paper and note on methodology

An improved method for quantification of cholesterol and cholesteryl esters in human monocyte-derived macrophages by high performance liquid chromatography with identification of unassigned cholesteryl ester species by means of secondary ion mass spectrometry

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Abstract The measurement of cholesteryl esters in human monocyte-derived macrophages using previously described high performance liquid chromatography methods is hampered by the presence in these cells of large amounts of triglycerides. We present a simple reversed phase high performance liquid chromatography protocol for quantification of cholesterol and cholesteryl esters in human monocyte/macrophages or other triglyceride-rich cells. Our method requires only lipid extraction and hydrolysis of triglycerides using a solution of ethanolic potassium hydroxide and is of sufficient sensitivity to allow measurement in 10^5 cells. Use of this protocol led to the isolation of eight previously unassigned cholesteryl ester peaks comprising 16% of the total cholesteryl ester content of human monocyte-derived macrophages. Using time-of-flight secondary ion mass spectrometry and synthesized authentic standards, seven of these peaks were found to comprise cholesterol esterified with polyunsaturated n–3 (ω3) (cholesteryl eicosapentaenoate, docosatrienoate, docosapentaenoate, and docosahexaenoate) and n–6 (ω6) (cholesteryl docosatetraenoate, eicosadienoate, and eicosatrienoate) fatty acids. The remaining peak was shown to be the cholesteryl ester of n–7 (ω7) palmitoleic acid by comparison with a commercially available standard. The identification of all the cholesteryl esters in cholesterol-loaded human monocyte-derived macrophages will assist future studies of lipid metabolism in these cells.—**Cullen, P., M. Fobker, K. Tegelkamp, K. Meyer, F. Kannenberg, A. Cignarella, A. Benninghoven, and G. Assmann.** An improved method for quantification of cholesterol and cholesteryl esters in human monocyte-derived macrophages by high performance liquid chromatography with identification of unassigned cholesteryl ester species by means of secondary ion mass spectrometry. *J. Lipid Res.* 1997. **38:** 401–409.

Supplementary key words cholesterol assay • HPLC • cell culture

For studies in cultured cells, accurate and precise measurement of nanomolar amounts of free cholesterol and cholesteryl esters is required. Up to now, the standard approach has been the fluorometric cholesterol oxidase method described independently in 1978 by Heider and Boyett (1) and Gamble et al. (2). The advantages of this method are its relative simplicity and its sensitivity in the picomole range, allowing the cholesterol concentration to be measured in as few as 10^4 cells. However, the procedure has two major disadvantages. First, the lack of an internal standard may lead to errors in the estimation of the efficiency of lipid extraction and hence in the calculation of the concentration of free cholesterol and cholesteryl esters. Second, the method does not allow quantification of individual cholesteryl ester species.

A number of chromatographic methods have been published for the measurement of cholesterol and cholesteryl esters in cultured cells. These include thin-layer chromatography (TLC) (3), gas–liquid chromatography (GLC) (4), and reversed phase high performance liquid chromatography (HPLC) (5–10).

HPLC has the advantage of allowing quantification

Abbreviations: AcLDL, acetylated low density lipoprotein; CHOD-PAP, cholesterol oxidase phenol aminophenazone; DCC, *N-N′*-dicyclohexylcarbodiimide; GLC, gas–liquid chromatography; HPLC, high performance liquid chromatography; TLC, thin-layer chromatography; TOF-SIMS, time-of-flight secondary ion mass spectrometry.

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of individual cholesteryl ester species. However, measurement of cholesterol and cholesteryl esters in human monocyte-derived macrophages using previously described HPLC protocols (5–8) is hampered by the presence in these cells of large amounts of triglycerides with retention times similar to cholesteryl esters. This not only makes it difficult to quantify the cholesteryl esters, but also reduces analytical sensitivity. A variety of methods may be used to deal with this problem, including enzymatic hydrolysis of triglycerides and selective removal of triglycerides by means of solid phase chromatography (11–13).

We describe here a simple, novel, and sensitive method for the HPLC measurement of the free cholesterol and cholesteryl ester content of cultured cells that contain large amounts of triglyceride such as human monocyte-derived macrophages. The procedure is based on the selective hydrolysis of cellular triglycerides using a solution of 15% ethanolic potassium hydroxide. Use of this method in human monocyte-derived macrophages led to the isolation of eight unassigned cholesteryl ester peaks, seven of which were identified by means of time-of-flight secondary-ion mass spectrometry (TOF-SIMS) and comparison with chemically synthesized authentic standards, and one of which (cholesteryl palmitoleate) was identified by TOF-SIMS analysis and by comparison with a commercially available standard. To our knowledge, these cholesteryl esters have not previously been described in human monocyte-derived macrophages in culture. This is also the first description of the use of TOF-SIMS to identify species of cholesteryl ester. Finally, our measurements show that the concentration of cholesterol in control macrophages, and of cholesterol and cholesteryl esters in macrophages that have been loaded with cholesterol by means of incubation in the presence of acetylated LDL (AcLDL), is more than five times greater than previously reported (14–17).

EXPERIMENTAL METHODS

Materials

All reagents were of analytical grade and were obtained from Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany), including the fatty acids used for chemical synthesis of the novel authentic cholesteryl ester standards. Cholesterol oxidase from *Nocardia erythropolis,* cholesterol esterase from bovine pancreas, and horseradish peroxidase were also purchased from Sigma. All solvents were of HPLC grade and were purchased from Baker GmbH (Gross-Gerau, Germany). Cell culture material was obtained from Life Technologies (Eggenstein, Germany).

Lipid extraction

Lipid extraction was essentially by means of a modification of the method of Hara and Radin (18). The cells were scraped from the culture flasks into 0.9% NaCl (2 ml per 75 cm^2 flask) and homogenized on ice by sonication for 10 sec with a Soniprep 150 sonicator (MSE, Fisons, England) set to maximum power. The protein concentration of the cell lysate was determined by the method of Lowry *et al.* (19). To a volume of cell suspension known to contain 1 mg of protein was added 100 µg of cholesteryl heptadecanoate in chloroform as internal standard. An equal volume of freshly prepared cold (−20°C) KOH in ethanol (150 g/l) was then added and the cell lysate was repeatedly vortexed until clear. An equal volume of hexane–isopropanol 3:2 (v/v) was added and the mixture was vortexed for 5 min followed by centrifugation at 800 *g* and 15°C for a further 5 min. The extraction procedure was repeated twice (total of three extraction procedures). The combined organic phase was transferred to clean tapered glass tubes and thoroughly dried under nitrogen at 40°C. The tubes were allowed to cool to room temperature, 100 µl of isopropyl alcohol–n-heptane–acetonitrile 35:12:52 (v/v) was added, and the sample was solubilized by placing in an ultrasound water bath for 5 min at room temperature (Laboson 200, Bender and Hobein, Germany). After centrifugation at 800 *g* for 5 min, the sample was introduced into the HPLC device using a Rheodyne 7125 (Rheodyne, Cotati, CA) 20 µl loop injector.

High performance liquid chromatography

HPLC was performed using a Kontron device (Neufahrn, Germany) equipped with a model 422 pump, a model 480 column oven, a model 440 diode array detector, and a 3 µm Spherisorb ODS2 250 × 4 mm column (Phase Separations, Queensferry, UK). Cholesterol and cholesteryl esters were eluted isocratically at a flow rate of 0.5 ml/min and at a temperature of 40°C using an eluent consisting of isopropanol–n-heptane–acetonitrile 35:12:52 (v/v) and detected by UV-absorption at 206 nm.

Measurement of cholesterol in the hospital laboratory

Total cholesterol was measured in the hospital laboratory by means of the CHOD-PAP method (Boehringer Mannheim, Mannheim, Germany) and an automated analyzer system (Hitachi-Boehringer Mannheim). The method was validated by regular analyses of reference sera supplied by the national German INSTAND proficiency testing program and the interna-

tional quality assurance program of the US Centers for Disease Control and Prevention.

Gas–liquid chromatography

The concentrations of free and total cholesterol in the cells were determined by GLC of the trimethylsilylether derivatives. Cells were harvested and homogenized as described above. Ten micrograms of 5β-cholestan-3α-ol was added to 100 μl of cell lysate as an internal standard. Lipid extraction was performed using the method of Klansek et al. (4). Derivatization was performed according to standard methods (20) and GLC was carried out using a Dani 8521a gas chromatograph (Monza, Italy) equipped with a programmable injector (PTV), a CP-Wax 57CB fused silica capillary column (25 m × 0.32 mm ID, 0.2 μm film thickness, Chromspec, Bridgewater, NJ), a flame ionization detector, and a chromatogram data processor MT2 (Kontron, Neufahrn, Germany).

Mass spectrometry

Mass spectrometry was carried out with a TOF-SIMS II (IONTOF, Münster, Germany) as previously described (21). HPLC fractions containing the peaks for analysis were collected and 10 μl was applied to a silver target that had been pre-etched for 2 min with 20% HNO_3. After evaporating the solvent, the sample was bombarded with $^{40}Ar^+$, 10^{11} ions/cm^2 (11 keV). Measurements were performed with typical acquisition times of 100 s. The approximate mass resolution was $M/\Delta M$ = 10,000, where M is the target ion mass and ΔM is the resolved mass difference at half-width full peak mode. The analytic sensitivity of the TOF-SIMS method was investigated using free cholesterol and cholesteryl palmitate. Cholesteryl palmitate at a concentration of 10 μg/ml was detected with a yield (number of secondary ions/number of primary ions) of 5×10^{-4}. Cholesterol at the same concentration was detected with a yield of 5×10^{-3}. Thus, in comparison with other organic substances, these compounds have a high probability of detection. The identity of the cholesteryl ester species was derived from the measured mass and confirmed by comparison with synthetic standards (except cholesteryl palmitoleate in which case a commercially available standard was used for identification).

Cell culture

Human monocytes were isolated by leukapheresis using a CS3000 cell separator (Baxter, Morton Grove, IL) and countercurrent flow elutriation (Beckman Instruments, Fullerton, CA) and cultivated for 14 days in RPMI 1640 supplemented with 20% human serum as described (22). Cholesterol loading was achieved by incubation of the cells for 48 h in the presence of 80 μg/ml AcLDL (23).

Synthesis of cholesteryl esters

Cholesteryl esters were synthesized using a modification of the method of Neises, Andries, and Steglich (24). A catalytic amount of 4-(dimethylamino)pyridine and 20 μmol cholesterol were added to 20 μmol of the respective fatty acid dissolved in 1 ml CH_2Cl_2. The resulting clear solution was cooled in iced water. Once a temperature of 0°C had been reached, 25 μmol of *N*-*N*′-dicyclohexylcarbodiimide (DCC) was added and the solution was stirred overnight under light exclusion at room temperature under nitrogen. Precipitated hydrolyzed DCC was then removed by filtration. The filtrate was extracted twice with an equal volume of CH_2Cl_2 and 5 ml of water. After centrifugation at 800 *g*, the lower organic phase was removed and dried over Na_2SO_4. The supernatant was removed, dried under nitrogen, and redissolved in isopropyl alcohol–n-heptane–acetonitrile 35:12:52 (v/v). Twenty microliters of this solution was applied to the HPLC for analysis as described above.

Measurements in the mouse macrophage cell line J774

In order to investigate the performance of our method with a second commonly used macrophage cell line and to allow comparison with published results (6), we performed HPLC on cholesterol-loaded J774 cells, a mouse macrophage-like cell line. J774 cells were maintained in DMEM medium containing 10% fetal calf serum. Cholesterol loading was carried out by incubating the cells for 48 h in the presence of 80 μg/ml AcLDL.

RESULTS

Hydrolysis of triglycerides using ethanolic KOH

Attempts by our laboratory to apply the HPLC methods of other authors (6) to the determination of intracellular cholesterol and cholesteryl esters in human macrophages proved unsuccessful because of the presence in these cells of other material that obscured the cholesteryl ester peaks (**Fig. 1**). Based on the results of TLC (not shown), we suspected that this material consisted of triglycerides. In order to investigate this hypothesis, triglyceride standards (triolein, trilinolein, and 1,3-dipalmitoyl-2-oleylglycerol) were applied to the HPLC column in the concentration range known to occur in cultured cells and were found to elute within the region of the interfering peaks. Treatment of the tri-

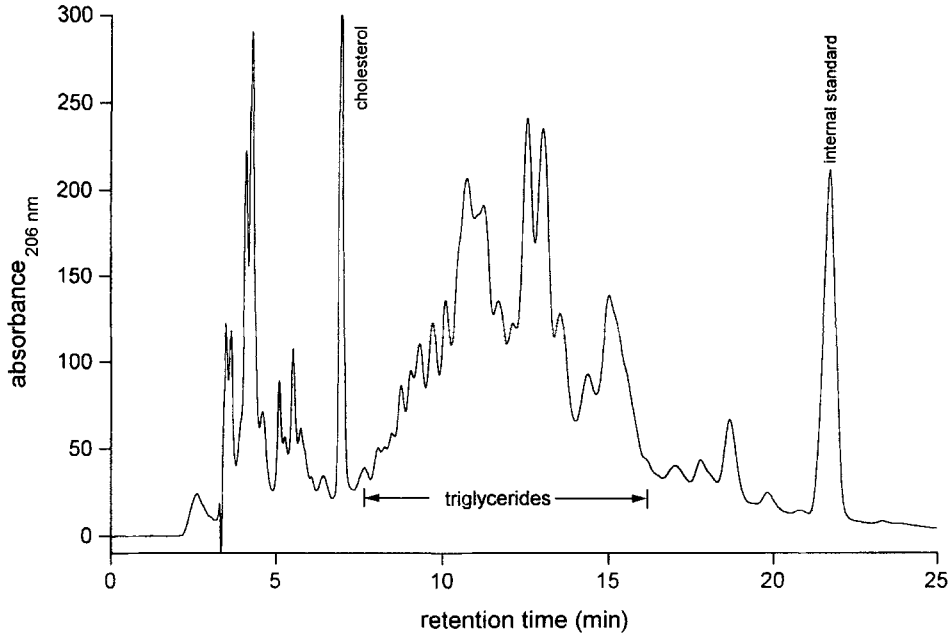

Fig. 1. HPLC chromatogram of lipid extract of cholesterol-loaded human monocyte-derived macrophages prior to treatment with a solution of ethanolic potassium hydroxide. The large amounts of triglyceride present obscure many of the cholesteryl ester peaks. Note that free cholesterol and the internal standard (cholesteryl heptadecanoate) occur outside the region obscured by triglycerides, allowing assay of free cholesterol in the cells without prior triglyceride hydrolysis.

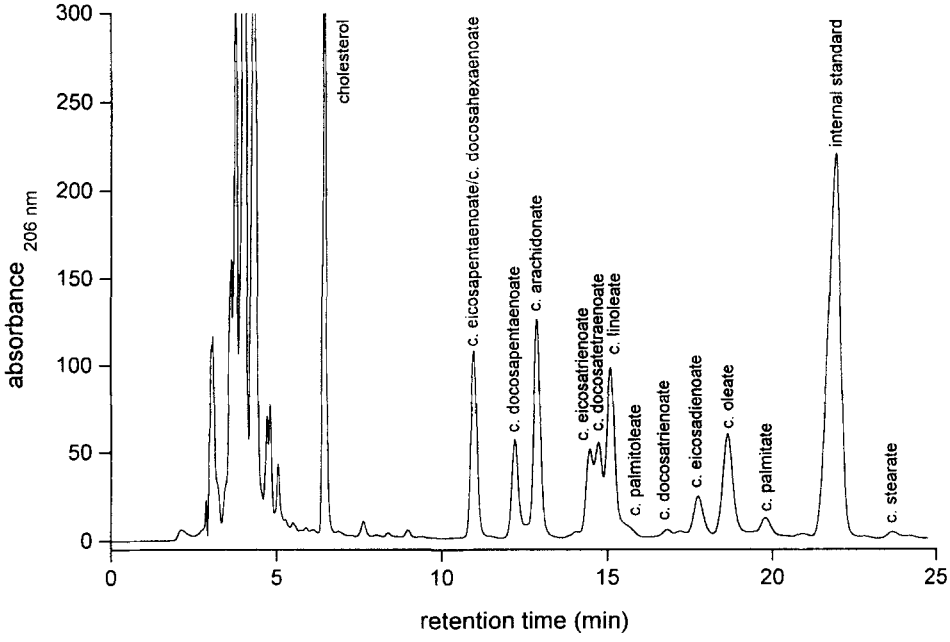

Fig. 2. HPLC chromatogram of lipid extract from same cells as shown in Fig. 1 after treatment of the extract with a solution of ethanolic potassium hydroxide as described in Methods. This procedure completely hydrolyzes triglycerides without producing hydrolysis of cholesteryl esters.

TABLE 1. Total, free, and esterified cholesterol concentrations in a serum sample

	CHOD-PAP	HPLC
	mg/dl	
Total cholesterol	2.43 ± 0.06	2.40 ± 0.05
Free cholesterol	—	1.59 ± 0.03
Cholesteryl ester	—	0.81 ± 0.02

Measurements of total, free, and esterified cholesterol concentrations in a sample of serum using reversed phase HPLC and enzymatic (CHOD-PAP) methods. Values given as mean ± SD, n = 10.

glyceride standards with a solution of ethanolic KOH as described in Methods led to their complete hydrolysis. We therefore treated the cell extracts with ethanolic KOH, which abolished the interfering peaks (**Fig. 2**).

Neither the free cholesterol nor the internal standard (cholesteryl heptadecanoate) peaks occur within the region obscured by cellular triglycerides (Fig. 1). Therefore it was possible to accurately assay the free cholesterol concentration in cells without hydrolyzing triglycerides. In order to investigate whether mild hydrolysis using ethanolic KOH also hydrolyzes cholesteryl esters, we performed a series of measurements of known amounts of cholesteryl heptadecanoate before and after treatment with ethanolic KOH. The calculated free cholesterol concentration, free cholesterol peak height, and internal standard peak height were identical before and after this treatment indicating absence of hydrolysis of cholesteryl esters.

Accuracy

To assess the accuracy of the method, serum samples were measured using HPLC and the results were compared to those obtained in the hospital laboratory. The total cholesterol concentration as estimated by reversed phase HPLC agreed with the value obtained in the hospital laboratory to within 2% (**Table 1**).

Precision

In order to assess the precision of the method, a sonicated suspension of pooled cholesterol-loaded human monocyte-derived macrophages was divided into 10 aliquots and assayed for content of cholesterol and selected cholesteryl esters. In addition, 10 aliquots of solutions containing low and high concentrations of cholesterol and cholesteryl ester standards were assayed. The results are shown in **Table 2.** The coefficient of variation for cultured cells varied between 2 and 5%. For the low standards (approximately 0.04 mg/ml) the coefficient of variation was in the range of 2% and for the high standards (approximately 0.4 mg/ml) the coefficient of variation was in the range of 1%. Table 2 also shows the concentrations of each of the quantified cholesteryl ester species in cholesterol-loaded human macrophages.

Linearity

Standard curves (**Fig. 3**) and serial dilutions of samples showed that measurement with our HPLC method closely fits a quadratic function over at least two orders of magnitude (from 0.01 to 1.0 mg/ml) for free cholesterol and all of the cholesteryl esters quantified. The concentrations of free cholesterol and cholesteryl esters in cultured macrophages lie well within this range (total cholesterol concentration approx 0.5 mg/ml, equivalent to 500 μg/mg cell protein).

Limits of detection

The limits of detection were estimated from the standard curve as the mean plus three times the standard deviation of the signals obtained in serial measurements of a sample containing none of the relevant analyte (25) and were as follows: cholesterol 5 μg/mg cell protein, cholesteryl arachidonate 8 μg/mg, cholesteryl linoleate 9 μg/mg, cholesteryl oleate 15 μg/mg, cholesteryl palmitate 11 μg/mg, and cholesteryl stearate 10 μg/mg. For human monocyte-derived macrophages, at least 0.1

TABLE 2. Precision of reversed phase HPLC measurement of cholesterol and cholesteryl esters in lipid extract of cholesterol-loaded human monocyte-derived macrophages and in standard solutions at the concentrations shown

	Free Cholesterol		Cholesteryl Arachidonate (20:4)		Cholesteryl Linoleate (18:2)		Cholesteryl Oleate (18:1)		Cholesteryl Palmitate (16:0)		Cholesteryl Stearate (18:0)	
	Conc	CV	Conc	CV	Conc	CV	Conc	CV	Conc	CV	Conc	CV
Cell extract	314	4.8	66	2.6	18	2.7	34	2.1	88	2.9	—	—
Low standard	44	1.6	57	1.3	47	1.2	48	1.0	45	2.2	47	1.7
High standard	330	0.6	363	0.8	379	0.5	419	0.6	387	1.0	421	1.0

Conc, concentration (μg/mg cell protein for cell extract; μg/ml for standards. CV, coefficient of variation in %; n = 10.

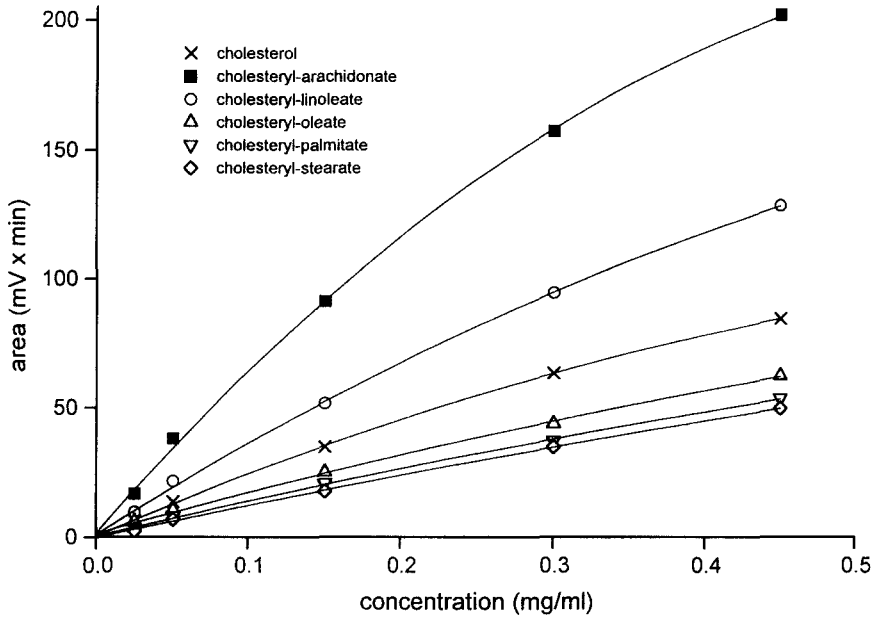

Fig. 3. Standard curves for free cholesterol, cholesteryl arachidonate, cholesteryl oleate, cholesteryl linoleate, and cholesteryl stearate showing excellent fit to a quadratic function (r^2 each case >0.999).

mg of cell protein must be applied to the HPLC column in order to permit accurate quantitation of the less abundant cellular cholesteryl esters such as cholesteryl stearate. This is equivalent to 10^5 cells in culture.

Identity of unassigned cholesteryl ester species

Analysis of unassigned HPLC peaks by means of TOF-SIMS led to identification of the following eight polyunsaturated cholesteryl esters: a) n–3 family (derivatives of α-linolenic acid): cholesteryl eicosapentaenoate (20:5, $\Delta^{5,8,11,14,17}$), cholesteryl docosatrienoate (22:3, $\Delta^{13,16,19}$), cholesteryl docosapentaenoate (22:5, $\Delta^{7,10,13,16,19}$), and cholesteryl docosahexaenoate (22:6, $\Delta^{4,7,10,13,16,19}$); b) n–6 family (derivatives of linoleic acid): cholesteryl docosatetraenoate (22:4, $\Delta^{7,10,13,16}$), cholesteryl docosadienoate (22:2, $\Delta^{13,16}$), and cholesteryl eicosatrienoate (20:3, $\Delta^{8,11,14}$). In addition, the cholesteryl ester of the n–7 fatty acid palmitoleic acid (16:1, Δ^{9}) was identified by TOF-SIMS and comparison with a commercially available standard. **Figure 4** (left frame) shows a representative eluted unassigned HPLC peak as analyzed by TOF-SIMS. Based on the observed mass, this compound was deduced to be cholesteryl eicosapentaenoate. This compound was then synthesized as described in Methods. The right frame of Fig. 4 shows the TOF-SIMS spectrum of the synthesized compound. The identity of all other unassigned peaks (apart from the cholesteryl palmitoleate peak) was derived in analogous fashion.

In the present analysis, HPLC quantification of cholesteryl esters in human macrophages was confined to the compounds cholesteryl arachidonate, linoleate, oleate, palmitate, and stearate. Comparison of the results of total cholesteryl ester determination performed with HPLC and GLC indicated that the previously unassigned polyunsaturated esters of cholesterol described in this report comprise 16% of the total cholesteryl ester content of human monocyte-derived macrophages loaded with AcLDL (**Table 3**).

Measurements in the mouse macrophage cell line J774

In contrast to human monocyte-derived macrophages, J774 cells contain virtually no triglycerides in the AcLDL-loaded state (**Fig. 5**), accounting for the success of the method of Vercaemst, Union, and Rosseneu (6) in assaying this cell type. The pattern of cholesteryl esters in J774 cells differs substantially from that in human monocyte-derived macrophages, despite the fact that cholesterol-loading was performed using the same lipoprotein as used in the human macrophages. The pattern of cholesteryl esters observed by us is in substantial agreement with that reported by Vercaemst et al. (6). However, the HPLC spectrum obtained by us shows

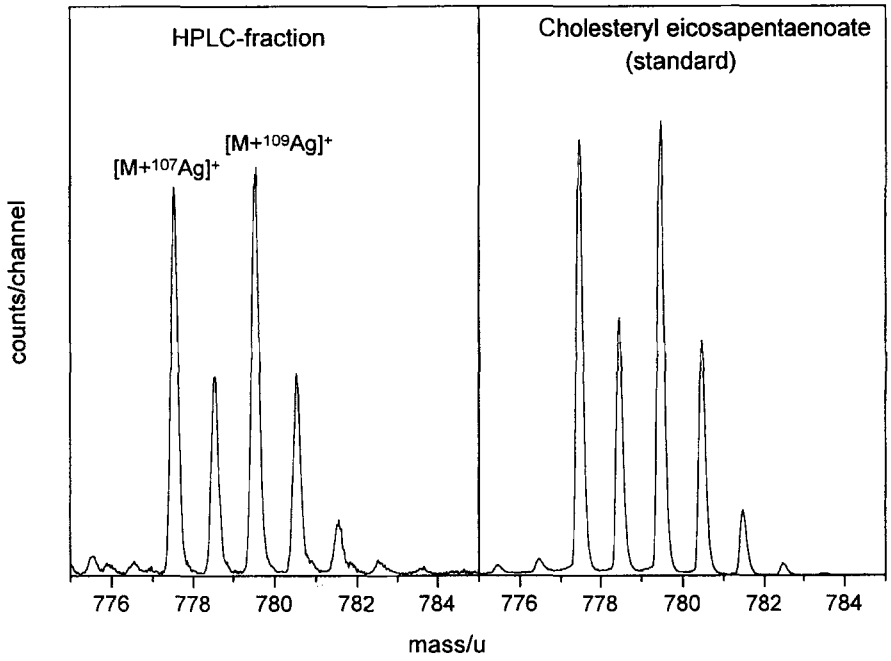

Fig. 4. The left frame contains a time-of-flight secondary ion mass spectrogram of an HPLC fraction containing an unidentified peak showing two major fragments of 777.5 and 779.5 atomic mass units. Based on the mass of the fragments obtained, this peak was calculated to consist of cholesteryl eicosapentaenoate. This compound was chemically synthesized as described in Methods and the synthesized compound was subjected to time-of-flight secondary ion mass spectrometry. This produced a pattern of fragments identical to those of the unknown peak (right frame) confirming the identity of the HPLC peak as cholesteryl eicosapentaenoate. All the unassigned cholesteryl ester peaks were identified in this fashion except for cholesteryl palmitoleate which was identified by comparison with a commercially available standard.

improved resolution and allows assignment of additional cholesteryl esters in J774 cells.

DISCUSSION

Macrophages in the atherosclerotic plaque accumulate cholesterol that is esterified for storage within cholesteryl ester droplets. For this reason methods are required for the quantification of intracellular free cholesterol and cholesteryl esters within these cells.

TABLE 3. Total, free, and esterified cholesterol concentrations in cholesterol-loaded human macrophages

	GLC	HPLC
	μg/ mg cell protein	
Total cholesterol	589 ± 15	542 ± 21
Free cholesterol	188 ± 7	206 ± 10
Cholesteryl ester	401 ± 8	336 ± 11

Measurements of free cholesterol and cholesteryl ester concentrations, and calculated total cholesterol concentration in cholesterol-loaded human macrophages using reversed phase HPLC and GLC. Values given as mean ± SD, n = 10.

Most investigators have determined the intracellular concentration of cholesterol and cholesteryl esters by means of enzymatic fluorimetry. However, the enzymatic method does not permit quantification of individual cholesteryl ester species.

Several chromatographic methods have also been described for the measurement of intracellular cholesterol and cholesteryl esters. TLC allows identification of phospholipids, triglycerides, cholesterol, and cholesteryl esters, but is difficult to quantify (3). GLC is regarded as the reference method for determination of free and total cholesterol in cells (4). Protocols also exist for the measurement of individual cholesteryl ester species by means of GLC, but these are time consuming and technically demanding.

A number of HPLC protocols have also been described for the measurement of cholesterol and cholesteryl esters in cells (5–8). We attempted to apply the protocol of Vercaemst et al. (6) to the measurement of cholesterol and cholesteryl esters in human monocyte-derived macrophages. We found that human macro-

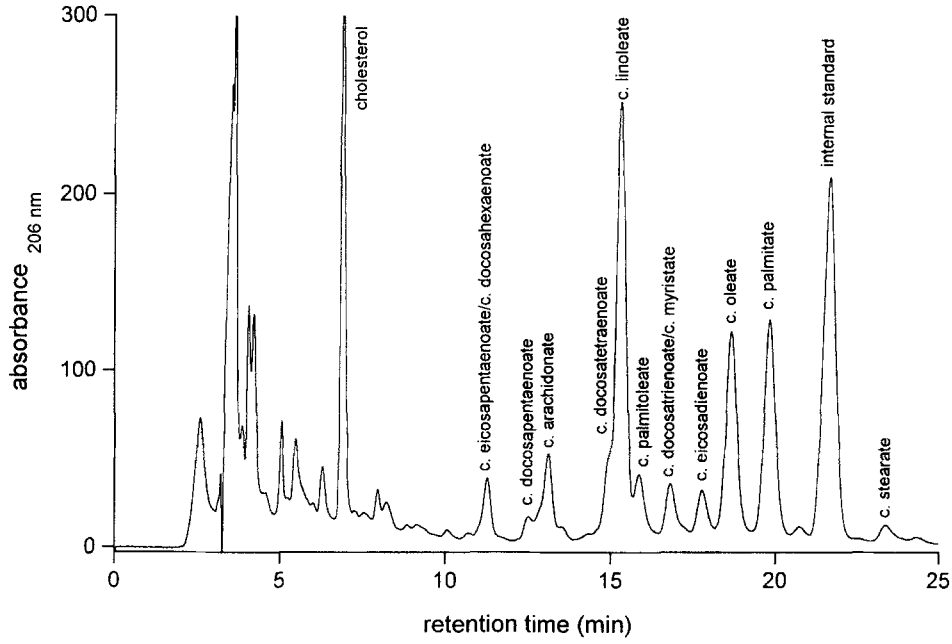

Fig. 5. Reversed HPLC chromatogram of lipid extract from cholesterol-loaded J774 macrophages showing absence of triglycerides and distribution of cholesteryl esters in this cell type.

phages, in contrast to J774 mouse macrophages in which this method of Vercaemst et al. (6) was validated, contain large amounts of triglycerides that elute from the chromatography column at a position similar to that of the bulk of the cholesteryl esters, rendering accurate cholesteryl ester quantification impossible. For this reason we developed a method for selectively hydrolyzing triglycerides by treatment with a solution of ethanolic KOH. Araki et al. (5) applied 0.1 N NaOH to rat macrophages prior to lipid extraction and obtained a smooth HPLC profile including cholesteryl ester peaks; however, they did not state whether NaOH was added to hydrolyze triglycerides. Our method produces complete hydrolysis of triglycerides while leaving cholesteryl esters intact as shown by identical free cholesterol and internal standard concentrations before and after treatment with ethanolic KOH. The concentration of cholesterol as measured by HPLC was slightly greater than that determined using the reference method of GLC. This can be attributed to the lesser resolution of HPLC that is unable to differentiate between cholesterol and other sterols such as lanosterol which are present in trace amounts.

Up to now, cholesteryl esters of palmitate, arachidonate, oleate, linoleate, and stearate have been described (in order of descending concentration) in cholesterol-loaded human monocyte-derived macrophages. We here describe for the first time that these cells also accumulate significant amounts of cholesteryl esters of several previously unassigned long-chain n–3 (ω3), n–6 (ω6), and n–7 (ω7) fatty acids. N–3 and n–6 fatty acids are derived from the essential fatty acids α-linolenic and linoleic acid, respectively. Palmitoleic acid is derived from palmitate by the action of Δ^9 desaturase. HPLC analysis of AcLDL showed that of the newly assigned cholesteryl esters, cholesteryl eicosapentaenoate (n–3, 20:5, $\Delta^{5,8,11,14,17}$) and cholesteryl docosatrienoate (n–3, 20:5, $\Delta^{13,16,19}$) were present only in the cells but not in AcLDL (data not shown). There are several possible explanations for this. These fatty acids may have been synthesized from precursors within the cells. Alternatively, they or their precursors may be incorporated into phospholipids or triglycerides within AcLDL. Comparison of the total cholesteryl esters as assayed by GLC and the sum of the concentrations of cholesteryl esters of oleate, linoleate, palmitate, arachidonate, and stearate as measured with HPLC indicates that these newly assigned cholesteryl esters comprise some 16% of total cellular cholesteryl ester in cholesterol-loaded human monocyte-derived macrophages (Table 3).

The HPLC method described here allows quantification of cholesteryl esters in cells containing large amounts of triglycerides and is of sufficient sensitivity to allow measurement in 10^5 cells. The estimates of intracellular cholesterol and cholesteryl ester in cholesterol-loaded human monocyte-derived macrophages obtained with our method, which were validated by comparison with GLC, are five to ten times greater than

those reported previously (14–17). We are unable to completely explain this discrepancy but feel it may be due, at least in part, to underestimation of cellular lipid content as a result of either incomplete lipid extraction or incomplete solubility of extracted lipids. These losses may go undetected due to the lack of a suitable internal standard for use with enzymatic or TLC methods of cholesterol and cholesteryl ester determination.

The identification of all the cholesteryl esters occurring in AcLDL-loaded human monocyte-derived macrophages will assist future studies of the lipid metabolism of these cells.

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