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Simultaneous determination of plasmatic phytosterols and cholesterol precursors using gas chromatography–mass spectrometry (GC–MS) with selective ion monitoring (SIM)

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Abstract

Phytosterols (β-sitosterol, cholestanol and campesterol) and cholesterol precursors (desmosterol and lathosterol), have been suggested as important biochemical markers of intestinal cholesterol absorption and liver biosynthesis, respectively, and as useful clinical parameters in the study of hypercholesterolemia, β-sitosterolemia, atherosclerosis and cardiovascular disease, including pharmacological response to hypolipidemic agents. We developed an optimised analytical method for the simultaneous analysis of cholestanol, desmosterol, lathosterol, campesterol and β-sitosterol in plasma using capillary gas chromatography coupled to mass spectrometry (GC–MS) with multiple selected ion monitoring (SIM). This method is based on the alkaline hydrolysis of sterol esters, extraction of free sterols and derivatization. The recovery of all sterols was in the range 76–101%. Within-day relative standard deviations (R.S.Ds.) and the between-day R.S.Ds. of cholestanol, desmosterol, lathosterol, campesterol and β-sitosterol were less than 8%, and their plasma levels in 161 normal subjects were (mean ± S.D.) 4.73 ± 2.57, 2.37 ± 1.04, 6.23 ± 3.14, 3.67 ± 1.95 and 5.92 ± 3.62 μmol/l, respectively.

Keywords: Phytosterols; Cholesterol precursors; Gas chromatography–mass spectrometry

1. Introduction

Evidence has been accumulated over the past years suggesting that the quantification of cholestanol, desmosterol, lathosterol, campesterol and β-sitosterol in plasma may be useful to reveal subgroups of patients with high or low absorption and/or synthesis of cholesterol. In moderate to severe hypercholesterolemic patients with coronary heart disease, increasing baseline levels of an index of cholesterol absorption were associated with a reduction in recurrence of major coronary events after statin treatment [1]. A possible explanation for this finding was that higher cholesterol absorption is associated to a lower cholesterol synthesis, a condition less favourable to the inhibition of cholesterol synthesis by statins. The measurement of plasma non-cholesterol sterols may predict the effectiveness of statin therapy in reducing cholesterol synthesis [2] and might help identify subjects with high cholesterol absorption and low synthesis who may benefit from a combination therapy with a cholesterol absorption inhibitor [3].

Accordingly, alteration in the ratio between absorption and synthesis of cholesterol have been described in several metabolic diseases, such as type 2 [4] and type 1 [5] diabetes mellitus and the metabolic syndrome [6]. Given the large number of patients currently treated with statins and/or inhibitors of cholesterol absorption and emerging concept of variations in the ratio between cholesterol absorption and cholesterol synthesis among different patient groups, a reliable assay of the markers of cholesterol absorption and synthesis has a great potential in clinical practice.

Several methods for sterol analysis from various biological matrixes have been described, including high performance liquid chromatography (HPLC) [7–9], gas liquid chromatography

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(GLC) [10–12], gas chromatography coupled to mass spectrometry (GC–MS) [13–16], and liquid chromatography coupled to mass spectrometry (LC–MS) [17,18].

A good chromatographic separation is achieved using capillary columns in GLC analysis, since HPLC methods usually present limits of resolution due to bad separation of plasma sterols. Furthermore, both HPLC UV detector and GLC flame ionisation detector are not able to assure peak purity. Since these detection systems are not structure-specific, there is a potential risk of interference from other analytes and from unrecognised co-elution components of plasma matrix. Other published methods including GC–MS and LC–APCI–MS have been proposed for sterol analysis in different biological samples, most of them using mass spectrometry only for qualitative analysis.

In this paper we describe a method for the simultaneous measurement of cholestanol, desmosterol, lathosterol, campesterol and β-sitosterol in human plasma, using GC–MS with selected ion monitoring (SIM).

2. Experimental

2.1. Sample collection

The study was performed on healthy normolipidaemic subjects. Blood samples were drawn into test tubes, containing ethylenediaminetetraacetic acid (EDTA) (1.0 mg ml^{-1}), separation of plasma was achieved by centrifugation of blood at 3500 rpm for 10 min at room temperature; plasma was then transferred into vials and stored in the dark at −80 °C until analyses.

2.2. Reagents

All chemicals and organic solvents used were of HPLC-grade. Double distilled water was used. Cholest-5-en-3β-ol (cholesterol), 5α-cholestane (internal standard), cholest-7-en-3β-ol (lathosterol), 24α-methylcholest-5-en-3β-ol (campesterol), 24α-ethylcholest-5-en-3β-ol (β-sitosterol), 5α-Cholestan-3β-ol (cholestanol), Cholest-5,24-dien-3β-ol (desmosterol), potassium hydroxide, and butylated hydroxytoluene (BHT), were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Hexane, ethanol, and toluene were purchased from Aldrich (Milwaukee, WI, USA). Silylation-grade pyridine and high purity derivatization solvents, *N*,*O*-bis-(trimethylsilyl) trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) were obtained from Supelco Inc. (Bellefonte, PA, USA).

2.3. Preparation of standard solutions

2.3.1. Internal standard

A stock solution of 5α-cholestane in toluene was prepared at a concentration of 1.0 mmol l^{-1}. Solutions containing 5α-cholestane (20, and 200 μmol l^{-1}) were prepared as working internal standard.

2.3.2. Standard solutions

A stock solution of each sterol was dissolved in toluene at a concentration of 1.0 mmol l^{-1}. Two sterols standard solution mixes were prepared: first mix concentrations were (1.25, 2.5, 5.0, 10, 20, 40 μmol l^{-1}) for each sterol; second mix concentrations were (6.25, 12.5, 25, 50, 100, 200 μmol l^{-1}) for cholestanol, desmosterol, lathosterol and campesterol; and (25, 50, 100, 200, 400, 800 μmol l^{-1}) for β-sitosterol. Internal standard solutions were (10, and 100 μmol l^{-1}), respectively, in order to obtain relative standard curves.

2.4. Analytical

2.4.1. Saponification and lipid extraction

To 200 μl of plasma in a glass tubes containing the internal standard (10 μmol l^{-1}) were added 1 ml of potassium hydroxide in ethanol (1.0 mol l^{-1}). Tubes were well mixed, flushed with N_2 and heated at 70 °C for 60 min in the dark. The reactions were stopped by cooling the tubes under running cold water. After cooling, the solution was diluted with water (1 ml) and the lipids were extracted twice with 2 ml of a solution of hexane and absolute ethanol (20:1, v/v), containing 12.5 mg l^{-1} butylated hydroxytoluene. The samples were vortexed and then centrifuged at 3500 rpm at 20 °C for 10 min to accelerate phase separation; the organic phase was transferred to small glass vials, dried completely under a steam of N_2 and derivatized as described below.

2.4.2. Preparation of trimithylsilyl (TMS) ether derivatives of sterols

The lipid extract was derivatized with 200 μl freshly prepared pyridine-BSTFA with 1% TMCS (1:1, v/v). Samples were then incubated at 70 °C for 60 min, and finally analyzed by GC–MS.

2.5. Experimental conditions

Samples were analyzed on a Perkin-Elmer (Norwalk, CT, USA) gas chromatograph coupled with a Perkin-Elmer Clarus 500 mass spectrometer. One microliter of each sample was injected into the gas chromatograph inlet via autosampler, controlled by the Turbomass 4.1 software. The injector temperature was held at 270 °C throughout the analysis while transfer line temperature was 230 °C. Separation was performed on a Restek (Bellefonte, PA, USA) Rtx®-1701 (14% cyanopropylphenyl–86% dimethylpolysiloxane) 60 m capillary column (0.25 mm ID, 0.25 μm film thickness), using helium as carrier gas. The initial column temperature of 90 °C was held for 3 min, then programmed at 25 °C min^{-1} to 260 °C and held for 28 min, then raised to 275 °C at a rate of 1 °C min^{-1} and maintained at this temperature for further 13 min, giving a total run time of 65.80 min. Injection was performed using a single tapered 2-mm glass inlet liner, packed with silanized glass wool in a programmed split mode. Before injection carrier gas flow was set to 4 ml min^{-1} with a split value of 5; after injection (1.5 min) the split value was set to 50 and carrier gas flow to 1 ml min^{-1} for 2 min, then raised to 1.5 ml min^{-1} at a rate of 0.25 ml min^{-1} and maintained at this flow rate.

The mass spectrometer operated in the electron impact mode at ionisation voltage of 70 eV, with an ion source temperature of 200 °C. Mass spectra in the full scan mode were recorded in the mass range of 100–500 amu. Selected ion monitoring was carried out by monitoring *m/z* 305 and 445 for cholestanol, *m/z* 351 and 456 for desmosterol, *m/z* 443 and 458 for lathosterol, *m/z* 382 and 472 for campesterol, *m/z* 129 and 486 for β-sitosterol, *m/z* 217 and 357 for 5α-cholestane. Peak identification was based on comparison with standards of retention times and mass spectra fragmentation.

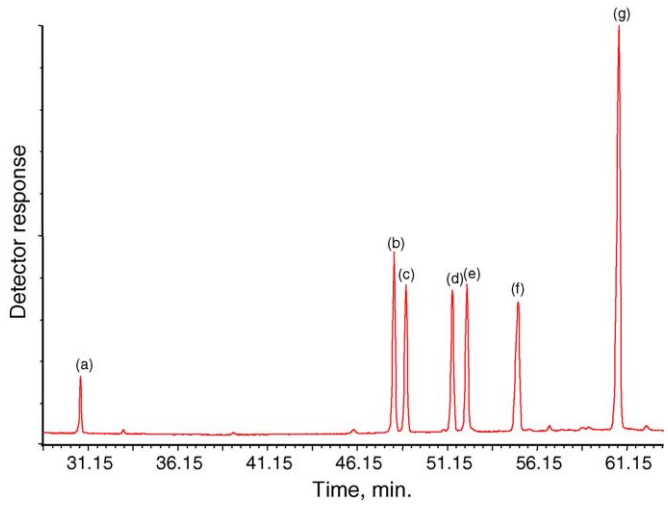

Fig. 1. Total ion chromatogram of sterol TMS ether from standard sample (40.0 μmol l^{-1}, I.S. 10.0 μmol l^{-1}). Peak identities are: (a) 5α-cholestane (I.S.); (b) cholesterol; (c) cholestanol; (d) desmosterol; (e) lathosterol; (f) campesterol; and (g) sitosterol.

3. Results and discussion

3.1. Gas chromatography–mass spectroscopy (GC–MS)

Simultaneous gas chromatographic separation of cholestanol, desmosterol, lathosterol, campesterol and β-sitosterol is achieved using a 60 m length 14% cyanopropylphenyl–86% dimethylpolysiloxane capillary column (0.25 mm ID, 0.25 μm film thickness). In Fig. 1 a typical chromatogram of each component in the standard mix prepared as described in Section 2.3.2 is shown. In Fig. 2 a typical chromatogram of a plasma control is shown (I.S. 10.0 μmol l^{-1}). Retention times of the TMS-ether derivatives of different sterols under study are shown in Table 1.

Both the polarity and the column physical characteristics are of crucial importance to obtain the best separation of plasma sterols. We have tested a DB1 100% dimethyl polysiloxane capillary column (30 m length, 0.25 mm ID, 0.25 μm film thickness) in order to shorten time analysis; however, this low polarity stationary phase has resulted to be unable to completely separate plasma sterols of interest, due to the complexity of biological matrix. The choice of a slightly polar stationary phase (14% cyanopropylphenyl–86% dimethylpolysiloxane) has resulted necessary for the resolution of each sterol peak, and of sterols from other plasma metabolites and impurities, but separation of cholestanol from cholesterol was not satisfactory [10,13]. An efficient elution of cholestanol is achieved with a non-constant flow chromatographic analysis, as a mobile phase flow ramp is required in order to obtain a delay for cholestanol peak retention time, as described in Section 2.5. Although the choice of a 60 m length capillary column requires relatively long time analysis, it is needed for simultaneous determination of cholestanol,

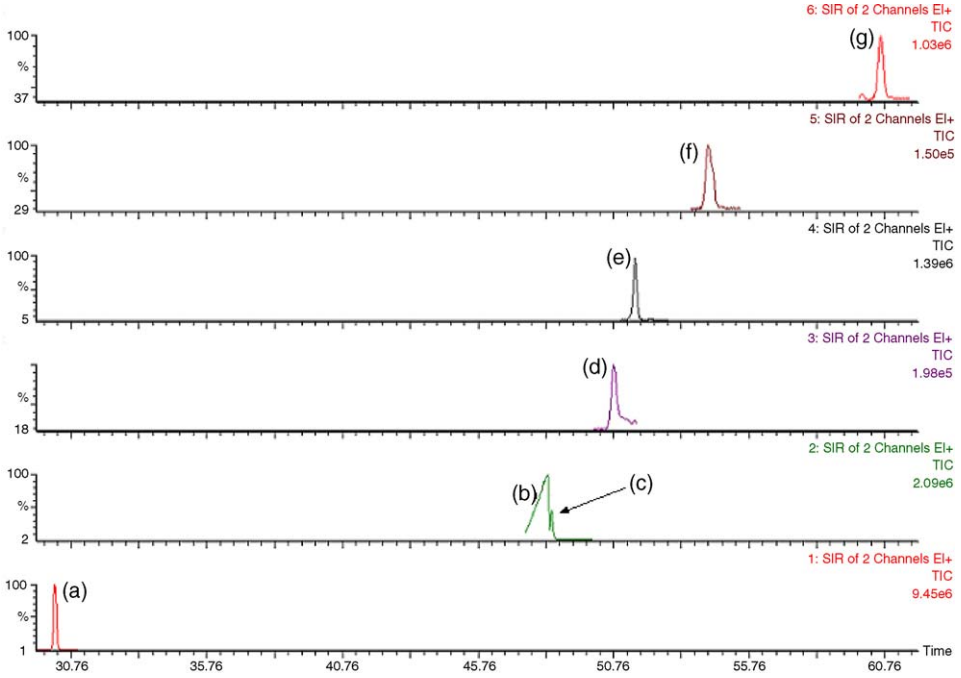

Fig. 2. SIM chromatogram of sterol TMS ether from plasma control sample (I.S. 10.0 μmol l^{-1}) Peak identities are: (a) 5α-cholestane (I.S.); (b) cholesterol; (c) cholestanol; (d) desmosterol; (e) lathosterol; (f) campesterol; and (g) sitosterol.

Table 1
Retention times (t_R) and analytical parameters for GC–MS of plasma phytosterols and cholesterol precursors assayed by the present method

Sterol	t_R (min)	LOD[a]	LOQ[b]	Low concentrations		High concentrations	
				Linear regression equation[c]	Correlation coefficient	Linear regression equation[c]	Correlation coefficient
Cholestanol	48.5	0.40	1.30	$Y = 0.16x + -0.07$	0.998	$Y = 0.10x + -0.30$	0.997
Desmosterol	51.2	0.30	0.90	$y = 0.15x + -0.08$	0.999	$y = 0.06x + -0.24$	0.998
Lathosterol	52.3	0.40	1.30	$y = 0.35x + -0.08$	0.999	$y = 0.19x + -0.42$	0.999
Campesterol	55.1	0.50	1.50	$y = 0.20x + 0.00$	0.999	$y = 0.17x + -0.45$	0.999
Sitosterol	60.7	0.20	0.70	$y = 2.2x + 0.48$	0.999	$y = 0.40x + 1.60$	0.999

[a] Limit of detection ($\mu mol\,l^{-1}$).
[b] Limit of quantitation ($\mu mol\,l^{-1}$).
[c] In the linear regression equation, x is expressed as $\mu mol\,l^{-1}$ and y is expressed as area units.

desmosterol, lathosterol, campesterol and β-sitosterol; furthermore, sterols elution temperature is approximately 270 °C, only 10 °C less than maximum programmable temperature of 14% cyanopropylphenyl–86% dimethylpolysiloxane capillary column. However, baseline noise, due to column bleeding, is minimised by use of mass spectrometry operating in SIM mode, as detection system for quantitative analysis. For this reason we have monitored those ions, which give a satisfactory intensity of response and, at the same time, are specific only for plasma sterols. This choice has permitted to obtain the best compromise between sensitivity and specificity of analysis, resulting in the highest value of signal-to-noise ratio, and minimising the risk of a potential error in quantitative determination of sterols, due to the co-elution of matrix impurities. On the other hand, most methods so far described use mass spectrometric detector mainly for sterols qualitative analysis, aiming at verifying purity of peaks and analytes structures [13,14].

Other published methods, including GC–MS and LC–APCI–MS, are specific and suitable for sterol analysis in various biological matrices, but usually they are used for determination of a limited number of analytes and often not from human plasma. Keller and Jahreis [15] reported a GC–MS method for quantitative determination of cholesterol, coprostanol, coprostanone, cholestanol and bile acids, but this study was applied to faeces samples. Another GC–MS method was described by Chevy et al. [16] for determination of cholesterol, lathosterol, 8-dehydrocholesterol, and 7-dehydrocholesterol from amniotic fluid. LC–APCI–MS was used to identify sitosterol and stigmasterol in soybean oil [17] and to quantify sitosterol and sitostanol in cultured CaCo-2 cells [18].

At the present, gas chromatography coupled with mass spectrometry remains one of the most sensitive and specific analytical techniques, available for simultaneous analysis of plasma sterols of clinical interest.

3.2. Limit of detection (LOD), limit of quantification (LOQ) and linearity

Limit of detection for this method was determined from repeated analyses of plasma sterols at low concentrations. Lowest sterol concentration that could be measured and reported with 99% confidence (three S.D.) was taken as the LOD. Limit of quantification was calculated as 10 times the standard deviation of replicates [10,19]. Calibration curves were obtained by plotting peak area ratio values (component peak area/internal standard peak area) against sterol concentration ratio (component amount/internal standard amount). A very good linearity

Table 2
Recoveries and reproducibility of plasma phytosterols and cholesterol precursors

Compound	Amount added[a]	Average recovery[b] ($n = 3$) (%)	Average intra day assay ($n = 8$) (%)	Average inter day assay (R.S.D.)[c] ($n = 48$) (%)
Cholestanol	2.5	89.15 ± 7.65	5.2	7.1
	5.0	91.24 ± 3.04		
Desmosterol	2.5	76.13 ± 5.37	2.4	6.3
	5.0	83.67 ± 1.17		
Lathosterol	2.5	94.4 ± 10.00	4.0	6.7
	5.0	101.13 ± 1.81		
Campesterol	2.5	83.33 ± 9.78	5.6	7.3
	5.0	94.13 ± 1.45		
Sitosterol	2.5	94.93 ± 12.71	5.0	6.7
	5.0	98.20 ± 6.68		

[a] Concentration are expressed in $\mu mol\,l^{-1}$.
[b] Recovery (%) = [(amount found − X_0)/amount added] × 100.
[c] Relative standard deviations.

was found in the concentrations ranges described in Section 2.3.2. In Table 1 are recorded LOD, LOQ, linear regression, and the respective correlation coefficients of the standard curves for each component in the present study. Standard calibration curves with high sterol concentrations (second mix) were built, for the determination of sterols level in pathological subjects, as reported in Section 2.3.2 (data not shown).

3.3. Recovery

In Table 2 are shown recovery results for this method, based upon replicate analyses of plasma samples spiked with known amounts (2.5 and 5.0 $\mu mol\,l^{-1}$) of sterol standards. Recovery results were higher in samples spiked with 5 $\mu mol\,l^{-1}$ standard level. With the exception of desmosterol (76%), recoveries were higher than 90%.

3.4. Reproducibility

Within-day and between-day precisions were evaluated using pooled human plasma. Within-day relative standard deviations (R.S.Ds.) of desmosterol, lathosterol, campesterol, and β-sitosterol were examined with eight replicate assays per day and the between-day R.S.Ds. by assays on six different days (Table 2). For all sterols the between-day standard deviations ranged from 0.09 $\mu mol\,l^{-1}$ for β-sitosterol to 0.20 $\mu mol\,l^{-1}$ for campesterol, the relative standard deviation for all components were ranged between 6.29 and 7.25%.

4. Conclusion

Measurement of plasma sterols is a useful tool in the investigation of patients with metabolic defects of cholesterol absorption and/or synthesis. This method combines the excellent power of separation performed by gas chromatography to the high sensitivity of mass spectrometric detection system operating in SIM mode, which improves signal-to-noise ratio. Furthermore, SIM mode highly increases the specificity of analysis, minimising the risk of an erroneous quantitative determination of sterols, due to the co-elution of impurities.

We applied this method to the measurement of plasma sterols in 161 healthy subjects and their levels were (mean ± S.D.) 4.73 ± 2.57, 2.37 ± 1.04, 6.23 ± 3.14, 3.67 ± 1.95 and 5.92 ± 3.62 μmol/l, for cholestanol, desmosterol, lathosterol, campesterol and β-sitosterol, respectively.

The results are compatible with those reported in other published methods such as GC–FID [10,11] and HPLC [7–9]. Furthermore, this method has resulted to be highly specific, sensitive, and reproducible, for the simultaneous measurement of cholestanol, desmosterol, lathosterol, campesterol and β-sitosterol in human plasma and is able to ensure a higher throughput.

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