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Simultaneous measurement of phytosterols (campesterol and β-sitosterol) and 7-ketocholesterol in human lipoproteins by capillary column gas chromatography

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Abstract

7-Ketocholesterol (a major cholesterol oxidation product) and phytosterols are important indicators of lipoprotein oxidation and lipoprotein metabolism respectively. We describe a simple, sensitive and reproducible method for the simultaneous measurement of these sterols in human lipoprotein samples by capillary column gas liquid chromatography. The method is suitable for clinical studies as small quantities of lipoprotein are required. Sterols are analysed after extraction from lipoprotein samples obtained by sequential flotation ultracentrifugation. The method involves briefly: extraction from lipoprotein samples using chloroform–methanol, saponification of sterol esters using cold potassium hydroxide, purification and derivatisation to trimethylsilyl ethers using BSTFA and 1% TMCS. Oxidation is prevented by drying under nitrogen and the use of powerful antioxidants. Separation is achieved using a DB-1 capillary column and a two-stage temperature ramp from 180–250°C and detection using FID. The identity of sterols can be confirmed by GC–MS. Phytosterols and 7-ketocholesterol are present at low concentration in all the major lipoproteins. Using [3,4-^{13}C]cholesterol and GC–MS we present evidence that cholesterol oxidation does not occur during the processing of lipoproteins using this technique.

1. Introduction

It has been suggested that the measurement of phytosterols, campesterol and β-sitosterol in human lipoproteins is useful in the study of lipoprotein metabolism. Miettinnen and co-workers [1,2] have shown that the levels of campesterol and β-sitosterol are proportional to the total and fractional dietary absorption of cholesterol, determined by continuous [^{14}C]cholesterol/[^{3}H]β-sitosterol feeding [3]. Determination of cholesterol absorption by the latter method is not ideal for large-scale studies since it is time consuming and involves the administration of radioactive isotopes. Therefore the measurement of phytosterols may provide a simple and convenient means of measurement of cholesterol absorption [1].

7-Ketocholesterol (7-KC) is the most abundant of the oxidation products of cholesterol [4] and can be used as an indicator of lipoprotein oxidation [5]. Free radical-mediated lipid peroxi-

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dation has been proposed as an important step in the development of atherosclerosis [6] and increased levels of products of free radical mediated reactions have been demonstrated in subjects with vascular disease and diabetes mellitus [7,8]. Cholesterol oxidation products are formed by free radical-mediated oxidation of cholesterol which occurs in association with the production of lipid peroxides [5], and have potentially atherogenic and thrombogenic properties [9]. Published methods for the determination of 7-ketocholesterol are complex [10] or not suitable for the measurement of native lipoproteins [11,12].

We describe a simple, specific and reproducible capillary column gas chromatographic (GC) method for the simultaneous determination of campesterol and β-sitosterol and 7-ketocholesterol in small volumes of human lipoproteins.

2. Experimental

2.1. Reagents

Analytical grade solvents (Aristar) and other reagents unless specified were obtained from BDH (Poole, UK). Standards of campesterol, cholesterol, β-sitosterol and 7-ketocholesterol were obtained from Sigma (Poole, UK). [^{3}H]Cholesterol and [3,4-^{13}C]cholesterol standards were obtained from Dupont Radiopharmaceuticals (Stevenage, Herts, UK) and MSD Isotopes (Montreal, Canada) respectively and were stored at −20°C. Silylation-grade pyridine and the derivatising reagent, N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) were obtained from Pierce and Warriner (Chester, UK). Size exclusion chromatography was performed using Biogel P100, exclusion limit 100 000 Da (Biorad Laboratories, Herts, UK).

2.2. Instrumentation

Lipoprotein separation was achieved using a Beckman 70.1 Ti fixed-angle rotor in a Beckman L8-70 ultracentrifuge. Chromatography was performed using a DB-1 fused-silica capillary column, 15 m × 0.25 mm I.D., 0.10-μm film thickness (J and W Scientific, Rancho Cordova, CA, USA) using a Hewlett-Packard gas chromatograph, Model 5790A. Peaks were integrated using a Hewlett-Packard integrator, Model 3390A. Mass spectrometry was performed using a Hewlett-Packard 5890 Series GC with Finnigan M.A.T. 1050 mass spectrometer.

Measurement of beta emission was performed using a LKB 1217 Rackbeta Liquid Scintillation Counter (LKB Instruments, Surrey, UK).

2.3. Sample preparation

Serum was obtained from ten healthy male volunteers after an overnight fast. Separation of serum was achieved by centrifugation of blood at 1000 *g* for 15 min within 1 h of venesection. Serum was mixed immediately with ethylenediaminetetraacetic acid (EDTA, disodium salt) as metal-ion chelator, butylated hydroxytoluene (BHT) as antioxidant and phenylmethylsulphonyl fluoride (PMSF) to prevent proteolysis. Final concentrations of each were 0.372 g/l, 0.022 g/l and 0.148 g/l, respectively. Serum was stored at −20°C until separated by ultracentrifugation (within 10 days). The effect of freezing on total and fractional cholesterol and triglycerides, free cholesterol, phospholipids and protein prior to ultracentrifugation has been studied and no significant changes were found after storage for 10 days [13].

2.4. Lipoprotein preparation

Separation of lipoproteins was achieved by sequential flotation ultracentrifugation as described previously [14]. Briefly, the density of 2 ml of serum was adjusted to 1.006 g/ml with 8 ml 11.05 g/l sodium chloride solution (NaCl). Specimens were centrifuged at 100 000 *g* for 24 h at 20°C. Very low density lipoprotein (VLDL) was recovered in the top 2 ml, the third milliliter being removed and used for measurement of refractive index using a refractometer. The density was then adjusted to 1.019 g/ml for the recovery of intermediate density lipoprotein

(IDL) by addition of 3 ml NaCl (71.74 g/l), to 1.063 for low density lipoprotein (LDL) by addition of 3 ml NaCl (245 g/l) and to 1.210 g/ml for high density lipoprotein (HDL) by addition of 3 ml 720 g/l sodium bromide solution. The density of each lipoprotein fraction was confirmed by refractometry and the identity of each fraction confirmed by qualitative assessment of apolipoprotein content using SDS polyacrylamide gel electrophoresis.

2.5. Lipid extraction

Internal standard, 25 μl of 0.025 g/l 5α-cholestane in chloroform was added to glass extraction tubes. A modified extraction of Folch et al. was then performed [15]. Lipoprotein samples (0.4 ml) were added, dropwise, to 2 ml of methanol while vortex-mixing. The mixture was left to stand for 10 min before addition of 4 ml of chloroform and 0.75 ml of water. Samples were then mixed for 60 min. The chloroform phase was separated after centrifugation at 1000 *g* for 20 min.

2.6. Saponification

The chloroform phase was evaporated to dryness by warming under nitrogen and the residue then redissolved in 2.5 ml of cold potassium hydroxide (1 mol/l) in methanol. Samples were flushed with nitrogen and covered with air-tight film. Saponification was allowed to proceed at room temperature and in the dark for 18–22 h.

2.7. Sterol extraction and purification

The alcoholic extract, together with 2.5 ml of water used to wash the saponification vessel, was mixed for 10 min with 3 consecutive volumes of chloroform (2.5 ml each). The chloroform phase was extracted each time and the combined phases were washed with 1.25 ml of aqueous potassium hydroxide (0.5 mol/l) and subsequently 2 volumes of distilled water. The mixture was then centrifuged for 30 min at 1000 *g*.

2.8. Derivatisation

The organic phase was concentrated under nitrogen, transferred to small glass reaction vials and evaporated to dryness. Derivatisation to trimethylsilyl (TMS) ethers was performed by addition of 20 μl of pyridine and 20 μl of BSTFA with 1% TMCS. Vials were left to stand at room temperature overnight.

2.9. Recovery of cholesterol and oxidation products

Recovery of cholesterol was calculated by addition of 0.32 mg of [7-^{3}H]cholesterol (0.02 mCi) to lipoprotein specimens. This was left to equilibrate with lipoprotein cholesterol at room temperature for 4 days. Lipoprotein-associated radiolabelled cholesterol was then separated from "free" cholesterol by size exclusion using Biogel P100. This fraction was then subjected to the lipid extraction technique described above. Recovery was calculated by measurement of beta emission using single sample incremental quench calibration, compared with samples not subjected to extraction.

2.10. Capillary column gas chromatography (GC)

Separation of cholesterol, campesterol, β-sitosterol and 7-ketocholesterol was performed using a DB-1 capillary column. Nitrogen (oxygen free) was used as the carrier and auxiliary gas at flow-rates of 1–2 ml/min. The injector split ratio was 3:1. The injector temperature was 270°C and detector temperature 300°C. Starting oven temperature was 180°C, which was increased by 8°/min to 220°C and then with 2°/min to a final temperature of 260°C. Peak height was used for quantitation. Concentrations of cholesterol and other sterols were calculated from standard curves of the appropriate sterol using 5α-cholestane as internal standard.

The limit of quantitation was determined by repeated analysis of sterols at low concentrations. The standard deviation of 10 measurements of a number of sterols was calculated. The

concentration at which the mean exceeded zero by more than 3 S.D. was taken as the limit of sensitivity.

In some lipoprotein specimens the presence of another cholesterol oxide, β-epoxycholesterol resulted in distortion of the campesterol peak. The effect of this distortion on the quantification of campesterol was studied by the addition of increasing quantities of β-epoxycholesterol to a mixture containing a known quantity of campesterol and 5α-cholestane as internal standard. Concentration of campesterol was 3.75 μmol/l. A range of concentrations of β-epoxycholesterol, 0–3.30 μmol/l, was investigated (concentration in lipoprotein samples rarely exceeds 0.5 μmol/l).

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

Cholesterol, campesterol, β-sitosterol and 7-ketocholesterol were provisionally identified by their retention times compared with commercially available standards. Their identities were confirmed using GC–MS. Helium was used as carrier gas (2 ml/min flow-rate). The ion source was held at 80°C and operated in the electron impact (EI) mode. Samples were applied via an on-column injector, with oven temperature 210–290°, ramping at 2°/min. Sterols were identified by their fragmentation pattern while scanning the mass range m/z 200–680.

2.12. Detection of artifactual oxidation

The possibility of artifactual oxidation during lipoprotein preparation, was investigated using a ^{13}C-labelled cholesterol standard. [3,4-^{13}C]Cholesterol (1 mg), was added to 8 duplicate LDL samples. In 4 samples this standard was added before, and in 4 samples, after lipid extraction. The standard contained approximately 0.1% of $^{13}C_2$-labelled 7-ketocholesterol. The concentrations of base ions of 7-ketocholesterol (472.7 ± 0.25) and [$^{13}C_2$]7-ketocholesterol (474.7 ± 0.25) at the retention time of 7-ketocholesterol standard in the final mixtures were determined by GC–MS. Quantitation was performed in the multiple ion (MI) mode, with a scan-time of 2.15 s. Artifactual oxidation of [$^{13}C_2$]cholesterol during the lipid extraction procedure would be detected as an increase in the intensity of the base peak ion of [$^{13}C_2$]7-ketocholesterol, 474.7, in relation to the base peak ion of unlabelled 7-ketocholesterol, 472.7.

3. Results

3.1. Chromatography of campesterol, β-sitosterol and 7-ketocholesterol

The retention times of the TMS-ethers of the sterols under study are shown in Table 1 and Fig. 1. Fig. 2 shows a typical chromatogram of LDL prepared by the described method and the posi-

Table 1
Retention times and fragmentation patterns of cholesterol, phytosterols and 7-ketocholesterol in native lipoproteins

Compound	Retention time	M	M − 15	M − 90	M − 105	Other
1 5α-Cholestane[a]	1.000	–	–	–	–	–
2 Cholesterol (Cholest-5en-3β-ol)	1.637	458 (12)	443 (5)	368 (34)	353 (18)	329 (52) [b]
3 Campesterol (24α-methyl cholest-5-en-3β-ol)	1.897	472 (5)	–	382 (45)	367 (30)	343 (100)
4 β-Sitosterol (24β-ethyl cholest-5-en-3β-ol)	2.126	486 (8)	–	396 (45)	381 (30)	357 (100)
5 7-Ketocholesterol (3β-hydroxycholest-5-en-7-one)	2.304	472 (40)	–	382 (35)	367 (100)	341 (15)

M = molecular ion, M − 15 = molecular ion − 15 Da etc.
Figures in brackets give size as percentage of base peak (100).
[a] Internal standard.
[b] Base peak ion for cholesterol 129 Da.

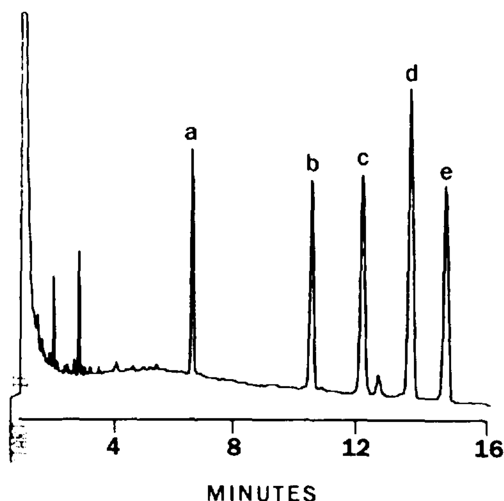

Fig. 1. Chromatogram of standard sterols showing relative retention times of (b) cholesterol, (c) campesterol, (d) β-sitosterol and (e) 7-ketocholesterol compared with (a) 5α-cholestane (I.S.).

tion of the cholesterol, phytosterol and 7-ketocholesterol peaks. Improved sensitivity and resolution of sterols was achieved using TMS-ethers compared with underivatised sterols. It is possible, using this technique to determine the concentrations of campesterol, β-sitosterol and 7-ketocholesterol in 0.4 ml of all 4 major lipoprotein fractions (representing 2 ml of serum). The limit of quantitation for each sterol was 0.14 μmol/l.

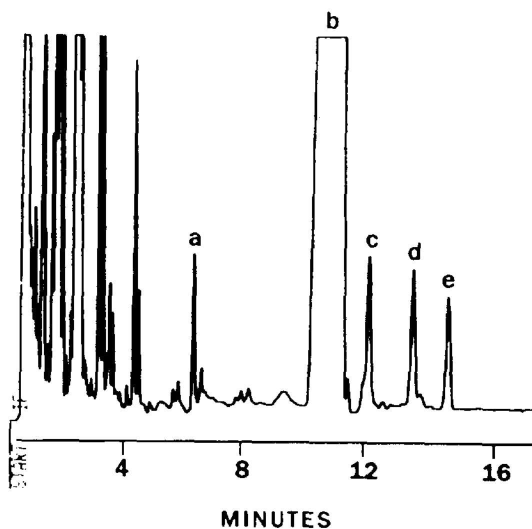

Fig. 2. Example of chromatogram of LDL sample using 5α-cholestane as internal standard (a). Shows relative retention times and typical relative amounts of trimethylsilyl ethers of cholesterol (b), campesterol (c), β-sitosterol (d) and 7-ketocholesterol (e).

Interference of β-epoxycholesterol with the peak of campesterol was investigated as described above. Results are shown in Table 2. The addition of a range of concentrations of β-epoxycholesterol had no effect on the quantitation of campesterol when peak height was used for integration.

3.2. Recovery, reproducibility and linearity

Recovery of the cholesterol extraction technique assessed by recovery of added [^{3}H]cholesterol was 74% for VLDL, 72.5% for IDL, 72.5% for LDL and 71% for HDL.

Linearity was excellent using known amounts of sterol standard added to patient samples, over the range 0.15–20 μmol/l for 7-ketocholesterol ($r = 0.998$, $p < 0.001$), campesterol ($r = 0.996$, $p < 0.001$) and β-sitosterol ($r = 0.996$, $p < 0.001$).

The recovery of 0.4 μg of 7-ketocholesterol, campesterol and β-sitosterol added to patient samples was 99, 95 and 98%, respectively.

Within-batch and between-batch coefficients of variation in 8 pooled lipoprotein samples measured on 4 separate occasions were: 7-ketocholesterol 13.5% and 7.0%, campesterol 6.4% and 6.3% and β-sitosterol 7.5% and 7.8%

Table 2
Effect of increasing concentration of β-epoxycholesterol on quantitation of campesterol

β-Epoxycholesterol[a] (μmol/l)	Campesterol[a] (μmol/l)
0.00	3.80
0.37	3.86
0.81	3.71
2.33	3.95
3.30	3.85

[a] Mean of 3 measurements.

(concentrations 0.41 μmol/l, 1.50 μmol/l and 1.05 μmol/l, respectively). At concentrations of 7-ketocholesterol, campesterol and β-sitosterol of 2.06, 4.49 and 1.98 μmol/l respectively, coefficients of variation were 16, 6.6 and 7.9%.

Table 1 shows the fragmentation patterns of sterols in patient samples.

3.3. Artifactual oxidation

The method for determination of the concentrations of base ions of 7-ketocholesterol and [$^{13}C_2$]7-ketocholesterol described above was sensitive and reproducible (C.V. of 10 repeated injections, 13.5%). Concentrations of m/z 472.7 and 474.7 were determined by integration of peak area. The concentrations were expressed as a ratio. The mean (±S.D.) ratio of 472.7/474.7 was 0.028 (±0.006) when $^{13}C_2$ was added before extraction and 0.027 (±0.005) when added after extraction. Therefore no artifactual oxidation occurred during the extraction stage. The formation of 7-KC during ultracentrifugation was not studied directly. However, there was no difference in concentration of 7-KC in samples left to stand at 20°C for 4 days (equivalent to the time and conditions of ultracentrifugation) and processed immediately.

Table 3
Concentrations of phytosterols and 7-ketocholesterol in lipoprotein fractions of 10 normal men

	Campesterol	β-Sitosterol	7-Ketocholesterol
Concentration of sterol (mmol/l)			
VLDL	0.66 (0.25)	0.37 (0.10)	0.66 (0.49)
IDL	0.79 (0.28)	0.49 (0.16)	0.58 (0.38)
LDL	7.23 (3.20)	3.82 (2.05)	1.97 (1.28)
HDL	2.92 (1.18)	1.78 (0.64)	1.17 (0.55)
Concentration of sterol/concentration of cholesterol[a] (mmol/mmol)			
VLDL	2.02 (1.03)	1.10 (0.51)	1.82 (1.90)
IDL	2.10 (0.93)	1.11 (1.04)	1.19 (0.81)
LDL	2.38 (0.77)	1.23 (0.38)	0.63 (0.34)
HDL	2.00 (0.75)	1.14 (0.42)	0.94 (0.78)

Means (S.D.).
[a] Determined enzymatically.

3.4. Levels of phytosterols and 7-ketocholesterol in human lipoproteins.

Levels of campesterol, β-sitosterol and 7-ketocholesterol in VLDL, IDL, LDL and HDL are shown in Table 3. Concentrations of sterols are expressed in μmol/l of whole serum and also as a ratio to cholesterol concentration in each lipoprotein fraction.

4. Discussion

Measurement of lipoprotein oxidation may be important in the investigation of atherosclerosis [6,9]. Many methods have been used for the measurement of free radical mediated lipoprotein oxidation [16], but few are sufficiently sensitive or specific for use in clinical studies. The present method has the advantage of allowing the measurement of 7-ketocholesterol in individual lipoprotein fractions, which may be more informative than the study of whole plasma [17]. The method is sufficiently sensitive to allow measurement of 7-KC in native lipoprotein fractions as well as the products of lipoprotein oxidation produced artifactually in in-vitro systems [5]. Specificity is excellent, in contrast to other methods [8,16], and confirmation of the identity of 7-KC is possible using GC–MS. Campesterol and β-sitosterol, plant sterols which may be indicators of dietary cholesterol absorption [1,2], can be measured within the same analytical run. Measurement of the concentrations of phytosterols may be useful for the study of cholesterol metabolism particularly in large-scale epidemiological studies [1]. This is of interest in the investigation of atherosclerosis since abnormalities of lipoprotein metabolism are implicated in this process [18]. Concentrations of campesterol and phytosterol are similar to published levels using similar methods, both in whole serum [19,20] and lipoproteins [2].

Modifications of previous methods [11,12] were necessary. The addition of lipoprotein samples to vortex-mixed methanol prior to chloroform–methanol extraction procedure was required to produce consistent recovery of lipid

from all lipoprotein classes. If lipoproteins were simply mixed with a 2:1 chloroform–methanol mixture recovery of cholesterol from VLDL and IDL was acceptable but recovery of cholesterol from LDL and HDL was less than 30% (data not presented). Mixing with vortex-mixed methanol is required for complete delipidation of lipoproteins [21]. The choice of solvent for lipid extraction is important. The use of diethyl ether for extraction of non-saponifiable lipid was associated with an artifactual increase in concentration of 7-KC of the order of 400–500% (data not presented). The use of ether should be avoided since it contains significant quantities of ether peroxide which is generated on exposure to light. Artifactual oxidation could not be avoided if ether was used despite the use of antioxidants and flushing with nitrogen. This problem did not occur if chloroform was substituted for diethyl ether in all extraction stages.

Previous studies have demonstrated that artifactual oxidation of cholesterol may occur during processing and that some cholesterol oxides are formed predominantly as artifacts [22,23]. 7β-Hydroxycholesterol, α-epoxycholesterol, β-epoxycholesterol and cholestane-triol were identified in some samples but at concentrations close to the limit of detection. Using the addition of [3,4-^{13}C]cholesterol to lipoprotein samples we have demonstrated that there was no increase in concentration of 7-ketocholesterol formed by autoxidation during sample processing.

We have measured the concentration of 7-ketocholesterol in native lipoprotein fractions. Care must be taken to prevent oxidation throughout the procedure as this may occur despite the addition of powerful antioxidants. Measurement of 7-ketocholesterol and phytosterols may be of interest in the investigation of the mechanisms of atherogenesis and the metabolism of cholesterol.

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