden, Germany).

Human serum and EDTA plasma samples were obtained from 60 healthy volunteers following the guidelines laid down in the Declaration of Helsinki. Samples were used for method development pool preparation (n = 10) and clinical verification (n = 50). None of the participants was vegetarian/vegan or treated with hypocholesterolemics. All participants signed an informed consent form before enrolment.

GC analyses were performed on HP-5, DB-17MS (Agilent Technologies, USA) and ZB-5MS (Phenomenex, USA) columns, while HPLC analyses were done using EclipseXDB C-18 column (Agilent Technologies, USA).

NCSs analyses were performed on Agilent 7890A GC instrument (Agilent Technologies, USA) equipped with FID. Additional analyses were done using Agilent GC/MSD system 6890N/5975C (Agilent Technologies, USA) and Agilent 1200 HPLC/DAD system (Agilent Technologies, USA).

2.2. Preparation of glassware

Borosilicate glassware (tubes, beakers, pipettes, volumetric flasks etc.) and labware made from high-density plastics (pipette tips, Pasteur pipettes etc.) were used for sample handling during the entire preanalytical and analytical process.

In order to avoid cross-contamination, rigorous glassware washing procedure employed tap water/distilled water wash followed by an overnight immersion in chromic acid. After regular wash, drying in the oven at 100 °C, glassware was rinsed twice with acetone, followed by n-hexane. We omitted the process of pre-analysis glass silylation described by some authors [11], since our preliminary method studies showed that this time- and the reagent-consuming step wasn't necessary for obtaining satisfactory derivatization yield.

2.3. Sample preparation and GC/FID method optimization

2.3.1. Experiment 1 - sample preparation without derivatization

Initially, we tested the sample preparation method without a derivatization step. Following the protocol of Matthan and co-workers [16], 150 μL of IS (5-α-cholestane) dissolved in chloroform (1 mg/mL) was added to 1 mL of sample and saponified with 5 mL of 0.5 M methanolic KOH solution for 1 h at 100 °C. After the addition of 2.5 mL of distilled water, the NCSs were extracted in 3 mL of petroleum ether and the tubes were vortexed and centrifuged at 1500 g for 15 min. The upper layer containing the nonsaponifiable materials was then transferred into clean glass tubes. Overall, the extraction procedure was repeated three times. Combined extracts were dried under nitrogen and re-suspended in 1 mL of chloroform prior to the GC/FID analysis [16].

GC/FID parameters were initially optimized on Agilent's HP-5 column ((5%-Phenyl)-methylpolysiloxane, 30 m × 0.32 mm × 0.25 μm). Initially, the isothermal run was performed with the following GC conditions: 5 μL injection volume, split injection mode (9:1), 300 °C inlet temperature, 280 °C column temperature, 310 °C detector temperature, 9.26 psi constant pressure and helium gas flow 0.78 mL/min. Total run time was 35 min. Finally, we optimized the following instrumental method: injection volume of 5 μL, split injection mode (9:1) and 280 °C inlet temperature were applied. The column temperature was set by multiple ramp as follows: initial temperature of 150 °C was held for 3 min, linear temperature ramp (rate 30 °C/min) was applied until reaching 280 °C held for 28 min and followed by another linear ramp (rate 10 °C/min) until reaching 295 °C which was held for 10 min. Detector temperature was 300 °C; constant pressure of 9.26 psi and helium gas flow of 1.23 mL/min. The total run time was