paper on methodology

Combination of an enzymatic method and HPLC for the quantitation of cholesterol in cultured cells

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Abstract The study of the cellular events that lead to the foam cell formation requires the development of fast, accurate, and sensitive methods to quantify cholesterol in cultured cells. Here we describe a procedure that allows the rapid determination of free and total cholesterol in a reduced number of cells, which makes it very suitable for cholesterol determination in cell cultures. The method consists of the enzymatic conversion of cholesterol to cholest-4-ene-3-one by cholesterol oxidase followed by the analysis of the sample by high performance liquid chromatography (HPLC) to detect this oxidized product. Due to the relatively high wavelength at which cholest-4-ene-3-one has its maximum absorption (240 nm), other cellular components do not interfere with the chromatographic procedure and prior lipid extraction is not required. Moreover, the duration of each chromatogram is about 3 min, contributing to the celerity of the method. All the cholesteryl esters used (oleate, palmitate, stearate and linoleate) were quantitatively hydrolyzed by incubation with cholesterol esterase; this was observed to occur with both pure standards and in cell homogenates. Sensitivity is enough to allow the determination of free and total cholesterol in less than 5×10^3 cells. We have applied this method to human monocyte-derived macrophages and the values obtained for free and total cholesterol are in close agreement with published data. **—Contreras, J. A., M. Castro, C. Bocos, E. Herrera, and M. A. Lasunción.** Combination of an enzymatic method and HPLC for the quantitation of cholesterol in cultured cells. *J. Lipid Res.* 1992. **33:** 931-936.

Supplementary key words free cholesterol • cholesteryl esters • cholest-4-ene-3-one • HPLC • human monocyte-derived macrophages

The study of foam cell formation, one of the key events in early atherosclerosis, requires the development of accurate methods to measure free and esterified cholesterol content of cells in culture. These methods should be both sensitive and rapid in order to reduce the number of cells used and the time required to analyze a relatively large number of samples. The former point can be a very limiting factor when studies are performed with cells that do not divide in culture, as is the case of human monocyte-derived macrophages, which are not easily obtained in great quantities.

There are several methods in the literature based on the enzymatic conversion of cholesterol to cholest-4-ene-3-one (hereafter referred to as cholestenone) by cholesterol oxidase and the reaction of the formed H_2O_2 to induce the oxidation of a chromogen (1) or a fluorogen (2, 3) by the catalytic action of peroxidase. Amongst them, fluorimetric methods are the most suitable for the determination of cholesterol in cultured cells because of their sensitivity. Nevertheless, the described methods involve either the extraction of lipids (2) or the use of a relatively high number of cells (3) to achieve good resolution.

The development of high performance liquid chromatography (HPLC) has provided an efficient tool for quantifying many substances present in a sample at low concentration. There are several methods that allow the determination of cholesterol and its different esters in plasma (4), lipoproteins (5, 6), or cells (7, 8). These methods allow the determination of individual cholesteryl esters which is interesting when studying the lipid composition of the samples, although they are time consuming and are not suitable for a small number of cells.

Here we describe a method that consists of the formation of cholestenone by enzymatic reaction and its separation and quantitation by HPLC. The method has the advantages of the enzymatic procedures and the sensitivity of HPLC, and allows the rapid measurement of free and total cholesterol in cultured cells.

MATERIALS AND METHODS

Reagents

Cholesterol oxidase from *Nocardia erythropolis* was purchased from Boehringer Mannheim GmbH (Germany).

Abbreviations: HPLC, high performance liquid chromatography; PBS, phosphate-buffered saline; LDL, low density lipoprotein; AcLDL, acetylated LDL; cholestenone, cholest-4-ene-3-one.

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Cholesterol esterase from *Pseudomonas fluorescens*, sodium cholate, Triton X-100, dithiothreitol (DTT), pure cholestenone, and cholesteryl esters were from Sigma Chemical Co. (St. Louis, MO). Cholesterol and other reagents were from Merck, (Darmstadt, Germany). RPMI 1640 was from Biochrom KG (Berlin, Germany), Plastic culture plates were from Nunc Inter Med, (Roskilde, Denmark) and Lymphoprep from Nycomed AS (Oslo, Norway). HPLC grade solvents were from Panreac (Barcelona, Spain). Proteins were determined either by the procedure of Lowry et al. (9) or by the Micro-BCA kit Assay from Pierce Chemical Co. (Rockford, IL).

Cells

Human monocytes were obtained from blood transfusion units from our hospital as described by Zanella et al. (10), with slight modifications. Basically, one unit of blood was centrifuged (2000 *g*, 10 min) to obtain a buffy coat that was sterilely transferred to the satellite bag with a plasma extractor so that erythrocytes could be used for transfusion. The buffy coat was centrifuged again (250 *g*, 10 min) and the resulting platelet-rich plasma was separated from the white cells. Plasma was then dialyzed in a low molecular weight cutoff dialysis bag against 5 l of 0.9% NaCl containing 1 g/l of $CaCl_2 \cdot 2H_2O$ to induce the formation of the clot which was removed by agitation with a glass pipette. The resulting platelet-free serum was used for the cell culture.

White cells were diluted with phosphate-buffered saline (PBS) and overlayered on Ficoll (Lymphoprep) according to Bøyum (11) to obtain the mononuclear cells. These cells were washed several times with PBS, diluted to 10^7 cells/ml with RPMI 1640, and plated in 24-well sterile plastic culture plates. After 2 h in the incubator, nonadherent cells (lymphocytes) were removed by gentle aspiration and the attached cells (monocytes) were washed three times with warm PBS and cultured in RPMI 1640 supplemented with 20% of autologous serum. Cells were cultured for 7 days under these conditions and then for 72 h in the presence of lipoproteins.

Low density lipoproteins (LDL) were isolated from human plasma by sequential ultracentrifugation as described (12), and acetylated low density lipoproteins (AcLDL) were obtained by treatment of LDL with acetic anhydride according to Basu et al. (13).

Solubilization of cells

Cells were washed three times with PBS and then an appropriate volume (usually 1 ml) of either 0.5% sodium cholate or 0.1% Triton X-100 was added to each well to obtain a solution containing from 0.05 to 0.2 mg cellular protein/ml. When sodium cholate was used, cells were sonified for 30 sec using an MSE sonifier. When Triton X-100 was used, sonication was not needed for cell disruption. Two 100-μl aliquots of the solution (5–20 μg cell protein) were taken for the measurement of free and total cholesterol and the rest of the sample could be used to measure protein or for other determinations.

Calibration curve and enzymatic procedure

Free cholesterol and cholesteryl oleate were dissolved in isopropyl alcohol (1 mg cholesterol/ml) and stored at −20°C as stock standard solutions. Calibration curves were obtained by diluting these standards in the same medium used for cell solubilization (either 0.5% sodium cholate or 0.1% Triton X-100) to obtain values ranging from 0 to 40 μg cholesterol/ml. Then, 0.1 ml of each sample (either standard or cell solution containing from 0.005 to 0.02 mg cell protein) was supplemented with 10 μl of a reaction mixture that contained 500 mM $MgCl_2$, 500 mM Tris buffer (pH 7.4), 10 mM dithiothreitol, and either 5% sodium cholate or 1% Triton X-100 (both detergents were needed to achieve maximum enzymatic activity, so when cholate was used to solubilize the cells, Triton X-100 was added to the reaction mixture and vice versa). Enzymes (either 0.4 U/ml of cholesterol oxidase for free cholesterol determination or cholesterol oxidase supplemented with 0.4 U/ml of cholesterol esterase for total cholesterol measurement) were added to each tube in 10 μl of 0.5% cholate. Final concentration of the reagents in the assay is summarized in **Table 1.** The tubes were incubated at 37°C for 30 min and then 100 μl of methanol–ethanol 1:1 was added to stop the reaction. Samples were kept cold for 30 min to allow protein precipitation and subsequently centrifuged in a Beckman Microfuge for 10 min. Fifty microliters of the supernatant was injected in the HPLC system.

Chromatographic procedure

Samples were analyzed using a System Gold Chromatographer (Beckman Instruments Inc., Palo Alto, CA) consisting of a Solvent Module 126, a 210A Injection Valve, and an Analog Interface Module 406, equipped with a Nucleosil 5 C-18 column (12 cm, 0.4 mm internal diameter) (Knauer FIOSA, Barcelona, Spain) and a precolumn module with an RCSS C-18 cartridge (Waters,

TABLE 1. Composition of the reaction mixture

Component	Concentration
Chemicals	
Sodium cholate	0.5% (w/v)
Triton X-100	0.1% (w/v)
Tris buffer, pH 7.4	50 mM
$MgCl_2$	50 mM
DTT	1 mM
Enzymes	
Cholesterol oxidase	0.4 U/ml
Cholesterol esterase[a]	0.4 U/ml

[a]For free cholesterol measurement, cholesterol esterase was omitted.

Milford, MA). Methanol–acetic acid 99:1 was used as eluent at a flow rate of 1.75 ml/min, and detection was achieved by monitoring absorbance at 240 nm in a Beckman 165 Detector. Data were analyzed with the System Gold software from Beckman.

Verification of the enzymatic reaction

In order to confirm the total hydrolysis of different cholesteryl esters and the conversion of all the cholesterol to cholestenone in our conditions, the following experiments were done. In Experiment 1, cells from 10 wells were dissolved in 5 ml of a solution containing all the chemicals needed for enzymatic reaction (see Table 1) and distributed in two tubes. One aliquot was incubated for 30 min at 37°C in the presence of the enzymes and the other without them. After incubation, cellular lipids were extracted from both tubes with hexane and this solvent was evaporated under a N_2 stream. Lipids were then analyzed by HPLC using acetonitrile–tetrahydrofuran 75:25 as eluent at a flow rate of 2 ml/min. Absorbance was monitored at 214 nm to detect free cholesterol and the different cholesteryl esters and at 240 nm to detect cholestenone. In Experiment 2, the cell suspension was replaced by a mixture of commercially available cholesteryl esters (15 μg free cholesterol, 20 μg cholesteryl oleate, 20 μg cholesteryl linoleate, 6 μg cholesteryl arachidonate, 10 μg cholesteryl palmitate, and 20 μg cholesteryl stearate) in 20 μl of sec-butanol and diluted to 500 μl with

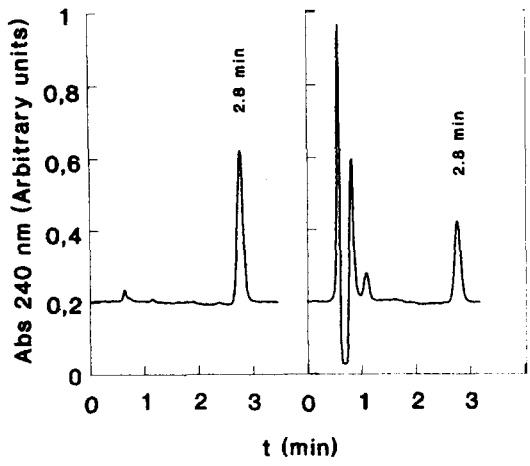

Fig. 1. HPLC separation of cholest-4-ene-3-one. Representative chromatogram of a cell homogenate treated for total cholesterol determination and processed as described in Methods (right panel). Pure choles-4-ene-3-one showed a retention time of 2.8 min under identical chromatographic conditions (left panel).

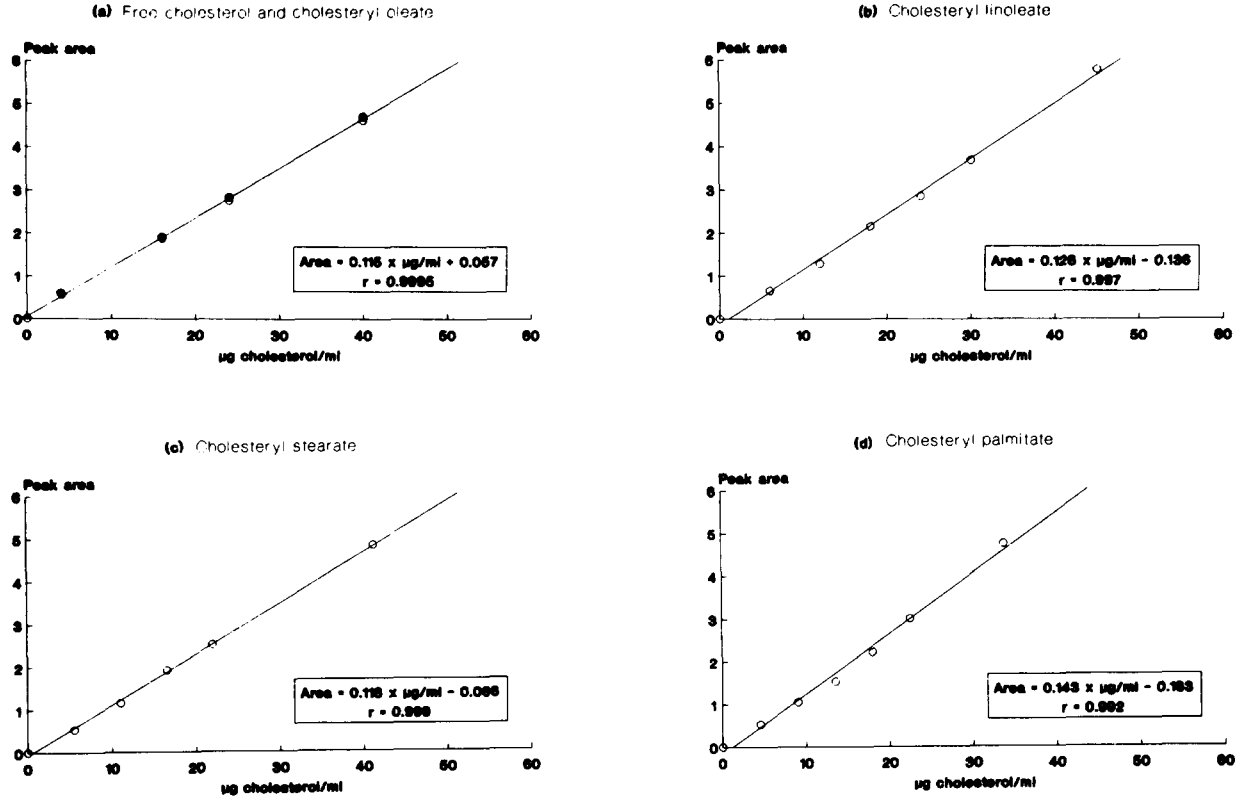

Fig. 2. Calibration curves for the quantitation of free and esterified cholesterol by HPLC. Cholestenone peak areas obtained (arbitrary units) are plotted as a function of cholesterol concentration in a standard solution. (a) Free cholesterol (○) and cholesteryl oleate (●) were used for the calibration curve. Equimolar concentrations of each lipid were used so that the concentration of cholesterol was the same at each point whether it was free or esterified. To assess linearity of the method with different cholesteryl esters, cholesteryl linoleate (b), cholesteryl stearate (c), and cholesteryl palmitate (d) were subjected to the same process.

the reaction mixture. Incubation was prolonged to 60 min in the presence or in the absence of enzymes. Lipids were extracted and analyzed by HPLC in the same way as in Experiment 1, but a 2.5 ml/min flow rate was used to elute the cholesteryl esters.

RESULTS

Detection of cholestenone and linearity

Fig. 1 shows a chromatogram of commercially available cholestenone (left) and a sample corresponding to a cell homogenate treated as for free cholesterol determination (right). It can be seen that cholestenone elutes promptly after injection (2.8 min) and without any interference. Furthermore, there are no retarded peaks that could disturb later analysis, so samples can be injected every 3 min.

As shown in **Fig. 2,** plots of cholestenone peak areas against cholesterol (free or esterified) concentration were linear within the range 0 to 40 μg cholesterol/ml, with correlation coefficients always greater than 0.990. In addition, values of peak areas were similar when identical molar concentrations of cholesterol were present, whether free or esterified with different fatty acids.

We also examined linearity and reproducibility at different cell concentrations. For this purpose, a cell homogenate was appropriately diluted with the reaction mixture to obtain different concentrations prior to the addition of the enzymes. As shown in **Fig. 3**, peak area values were linearly correlated to the dilution factor of the sample and the adjusted line passed through the point of origin.

Accuracy of the method was tested by analyzing a 1/100 dilution of the control serum Precinorm L QCS, lot no. 170101 (Boehringer Mannheim, Mannheim, Germany). The observed value was 188.2 ± 4.27 mg/dl (mean ± SD, n = 4) which is close to its assigned value (190 mg/dl) for enzymatic colorimetric methods.

Verification of cholesteryl ester hydrolysis

As shown in **Fig. 4a,** incubation of the cell homogenates with the reagents and enzymes resulted in the loss of peaks absorbing at 214 nm and the appearance of a new component at 240 nm, which indicates the total hydrolysis of all the cholesteryl esters present in the cells and the conversion of free cholesterol to cholestenone. Similar results were obtained when known cholesteryl ester standards were used instead of the cell homogenate (Fig. 4b).

Determination of free and total cholesterol in cultured cells

Human monocyte-derived macrophages were cultured for 7 days as described above and then incubated for 72 h

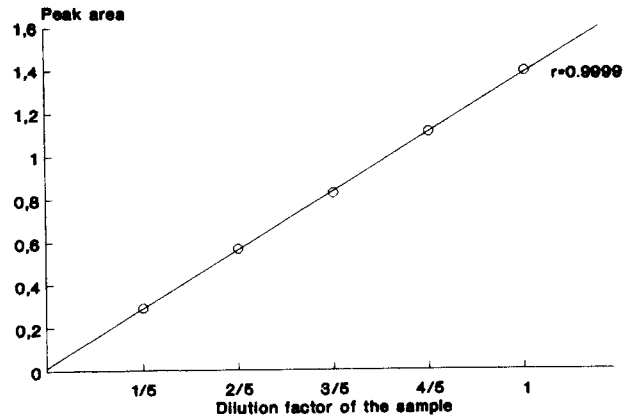

Fig. 3. Effect of sample dilution on cholesterol determination by HPLC. Cholestenone peak areas (arbitrary units) of a cell homogenate are plotted against dilution factor of the sample.

in the presence of 200 μg/ml of either LDL or AcLDL. Incubations were in quadruplicate for each condition. Then, the cells were washed and the free and total cholesterol content was determined. Esterified cholesterol was calculated as total minus free cholesterol for each sample. As expected, AcLDL increased the macrophage content of esterified cholesterol several-fold in comparison to cells incubated with native LDL, whereas only a slight elevation of free cholesterol was observed (**Table 2**). Values obtained are in close agreement with previous observations by others (14, 15).

To test the intra- and interassay coefficients of variation, a large batch of cells was dissolved in Triton X-100. Free and total cholesterol content were determined in 20 aliquots each within 1 day and analyses were repeated in triplicate over 7 more days. Calibration curves were prepared daily. The intra-assay coefficients of variation were 2.53% and 3.71% and day-to-day coefficients of variation were 5.61% and 6.52% for free and total cholesterol, respectively.

DISCUSSION

In the present report we describe an accurate, sensitive, and relatively rapid method for the quantitation of free and total cholesterol in cultured cells. It consists of the enzymatic oxidation of cholesterol to cholest-4-ene-3-one and the separation and detection of this latter product by HPLC. When total cholesterol measurement is desired, cholesteryl esters are previously cleaved by a cholesteryl ester hydrolase.

To achieve a complete cleavage of cholesteryl esters and oxidation of free cholesterol to cholestenone, both sodium cholate and Triton X-100 were needed in the medium, as well as Mg^{2+} and a buffer such as Tris, pH 7.4. Since many of these components interfere with the Lowry procedure for protein determination, cells must be sonicated

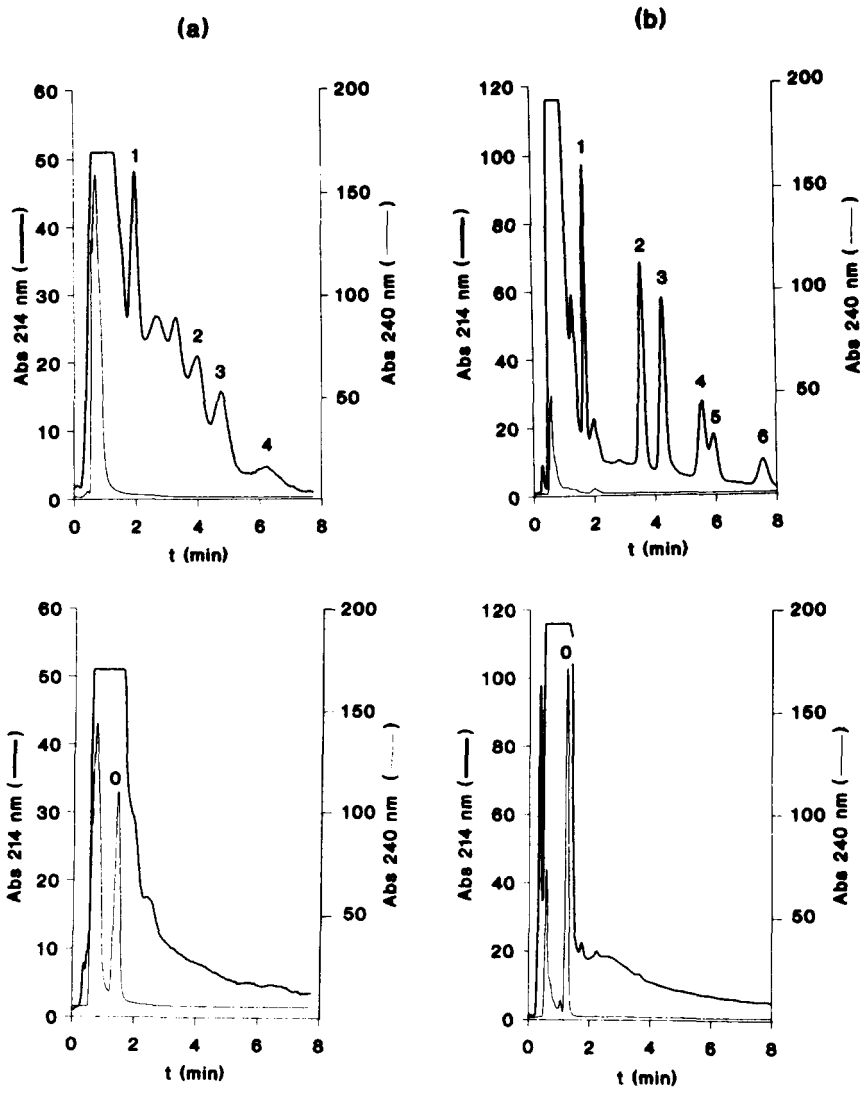

Fig. 4. Verification of cholesteryl ester hydrolysis. An extract of human macrophages (a) or a mixture of cholesterol and several cholesteryl esters (b) were incubated in the absence (top panels) or in the presence (bottom panels) of cholesterol esterase and cholesterol oxidase and then lipids were extracted and analyzed by HPLC. Peaks: 0, cholest-4-ene-3-one; 1, free cholesterol; 2, cholesteryl arachidonate; 3, cholesteryl linoleate; 4, cholesteryl oleate; 5, cholesteryl palmitate; and 6, cholesteryl stearate. (See text for details.)

in a medium containing sodium cholate alone and the rest of the components must be added to the aliquots directed to cholesterol quantitation. Sonication can be avoided by using Triton X-100 to dissolve the cells and then an alternative method for protein determination, such as the Micro-BCA kit from Pierce, which is not affected by the detergent, must be used.

The main source of error of enzymatic methods for the determination of total cholesterol is the efficiency of cholesteryl ester cleavage (16). To assess the extent of this hydrolysis and oxidation of the different cholesteryl esters in the conditions described herein, several experiments were undertaken. First, treatment of a cell extract for total cholesterol determination apparently resulted in the complete hydrolysis of the cholesteryl esters present in the cells, and subsequent oxidation of the resulting free cholesterol to cholestenone, as judged by the disappearance of the peaks absorbing at 214 nm and the appearance of the

TABLE 2. Content of free and esterified cholesterol in human monocyte-derived macrophages

Lipoprotein Added	Cellular Cholesterol	
	Free	Esterified[a]
	μg/mg cell protein	
LDL	24.6 ± 1.6[b]	8.0 ± 1.6
AcLDL	27.2 ± 1.8	41.1 ± 4.8

[a]Calculated as total minus free cholesterol for each well.
[b]Values correspond to the average ± SD of quadruplicate wells.

new component at 240 nm. This was clearly observed for the main cholesteryl esters in macrophages, e.g., cholesteryl oleate and cholesteryl linoleate (Fig. 4a) (8). Since saturated cholesteryl esters (palmitate or stearate) are the most insoluble in aqueous solutions, and thus the most inaccessible to the esterase, we tested the efficiency of their cleavage directly with a mixture of purified cholesteryl esters that contained cholesteryl arachidonate, linoleate, oleate, palmitate, and stearate. Results shown in Fig. 4b demonstrate that all cholesteryl esters were completely hydrolyzed regardless of the acyl group. On the other hand, equivalent cholesterol concentrations of different purified cholesteryl esters gave similar cholestenone values (Fig. 2). Therefore, with the proposed reagent concentrations and incubation conditions, the two enzymatic reactions were complete, and thus the final concentration of cholestenone in the tube corresponds quantitatively to the original amount of cholesterol in the sample.

Our routine calibration curves were of free cholesterol and cholesteryl oleate separately in concentrations ranging from 0 to 40 μg cholesterol/ml (though the method is linear at least up to 80 μg/ml, data not shown). This range is wide enough to hold samples with a cholesterol content up to 400 μg cholesterol/mg cell protein, provided that they are dissolved at a rate of 0.1 mg cell protein/ml.

The high sensitivity of cholestenone detection by HPLC makes this method very suitable for cholesterol determination in cultured cells, since it allows determination of cellular cholesterol corresponding to as little as 5 μg of cell protein. This is of special interest for cells that are difficult to obtain in great quantities. We have estimated that 5×10^3 cells are sufficient for the determination of free and total cholesterol by this method. We have applied the method for the measurement of cholesterol content in human monocyte-derived macrophages and the values obtained are in close agreement with values observed by others (14, 15), both in cells exposed to native LDL and in cells overloaded with cholesterol by incubation with AcLDL.

Among the advantages over other chromatographic procedures the following are worth mentioning: *1)* No lipid extraction is needed, which avoids the use of internal standards, and even sonication can be avoided if Triton X-100 is used for cell solubilization. *2)* Detection is made at a wavelength at which cellular components do not interfere. This means clean and easily integrable chromatograms. *3)* The chromatographic process is very short, taking only 3 min per sample, which contributes to the rapidity of the method.

In conclusion, the proposed method allows the quantitation of free and total cholesterol of cells in culture in a sensitive and rapid way. With it, it is possible to quantify the content of free and esterified cholesterol of less than 5000 cells and one person can easily run 30 samples in one day.

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