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Concurrent quantification of cellular cholesterol, cholesteryl esters and triglycerides in small biological samples Reevaluation of thin layer chromatography using laser densitometry

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Abstract

Absolute specificity and high accuracy is required for the quantitation of cholesterol, cholesteryl esters and triglycerides in small biological samples, particularly in a limited number of cells. Both can be achieved through thin-layer chromatography and molybdatophosphoric acid staining, while the shortcomings of traditional spot detection are overcome by laser densitometry. The major advantage of the proposed technique is the concurrent assay of nanogram quantities of cholesterol, cholesteryl esters and triglycerides. Our assay is at least ten-fold more sensitive than common thin-layer chromatography-based techniques and at least four-fold more sensitive than common enzymatic methods. The present low-cost assay is highly reproducible and may be particularly suitable for the routine lipid analysis of a 10% aliquot of relatively small tissue and cell samples, equivalent, for instance, to $\geq 10^4$ human monocytes.

Keywords: Cholesterol; Cholesteryl esters; Triglycerides; Fatty acids

1. Introduction

Cell biology and modern pathophysiological research in the human demand the specific analysis of cellular lipids such as cholesterol, cholesteryl esters and triglycerides in tiny samples. Conventional enzymatic or colorimetric assays, while quite suitable for classical clinical chemistry are of limited sensitivity or specificity, and the major lipid classes have to be determined separately. This increases sample size as well as labor and cost. Highly sensitive techniques for lipid quantitation with high specificity such as high-performance liquid chromatography (HPLC), gas chromatography (GC) and mass spectroscopy (MS) require extensive sample preparation and may not be suited for routine analysis. Thin layer chromatography (TLC), however, provides a cost effective, specific and sensitive assay for lipids, provided that spot detection and quantitation can be adequately standardized. Densitometers with conventional light sources have replaced manual scraping and thereby have greatly enhanced the potentials of TLC. However, reflectance densitometry of TLC plates suffers from high noise levels because of the granular nature of the chromatography media [1]. Since reflectance methods only see the surface of the TLC plate they also

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fail to detect and correct for heterogeneous distribution of the samples in the gel layer. Accurate and sensitive transmission densitometry of TLC plates, however, requires a laser source because of the high background. Sensitivity was further increased by staining the lipids with molybdatophosphoric acid. We reduced the detection limits for cholesterol, cholesteryl oleate, and triolein to the low nanomolar range which is approximately 100-fold lower than reported for charring with sulfuric acid [2] and many other staining techniques for lipids [3]. Simple regression methods for standard curves are presented and limitations in the quantitation of cholesteryl esters and triglycerides are discussed.

2. Experimental

2.1. Reagents

All chemicals were of the highest purity commonly available. Organic solvents, cholesterol, cholesteryl stearate, cholesteryl linoleate, and all triglycerides were bought from Fluka (Buchs, Switzerland). Cholesteryl oleate, cholesteryl palmitate, cholesteryl arachidate and cholesteryl arachidonate were purchased from Sigma (Buchs, Switzerland), and molybdatophosphoric acid hydrate from Merck (Darmstadt, Germany).

2.2. Isolation of human monocytes

Mononuclear cells were isolated from whole blood donations of six apparently healthy normolipidemic middle-aged male volunteers between May and September 1995. Monocytes were purified by density gradient centrifugation as described by Jungi et al. [4] except that autologous serum was used for cell culture instead of AB serum. Following overnight culture, cells were dislodged by chilling for 60 min at 4°C and vortexing the flasks. Three cell samples (10^5 cells) were extracted and their lipid content analyzed. The final cell population consisted of 70–88% monocytes characterised by non-specific esterase staining.

2.3. Lipid extraction

Lipids were extracted by a modification of the method described by Bligh and Dyer [5]. Briefly, the volume of the cell suspension was adjusted to 800 μl of water and 3 ml methanol–dichloromethane [2:1, v/v, 0.001% 2,6-di-*tert.*-butyl-4-methylphenol (BHT)] were added. The sample was vortexed vigorously. Phase separation was achieved by adding 1 ml dichloromethane and 500 μl water. The aqueous phase was reextracted twice with 1 ml dichloromethane. The combined organic phase was evaporated under nitrogen at 40°C and stored at −20°C.

2.4. Thin-layer chromatography

TLC was performed according to a modification of the method described by Christie [2]. Lipid samples were resuspended in 100 μl methanol–methylene chloride (1:1, v/v), sealed and kept on ice to avoid evaporation. Samples were spotted on TLC plates (SIL G-25, layer thickness: 0.25 mm, Macherey-Nagel AG, Oensingen, Switzerland; band width: 5 mm, distance between bands: 6 mm) with a CAMAG Linomat IV (Muttenz, Switzerland). All TLC plates were run in methanol prior to use in order to minimize background staining. The first separation was performed in heptane–diethyl ether–acetic acid (70:20:4, v/v) at a length of 100 mm from the origin. Plates were then allowed to dry at room temperature. In order to separate triglycerides and cholesteryl esters the plates were redeveloped in heptane at a distance of 150 mm from the origin and dried at room temperature. Developing chambers were allowed to equilibrate for 15 min, but no paper lining was used for gas room saturation. Spots were visualized by molybdatophosphoric acid staining [6] and heating of TLC plates at 120°C for 7 min. The optical density of the plates was analyzed with a Personal Laser Densitometer (Molecular Dynamics, Sunnyvale, CA, USA). Data was collected in high resolution mode with a 50-μm pixel size (200 data points/cm) and a 12-bit signal resolution (4096 levels of density resolution). Spot integration was performed with Image Quant® Software supplied with the laser densitometer. During the integration process, an optical density (OD) was assigned to

every pixel of the computer image. We outlined areas of interest with rectangles. Only areas circumscribed by the rectangles were integrated. We determined background values for each rectangle with the Image Quant local background option. Briefly, the program calculated the background of an area of interest as the average OD of all pixels forming the rectangular perimeter of that area. This background value was subtracted from the OD value of each pixel in the area of interest. Finally, the sum of all background-corrected pixel values within an area of interest was assigned to the spot or blank.

2.5. Data analysis

Standard curves were fitted with the curve fit program supplied with the SigmaPlot graphics program (Jandel Scientific, San Rafael, CA, USA) which uses the Marquart–Levenberg algorithm.

3. Results

3.1. Calculation of standard curves

The regression of standard curves improved significantly when identical rectangles were used for the integration of spots of the same lipid standard. The correlation coefficients of standard curves also increased significantly when we used the local background procedure instead of a single background value for all spots. The local background procedure took into account local variations in the staining of a plate and therefore provided more accurate estimates for the background of each spot. In areas that did not contain any lipid, e.g., blanks, this procedure can yield a negative result if the average pixel value of the integration area perimeter is greater than the average pixel value of the integration area itself.

Standard curves for nanogram quantities of lipid, i.e., 10–125 ng/spot (Fig. 1A, $n=8$) were calculated by linear regression through the origin [$f_1(x)=ax$]. In all cases the mean correlation coefficients for cholesterol, cholesteryl oleate and trioleate were 0.998, 0.996 and 0.998, respectively, or better.

Standard curves for lipid levels in the microgram range, i.e., 0.25–2.5 μg lipid/spot (Fig. 1B, $n=6$)

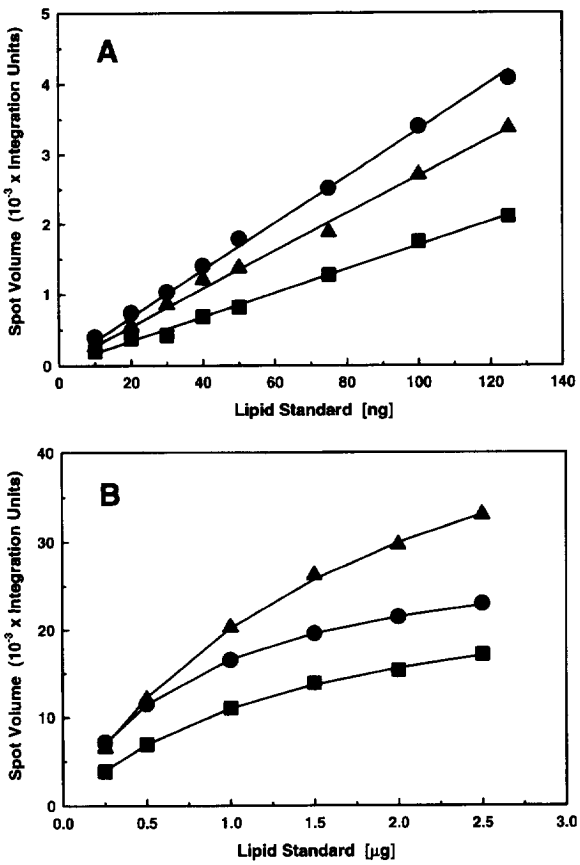

Fig. 1. Standard curves for cholesterol, cholesteryl oleate and trioleate; 5–50 μl of a mixture of cholesterol (●), trioleate (■), and cholesteryl oleate (▲) were spotted on a TLC plate. The concentration of each standard in the mixture was 2.5 μg lipid/ml (panel A) and 50 μg lipid/ml (panel B), respectively. TLC plates were developed, stained and analyzed as described under Section 2. Standard curves (solid lines) were obtained by linear regression through the origin (panel A) and hyperbolic regression (panel B), respectively.

were fitted to the hyperbolic function $f_2(x)=bx/(c+x)$. In all cases the mean correlation coefficients for cholesterol, cholesteryl oleate and trioleate were 0.999.

3.2. Sensitivity

TLC plates with 5 through 50 ng lipid/spot were developed and stained. In addition to the six lanes used for standards, areas of interest were also

Table 1
Reproducibility of reference sample analysed on different days

Aliquot No.	Cholesterol ($\mu g/10^5$ cells)	Cholesteryl esters[a] ($\mu g/10^5$ cells)	Triglycerides[b] ($\mu g/10^5$ cells)
1	0.261	0.444	0.482
2	0.275	0.421	0.495
3	0.301	0.389	0.585
4	0.310	0.426	0.575
5	0.279	0.447	0.451
Mean±S.E.	0.285±0.009	0.425±0.010	0.518±0.027

Human monocytes were isolated from a healthy normolipidemic male donor as described under Section 2. Five cell samples with 105 cells/sample were prepared. The dried lipid extracts were resuspended in 100 μl methanol–methylene chloride (1:1, v/v) and 10-μl samples were spotted on TLC plates. The lipid content of each cell sample was determined individually and on different days within 2 weeks. The values represent five independent determinations.
[a] Determined as cholesteryl oleate equivalents.
[b] Determined as triolein equivalents.

marked with identical rectangles in three lanes that did not contain any lipid samples. Since integration of these blanks may yield negative values (see Section 2), absolute values for the three blanks were averaged in order to estimate the noise of our measurement. We defined the limit of detection for our assay as the value or signal that is 3-fold greater than its corresponding noise. Detection limits were 5 ng for cholesterol, 10 ng for cholesteryl oleate, and 20 ng for trioleate.

3.3. Reproducibility and lipid recovery

Six identical samples with 5 μg of cholesterol, cholesteryl oleate and trioleate, respectively, were prepared from the same stock solution. The samples were extracted on the same day and spotted on the same TLC plate as described under Section 2. We obtained fair coefficients of variation (recovery in percent±SE): cholesterol 1.6% (100.2±1.6), cholesteryl oleate 1.5% (94.6±1.4), and trioleate 2.5% (99.5±2.4). When a reference sample of human monocytes was repeatedly analyzed on different days (Table 1), we found the following coefficients of variation: cholesterol 3.2%, cholesteryl ester 2.4%, triglycerides 5.2%.

To test the recovery of our procedure we supplemented human monocytes with lipid standards and determined the lipid content of these samples. The lipid recovery was within the above mentioned coefficients of variation, i.e., practically quantitative (Table 2). Spot purity was verified by two-dimensional TLC. Lipid extracts of 10^5 human monocytes were separated by TLC as described above. Plates were than developed in the second dimension with either toluene–diethyl ether (95:5, v/v) or with methanol–methylene chloride–water (2:1:0.5, v/v). As expected, in neither case were further lipids

Table 2
Recovery of lipids from samples supplemented with 0.5 μg of cholesterol, cholesteryl oleate and trioleate

	Cholesterol [$\mu g/10^5$ cells]	Cholesteryl oleate [$\mu g/10^5$ cells]	Trioleate [$\mu g/10^5$ cells]
Monocytes	0.361±0.010	0.600±0.045	0.837±0.084
Monocytes+ lipid standards	0.828±0.030	1.160±0.069	1.362±0.048
Recovery	96±4%	105±10%	98±10

Human monocytes were isolated and their lipid content was determined as described under Section 2. Six cell samples with 10^5 cells/sample were prepared, three of which were supplemented with 0.5 μg cholesterol, 0.5 μg cholesteryl oleate and 0.5 μg trioleate each, prior to lipid extraction. The dried lipid extracts were resuspended in 100 μl methanol–methylene chloride (1:1, v/v) and 10 μl samples were spotted on TLC plates. Recovery of extracted lipids was calculated (100%=μg lipid/10^5 monocytes+0.5 μg lipid standard) and is given in percent. Results are given as the mean±S.D. of a representative experiment.

detected by laser densitometry in the cholesterol, triglyceride or cholesteryl ester lanes.

3.4. Intensity of staining depends on degree of unsaturation

Molybdatophosphoric acid is a nonspecific stain for reducing substances [6]. Although it is a sensitive stain for lipids the intensity of staining may vary with the number of double bonds in the lipid molecules. We therefore tested the effect of the degree of unsaturation on the staining of the most common cholesteryl ester and triglycerides found in humans. The relative staining intensities of cholesteryl ester and triglyceride standards compared to cholesteryl oleate and triolein, respectively. They were constant over the concentration range tested (Table 3). However, the lipid mass of cholesteryl palmitate, stearate, and arachidate spots were underestimated by 19%, 16%, and 21%, respectively (Table 3). The amount of lipid in cholesteryl linoleate spots was overestimated by 13%. Cholesteryl arachidonate stained as efficiently as cholesteryl oleate. When the relative intensity of staining was calculated for equimolarity rather than equal lipid mass we obtained 0.78 for cholesteryl palmitate, 0.84 for cholesteryl stearate, 1.14 for cholesteryl linoleate, 0.83 for cholesteryl arachidate, and 1.04 for cholesteryl arachidonate.

In contrast, the relative intensity of staining of triglyceride standards varied greatly (Table 3). Tristearin which contains no double bonds did not stain at all, neither did trimyristin, tripalmitin nor triarachin (not shown). The lipid content of trilinolein and trilinolenin spots was overestimated 2.5-fold and 3.2-fold, respectively, compared to triolein. At equimolarity the relative intensity of staining was 0 for tristearin, 1.00 for triolein, 2.44 for trilinolein, and 3.18 for trilinolenin.

Interestingly, trilinolein, composed of n-6 fatty acids with a total of 6 double bonds, stained 2.4-fold more intensely than triolein, composed of three n-9 fatty acid moieties with a total of three double bonds. Despite the three additional double bonds of the three γ-linolenic acid moieties of trilinolenin, the intensity of staining increased by less than 40% compared to trilinolein. Thus, the staining intensity of triglycerides is not a simple function of the number of double bonds, but may also depend on their intramolecular position.

3.5. Lipid analysis of human monocytes

The results of the lipid analysis of monocytes from six normolipidemic donors are shown in Table 4. The triplicate analysis shows good reproducibility for cholesterol and cholesteryl esters, with standard deviation of the triplicates between 4–8% and 2–

Table 3
Intensity of staining and R_F values of cholesteryl esters and triglycerides as a function of fatty acid chain length and degree of unsaturation

	R_F Value	Relative intensity of staining
Cholesteryl palmitate	0.68	0.81±0.07
Cholesteryl stearate	0.69	0.84±0.05
Cholesteryl oleate	0.68	1.0
Cholesteryl linoleate	0.66	1.13±0.02
Cholesteryl arachidate	0.70	0.79±0.05
Cholesteryl arachidonate	0.65	1.01±0.04
Tristearin	N.D.	0
Triolein	0.39	1.00
Trilinolein	0.36	2.46±0.22
Trilinolenin	0.33	3.23±0.40

Lipid standards were applied to a TLC plate at 1, 2, 3 and 4 μg/spot. The plate was developed and stained as described under Section 2. For each lipid concentration the intensity of staining of cholesteryl esters and triglycerides was determined as the relative integration value of each spot compared to the value the corresponding cholesteryl oleate and triolein spot, respectively. Results are given as the mean ±S.D. of three experiments.
N.D.= No spot was detected.

Table 4
Lipid levels in human monocytes

Donor	Cholesterol [μg/10^5 cells]	Cholesteryl esters[a] [μg/10^5 cells]	Triglycerides[b] [μg/10^5 cells]
1	0.419±0.032	0.600±0.011	0.377±0.040
2	0.498±0.036	1.325±0.083	0.351±0.068
3	0.335±0.018	0.741±0.013	0.514±0.049
4	0.513±0.021	0.941±0.051	0.534±0.032
5	0.304±0.022	0.259±0.033	0.268±0.056
6	0.385±0.026	0.182±0.017	1.411±0.037
Mean	0.409	0.675	0.409
Range of means	0.304–0.513	0.182–1.325	0.268–0.534

Human monocytes were isolated from six healthy normolipidemic male donors as described under Section 2. Cells (10^5) from each blood sample were extracted in triplicate. The dried lipid extracts were resuspended in 100 μl methanol–methylene chloride (1:1, v/v) and 20-μl samples were potted on TLC plates. Results are given as the mean ±S.D. of the triplicates.

[a] Determined as cholesteryl oleate equivalents.

[b] Determined as triolein equivalents.

13% respectively. The triglyceride values showed higher variations (standard deviation 9–21%). The reason for this higher variability is unclear.

All measured values were at least 3-fold greater than the detection limit determined for the corresponding lipid. Also, we only applied 20% of each sample to the TLC plate leaving 80% for other determinations, such as phospholipids, lipophilic vitamins etc. Theoretically, lipid analysis could therefore be performed in as few as 7000 monocytes with this assay, but this was not tested experimentally.

Cholesterol and triglyceride levels in monocytes of the six donors revealed relatively small (2-fold) differences between individuals. In contrast, cellular cholesteryl ester levels appeared to vary greatly (up to 8-fold) between the 6 individuals tested. We observed no striking correlation between cellular cholesteryl ester levels and total plasma cholesterol (not shown). Thus, factors determining cholesterol ester levels in human monocytes have yet to be investigated in more detail.

4. Discussion

4.1.1. Specificity

In routine analyses, only enzymatic assays have a sufficient specificity to determine cholesterol, cholesteryl esters, and triglycerides in whole cells or cell homogenates. However, commercially available cholesterol and triglyceride kits, although easy to use, require one assay per lipid of interest, increasing the cost and time of analysis. Furthermore, enzymatic assays are prone to interference due to the heterogeneous nature of the sample. Ascorbic acid, for example, is a substrate for most peroxidases [7]. In our hands, commercially available enzymatic assays underestimated cholesterol and triglyceride levels by 20% and 80%, respectively, when the ascorbic acid concentration in human serum was increased to 1 m*M* by in vitro supplementation. Monocytes have an intracellular ascorbic acid concentration of >3 m*M* [8]; i.e., 60-fold higher than the desirable plasma levels of ≥50 μ*M* vitamin C [9]. Ascorbic acid in monocytes may therefore be an important source of error in the determination of cellular lipid levels by peroxidase-based assays. Potentially, other antioxidants, e.g., glutathione, might interfere with the intermediary H_2O_2 formation as well. Interfering water-soluble substances can be eliminated by extracting the lipids prior to the enzymatic assays as described by Heider and Boyett [10]. Lipid-soluble substances, e.g., α-tocopherol, ubiquinol, carotinoids, polyphenols, many pharmacological agents, etc., are concentrated in lipophilic phases such as membranes, organelles and lipoproteins. They could still be present in lipid extracts and may interfere with peroxidase-based assays. The risk of lipid-soluble interferences is practically eliminated if the lipid extract is further separated by TLC and individual lipids are visualized by nonenzymatic

staining. We chose the molybdatophosphoric acid stain because of its high sensitivity for reducing lipids [6]. Compared to cholesteryl oleate the intensity of staining of the other cholesteryl ester standards varied only a little, irrespective of the number of double bonds or the chain length of the fatty acid. Comparison of lipid content of a cholesteryl ester spot from a biological sample, to the cholesteryl oleate spot of the lipid standard, will therefore yield a good estimate of the cholesteryl ester content of that sample. The accuracy of the present technique in determining cholesterol as well as cholesteryl ester concentrations of small biological samples may be related to the fact that the cholesteryl moiety is the major contributor to the staining of cholesteryl esters.

The determination of the triglyceride content of the same samples, however, may not be as accurate because of the variable staining of different triglyceride species. Results should therefore be expressed as triolein equivalents rather than as triglyceride mass. Nevertheless, significant differences in triolein equivalents between samples are more likely to reflect a change in total triglyceride mass than a change in fatty acid composition. That is due to two reasons: oleic acid is one of the major fatty acids found in human polymorpho- and mononuclear cells [12]. It constitutes 48.1% of the total fatty acids in adipose tissue and 39.6% of fatty acids in the triglyceride fraction of human plasma [13]. Only a major change in fatty acid composition will therefore result in a similar major change in staining intensity as observed for pure triolein and trilinolein. Furthermore, in a given population the fatty acid composition of cells or tissue can be expected to vary only marginally with regard to the major fatty acids. The content of the major fatty acids in human lymphocytes differs by less than 10% in both young and old males [14]. Between young and old males the difference in concentration of any single major fatty acid was less than 20%. Even dietary supplementation with α-linolenic acid does not alter the concentration of any of the major fatty acids in serum and monocytes by more than 20% [15]. Dietary n-3 fatty acid supplementation reduced the stearic acid and arachidonic acid content in human monocytes from 21.1% to 16.5%, and 22.6% to 15.0%, respectively, of total fatty acids [16]. The content of the other major fatty acids remained unchanged while homo-γ-linolenic, eicosapentaenoic and docosahexaenoic acid increased by 1–2% each. Even this physiologically important change in fatty acid composition would not result in a major increase in triolein equivalents unless the triglyceride mass of the monocytes had changed as a result of the diet. A significant change in triolein equivalents (e.g., 50% or more) is therefore a strong indicator of a change in total cellular triglyceride mass. Nevertheless, it would be prudent to either confirm that the measured effect was not caused by a major change in fatty acid composition or to determine the triglyceride mass by a different procedure.

4.1.2. Sensitivity

Our assay was optimized with regard to quantitative yields and to high sensitivity. The detection limits were 5 ng for cholesterol, 10 ng for cholesterol oleate and 20 ng for triolein. The enzymatic cholesterol assay described by Heider and Boyett [10], to our knowledge, is the only enzymatic assay for cholesterol with a sensitivity in the picomolar range. However, reliable cholesterol measurements can only be performed on samples with more than 50 pmol or 19 ng of cholesterol [10], which is still 4-fold more than required by our method. Also, three separate measurements per sample are required for the determination of cholesterol, cholesteryl ester and triglycerides with enzymatic assays. Not only would this significantly increase cost and labor, but material could be too scarce in typical cell specimens for three independent measurements.

In our assay, lipid extraction, TLC and a sensitive lipid stain provided high sensitivity and outstanding specificity without the use of costly enzymatic reagents. Also, our method allows for the simultaneous, i.e., less tedious, determination of several lipids in a single sample. Finally, the previous laborious spot scraping and elution became superfluous with the use of the laser densitometer. Spot volumes were determined directly on the TLC plate and compared to the respective standard spots. The use of an internal standard becomes obsolete because sample loss during the extraction and spotting process is negligible. We found that simple linear regression $[f_1(x)=ax+b]$ yielded satisfactory approximations of the standard curves for low lipid mass

levels within a narrow mass range. Better fits were obtained with the hyperbolic function [$f_2(x)=bx/(c+x)$], particularly if a wide range of volumes was spotted for any given standard or standard mix. The reason for the observed deviation of the Lambert–Beer law is unclear, but may be due to the light scattering nature of the TLC plates [11].

The presented assay is versatile and easily transferable to the analysis of other lipids, such as phospholipids, with comparable standard errors (results not shown). The reproducibility of the assay (Table 1) is high and allows for the detection of statistically significant and potentially biologically important differences in the cellular lipid levels between individuals (Table 2).

4.1.3. Lipids in monocytes

Although the accumulation of cholesteryl esters in lipid-accumulating monocytes (foam cells) is the most characteristic pathological alteration, the uptake of cholesterol and triglycerides might modulate atherogenesis as well. The accurate assay of all neutral lipids in monocytes is therefore a prerequisite for the study of atherogenic foam cells in cell culture as well as in circulating monocytes of atherosclerosis-prone subjects [17]. We are aware of only one report that investigated the lipid composition in whole cell extracts of human monocytes [18]. Unfortunately, data were only reported as μg cholesterol (or cholesteryl ester)/mg protein, without an indication of the ratio of cell number/mg protein (or ideally of the lipid/DNA ratio) of the samples. Also, cells were incubated in serum-free medium for 18 h prior to harvest and extraction. A comparison of values reported earlier with our results from circulating monocytes is difficult at best. The previously reported cholesterol/cholesteryl ester mass ratio of 11:1 differs markedly from the ratios (2:1 to 1:2.5) that we observed in our cell samples. This difference may be a direct consequence of the lipid-free environment to which the monocytes were subjected prior to analysis in the reported study. To our knowledge the present study is the first on cholesterol, cholesteryl esters and triglycerides in whole cell extracts of circulating human monocytes that are purified in the presence of autologous serum lipoproteins.

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