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Note

Simplified gas chromatographic method for the simultaneous determination of phytosterols and cholesterol[a]

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The simultaneous determination of phytosterols and cholesterol is important both for the diagnosis of phytosterolaemia and for the control of dietary treatment of hypercholesterolaemia with plant sterols.

Phytosterolaemia is a rare case of impaired lipid metabolism characterized by accumulation of plant sterols and cholesterol in blood and tissues. Under physiological conditions, the phytosterol content represents *ca.* 2% of the individual plant sterols (primarily campesterol and β-sitosterol) in relation to the total cholesterol content. In phytosterolaemia, elevated concentrations of free and esterified sterols have been found and a detailed gas chromatographic (GC) determination of individual steryl esters has been carried out [1].

Plant sterols are known as effective inhibitors of cholesterol absorption in the gut and their hypocholesterolaemic effect has frequently been studied [2–6].

Intact steryl esters have been determined using capillary GC or reversed-phase liquid chromatography [1,7–9]; free sterols have been determined in the unsaponifiable residue of a lipid extract, using GC after derivatization [10–13] or high-performance liquid chromatography [14,15]. Free sterols were separated without derivatization on a non-polar capillary column with sufficient resolution [16].

We propose a simple, rapid and reproducible capillary GC method for the determination of cholesterol and plant sterols in serum and dietary supplement samples.

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EXPERIMENTAL

Reagents

Analytical-reagent grade solvents were supplied by Lachema (Brno, Czechoslovakia) and were further distilled, if necessary. Standards of campesterol, cholesterol, β-sitosterol, stigmasterol and 5α-cholestane were obtained from Sigma (St. Louis, MO, U.S.A.), and their stock solutions in chloroform (10 mg/ml) were stored at −20°C. Silica gel 60 $HF_{254+366}$ for thin-layer chromatography (TLC) (Merck, Darmstadt, F.R.G.) was used for the preparation of TLC plates.

Sample preparation

A 0.5-ml volume of serum and 0.1 ml of 5α-cholestane were extracted with chloroform–methanol (2:1, v/v), filtered, dried and saponified with 5 *M* potassium hydroxide solution in 50% methanol at 90°C for 30 min. The unsaponifiable residue was extracted with hexane–diethyl ether (1:1, v/v) and dried and the residue was dissolved in a small amount of chloroform and analysed by GC.

A 10-mg amount of dietary supplement was dissolved in 0.5 ml of cholesterol stock solution and the sterol fraction was isolated by TLC using heptane–diethyl ether–acetic acid (85:15:1, v/v/v) as the mobile phase. The sterol fraction was scraped off and the sterols were isolated by a dry column technique with chloroform–methanol (2:1, v/v). The dried samples were dissolved in a small amount of chloroform and analysed by GC.

Apparatus

A Chrompack (Middelburg, The Netherlands) Model 438 A gas chromatograph interfaced to an IBM PC/2 Model 30 computer was used. The instrument was equipped with a split/splitless capillary injector heated to 260°C (splitting ratio 1:150) and a flame ionization detector heated to 290°C. The flexible WCOT quartz column (10 m × 0.26 mm I.D.) was coated with chemically bonded, non-polar CP-Sil 5 CB liquid phase (Chrompack). The oven temperature was programmed from 220 to 245°C at 5°C/min. The hydrogen carrier gas flow-rate was maintained with a head pressure of 50 kPa.

RESULTS AND DISCUSSION

The chromatographic conditions were optimized to attain a sufficient peak resolution within the shortest possible time. Chromatograms of the calibration mixture, the sterol fraction of a dietary supplement and the serum sample are shown in Fig. 1.

The purity of the reference standards was checked by multiple analysis of the individual stock solutions and the mass correction factors f_w were calculated from the equation [17]

$$f_w = \text{mass (\%)} / \text{area (\%)} \quad (1)$$

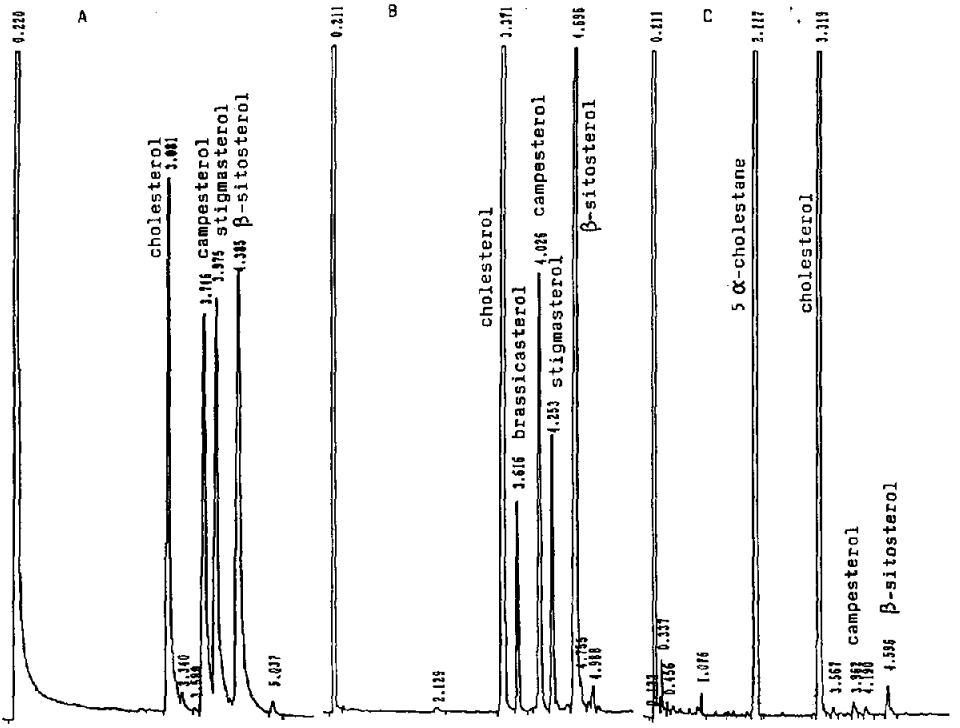

Fig. 1. Examples of chromatograms. (A) Standard sterol mixture; (B) sterol fraction of the dietary supplement with cholesterol as internal standard; (C) plasma sterols with 5α-cholestane as internal standard. Numbers at peaks indicate retention times in min.

The molar correction factors f_m for the individual plant sterols were calculated according to the equation

$$f_{mi} = \frac{f_{wi}}{f_{wc}} \cdot \frac{M_i}{M_c} \tag{2}$$

where f_{wi}, f_{wc} are the mass correction factors of the individual phytosterols and cholesterol, respectively, and M_i, M_c are the respective molecular masses. As observed earlier, the f_m values may depend on the amount injected, resulting in a non-linear calibration dependence [18]. Therefore, the f_m values were calculated for the whole calibration range. The statistical evaluation of all the calibration data is summarized in Table I.

A linear dependence of the detector response on the concentration of the test compounds was observed in the range 1–10 μg/μl using a packed column [19]. A concentration range of 0.05–10 μg of the test compound in the injected sample volume (1 μl) was studied, which corresponds, according to the splitting ratio, to the actual range of 0.33–66.6 ng of the test substance. It is apparent from Table I

TABLE I

STATISTICAL EVALUATION OF f_m VALUES OF INDIVIDUAL PHYTOSTEROLS RELATED TO CHOLESTEROL

Amount of cholesterol injected, 10 μg.

Amount of sterol injected (μg)	Campesterol		Stigmasterol		Sitosterol	
	f_m[a]	R.S.D.[b] (%)	f_m[a]	R.S.D.[b] (%)	f_m[a]	R.S.D.[b] (%)
10.0	1.04	0.5	1.05	0.4	1.04	0.4
5.0	1.04	0.6	1.05	0.4	1.03	0.3
2.5	1.05	0.4	1.05	0.8	1.01	0.7
2.0	1.03	0.6	1.06	0.6	1.02	0.6
1.25	1.02	0.8	1.04	0.9	1.01	0.1
1.0	1.01	0.3	1.03	0.4	0.98	0.9
0.5	1.00	0.4	0.99	0.7	0.96	0.2
0.25	0.94	1.2	0.84	1.1	0.76	1.7
0.125	0.76	1.7	0.69	1.3	0.58	1.0
0.05	0.68	3.8	0.53	2.8	0.38	4.3

[a] Each value represents the mean of three measurements.

[b] Relative standard deviation.

that the calibration dependence is linear over the range 0.5–10 μg for campesterol, stigmasterol and β-sitosterol. With low concentrations (0.05–0.5 μg/μl) the f_m values tend to be smaller than unity because of integration errors caused by tailing peaks. Within this range, the determination is subject to a systematic error and can only be considered as semi-quantitative. However, these values represent the physiological levels of phytosterols related to blood serum cholesterol level and such a determination is sufficient for the diagnostic purposes.

The method was used for the monitoring of patients with hypercholesterolaemia fed with a dietary supplement containing 2 g of plant sterols three times a day for three weeks. Cholesterol was determined with 5α-cholestane as internal standard (Fig. 1C). Only campesterol and β-sitosterol levels were measurable together with cholesterol and their amount varied from 0.5 to 2.1% of the total cholesterol content.

The qualitative analysis of the dietary supplement was made after previous TLC separation of the sterol fraction according to the GC elution times. The main components of this supplement were β-sitosterol (490 mg), campesterol (190 mg) and stigmasterol (140 mg in 1 g). For the quantitative determination of individual plant sterols, cholesterol can be used as an internal standard, as it is not present in the dietary supplement and has the same TLC mobility.

The proposed method is simple, rapid (analysis time 5 min), reproducible and suitable for the simultaneous determination of cholesterol and plant sterols in various biological samples.

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