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Separation of cholesterol esters by silver ion chromatography using high-performance liquid chromatography or solid-phase extraction columns packed with a bonded sulphonic acid phase

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ABSTRACT

Two methods for the separation of cholesterol esters, based on the number of double bonds in their fatty acid moieties, are presented. Silver ion chromatography, usually performed on thin-layer chromatographic plates, was made suitable for high-performance liquid chromatography (HPLC) and solid-phase extraction. Separation on a bonded sulphonic acid phase loaded with silver ions was achieved with cholesterol esters containing up to six double bonds in their fatty acid moieties. No cross-contamination between fractions with different numbers of double bonds was detected with the HPLC method, as was demonstrated by subsequent gas chromatographic analysis of the fatty acid moieties, following transmethylation. For adequate separations with the solid-phase extraction columns it proved important to avoid overloading. The methods may be of use for the off-line analyses of the sterol compositions of the isolated fractions, which each contain sterol esters with an equal number of double bonds in their fatty acid moieties.

INTRODUCTION

Cholesterol is a structural constituent of cell membranes, and a substrate for the synthesis of steroid hormones and bile acids. Cholesterol is transported and stored in the form of cholesterol esters (CE). Deposition of CE in the arterial wall leads to atherosclerosis [1,2]. At least three enzymes are responsible for cholesterol esterification: acyl-CoA:cholesterol O-acyltransferase (ACAT; EC 2.3.1.26) exerts its function intracellularly (CE for storage and internal secretion), lecithin:cholesterol acyltransferase (LCAT; EC 2.3.1.43) esterifies cholesterol in plasma (for reverse transport) and at pH 6.2 pancreatic sterol ester acylhydrolase (cholesterol esterase; EC 3.1.1.13) functions as a sterol esterifying enzyme in the

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intestinal lumen. The enzymes have different fatty acid specificities [3–6]. Inhibition of intestinal ACAT is a potential means of lowering cholesterol absorption and thereby its plasma concentration [7]. LCAT deficiency leads to, amongst other effects, premature atherosclerosis [8]. Cholesterol esterase may be involved in the selectivity of sterol absorption in the gut by influencing the local equilibrium between free and esterified sterol. Under normal conditions dietary cholesterol is about 50% absorbed, whereas for phytosterols this amounts to only 5% [9]. Patients with sitosterolaemia suffer from premature atherosclerosis and have both increased gastrointestinal absorption and prolonged plasma clearance of sitosterol [10].

Isolation of the total CE fraction is usually performed by thin-layer chromatography (TLC) [11], high-performance liquid chromatography (HPLC) [12,13], or solid-phase extraction [14]. Following transmethylation, analysis of its fatty acid composition is done by gas chromatography with flame ionization detection (GC–FID). These methods are, however, not suitable for the examination of CE in samples that also contain other sterol esters (*e.g.*, phytosterol esters), such as gastrointestinal contents and faeces [15]. Analysis of intact sterol esters by liquid chromatography–mass spectrometry (LC–MS) [16] and high temperature GC–FID [16] have been developed, but suffer from quantification difficulties. A completely different approach is to isolate sterol esters on the basis of the properties of their fatty acid moieties and to analyse the sterol part after hydrolysis. Separation of fatty acids, as either their methyl esters (FAME), CE and triacylglycerols [11], on the basis of the number of double bonds has been achieved by silver ion TLC. These methods are laborious, however. They should be performed under at least two different development conditions to allow for adequate separation between fractions with 0–2 and those with 3–6 double bonds, while elimination of silver ions also deserves special attention [11]. Separations of FAME by solid-phase extraction [17] and HPLC [18] and triacylglycerols by HPLC [18,19], based on this principle, have been succesfully developed and do not seem to suffer from these disadvantages.

In this paper we describe HPLC and solid-phase extraction methods that employ a covalently bound sulphonic acid stationary phase to which silver ions are coupled. They were validated by investigating human and sheep plasma total CE and analysing the isolated fractions by GC–FID.

EXPERIMENTAL

Materials

Bond Elut SCX (*p*-propylbenzenesulphonic acid, 500 mg) disposable columns were kindly donated by R. A. Calverly of Analytichem International (Cambridge, U.K.); they are also available from Analytichem, Bètron Scientific (Rotterdam, The Netherlands). All reagents were of HPLC grade and were purchased from Fisons (Loughborough, U.K.).

Isolation and characterization of plasma total cholesterol ester fractions

Isolation. Human and sheep plasma total CE fractions were isolated by solid-phase extraction [14] and HPLC [12,13], respectively.

Base-catalysed transmethylation. Part of each plasma total CE fraction was dissolved in 0.4 ml of hexane containing 0.2 g/l butylated hydroxytoluene. A 40-μl volume of methyl acetate and 40 μl of sodium methoxide (1 mol/l in methanol) were added. The mixtures were heated at 60°C for 30 min with occasional shaking. After the addition of 4 μl of acetic acid the samples were evaporated to dryness at 40°C under a stream of nitrogen and the residues dissolved in 1 ml of hexane. The tubes were centrifuged and the hexane layers transferred to other tubes. After evaporation to dryness at 40°C under a stream of nitrogen, the FAME were taken up in a small volume of hexane for analysis by GC–FID.

Gas chromatography. Analyses of FAME compositions were performed by GC–FID as described elsewhere [17]. It was assumed that equal FAME masses give rise to equal peak areas. For the calculation of the original CE composition, the peak area of each FAME was multiplied by the molecular weight of its CE and divided by the molecular weight of that FAME. These "corrected" peak areas were normalized to obtain the original CE composition in grams per 100 g.

Silver ion high-performance liquid chromatography of the plasma total cholesterol ester fractions

For HPLC analyses, a Spectra-Physics (St. Albans, U.K.) Model 8700 ternary solvent delivery system was used in combination with an ACS Model 750/14 light-scattering detector (Applied Chromatography Systems, Macclesfield, U.K.). A stream splitter (*ca.* 10% to detector and 90% to outlet) was inserted between the column and detector. A 250 mm × 4.6 mm I.D. column, packed with Nucleosil 5SA (5-μm particles; HPLC Technology, Macclesfield, U.K.), was utilized in the silver ion form as described elsewhere [18,19]. The CE (*ca.* 1 mg) were applied to the column in 10 μl of dichloroethane. The solvent reservoirs contained dichloroethane–dichloromethane (1:1, v/v) (A), acetone (B) and acetone–acetonitrile (9:1, v/v) (C). The gradient was started at 100% solvent A, going in 20 min to 50% A and 50% B, then in a further 20 min to 40% B and 60% C and in the final step, again in 20 min, to 100% C. The column was regenerated with solvent A for 15 min. The flow-rate was 1 ml/min. The separated fractions were collected, evaporated to dryness at 40°C under a stream of nitrogen and characterized by transmethylation and subsequent GC–FID analysis as described above.

Atmospheric pressure silver ion chromatography of the plasma total cholesterol ester fractions

SCX extraction columns in the silver ion form were prepared as described elsewhere [17]. Briefly, the column, wrapped in aluminium foil to the level of the top of the adsorbent bed, was loaded with 20 mg of silver nitrate in 250 μl of acetonitrile–water (10:1, v/v). The column was then flushed with 5 ml of aceto-

TABLE I

SOLVENT ELUTION SCHEME FOR THE ISOLATION OF CHOLESTEROL ESTER FRACTIONS, USING A BOND ELUT SCX SOLID-PHASE EXTRACTION COLUMN LOADED WITH SILVER IONS

No. of db[a]	Elution mixture (ml)			
	Dichloromethane	Acetone	Acetonitrile	Total volume
0	5.0			5.0
1	4.5	0.5		5.0
2		5.0		5.0
3		9.7	0.3	10.0
4		9.4	0.6	10.0
5		4.4	0.6	5.0
6		3.0	2.0	5.0

[a] Number of double bonds present in the fatty acid moieties of the cholesterol esters that are subsequently eluted, using the indicated solvent mixtures.

TABLE II

HUMAN AND SHEEP PLASMA CHOLESTEROL ESTER COMPOSITIONS AS DETERMINED BY GC–FID

Quantification of CE by GC–FID was performed by assuming that equal masses of FAME give rise to equal peak areas (see also Experimental).

Cholesterol ester	Human plasma (g per 100 g)	Sheep plasma (g per 100 g)
14:0	0.97	1.33
15:0	0.32	1.36
16:0	13.63	4.86
18:0	1.54	0.92
16:1,*n*−7	5.08	4.46
18:1[a]	22.45	3.89
18:2,*n*−6	45.46	31.99
18:3,*n*−6	0.73	1.47
18:3,*n*−3	0.68	43.96
20:3,*n*−9	0.21	N.D.[b]
20:3,*n*−6	0.93	N.D.
18:4,*n*−3	N.D.	3.01
20:4,*n*−6	6.28	0.52
20:4,*n*−3	0.10	0.79
20:5,*n*−3	0.99	1.44
22:6,*n*−3	0.63	N.D.

[a] Sum of 18:1,*n*−9 (major component) and 18:1,*n*−7 (minor component).

[b] Not detectable.

nitrile, 5 ml of acetone and 10 ml of dichloromethane by using slight pressure. The CE sample (less than 0.5 mg) was applied to the column in about 100 μl of dichloromethane. The solvent elution scheme is shown in Table I and is the same as described previously for FAME [17]. Fractions were collected by draining at atmospheric pressure. After evaporation to dryness at 40°C under a stream of nitrogen they were transmethylated and characterized by GC–FID as described above.

RESULTS AND DISCUSSION

The CE compositions of the human and sheep plasma total CE fractions, as calculated from data obtained by GC–FID analyses, are shown in Table II. Cholesteryl linoleate (18:2,$n-6$) is the major constituent of human plasma CE, whereas in sheep plasma CE cholesteryl linolenate (18:3,$n-3$) is the quantitatively most important component. Fig. 1 shows the corresponding HPLC light-scattering detection (HPLC–LSD) results for (A) human and (B) sheep plasma CE. Collection and subsequent GC–FID analyses of the HPLC–LSD fractions (for fraction sizes, see Fig. 1) showed excellent qualitative agreement with expectations based on the GC–FID composition. HPLC–LSD separation characteristics were conserved with the injection of up to at least 1.25 mg of total CE. On the basis of the GC–FID data, several HPLC–LSD fractions were expected to be composed of more than one component (*e.g.*, the db-3 and db-4 fractions, Table II), which indeed became apparent in the chromatograms (Fig. 1). As *cis*- and *trans*-fatty acids become separated on silver ion columns, the small peak in the db-1 fraction of sheep plasma (Fig. 1B) will probably be the *trans*-isomer of 18:1. This was not investigated further. Comparison of relative HPLC–LSD peak areas with the corresponding calculated sums of reference GC–FID data (Table III) shows that quantification by HPLC–LSD, although grossly in agreement, is inaccurate, but reasonably precise.

Plasma CE fractions obtained by SCX minicolumn extraction showed cross-overs of less than 5% in the GC–FID analyses. With human plasma CE this may, however, lead to considerable contamination of, *e.g.*, the db-3 fraction with both cholesteryl linoleate (db-2) and cholesteryl arachidonate (db-4). Loading with more than 0.5 mg of total CE caused even higher cross-overs.

Thus, using essentially the same method as originally developed for the separation of FAME, it proved possible to isolate CE on the basis of the number of double bonds in their fatty acid moieties by both HPLC and atmospheric pressure solid-phase extraction. Replacement of the methyl group by a cholesteryl group obviously does not influence the separation characteristics in silver ion chromatography, at least not under the conditions of atmospheric pressure extraction, *i.e.*, the cholesterol moiety does not appear to complex with the silver ions under these conditions. It may therefore be expected that the separation of complex sterol ester mixtures by the minicolumn procedure will permit the off-

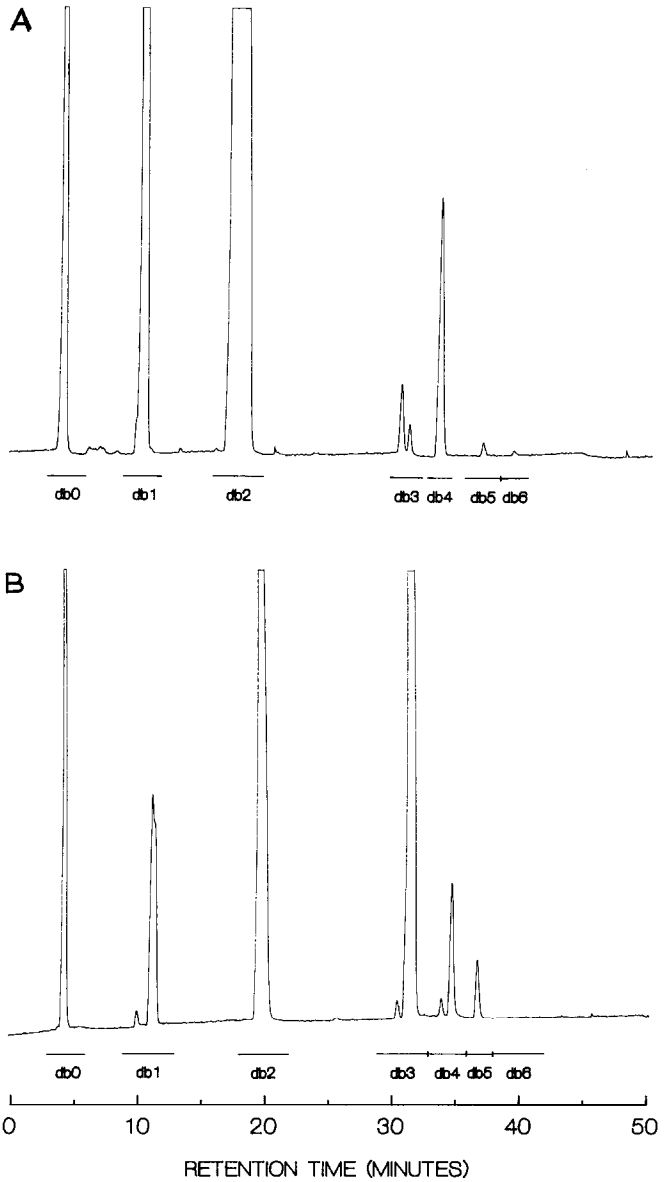

Fig. 1. High-performance liquid chromatograms of (A) human and (B) sheep plasma total cholesterol ester fractions. Separation was performed on an HPLC column loaded with silver ions with light-scattering detection.

line determination of the sterol compositions of the isolated fractions, and that each of these fractions contains sterol esters with an equal number of double bonds in their fatty acid moieties. Whether further separation based on the sterol

TABLE III

COMPARISON OF QUANTITATIVE DATA OF TOTAL CHOLESTEROL ESTER FRACTIONS WITH EQUAL NUMBERS OF DOUBLE BONDS AS CALCULATED BY GC–FID WITH DATA OBTAINED FROM HPLC–LSD

Fraction[a]	Human plasma (g per 100 g)			Sheep plasma (g per 100 g)		
	GC–FID[b]	HPLC–LSD[c]		GC–FID[b]	HPLC–LSD[c]	
		1	2		1	2
db-0	16.46	14.9	15.0	8.47	12.7	13.5
db-1	27.53	24.0	22.5	8.35	9.0	7.5
db-2	45.46	57.1	58.3	31.99	32.6	32.0
db-3	2.55	0.8	0.8	45.43	41.1	43.1
db-4	6.38	3.1	3.4	4.32	3.3	2.8
db-5	0.99	0.1	0.1	1.44	1.2	1.1
db-6	0.63	0.0	N.D.[d]	N.D.	N.D.	N.D.

[a] Sum of CE with equal numbers of double bonds in their fatty acid moieties, *e.g.*, db-0 = sum of CE containing fatty acids with zero double bonds.

[b] Sum of CE in indicated fractions as calculated from the data given in Table II.

[c] HPLC–LSD analyses were performed twice; quantification was performed by normalizing peak areas.

[d] Not detectable.

moiety occurs on HPLC and whether on-line quantification by LSD is feasible remain to be established.

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