paper on methodology

Quantification in the subnanomolar range of phospholipids and neutral lipids by monodimensional thin-layer chromatography and image analysis

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Abstract A sensitive, rapid, and simple method is presented for the simultaneous quantitation of the major phospholipids and neutral lipids using an image analyzer after separation by one-dimensional thin-layer chromatography. A clear-cut separation of the lipids was achieved on one EDTA-impregnated chromatoplate with five step-wise developments. An image was acquired, and the integrated optical densities of the individual spots were quantitated by a camera-equipped image analyzer against an internal standard of cholesteryl formate. Calibration curves for each lipid followed linear or hyperbolic functions permitting quantification of as little as 0.015 nmol of lysophosphatidylcholine and as much as 7.50 nmol of triglyceride from a single application. Quantitation of the major lipids in human plasma by TLC and image analysis and standardized enzymatic methods were well correlated. The method is suitable for routine analysis of biological samples having lipid profiles as different as rat hepatocyte subcellular fractions, and very low density lipoproteins secreted by rat hepatocyte suspensions.—**Ruiz, J. I., and B. Ochoa.** Quantification in the subnanomolar range of phospholipids and neutral lipids by monodimensional thin-layer chromatography and image analysis. *J. Lipid Res.* 1997. **38:** 1482–1489.

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The most popular methods to quantify phospholipids (PL) in biological materials involve their chromatographic isolation, followed by colorimetric assay of some products of specific reactions (1–3). Although the sensitivity of these techniques satisfies most of the needs of lipid biochemists, the number of operational steps required to obtain the analytical result is a major drawback. Existing lipid separation techniques, including pre-packed column separation (4, 5), colorimetric (6, 7) and fluorimetric (8) procedures and solubilization of detergent–dye complexes (9), are generally time consuming and unsuitable for routine analysis of the major lipids in small amounts of biological samples. To increase the rate of analysis, two methods have been developed recently: Iatroscan (10, 11) and high-performance liquid chromatography (HPLC) (12, 13). Iatroscan is a thin-layer chromatography (TLC) technique in which lipid classes are separated on quartz rods impregnated with silica and detected by a flame-ionization detector (10, 11). Though this method is satisfactory for neutral lipids, it is inadequate for the analysis of polar lipids; moreover, calibration of the system poses a serious problem. Many common polar and neutral lipids are well separated by HPLC, but difficulties with the resolution of some lipid classes remain, and the complexity of the system, standardization, and detection are serious disadvantages. Lipids have traditionally been separated by TLC for its simplicity and rapidity, and the variety of solvent systems available makes it suitable for most purposes. TLC separations are usually carried out using simple development systems that necessitate different chromatoplates to estimate neutral and polar lipids. Recently, image analyzers have improved the possibilities of in situ quantification, in comparison to the classical densitometric scanning (14–21).

Abbreviations: apo, apolipoprotein; BSA, bovine serum albumin; CE, cholesteryl ester; CF, cholesteryl formate; CLn, cardiolipin; DG, diglyceride; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; FC, free cholesterol; FFA, free fatty acid; HEPES, N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]; HPLC, high performance liquid chromatography; IOD, integrated optical density; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, phospholipid; PS, phosphatidylserine; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SPH, sphingomyelin; TG, triglyceride; TLC, thin-layer chromatography; VLDL, very low density lipoprotein.

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We present herein a new combination of solvent systems for the resolution of the major phospholipids and neutral lipids in one-dimension on one chromatoplate. Subnanomolar concentrations of the individual lipids can then be quantified from a single application with a camera-equipped image analyzer, provided that an internal standard is included in each application and calibration curves for all lipids under estimation are included in each plate. A complete lipid analysis of 24 samples takes no more than 90 min. The main advantages of the present method over other conventional procedures are operational simplicity, rapidity, and sensitivity, with the maintenance of excellent reproducibility. The concentrations of total cholesterol, triglycerides, and phospholipids in human plasma obtained by this method and by standardized enzymatic methods correlated well. This method is suitable for routine use. We present data using this technique on the lipid composition of some common rat hepatocyte subcellular fractions, and very low density lipoproteins secreted by rat hepatocytes cultured in suspension.

MATERIALS AND METHODS

Materials

Standards for cholesteryl oleate, triolein, diolein, free cholesterol (FC), and oleic acid were obtained in 99% purity from Nu-Chek Prep Inc. (Elysian, MN). Sigma Chemical Co. (St. Louis, MO) supplied standards for phosphatidylethanolamine (PE), phosphatidylcholine (PC), lysophosphatidylcholine (LPC), all from egg yolk; phosphatidylserine (PS) and sphingomyelin (SPH) from bovine brain; phosphatidylinositol (PI) from soybean, and cardiolipin (CLn) from bovine heart; cholesteryl formate (CF), bovine serum albumin (BSA) (essentially fatty acid free), soybean trypsin inhibitor, Dulbecco's modified Eagle's medium (DMEM), phosphorylase *b* from rabbit muscle, and Cab-O-Sil (fumed silica). Collagenase A was from Boehringer (Mannheim, Germany). TLC precoated silica gel G-25 plates (20 × 20 cm, 0.25 mm thick, glass-backed) were purchased from Scharlau (Barcelona, Spain) or Macherey-Nagel (Posfach, Germany). Percoll and density marker beads were from Pharmacia (Uppsala, Sweden) and reagents for electrophoresis from Bio-Rad (Richmond, CA).

Apparatus

For acquisition and processing of an image of the TLC plate, an image analysis system Bio Image equipped with a Kodak Videk Megaplus digital camera and a commercial software Whole Band Sun View from Bio Image Corporation (Ann Arbor, MI) was used.

Standard assay procedure for lipid separation and quantification

Separation of PL and neutral lipid species was performed on one EDTA-impregnated TLC plate using five developing solvent mixtures in the same direction. The plate was impregnated with 1 mM EDTA, pH 5.5, by an ascending development, and dried in the air overnight and then at 110°C for 1 h. EDTA-treated plates can be stored for several months. Before separation, the plate was washed overnight in chloroform–methanol–water 60:40:10 (v/v/v) in the same direction as the impregnation, and dried under a stream of hot air and activated at 110°C for 30 min. Portions of 1 μl of each sample in toluene were spotted 1 cm from the edge of the plate in 0.3 cm bands. Thus, 34 samples, 0.2 cm apart, can be applied to a standard 20 × 20 cm plate. Applications were first concentrated as fine bands with chloroform–methanol–water 60:40:10 (v/v/v), allowing the solvent to move to 1 cm, and dried by directing a hot air stream towards the glass back of the plate, ensuring complete removal of water. Plates were developed in a step-wise fashion in chambers saturated with *i*) chloroform–methanol–water 65:40:5 (v/v/v) to 2 cm; *ii*) ethyl acetate–2-propanol–ethanol–chloroform–methanol–0.25% KCl 35:5:20:22:15:9 (v/v/v/v/v/v) to 5 cm; *iii*) toluene–diethyl ether–ethanol 60:40:3 (v/v/v) to 7.5 cm; *iv*) n-heptane–diethyl ether 94:8 (v/v) to 10.5 cm; and *v*) pure n-heptane to 12.5 cm. A thorough drying of the plate between developments is crucial. After TLC, lipids were charred according to Bitman and Wood (22), by dipping the plate in a solution of 10% cupric sulfate (w/v) in 8% phosphoric acid (v/v) for 10 sec. After wiping dry the glass back, the plate was thoroughly dried under a stream of hot air until the lipid spots became evident and immediately heated at 200°C for 2 min. An image of the plate was acquired with an image analyzer equipped with a high resolution camera operating in transmissive mode, and was digitalized in 1024 × 1024 pixels. At the beginning of each session, the light emission was calibrated (equalization step), and the size and optical density for each pixel was assigned using a 24-well grey scale template (calibration step) following the supplier's instructions. Spots were quantified as integrated optical densities (IOD) against an internal standard of cholesteryl formate (0.2 μg/μl), which had been included in every application. Standards for each of the lipid classes to be quantitated were applied to every plate in the range given in Table 1, and the calibration curves were constructed by plotting the IOD of the lipid standard, corrected by the IOD of cholesteryl formate, versus the amount of lipid loaded.

Isolation and incubation of rat hepatocytes and preparation of subcellular fractions

Hepatocytes were isolated by collagenase digestion (23) from female Sprague-Dawley rats (180–200 g), housed in a temperature- and light-controlled room (light from 07.00 to 19.00) and maintained on a standard diet ad libitum with free access to water. Cells were routinely 90–95% viable as assessed by the Trypan blue exclusion test and by lactate dehydrogenase cell retention (24). Hepatocytes (1 g) were incubated at a cell density of 5×10^6 cells/ml, in DMEM containing 2.5% BSA, 20 mM Na HCO_3, and 10 mM N-[2-hydroxyethyl]-piperazine-N′-[2-ethanesulfonic acid) (HEPES), pH 7.4. After 4 h at 37°C under continuous gassing with O_2/CO_2 (19:1), the cells were pelleted (50 g_{av} for 3 min) and the medium was removed and set aside for isolation of VLDL. Cells were washed with ice-cold 0.25 M sucrose, pH 7.4, and homogenized in 6 vol of 0.25 M sucrose, pH 7.0, with 20 strokes of a Teflon pestle in a Elvejhem-Potter homogenizer. Microsomes were obtained by differential centrifugation (500 g_{max}, 10 min; 22,000 g_{max}, 10 min; 105,000 g_{max}, 60 min, twice). The supernatant from the first 105,000 g_{max} centrifugation was retained as the cytosol. The lysosomal fraction was obtained from the 22,000 g_{max} pellet according to Yamada, Hayashi, and Natori (25) with some minor modifications. Briefly, the pellet was suspended in 0.25 M sucrose, 20 mM HEPES, pH 7.0, and 1 mM $CaCl_2$ to a total volume of 5.5 ml. This crude lysosomal fraction was thoroughly mixed with 4.5 ml of isotonic Percoll (9 ml of Percoll plus 1 ml of 0.25 M sucrose buffered with 20 mM HEPES, pH 7.0) and centrifuged at 44,000 g_{max} for 90 min. The upper 8 ml were discarded, and the 1 ml remaining at the bottom of the tube containing the lysosome-enriched cell fraction was collected, diluted with 0.25 M sucrose, 20 mM HEPES, pH 7.0, and washed twice at 10,000 g_{max} for 30 min. All centrifugations were performed at 4°C.

Isolation of VLDL secreted by hepatocyte suspensions and apolipoprotein (apo) B determination

Medium samples from cell incubations were adjusted to a density of 1.006 g/ml with solid KBr, and a preservation cocktail (final concentrations of 0.02% ethylene diaminetetraacetic acid (EDTA), 0.02% NaN_3, and 0.1 mM phenylmethylsulfonyl fluoride) was added. The samples were centrifuged for 18 h at 10°C at 105,000 g_{av}. The white uppermost band was then collected and designated VLDL. ApoB-100 and apoB-48 were separated by SDS polyacrylamide slab gel electrophoresis after concentration of the VLDL by adding 5 mg/ml Cab-O-Sil (26). Apolipoproteins were solubilized from the Cab-O-Sil pellet by boiling in 75 μl of an extraction buffer containing 2% SDS and 8 M urea (pH 6.8) for 2×15 min and separated on a 2–6% polyacrylamide gradient gel. The protein bands were stained with Coomassie Brilliant Blue R-250 and quantitated with a Bio-Image image analyzer using phosphorylase *b* as a standard. Proteins from cell homogenates and subcellular fractions were quantified by the dye-binding method of Bradford (27) using BSA as the reference protein. Lipids from VLDL and subcellular fractions were extracted twice following the method of Folch, Lees, and Sloane Stanley (28).

RESULTS AND DISCUSSION

The method described provides a relatively simple, sensitive, and reliable means of determining the content of the major lipids in samples of very different origin by a combination of monodimensional separation of lipids on one TLC plate and image analysis of the individual spots. A reduced-scale photograph of a representative chromatoplate showing the separation of lipids from rat hepatocyte homogenates and standards is depicted in **Fig. 1.** Five lipids of low polarity, CE, TG, DG, FC, and FFA, together with the internal standard CF were well separated. A clear-cut separation of the seven major phospholipids CLn, PE, PI, PS, PC, SPH, and LPC was also achieved. With this method, phosphatidylglycerol migrates just above PE. Separation of CLn from PE with the combination of solvents used here is not achieved unless the plate is impregnated with EDTA, as described. The final development with pure n-heptane separates hydrocarbons from cholesteryl esters. Rapidity, accuracy, and low cost are the major features of the separation protocol presented. The separation of all the lipids mentioned takes no more than 1 h. Another major advantage is that aliquoting of the sample for separation of polar and nonpolar lipids with two TLC systems is avoided, whereby the major phospholipids and neutral lipids are quantitated from a single application of the lipid extract.

Image analysis gives more accurate quantification of the whole spot than densitometric scanning procedures, and moreover, problems derived from light detection through a scattering medium are greatly overcome (29, 30). In addition, the speed of analysis is greatly improved. Calibration curves for each neutral lipid (CE, TG, and FC) or phospholipid (CLn, PE, PI, PS, PC, LPC, and SPH) class to quantitate are shown in **Fig. 2.** For each lipid, the dependence of the IOD, corrected by the IOD of the internal standard, on the

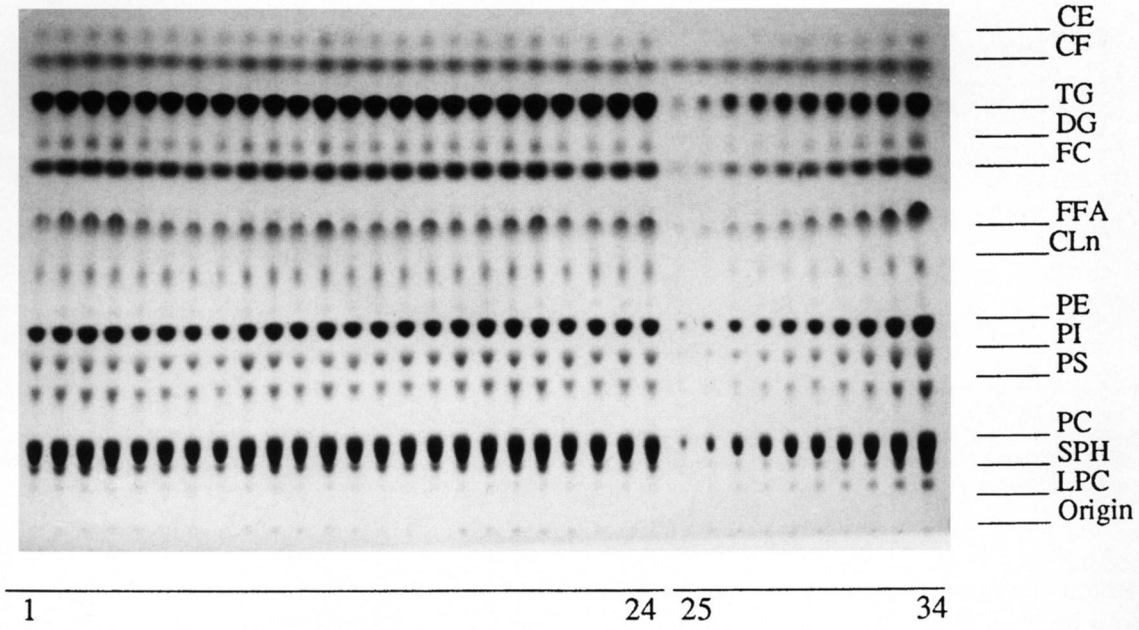

Fig. 1. Representative thin-layer chromatogram of neutral lipids and phospholipids. A lipid extract portion (1 μl) from homogenates of eight different rat hepatocyte populations (lanes 1–8) was spotted in triplicate (lanes 9–16 and 17–24) on one EDTA-impregnated TLC plate. The lipid standards in the ranges shown in Table 1 were spotted in lanes 25–34. The plate was developed and the lipids were charred according to the procedures described in the Materials and Methods Section.

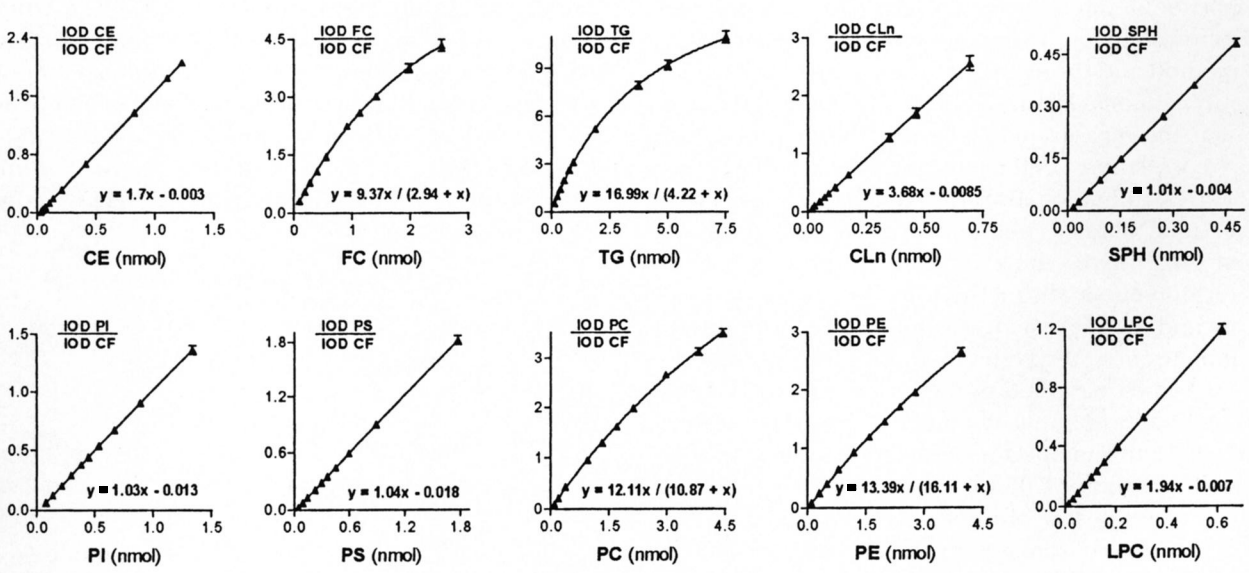

Fig. 2. Calibration curves for neutral lipids and phospholipids. The application of the standards for CE, TG, FC, CLn, PE, PI, PS, PC, SPH, and LPC in various concentrations on EDTA-impregnated TLC plates, the development of the plates, and the quantitation of the lipid spots were performed as described in the standard assay procedure subsection. For each lipid class, the integrated optical densities, relative to those of the internal standard, were plotted versus the amount of lipid loaded. The concentration of the internal standard cholesteryl formate was kept constant to 0.2 μg. Each calibration curve with SD was calculated from 130 curves accumulated over 4 months.

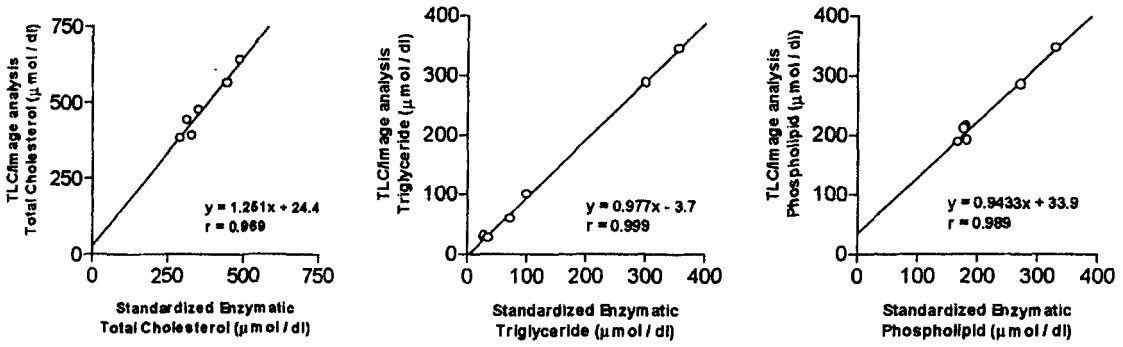

Fig. 3. Comparison of the lipid composition of human plasma as quantified by TLC/image analysis and by standardized enzymatic assays. Aliquots of human plasma (n = 6), obtained from the Cruces Hospital blood bank (Baracaldo, Spain), were assayed in the standardized lipid laboratory at Cruces Hospital for TG (Unimate 7 TRIG, Roche, Spain), total cholesterol (Unimate 7 CHOL, Roche, Spain), and phospholipids (PL Menagent, Menarini Diagnostics, Italy), using a Hitachi 911 autoanalyzer and the Decission level 2 and 3 from Beckman as references. Other aliquots were quantified for TG, FC, CE and individual PL according to the procedure described in the standard assay procedure subsection.

amount of lipid followed a linear or hyperbolic function. It must be pointed out that, though the charring method adopted here was designed by the authors to minimize the IOD dependence on the fatty acyl constituent of lipids (22), biologically derived standards should be used for calibration. Reproducibility of the method is excellent, with an intra-assay coefficient of variation (%) not exceeding 5% (3 replicates containing 8 different amounts of each lipid) and an inter-assay coefficient of variation less than 8% (130 calibration curves performed over 4 months, Fig. 2). Additionally, the accuracy of this method was demonstrated by the comparison of values for total triglycerides, cholesterol, and phospholipids in human plasma obtained using this method and those obtained using standardized enzymatic methods. **Figure 3** shows the good agreement between the measurements by the two methods, with linear correlation coefficients (r) between 0.969 and 0.999. Determination by TLC–image analysis yielded higher total plasma cholesterol values than those obtained obtained by the enzymatic method. A positive bias for non-enzymatic methods has been reported previously and, as suggested, this could be partly due to incomplete cholesteryl ester hydrolysis (i.e., see ref. 31). Lipids can be quantified in the ranges shown in **Table 1.** As only a tiny quantity of sample is needed to perform the analysis, this method is suitable for routine analysis of small amounts of biological materials, and it also allows the acyl profile of individual lipids to be studied by HPLC or gas chromatography after their resolution (J. I. Ruiz, unpublished observation). Quantitative data for DG and FFA were not obtained in this work. However, DG quantification in liver cells following the standard assay procedure here described and using 1,2-dioleylglycerol to construct the calibration curve was reported by us elsewhere (32).

We present data using this TLC–image analysis method on the lipid composition of different subcellular fractions from rat hepatocytes, and VLDL secreted by rat hepatocytes cultured in suspension. **Table 2** shows the defined lipid profile of hepatocyte fractions, which is basically in accordance with those reported for rat liver subcellular fractions (i.e., see refs. 12, 33, 34). The same classes of lipids were found in lysosomes, cytosol, microsomes, and cell homogenate, except CLn, which was only detected in the homogenate because of its mitochondrial origin. PL were the major lipid constituents in lysosomal and microsomal fractions and cell homogenates, scoring about 90 mol % (the sum of nmol of all PL/mg protein divided by the total nmol/mg protein for neutral lipids plus phospholipids), whereas neutral lipids accounted for more than 60 mol % (the sum of nmol of all neutral lipids/mg protein divided by the total nmol/mg protein for neutral lipids plus phospholipids) in the cytosol. The relative contri-

TABLE 1. Range of detection for the major phospholipids and neutral lipids

Lipid	nmol
Cholesteryl esters	0.03–1.23
Triglycerides	0.10–7.50
Free cholesterol	0.06–2.53
Cardiolipin	0.02–0.69
Phosphatidylethanolamine	0.10–3.95
Phosphatidylinositol	0.08–1.33
Phosphatidylserine	0.05–1.78
Phosphatidylcholine	0.09–4.43
Sphingomyeline	0.015–0.48
Lysophosphatidylcholine	0.015–0.62

The application of each lipid standard on EDTA-impregnated TLC plates, the development of the plates, and the quantitation of the lipid spots were performed as described in the standard assay procedure subsection. Data represent the minimum and maximum amount of lipid that can be quantified accurately in one spot.

TABLE 2. Lipid composition of the homogenate and the lysosomal, cytosolic, and microsomal subcellular fractions from isolated rat hepatocytes

Component	Cellular Fraction			
	Homogenate	Lysosomes	Cytosol	Microsomes
Neutral lipids	(100)[a]	(100)	(100)	(100)
CE	2.6 ± 0.4 (5.6)	4.5 ± 0.9 (24.0)	3.7 ± 0.6 (7.0)	2.0 ± 0.1 (3.1)
TG	27.1 ± 2.5 (58.7)	7.1 ± 1.6 (38.0)	45.1 ± 4.1 (85.6)	23.4 ± 2.3 (36.1)
FC	16.5 ± 1.3 (35.7)	7.1 ± 2.1 (38.0)	3.9 ± 0.3 (7.4)	39.4 ± 3.7 (60.8)
Phospholipids	(100)	(100)	(100)	(100)
PE	73.5 ± 7.9 (25.4)	62.9 ± 5.7 (36.7)	8 ± 0.8 (24.5)	117 ± 10 (21.5)
PI	25.1 ± 1.5 (8.7)	14.4 ± 3.0 (8.4)	2.9 ± 0.3 (8.9)	52.4 ± 6.6 (9.7)
PS	10.3 ± 1.4 (3.5)	4.9 ± 1.1 (2.9)	2.1 ± 0.1 (6.4)	23.1 ± 2.0 (4.3)
PC	164 ± 15 (56.5)	81 ± 12 (47.2)	18 ± 1 (55.2)	321 ± 33 (59.2)
SPH	12.2 ± 1.6 (4.2)	6.9 ± 1.9 (4.0)	1.4 ± 0.2 (4.3)	27.1 ± 3.6 (5.0)
LPC	1.1 ± 0.15 (0.4)	1.4 ± 0.4 (0.8)	0.2 ± 0.01 (0.6)	1.6 ± 0.4 (0.3)
Cln	3.7 ± 0.15 (1.3)			

The preparation and lipid analysis of the cell fractions were performed according to the procedure described in the Materials and Methods Section. For quantification, the value of the ratio IOD of the lipid/IOD of cholesteryl formate was interpolated in the corresponding calibration curve that is included for all lipids under estimation in each plate. Values are given as nmol/mg protein and represent means ± SE of five independent preparations.

[a]The values in parentheses are mol % values of each lipid class with respect to neutral lipids or PL.

bution of CE, TG, and FC to neutral lipids in the fractions studied was heterogeneous. TG was quantitatively the most important lipid in the cytosol, while FC comprised as much as 60 mol % of the total neutral lipids in the microsomes. Another notable point is the variability of the CE:FC mol ratio between the fractions, being about 1:1 in the cytosol and less than 1:15 in microsomes. Regarding the phospholipid profile, there was a higher percentage of PE in lysosomes compared to other fractions and this was compensated for by a decrease in the major phospholipid, PC. In lysosomes from alveolar macrophages and tissues of animals suffering from certain inherited or drug-induced lipid storage diseases, substantial amounts of bis(monoacylglycero)phosphate may be present (35). As interferences with the separation and/or quantification of lipids might occur, appropriate evaluation of the procedure should be performed.

It is known that rat hepatocytes in suspension secrete VLDL with characteristics that differ from those of circulating lipoproteins, showing features more similar to nascent particles (36, 37). Each VLDL particle is known to carry one copy of apoB (38), either apoB-48 or apoB-100. As shown in **Table 3,** under our experimental conditions, cell suspensions secreted almost 5-fold more lipoprotein particles containing apoB-48 than apoB-100. The whole VLDL fraction was characterized by a high level of neutral lipids and PC, the latter representing more than 75 mol % of PL. The relatively high percentage of PE and PI is also to be noted. This may be due, as suggested by Hamilton and Fielding (39), to the lack of remodelling agents in the in vitro system used. Considering the lipid and apolipoprotein data reported here for VLDL, and according to the equations given for a model particle whose components partition into two phases (40), the radius of the VLDL particles secreted by rat hepatocyte suspensions was found to average 24.0 ± 1.1 nm. This value agrees closely with those reported by other authors resulting from direct analysis (41–43).

In conclusion, we present a method that permits the quantification of the major phospholipids and neutral lipids in biological materials with marked differences in their lipid composition, based on monodimensional

TABLE 3. Lipid and apolipoprotein composition of VLDL secreted by rat hepatocytes cultured in suspension

Component	Content	Mean Mol % of Total Lipids	Mean Mol % of Neutral Lipids or PL
Neutral lipids		68.3	100
CE	0.58 ± 0.04	2.4	3.5
TG	14.40 ± 1.19	58.5	85.7
FC	1.82 ± 0.12	7.4	10.8
Phospholipids		31.7	100
PE	0.63 ± 0.11	2.6	8.1
PI	0.54 ± 0.04	2.2	6.9
PS	0.15 ± 0.04	0.6	2.0
PC	6.05 ± 0.50	24.6	77.6
SPH	0.34 ± 0.02	1.4	4.4
LPC	0.09 ± 0.02	0.4	1.1
Apolipoprotein B			
ApoB-100	109.3 ± 37.5		
ApoB-48	513.2 ± 169.7		

The preparation and analysis of VLDL were performed according to the procedure described in the Materials and Methods Section. Lipid contents are expressed as nmol/mg cell protein and apolipoproteins as fmoles phosphorylase *b*/mg cell protein secreted into the medium for a 4-h incubation period. Values represent means ± SE of five independent experiments.

separation by TLC followed by image analysis of the individual spots. The analytical procedure is relatively simple and its speed, sensitivity and reproducibility makes it suitable for those lipidologists working with a large number of samples containing very small amounts of lipid.

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