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Online solid-phase extraction–liquid chromatography–mass spectrometry to determine free sterols in human serum

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ABSTRACT

An automated method for analyzing free non-cholesterol sterols in human serum using online solid phase extraction–liquid chromatography–mass spectrometry is proposed herein. The method allows the determination of three phytosterols (sitosterol, stigmasterol and campesterol) and two cholesterol precursors (desmosterol and lanosterol). The analysis of sterols in human serum is critical in the study of cholesterol-related disorders, such as inherited familial hypercholesterolemias. Special effort was made to isolate the analytes from the serum lipoproteins, their natural conveyance through the bloodstream. The sample treatment consisted of a Bligh–Dyer extraction followed by dilution of the extract. This treatment allowed the sample to be injected into the online system and ensured the correct detection of the analytes, while avoiding the matrix effects commonly related to serum samples.

The analytical performance showed linear ranges that covered two orders of magnitude, with correlation coefficients above 0.99. Limits of detection and quantification ranged from 0.2 ng/mL to 13 ng/mL and from 1.0 ng/mL to 43 ng/mL, respectively. Recovery when spiking serum with a half or a tenth of the average concentration reported in human serum ranged from 99% to 111% and from 102% to 120%, respectively. Intra-day precision and inter-day precision were below 20%.

1. Introduction

Sterols are metabolites related to cholesterol metabolism. The analysis of these compounds in human serum is an essential step in the diagnosis of cholesterol-related disorders, such as inherited familial hypercholesterolemias, cerebrotendinous xanthomatosis and sitosterolemia [1]. Cholesterol and related sterols are regulated in the human body *via* a complex set of mechanisms. Cholesterol can be absorbed through diet at intestinal level [2]. The rate of cholesterol consumption can be inferred from the presence of phytosterols such as sitosterol, stigmasterol and campesterol in human serum, since they undergo the same absorption and secretion processes [3]. Cholesterol is also endogenously produced and regulated in the liver. Some sterols such as desmosterol and lanosterol are intermediate products (precursors) in this reaction mechanism. Since these compounds leak into the bloodstream at a rate proportional to their formation in the cholesterol synthetic pathway, the circulating levels of these endogenous precursors reflect the rate of cholesterol synthesis [4,5].

Sterols have a non-polar structure and are carried through the circulatory system by lipoproteins. Lipoproteins are aggregates of lipids surrounded by a 2-nm amphiphilic layer composed of phospholipids, apoproteins and free cholesterol [6]. Therefore, the analysis of sterols involves the breakage of these aggregates.

The extraction of sterols has been typically performed using the Bligh–Dyer method [7,8], where a mixture of chloroform and methanol solubilizes the lipids from the lipoproteins, allowing the apoproteins to be separated. Sterols have been traditionally analyzed using gas chromatography, *via* a time-consuming derivatization step [4,9–11]. Liquid chromatography (LC) coupled to mass spectrometry (MS) avoids this derivatization step, diminishing the sample processing time [8,12]. However, the sample cleanup prior to LC–MS analysis is still strongly recommended and a solid phase extraction (SPE) step is generally performed [8,13].

An interesting choice is the use of hyphenated techniques that couple SPE to LC–MS using a switching valve, which is common in the analysis of biological samples and enables rapid and reliable sample preparation [14–17]. Several publications dealing with online systems are focused on determining drugs in plasma or urine; but scarce literature has been found related to the determination of endogenous metabolites [18,19]. A previous work of the authors aimed at the reduction of the sample processing time for determining free and bonded sterols in human serum [20,21] and another contribution demonstrated the capacity of coupling a Restricted Access Material (RAM) cartridge to LC–MS for determining bile acids

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in human serum [22]. To the best of our knowledge, there is not such a system to determine sterols in human serum, so the present contribution aims at the routine analysis of sterols in a faster way by using an online system.

2. Materials and methods

2.1. Chemicals and reagents

The sterols under study (Fig. 1) were provided by Sigma-Aldrich (Madrid, Spain): desmosterol (purity 85%, Chemical Abstract Service identification number: 303-04-2), campesterol (65%, 474-62-4), stigmasterol (95%, 83-48-7), lanosterol (97%, 79-63-0), sitosterol (95%, 83-46-5), and cholesterol-26,26,26,27,27,27-D6 (internal standard, IS, 97%, 60816-17-3). The solvents used were methanol (supragradient LC–MS grade, 67-56-1), 2-propanol (LC–MS grade, 67-63-0), and chloroform (HPLC grade, 67-66-3). The solvents were supplied by Sharlab (Barcelona, Spain). Water was obtained from a Milli-Q Plus 186 device from Millipore (Billerica, MS, USA).

Stock solutions of 1000 mg/L in methanol were prepared for each analyte. A stock solution of the IS at 100 mg/L was prepared in 2-propanol. A working solution with all of the sterols in methanol, at a concentration of approximately 10 mg/L, was prepared and stored in the dark at 4 °C.

2.2. Pooled serum

The pool of serum was obtained by mixing individual serum samples from 150–200 informed healthy volunteers. The pool was provided by the Lipids Unit of the Aragon Institute of Health Sciences (Zaragoza, Spain), and stored in the dark at −80 °C prior to the analysis.

2.3. Sample preparation

The sample was prepared using the following method: 300 μL of serum were extracted through a modification of the Bligh–Dyer method [23]. A volume of 1 mL of chloroform–methanol (1:2, v/v) was mixed with 30 μL of the IS solution and then added to the serum. The mixture was then centrifuged at 9000 rpm (7500*g*) for 10 min in a Microfuge® 18 Centrifuge, Beckman Coulter™ (Brea, CA, USA). The lower organic phase was recovered using a glass Pasteur pipet and transferred to a 2-mL vial. The extract was then accurately weighed, and 1 mL of methanol was added. The resulting extract was filtered using a 0.22-μm Nylon syringe filter. After this sample treatment, the final serum sample extract was ready to be injected into the online system described below.

2.4. Online system setup

Fig. 2 illustrates the online system. The mobile phases were delivered using three pumps; a 600E Controler Multisolvent Delivery System (pump 1) with a 717 plus Autosampler (Waters, Milford, MS, USA), a Kontron 322 System auxiliary binary pump (Kontron Instruments, Neufahrn, Germany) (pump 2) and an Alliance® 2795 Separations Module (Waters) (pump 3). The fluidic paths were controlled using a Cheminert C72 × 1690ED 10-port valve controlled by microelectric actuators, purchased from VICI® Valco Instruments (Houston, TX, USA). The analytes were detected using a Quattro micro™ API Mass Spectrometer (Waters). All these instruments were connected to operate simultaneously. This is explained in detail in the Supplementary information section S1.

Table 1 shows the steps of the online method, flow rate and composition of pumps 1 and 3, as well as the valve position program. The online method comprises four steps: loading, washing, eluting and detection.

2.4.1. Loading

For loading, 300 μL of the treated serum were injected using the 717 plus Autosampler and transferred to a 20 × 4 mm BioTrap 500C18 cartridge, supplied by ChromTech Ltd. (Cheshire, UK) at a 2.0 mL/min flow of 60% methanol and 40% water. A 2-μm filter was included before the cartridge to prevent clogging. This step lasted 4 min.

2.4.2. Washing

The washing step consisted of a 2.0 mL/min flow of water. The elution of large, non-retained molecules was monitored at 220 nm using a 2487 dual λ absorbance detector (Waters). Their elimination was considered to be complete when the UV signal had fallen below 0.05 absorbance units. Then, the valve was automatically switched to the eluting position.

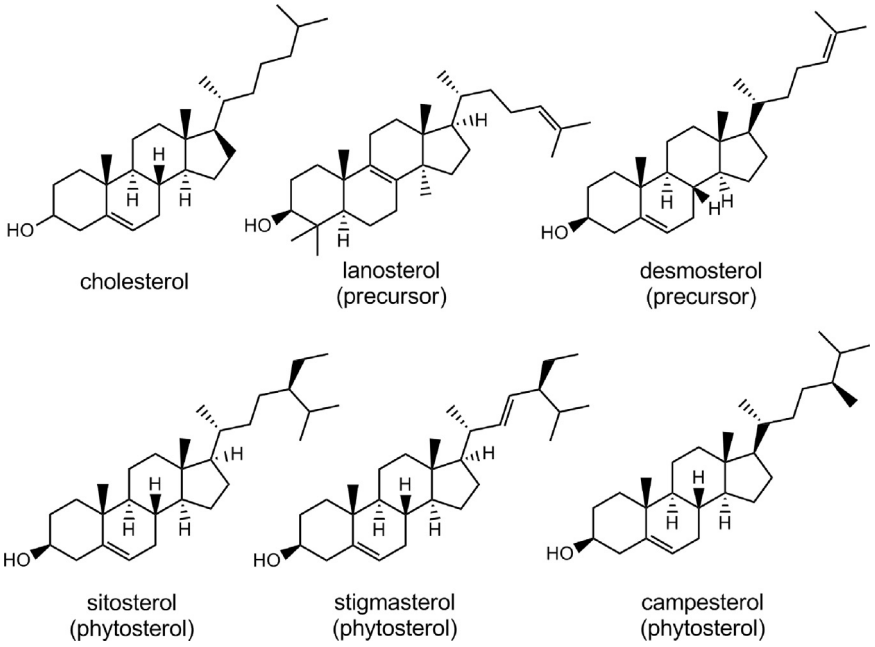

Fig. 1. Chemical structures of the sterols under study and cholesterol.