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SEPARATION AND QUANTITATION OF FREE CHOLESTEROL AND CHOLESTERYL ESTERS IN A MACROPHAGE CELL LINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method for the direct high-performance liquid chromatographic (HPLC) determination of free cholesterol and the individual cholesteryl esters in cell culture experiments is described The murine macrophage-like J774 cell line was loaded with cholesterol by incubation with low-density lipoproteins After extraction of the cellular lipids with hexane–isopropanol (3 2 v/v), the cholesteryl esters were identified and quantified by isocratic HPLC Unesterified cholesterol and its esters were eluted with acetonitrile–isopropanol (50 50 v/v) on a Zorbax ODS column within 25 min and detected at 210 nm Cholesteryl heptadecanoate was used as an internal standard The detection response is linear in the analytical range of interest, the overall coefficients of variation are less than 8% and the detection limit is between 50 and 150 ng The results demonstrate that HPLC is suitable for the determination of cellular cholesteryl ester profiles and could usefully contribute to the understanding of the mechanism of foam cell formation during the development of atherosclerosis This method can also be applied to all experimental systems involving the study of cholesteryl esters

INTRODUCTION

Cholesteryl esters contribute to the transport and storage of cholesterol in mammals and the study of their metabolism in cultured cells is becoming increasingly important Foam cells whose cytoplasms are filled with cholesteryl ester inclusions are a prominent feature of the atherosclerotic plaque [1, 2] Several studies [3–5] have demonstrated that macrophages are the precursors of the lipid-laden foam cells and play an important role in the pathogenesis of

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atherosclerosis Modified low-density lipoproteins (LDL) such as acetylated LDL are taken up by macrophages through a high-affinity scavenger receptor The uptake process is not down-regulated so that high levels of cholesteryl esters accumulate intracellularly, converting the macrophages into foam cells [6] The study of the intracellular lipoprotein metabolism and of the "in vitro" formation of foam cells requires a sensitive and accurate method for the determination of the cellular free cholesterol and cholesteryl esters

Until now, the quantification in cells of total and free cholesterol and of the cholesteryl esters was mostly based on enzymatic assays [7, 8], gas chromatography (GC) [9, 10] or thin-layer chromatography (TLC) [11, 12] Such methods quantify only the total mass of cholesteryl esters and do not differentiate between these components Recently, Snow et al [13] and Yla-Herttuala et al [14] reported two methods for the characterization and quantification of the individual cholesteryl esters in cultured macrophages and in human aortic intima However, both methods are destructive, time-consuming and complicated In these techniques, after extraction the cellular lipids are isolated by TLC, the cholesteryl ester fraction is eluted and hydrolysed and the resulting fatty acids are transesterified and determined by GC The determination of cholesteryl esters in plasma or lipoproteins by high-performance liquid chromatography (HPLC) has been reported previously [15–20], but HPLC has not yet been applied to the quantification of cholesterol and its esters in cell culture experiments

In this paper, we describe a relatively simple and rapid reversed-phase HPLC method that permits the separation, characterization and quantification of free cholesterol and of the individual cholesteryl esters in cultured cells

EXPERIMENTAL

Materials

The chromatographic standards, cholesterol and the cholesteryl esters of palmitate, palmitoleate, oleate, myristate, stearate, linoleate, arachidonate and heptadecanoate, were obtained from Sigma (St Louis, MO, U S A) Hexane, isopropanol, chloroform and acetonitrile were HPLC-grade solvents obtained from Merck (Darmstadt, F R G) Dulbecco's modified Eagle's medium (DMEM), foetal calf serum (FCS), penicillin–streptomycin solution, gentamycin, six-well plates and cell culture flasks were obtained from Gibco (Ghent, Belgium) Bovine serum albumin (BSA) was purchased from Sigma

Lipoprotein isolation and modification of LDL

Blood was collected in 0 2% EDTA and LDL were isolated by sequential ultracentrifugation in the density range 1 019–1 063 g/ml [21] The lipoproteins were dialysed against 0 15 *M* NaCl–0 01% EDTA (pH 7 4), filtered through a 0.22-μm Millipore filter and stored at 4°C Lipoprotein-deficient serum (LPDS, density > 1 21 g/ml) was obtained from foetal calf serum after

ultracentrifugation at 110 000 *g* for 48 h, filtration through a 0 22-μm Millipore filter and storage at −20 °C The LDL were acetylated according to the method of Basu et al [22]

Cell culture experiments

The murine cell line J774 obtained from ATCC was routinely grown in DMEM containing 10% FCS supplemented with gentamycin (25 μg/ml) in a humified 5% carbon dioxide incubator at 37 °C The medium was changed twice a week For the incubation experiments, J774 cells were seeded at a concentration of $5 \cdot 10^5$/ml in 35-mm plates (3 ml per dish) in DMEM supplemented with 10% FCS for 18 h Cells were subsequently washed with DMEM, incubated for 48 h in 1 5 ml of DMEM containing 10% LPDS, with or without lipoproteins at a concentration of 100 μg/ml

Lipid extraction from lipoproteins and cells

Total lipid extracts from lipoproteins were prepared by the addition of 5 ml of hexane–isopropanol (3 2, v/v) to 50 μl of lipoprotein solution (protein concentration 1–4 mg/ml) containing 50 μl of cholesteryl heptadecanoate (1 2 mg/ml in chloroform) [23] The mixture was vortex mixed for 1 min and centrifuged at 1800 *g* for 15 min The supernatant was dried under nitrogen at room temperature, the residue dissolved in a mixture of 10 μl chloroform and 40 μl of elution solvent and an aliquot of 20 μl was injected for HPLC analysis

For the extraction of cellular lipids, the cell monolayer of a 35-mm well was washed once with phosphate-buffered saline (PBS)–0 2% BSA and twice with PBS alone The cells were subsequently scraped in PBS with a "rubber policeman", centrifuged at 650 *g* and the lipids were extracted from the cell pellet as described for the lipoproteins The cell residues were subsequently dissolved in 0 1 *M* sodium hydroxide solution and the protein content was determined by the method of Lowry et al [24]

Reversed-phase high-performance liquid chromatography

HPLC analysis was performed on a Hitachi 655A-11 liquid chromatograph equipped with an L-5000 LC controller, a 655 A-52 column oven, a D-2000 chromatographic integrator and a Schoeffel SF770 variable-wavelength detector The separation was carried out on a 5-μm Zorbax ODS column (25 cm × 4 6 mm I D , DuPont, F R G) The cholesteryl esters were eluted isocratically at a flow-rate of 1 2 ml/min with acetonitrile–isopropanol (50 50 v/v) The solvent was filtered through a 0 22-μm Millipore filter and degassed by sparging with helium before use The cholesteryl esters were separated at 45 C and detected by UV absorption at 210 nm

The standard mixture contained 35 0 mg of cholesterol, 20 0 mg of cholesteryl arachidonate, 100 0 mg of cholesteryl linoleate, 100 0 mg of cholesteryl palmitoleate, 100 0 mg of cholesteryl myristate, 100 0 mg of cholesteryl oleate,

25 0 mg of cholesteryl palmitate and 42 0 mg of cholesteryl stearate in 100 ml of chloroform It was kept stored under nitrogen at 4 °C

Calibration graphs for cholesterol and the various cholesteryl esters were obtained by extracting different amounts with hexane–isopropanol as described and plotting the ratios of the peak heights to that of the internal standard against concentration The relationship between the ratio of the peak heights and the cholesteryl ester concentration was calculated by the least-squares method

Gas chromatography

The lipids obtained after the extraction of LDL were further separated by TLC on silica gel plates using light petroleum (b p 60–80 °C)–diethyl ether–acetic acid (80 20 1, v/v/v) The cholesteryl ester fraction was recovered and the fatty acids analysed by GC as described previously [25]

RESULTS AND DISCUSSION

Duncan et al [15] first used HPLC for the determination of serum free and total cholesterol, but the individual cholesteryl esters were not separated An alternative HPLC procedure using laser-based optical activity detection was presented by Kuo and Yeung [16] The chromatographic conditions used yielded no separation between the linoleate–palmitoleate and oleate–palmitate pairs while the total analysis time was about 45 min Bernert et al [17] determined the linoleate/oleate ratio in serum cholesteryl esters using an isocratic HPLC system, but the analysis of the other cholesteryl esters was not evaluated Recently, Chu and Schroepfer [18] reported an HPLC method for cholesteryl esters on a C_6 Spherisorb reversed-phase column The total analysis time was 35 min and most cholesteryl esters did not show complete baseline resolution Billheimer et al [26] reported on the isocratic separation of steryl esters by reversed-phase HPLC with the solvent system acetonitrile–isopropanol This system, however, required a relatively high flow-rate of 2 0 ml/min for the analysis of plant steryl esters

With our HPLC procedure, we obtained an elution pattern similar to that reported by Carroll and Rudel [19], who used a linear gradient system of water in tetrahydrofuran The use of tetrahydrofuran in the low-UV range is limited, however, owing to a variable cut-off limit Baseline instability might therefore result both from the use of a gradient and from the presence of tetrahydrofuran while operating at short wavelengths Our isocratic procedure permits the resolution of unesterified cholesterol and of the major cholesteryl esters Fig 1a illustrates a typical HPLC elution profile of the standard mixture, showing baseline resolution of free cholesterol and of the arachidonate, linoleate, myristate, oleate, palmitate, heptadecanoate and stearate esters in approximately 25 min Cholesteryl heptadecanoate appears to be a suitable internal standard

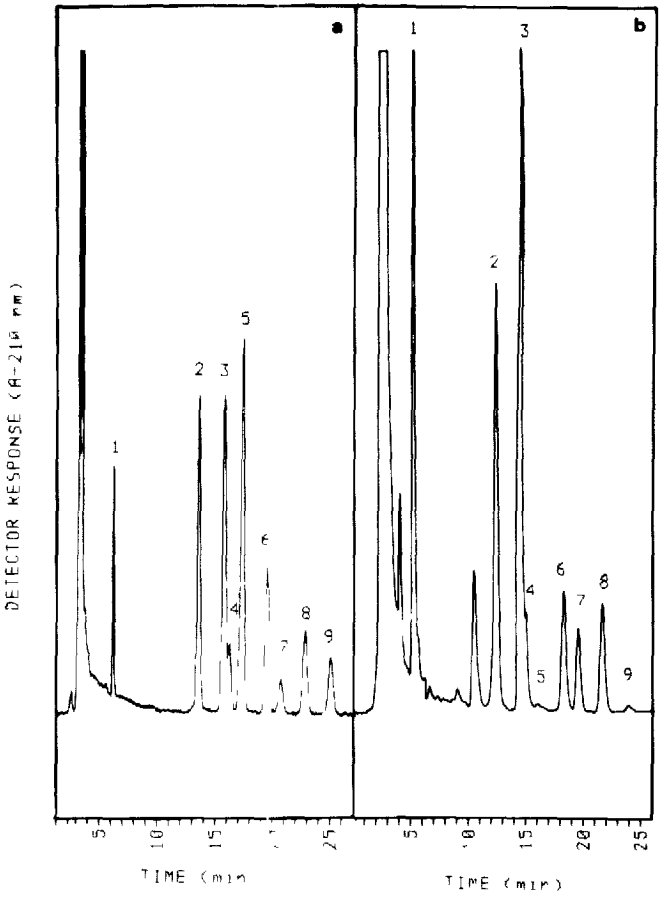

Fig 1 (a) HPLC separation of free cholesterol and cholesteryl ester standards (b) HPLC separation of unesterified cholesterol and cholesteryl esters extracted from human low-density lipoproteins Chromatographic conditions as described under Experimental Peaks 1 = free cholesterol (7 μg), 2 = cholesteryl arachidonate (4 μg), 3 = cholesteryl linoleate (20 μg), 4 = cholesteryl palmitoleate (20 μg), 5 = cholesteryl myristate (20 μg), 6 = cholesteryl oleate (20 μg), 7 = cholesteryl palmitate (5 μg), 8 = cholesteryl heptadecanoate (30 μg), 9 = cholesteryl stearate (8 4 μg)

as it is not a usual constituent of biological fluids or cells and as it migrates as a well separated peak between cholesteryl palmitate and stearate Only the cholesteryl ester linoleate–palmitoleate pair does not give baseline resolution, but this can be improved either by decreasing the flow-rate to about 0 5 ml/min or by using a lower isopropanol concentration in the elution solvent

The mean retention time, standard deviation and coefficients of variation (C V s) of free cholesterol and the individual cholesteryl esters are given in Table I. The C V of the retention time is lower than 2% for all the compounds,

TABLE I

REPRODUCIBILITY OF RETENTION TIMES OF CHOLESTEROL AND CHOLESTERYL ESTERS

Values obtained from duplicate determinations conducted on five days ($n=10$) CE = cholesteryl ester

Component	Retention time (mean ± S.D.) (min)	C.V. (%)
Cholesterol	9.47 ± 0.07	1.4
CE 20:4	13.08 ± 0.18	1.5
CE 18:2	15.36 ± 0.20	1.5
CE 16:1	17.92 ± 0.21	1.5
CE 14:0	18.89 ± 0.23	1.5
CE 18:1	19.29 ± 0.25	1.4
CE 16:0	20.66 ± 0.26	1.4
CE 17:0	22.83 ± 0.28	1.3
CE 18:0	25.29 ± 0.38	1.6

TABLE II

CHARACTERIZATION OF CALIBRATION GRAPHS OF CHOLESTEROL AND CHOLESTERYL ESTERS

CE = cholesteryl ester; r = correlation coefficient

Component	Slope	Intercept	r
Cholesterol	0.3439	−0.0189	0.9995
CE 20:4	1.3076	0.0624	0.9981
CE 18:2	0.3009	0.0641	0.9992
CE 16:1	0.1516	−0.0279	0.9952
CE 14:0	0.1189	0.0076	0.9952
CE 18:1	0.1243	0.0206	0.9974
CE 16:0	0.1241	0.0068	0.9998
CE 18:0	0.2295	0.0142	0.9996

demonstrating a good inter-assay reproducibility. The absorbance of the cholesteryl esters in the low-UV range varies with both the chain length and the number of double bonds in the fatty acyl chain. To achieve maximum sensitivity for all compounds, an optimum wavelength of 210 nm was selected.

The plots of peak-height ratio versus concentration were linear for each component in the analytical range used. The equation parameters of the calibration graphs together with the correlation coefficients are summarized in Table II, all the correlation coefficients are higher than 0.995.

Assuming a signal-to-noise ratio ≥ 2, the detection limits correspond to 50 ng for the polyunsaturated esters and free cholesterol and 150 ng for the mono-

unsaturated and saturated cholesteryl esters The sensitivity was adequate for monitoring relevant changes in cholesteryl ester composition

In order to investigate the intra-assay precision of the method, ten aliquots of a standard mixture prepared from equal amounts of cholesteryl esters in the low and high concentration ranges were extracted For inter-assay determination, aliquots of the standard mixture were extracted in duplicate over a 5-day period Table III summarizes the analytical precision for the quantification of free cholesterol and the cholesteryl esters These data show that the intra- and inter-assay C V s were low in both concentration ranges At the higher concentration of 40 mg/dl the intra-assay precision was less than 3.0% for all components while the inter-assay C V s remained below 3 4%. Intra- and inter-assay C V s were higher for the low concentration range but still remained below 8% The analysis of five extracted standard cholesteryl esters with a concentration of 50 mg per 100 ml in chloroform was compared with that obtained by direct injection of the unextracted standard This yielded an overall analytical recovery of about 98%

The HPLC method was further validated by comparing the cholesteryl ester composition of LDL analysed by HPLC with that obtained by GC Fig 1b shows the HPLC separation of free cholesterol and cholesteryl esters from the hexane–isopropanol extract of human LDL The data in Table IV indicate that a similar composition is obtained by the two methods for the LDL cholesteryl esters

The major objective of this study was to apply the HPLC separation of cholesteryl esters to cell culture experiments Until now, the isolation and quantitation of unesterified cholesterol and of the individual cholesteryl esters by HPLC had only been applied to serum or isolated lipoprotein fractions [15–19] This HPLC procedure determines unesterified cholesterol and the indi-

TABLE III

PRECISION OF THE HPLC DETERMINATION OF CHOLESTEROL AND CHOLESTERYL ESTERS

Values obtained after extraction of 50 μl of a standard mixture containing 5 or 40 mg/dl cholesterol and cholesteryl ester CE = cholesteryl ester

Concentration (mg/dl)	C V (%)							
	Cholesterol	CE-20 4	CE-18 2	CE-16 1	CE-14 0	CE-18 1	CE-16 0	CE-18 0
Intra-assay (n = 10)								
5	3 8	5 3	5 6	6 0	5 0	3 2	3 3	4 3
40	0 8	1 1	2 9	1 0	1 8	2 3	1 3	1 1
Inter-assay (n = 10								
5	6 4	7 2	7 3	8 0	6 7	6 1	6 4	7 4
40	3 3	2 3	3 4	2 3	3 0	2 8	2 7	2 1

TABLE IV

COMPARISON OF CHOLESTERYL ESTER PERCENTAGE COMPOSITION OF HUMAN LDL DETERMINED BY HPLC AND GC

CE = cholesteryl ester

Method	CE-20 4	CE 18 2	CE-16 1	CE-14 0	CE-18 1	CE-16 0	CE-18 0
GC	6 1	58 2	3 5	0 6	19 0	11 7	0 9
HPLC	7 1	57 0	4 1	0 6	18 1	12 1	1 0

TABLE V

TOTAL CHOLESTEROL AND CHOLESTERYL ESTER ACCUMULATION IN J774 MACROPHAGES

Values are mean of a minimum of five different experiments 100 μg LDL or acetylated LDL were added per ml of medium and incubated for 48 h N D = Not detected, CE (%) = percentage of esterified cholesterol

Lipoprotein	Total cholesterol (μg/mg)	CE (%	Cholesteryl ester distribution (%)						
			CE-20 4	CE-18 2	CE-16 1	CE-14 0	CE-18 1	CE-16 0	CE-18 0
LPDS	15	N D	N D	N D	N D	N D	N D	N D	N D
LDL	52	22	N D	25 8	N D	N D	47 6	26 5	N D
Acetyl-LDL	140	55	0 9	34 9	8 7	7 6	29 9	16 3	1 7

vidual cholesteryl esters directly after cellular extraction, avoiding time-consuming isolation and derivatization procedures and minimizing losses during handling As this technique is not destructive, the separated components can easily be recovered for further analysis or characterization

The murine macrophage-like J774 cell line was incubated with native and acetylated low-density lipoproteins and the cellular cholesterol accumulation as well as the cholesteryl ester composition were determined The total amount of cholesterol and the percentage of esterified cholesterol together with the cholesteryl ester composition of the J774 macrophages cultured under various conditions are summarized in Table V When cells were incubated for 48 h with LPDS no measurable cholesteryl esters were found, as only unesterified cholesterol was detected at a concentration of 15 μg/mg cell protein Fig 2a represents the elution pattern of the hexane–isopropanol extract of J774 macrophages cultured in the absence of lipoproteins Loading the J774 macrophages with native low-density lipoproteins results in a modest increase in cellular cholesterol up to 52 μg/mg cell protein with 22% of cholesteryl esters Only cholesteryl oleate, linoleate and palmitate were detected in significant amounts When the cells were incubated for 48 h with 100 μg/ml acetylated LDL, the total cholesterol accumulation increased 2–3 fold over that induced by native LDL (140 μg/mg), with 55% of the total cholesterol consisting of cholesteryl esters The cellular accumulation of different cholesteryl esters is

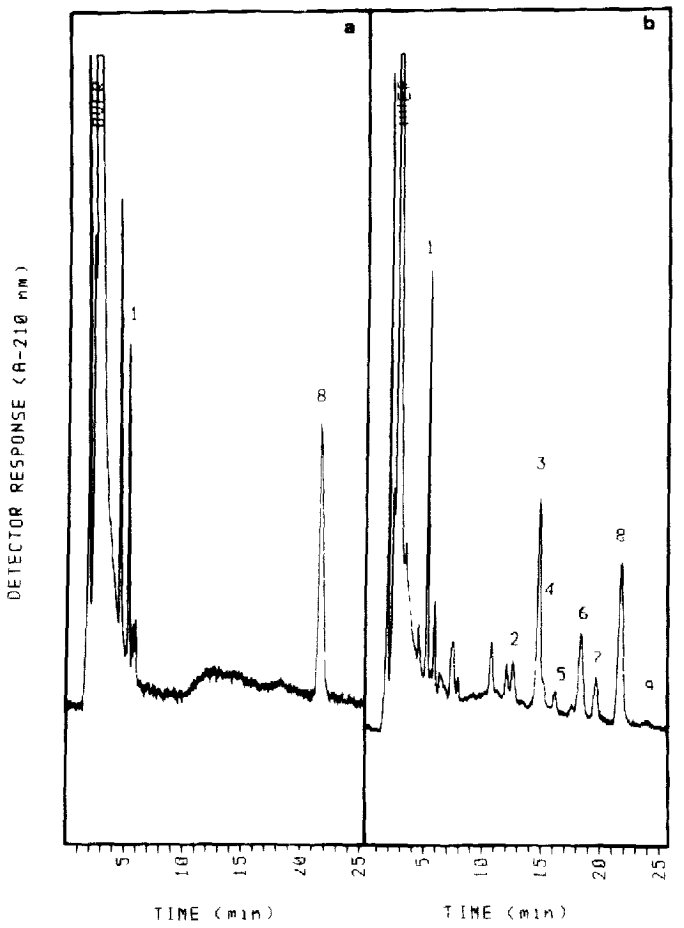

Fig 2 (a) HPLC elution pattern of the hexane–isopropanol extract of the murine J774 cell line after 48 h of incubation in DMEM containing 10% LPDS (b) HPLC elution pattern of the hexane–isopropanol extract of the J774 cell line after 48 h of incubation in DMEM containing 10% LPDS and 100 μg/ml acetylated LDL Peaks as in Fig. 1

demonstrated in Fig 2b This chromatogram clearly shows the presence of unesterified cholesterol and of cholesteryl arachidonate, linoleate, palmitoleate, myristate, oleate palmitate and stearate Cholesteryl linoleate and oleate represent about 65% of the total cholesteryl ester content followed by cholesteryl palmitate (16%) and by smaller amounts of cholesteryl arachidonate, palmitoleate, myristate and stearate The cholesteryl ester accumulation results in the formation of foam cells, as confirmed by staining with Oil Red O and by electron microscopy (data not shown) Further, as the composition and physical properties of the cytoplasmic cholesteryl ester droplets affect their ability to be cleared from the cell and presumably from the atherosclerotic

plaque, the determination of the cholesteryl ester composition within the cells is highly relevant [13]

This paper clearly demonstrates that the separation and quantification of the different individual cholesteryl esters by HPLC can usefully contribute to the monitoring of changes in cholesteryl ester composition and accumulation in macrophages and will be helpful in elucidating the mechanisms involved in foam cell formation

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