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A QUANTITATIVE DENSITOMETRIC METHOD FOR THE RAPID SEPARATION AND QUANTITATION OF THE MAJOR TISSUE AND LIPOPROTEIN LIPIDS BY HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY

I. SAMPLE PREPARATION, CHROMATOGRAPHY, AND DENSITOMETRY

GERD SCHMITZ and GERD ASSMANN*

Zentrallaboratorium der Medizinischen Einrichtungen der Westfälischen Wilhelms-Universität, Domagkstrasse 3, and Institut für Arteriosklerosеforschung an der Universität Münster, 4400 Münster/Westf. (F.R.G.)

and

DAVID E. BOWYER

Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP (U.K.)

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SUMMARY

A rapid method for the separation and quantitation of the major lipids of tissues and lipoproteins by automated high-performance thin-layer chromatography is presented. Solvent systems for one-dimensional separation of neutral lipids, of cholesteryl esters, and of phospholipids are described. Separated lipids are measured following treatment with methanolic sulphuric acid containing manganese chloride and scanned in fluorescence or absorption mode. Absolute quantitation is obtained by the use of an internal standard and by references to standards for each lipid run on the same plates as samples. The method described here is particularly suitable for the rapid quantitation of small amounts of lipid (0.01—0.02 nmol per sample), for example in tissue culture studies; 100 μg of fibroblast or macrophage protein are sufficient for complete lipid analysis. The coefficients of variation due to the sample preparation, application to the plates and densitometry are in the range 7.2—9.1%.

The method was compared with enzymatic determinations for cholesterol and gave correlation coefficients of 0.95 for total cholesterol and 0.91 for unesterified cholesterol. Phospholipid estimation was compared with large-plate thin-layer chromatography and

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phosphorus analysis and gave correlation coefficients of 0.90 for phosphatidylcholine and 0.89 for sphingomyelin.

INTRODUCTION

There is a need in many studies of tissue and lipoprotein lipids for rapid separation and subsequent estimation of small amounts of material. For example, in studies of cells in culture, where the concentration of most lipids is of the order of femtomoles (10^{-15}) per cell, unless very sensitive methods of analysis are available, large cell populations must be used. This may complicate experimental conditions. Similarly, in studies of influx and efflux of lipoprotein lipids in cells, there is a need for sensitive methods for analysing the individual lipids of both cells and lipoproteins.

Various methods have been described including enzymatic fluorimetric methods [1], gas—liquid chromatography (GLC) [2—6], high-performance liquid chromatography (HPLC) [7—9], thin-layer chromatography (TLC) with subsequent microchemical assay [10, 11], Iatroscan [12], and high-performance thin-layer chromatography (HPTLC) followed by quantitative densitometry on the plate [13—15].

TLC offers relatively simple separation systems, which principally allow inexpensive analysis of multiple samples at the same time with sufficient sensitivity. However, in situ quantification of the separated compounds on a TLC plate as done by scanning densitometry presents various difficulties and in the past has often had a poor reputation. Thus, satisfactory quantification is dependent upon consistent chromatographic separations, the homogeneity of impregnation of the plates with detection reagent and uniform background staining. In addition, the densitometric quantification of a spot depends not only on the mass of material in the spot, but also upon the area which the spot occupies, and the signal per unit mass is also related to the absorption and/or fluorescence spectra of the material and these may be different for unknowns and standards.

Due to the recent availability of HPTLC plates [16, 17], automated sample application systems working in the nanolitre range and improved separation systems (Camag linear development chamber), certain disadvantages (e.g. poor reproducibility, irregular spot shape, etc.) of TLC are minimized. The high performance of the plates ensures excellent and reproducible separation of the lipids. The total mass of material added to the HPTLC plates can be kept very low, thus reducing the dependance of the integrated signals on the spot geometry. The method is fast and the cost of expendable materials is small, giving a low cost per analysis.

We have, therefore, extensively re-investigated the technique of quantitative HPTLC and included the experiences of various published procedures [13—15] to elaborate a method which avoids most of the difficulties enumerated above and gives fast, accurate, and reproducible analysis of the major tissue and lipoprotein lipids combined with fully automated data calculation.

MATERIALS AND METHODS

Thin-layer plates, organic solvents, and lipid standards

High-performance thin-layer plates, 10 × 20 cm from Merck (Silica Gel 60, Cat. No. 5641) were used for the separation of the lipids. If a documentation by ultraviolet (UV)-sensitive films (see below) was required, plates with fluorescence indicator F 254 (Silica Gel 60 F 254, Cat. No. 5642) were used. All chromatography plates were prewashed before chromatography in a solvent with the same composition as that used for the separation. Before sample application the plates were activated in an oven for 1 h at 110° C. Drying the plates in this way markedly improved the separation. Plates were stored and handled carefully in order to avoid touching the surface and to prevent contact with dust.

Diethyl ether, acetic acid methyl ester and *n*-propanol were laboratory grade. Chloroform, methanol, *n*-hexane, and *n*-heptane were LiChrosolv quality (Merck). Satisfactory chromatography may also be achieved using freshly redistilled laboratory grade reagents. Pure lipid standards were purchased from Sigma, Munich (cholesterol No. CH 8253, triolein No. T 7502, cholesteryl formate No. C 9398, cholesteryl stearate No. C 9503, cholesteryl oleate No. C 9253, cholesteryl linoleate No. C 9003, phosphatidylethanolamine No. P 4513, phosphatidylcholine No. P 6267, sphingomyelin No. S 7004).

Sample application

For all applications of lipids to the thin-layer plates, the Camag Nanomat (Camag, Muttenz, Switzerland) was used with disposable 0.5-µl Microcap pipettes or the 100-nl and 200-nl platinum capillary. Spots were applied at a constant distance apart, 0.5 cm or 1.0 cm.

Chromatographic separation

The separation of the neutral lipids was performed as a one-step procedure with 4.5 ml of *n*-hexane—*n*-heptane—diethyl ether—acetic acid (63:18.5:18.5:1, v/v) in the Camag HPTLC linear developing chamber (Cat. No. 28520) at room temperature for 10 min.

For cholesteryl ester separation, one-step chromatography on 10 × 20 cm HPTLC plates in chloroform—*n*-heptane (40:60, v/v) was used. The separation was done in the linear developing chamber with 4.5 ml of solvent at 4° C for 15 min. Chilled solvents gave an improved separation.

Phospholipid separation was done with a one-step procedure as previously described [18], using 10 × 20 cm HPTLC plates. The solvent was acetic acid methyl ester—*n*-propanol—chloroform—methanol—43 mmol/l potassium chloride in distilled water (25:25:25:10:9, v/v). This separation was done with 4.5 ml of solvent in the linear developing chamber at room temperature for 20 min.

Detection of separated lipids

Following chromatography, spots were detected using the manganese chloride—sulphuric acid reagent containing 3.2 g of manganese chloride, 480 ml of methanol, 32 ml of concentrated sulphuric acid and 480 ml of deionized

water [19]. Following chromatography, the plates were dried for 10 min in an oven at 110°C and then immediately immersed in a tank filled with reagent for 20 sec. The plates were then placed horizontally on a paper towel to remove the excess reagent from the back. They were then placed in an oven on a PTFE block to ensure even heating at 110°C for precisely 30 min.

Documentation of separated lipids on the chromatography plate

The charred plates can be easily documented on UV-sensitive films (Technifax DZO blue) using a Technifax apparatus (Technifax, A. Leistenschneider, Düsseldorf) as follows: the back of the thin-layer plate is carefully cleaned and the plate layed on the UV-light source. A sheet of film is then placed on the coated side of the HPTLC plate and exposed for 2 min. The film is then developed in ammonia vapour for 1 min.

TLC scanning and quantitation

Quantitation of the lipids on the HPTLC plates was performed with a Shimadzu CS 910 TLC scanner (Shimadzu Seisakusho, Japan) working semi-automatically or with the fully automated Camag TLC scanner. Both instruments were interfaced with a Spectra Physics Basic integrator SP 4100 equipped with a Kerr minifile 4100 D for data storage (Spectra Physics, Darmstadt, F.R.G.).

Plates were scanned either by fluorescence excitation or by absorption. For fluorescence scanning, the emitted fluorescence light was scanned in the reflection mode. For absorption scanning in preliminary experiments either reflected or transmitted light was scanned. However, the reflection mode is far superior, because it is not dependent on slight differences in thickness and dispersion of particles of the gel and, therefore, was used in all subsequent experiments with absorption scanning.

The light sources used for fluorescence scanning were a xenon lamp (Shimadzu) or a mercury lamp (Camag). The excitation wavelengths selected by the monochromator depended on the lamp used as follows: 370 nm for the xenon lamp and 366 nm for the mercury lamp. The light sources used for absorption scanning were a xenon lamp (Shimadzu) or a deuterium lamp (Camag).

Slit conditions were selected according to the spot size. The slit height should always cover the whole spot diameter (0.4—0.6 cm). The slit width was kept constant at 0.1 mm for the Shimadzu scanner or 0.2 mm for the Camag TLC scanner. A scanning speed of 60—80 cm/min was used and gave good peak resolution.

The densitometric signals of all tracks (2—3 scans per track) were integrated in the SP 4100 Basic integrator and stored on the floppy disc. For the data manipulation and standard correction, a Basic computing program has been elaborated and is described elsewhere [20].

Internal standard correction

In this method, an internal standard was used to correct for inevitable slight losses of material during extraction and chromatography. Various internal standards were used.

Cholesteryl formate. This was obtained from Sigma, Munich (Cat. No. C 9398) and made up as a stock solution containing 10 mg/dl (24.1 nmol/l) in chloroform. The standard was added according to the lipid content of the samples being analysed, so that the mass of standard was approximately equal to the mass of the major lipid in the sample (for example, for cells such as cultured fibroblasts, about 50 μg internal standard per mg protein).

[^{14}C] Cholesteryl oleate and [^{14}C] phosphatidylcholine. These were obtained from NEN Chemicals, Munich: cholesteryl [1-^{14}C] oleate (Cat. No. NEC 638; 50—60 mCi/mmol) and L-α-[*dipalmitoyl*-1-^{14}C] phosphatidylcholine (Cat. No. NEC 682; 60—100 mCi/mmol). The volume added to each sample was sufficient to give about 5500 dpm/μl of the application volume. Radioactivity was measured directly on the plate using a Berthold TLC linear analyser Type LB 2832 (Laboratorium Prof. Dr. Berthold, Wildbad, F.R.G.) at gain 3, voltage 550 V and scanning time 180—900 sec.

Lipid extraction

Cells from tissue cultures. Medium was removed from the dishes and the cells were washed three times with 2 ml of Dulbecco's modified Eagle medium (DMEM, Flow No. 10-331-24) and once with 2 ml of Dulbecco's phosphate-buffered saline (PBS, Flow No. 18-610-54). The cells were harvested with a rubber policeman in 500 μl of PBS and transferred to a siliconized conical glass tube. The cells were sonicated with a Branson sonifier (Branson Scientific) three times for 20 sec at 30 W, in tubes chilled in ice water. Defined volumes (0.3—0.7 ml) of the sonicated samples containing 100—400 μg cell protein were delipidated by a modification of the Folch procedure [17] with 5 volumes of chloroform—methanol (2:1, v/v) containing the appropriate internal standard. The two phases were separated by centrifugation at 1100 *g* for 10 min in a laboratory centrifuge. The lower phase was transferred to a conical glass tube and the upper phase was reextracted for 1 h, with repeated vortexing, with an equal volume of chloroform—methanol—normal saline (86:14:1, v/v) to ensure a good recovery of lipids [10]. The two phases were again separated by centrifugation and the first and the second lower phases (about 3 ml + 2 ml) were mixed and evaporated at 40°C under vacuum. To avoid oxidation, butylated hydroxytoluene (Fluka, Heidelberg, F.R.G.) at a concentration of 0.005% was present in all organic solvents [21]. The dried samples were stored in a desiccator overnight and redissolved in 50 μl of chloroform—methanol (2:1, v/v) as solvent.

Medium. A defined volume of medium was subjected to ultracentrifugation at d = 1.21 g/ml potassium bromide for 48 h at 220,000 *g* using a 50.3 Ti rotor in a Beckman ultracentrifuge. The 1.21 g/ml potassium bromide supernatant was dialysed against 1 mmol/l ammonium bicarbonate, 0.01 mmol/l EDTA, pH 8.6 for 24 h. Samples were then dialysed for at least 1 h against distilled water and lyophilized. The samples were then redissolved in 1 ml of normal saline with vortexing. Protein was determined in an aliquot and 700—1000 μl containing 250—500 μg protein were delipidated after addition of an appropriate amount of internal standard as described for cells.

Lipoprotein fractions. Appropriate volumes of lipoprotein fractions obtained after ultracentrifugation were analysed for protein and 10—30 μl of lipoprotein

fractions were delipidated as described for cells with 5 volumes of chloroform—methanol (2:1, v/v) after addition of 500 μl of normal saline and internal standard (1 μg internal standard per 1—10 μg of protein depending on the lipoprotein fraction).

Other methods

Protein was determined according to the method of Lowry et al. [22]. Cholesterol and triglycerides were measured using enzymatic methods. The analysis for total cholesterol was performed with cholesterol oxidase—para-aminophenazon (CHOD—PAP) reagent (Boehringer Mannheim, Cat. No. 148 393) using a Cobas-Bio centrifugal analyser (Hoffmann-La Roche, Zürich, Switzerland). Free cholesterol was measured using the CHOD—PAP method without cholesteryl ester hydrolase (Boehringer Mannheim, Cat. No. 310 328). Esterified cholesterol was calculated by difference. Triglycerides were measured using an enzymatic method (Boehringer Mannheim, Cat. No. 475 429). Phospholipids were also measured following separation on standard TLC plates using the solvent system described above and analysis for phosphurus [23].

Mouse peritoneal macrophages were prepared as described by Ho et al. [24]; the preparation of acetyl-LDL (low-density lipoproteins) was performed according to the description of Brown et al. [4] and Goldstein et al. [25].

RESULTS

Chromatographic separation

The separation of the neutral lipids is shown in Fig. 1. It was demonstrated by co-chromatography of pure lipids that the separated spots of cholesterol, triglycerides and cholesteryl esters and the internal standard were not contaminated by other lipids such as diglycerides as may occur in certain solvent systems.

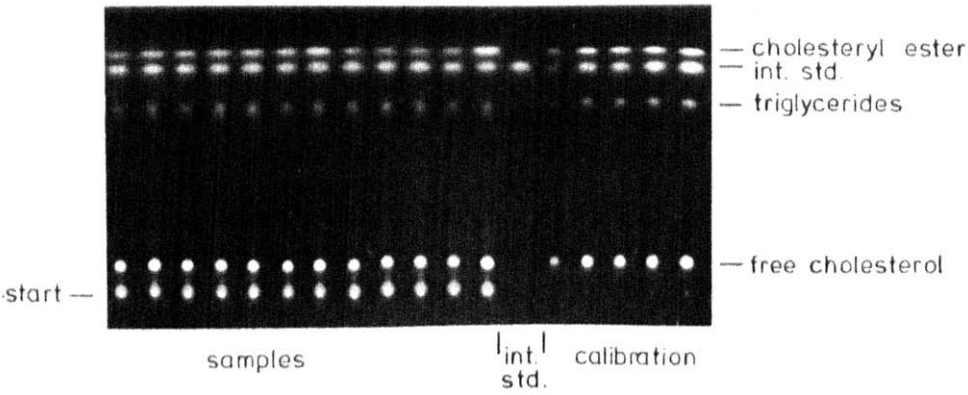

Fig. 1. Separation of the neutral lipids on HPTLC plates in *n*-hexane—*n*-heptane—diethyl ether—acetic acid (63:18.5:18.5:1, v/v). The external standards consisting of cholesterol, triolein, cholesteryl formate (internal standard), and cholesteryl linoleate for calculating the calibration curves are applied on the first five tracks (cholesterol 0.008—0.155 nmol; triolein 0.018—0.36 nmol; cholesteryl formate and cholesteryl linoleate 0.013—0.26 nmol). Track 6 contains the internal standard in the concentration as added to the samples (0.104 nmol/spot). The samples are applied on the remaining tracks. For documentation purposes, the HPTLC plates have been overloaded with the samples.

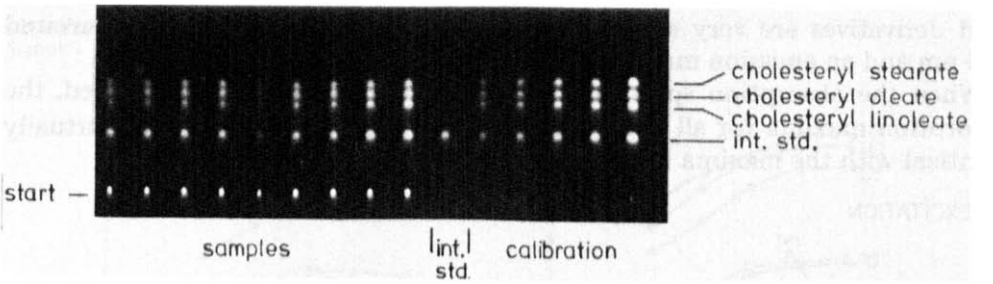

Fig. 2. Separation of the cholesteryl esters (cholesteryl stearate, cholesteryl oleate, cholesteryl linoleate, and the internal standard cholesteryl formate) on HPTLC plates in *n*-heptane—chloroform (60:40, v/v). The external standards are applied in masses between 0.039 nmol and 0.26 nmol. The concentration of the internal standard applied on track 5 is 0.104 nmol per spot. For documentation purposes, the HPTLC plates have been overloaded.

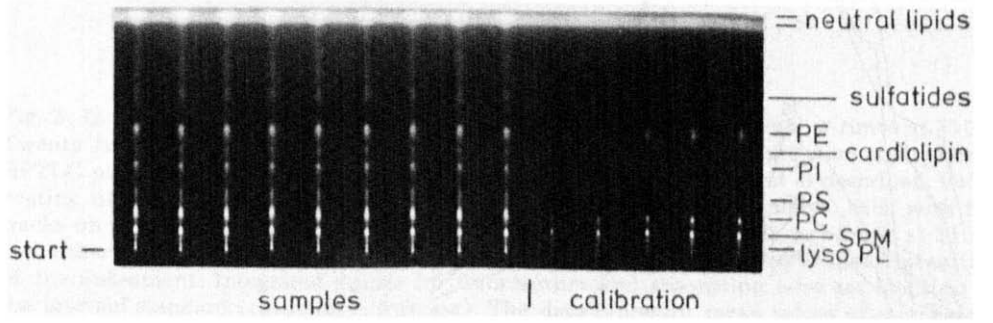

Fig. 3. Separation of phospholipids on HPTLC plates in *n*-propanol—acetic acid methyl ester—chloroform—methanol—43 mmol/l potassium chloride in distilled water (25:25:25:10:9, v/v). This chromatography system separates lysophospholipids (lyso PL), sphingomyelin (SPM), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), cardiolipin, phosphatidylethanolamine (PE), and sulphatides. The external standard consists of SPM, PC, and PE (0.039—0.39 nmol). For documentation purposes, the HPTLC plates have been overloaded.

The separation of cholesteryl esters is shown in Fig. 2. The solvent system separates the cholesteryl esters according to the degree of unsaturation of the fatty acids, thus permitting resolution of the major saturated esters, palmitate and stearate, from the major monoene, oleate and from the major diene, linoleate.

The separation of phospholipids is shown in Fig. 3. The solvent system separates lysophosphatidylcholine, sphingomyelin, phosphatidylcholine, phosphatidylserine, phosphatidylinositol, cardiolipin, phosphatidylethanolamine and sulphatides.

On each plate the external standards are applied in the expected concentration range of the samples (0.01—0.5 nmol per spot).

Fluorescence-absorption measurements

Characteristics of the fluorescent lipid derivatives. Using the described staining procedure, the in situ fluorescence characteristics of all fluorescent

lipid derivatives are very similar, all showing an excitation maximum around 366 nm and an emission maximum around 410 nm (Fig. 4).

When the absorption spectrum of the lipid derivatives was determined, the absorption maxima for all compounds were found at 366 nm. This is virtually identical with the maxima for fluorescence excitation.

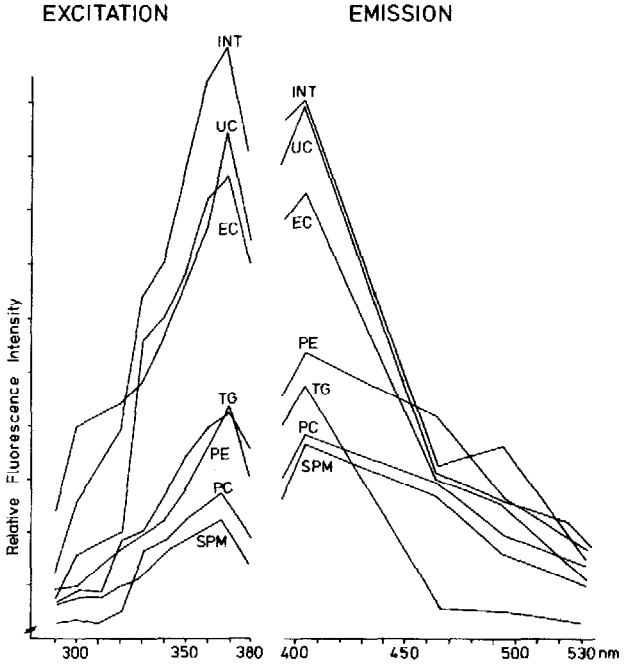

Fig. 4. In situ excitation and emission spectra of the quantitated lipids on the HPTLC plates immersed in the detection reagent for 20 sec and then heated at 110°C (see Methods) for 20 min. INT = cholesteryl formate; UC = unesterified cholesterol; EC = esterified cholesterol; TG = triglycerides; PE = phosphatidyl ethanolamine; PC = phosphatidylcholine; SPM = sphingomyelin. The HPTLC plates were measured at identical settings of the instrument.

Effect of heating time on the fluorescence intensity. Heating of the HPTLC plate after treatment with the detection reagent induces the formation of fluorescent derivatives. This process is time-dependent with the maximum effect reached after about 40 min with heating at 110°C (Fig. 5A). Longer heating time ashes the derivatives and the fluorescence ultimately decreases to zero. On the other hand, absorption (Fig. 5B) increases until it reaches a maximum at 60 min.

Comparison of calibration curves measured by fluorescence and absorption. The calibration curves for the major neutral lipids and phospholipids are given in Fig. 6 and the regression equations are shown in Table I. For all compounds whose separation is demonstrated in Figs. 1–3, there is a linear relationship between the logarithm of the integrated signal and the logarithm of the corresponding mass of compound over a wide range. At concentrations exceeding 0.3 nmol either fluorescence quenching or absorption saturation causes non-linearity of the calibration curves.

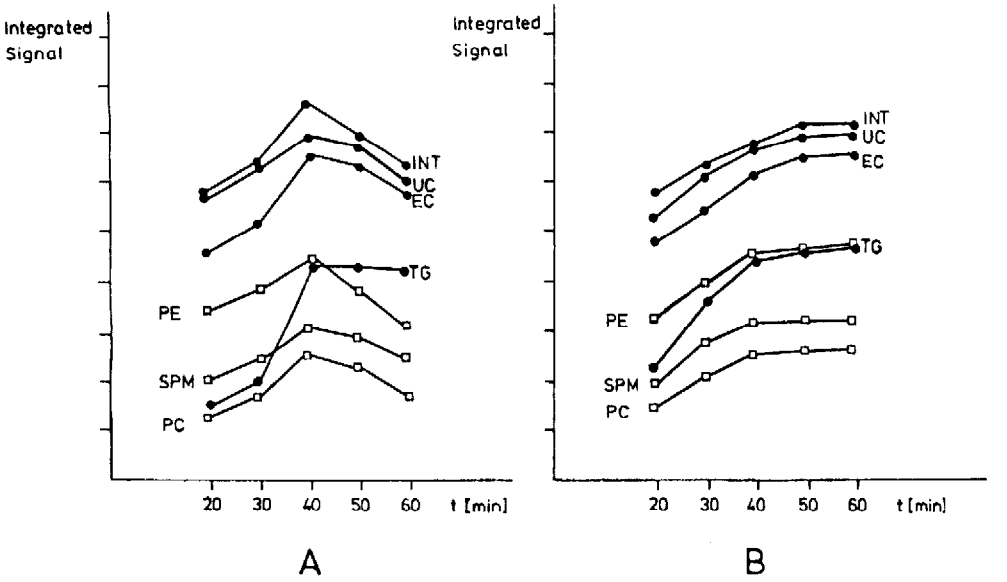

Fig. 5. In situ fluorescence emission and absorption after different heating times at 110°C. Twenty tracks with identical concentrations of a lipid standard mixture were applied on an HPTLC plate, chromatographed, and immersed in the detection reagent as described. Before heating of the plate at 110°C, the HPTLC plate was cut into five pieces, each with four tracks on it. Then the glass pieces were heated for either 20, 30, 40, 50, or 60 min at 110°C, and either fluorescence (panel A) or absorption (panel B) was measured at identical settings of the instrument. Integrated signals for fluorescence and absorption were set identical for the internal standard (cholesteryl formate). The data represent mean values of each heating time. (•), neutral lipid separation; (□), phospholipid separation. Abbreviations as in Fig. 4.

Within the cholesteryl ester class, the degree of unsaturation of the fatty acid does not significantly affect the shape of the calibration curve. Cholesteryl linoleate, oleate, stearate and palmitate exhibit practically the same response for each concentration applied and reveal identical calibration curves (not shown). However, in both the fluorescence and absorption measurement, cholesteryl formate (used as an internal standard) showed a slightly higher signal response per mass as compared to the other cholesteryl esters tested (Fig. 6, upper plots).

For triglycerides containing unsaturated fatty acids, signal intensity is slightly higher than that of the saturated homologues (not shown). Since most of the tissue and lipoprotein triglycerides contain oleic acid, triolein was used as a standard.

For phosphatidylcholine and sphingomyelin substituted with different fatty acids, the degree of unsaturation did not significantly affect the calibration curves (not shown).

Standards separated on different plates yielded almost identical shapes of calibration curves, although the absolute value of the integrated signal varied depending on the plate and the sensitivity setting of the scanner. It is possible, therefore, to calibrate each plate from a single standard. Thus, as an example the 0.1-μg standard can be applied to the plate and the sensitivity for the scanner is set to give 50,000 units of integrated signal before measuring the whole plate.

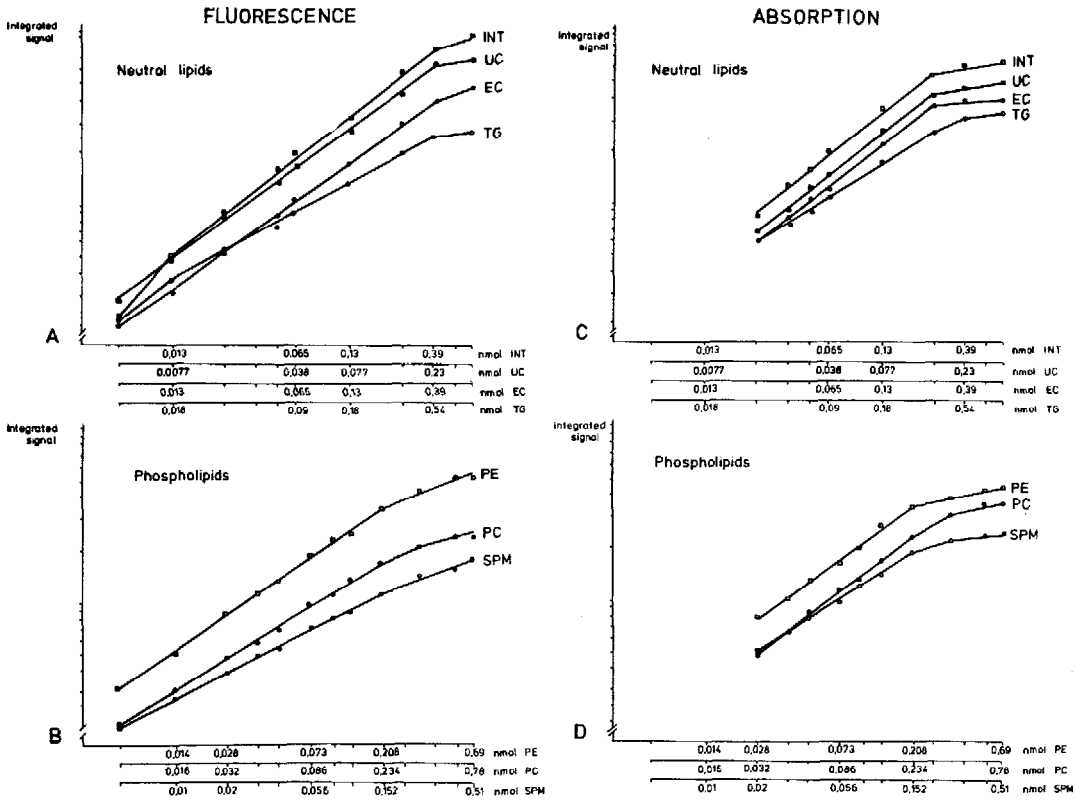

Fig. 6. Calibration curves of the lipids usually quantitated, separated on an HPTLC plate, immersed in the detection reagent for 20 sec and heated for 40 min at 110°C. The plates were measured either in the fluorescence (panels A and B) or absorption (panels C and D) mode. Panels A and C: □ = cholesteryl formate, ■ = unesterified cholesterol, ● = esterified cholesterol, ○ = triglycerides. Panels B and D: □ = phosphatidylethanolamine, ● = phosphatidylcholine, ○ = sphingomyelin. Abbreviations as in Fig. 4.

TABLE I

REGRESSION EQUATIONS AND CORRELATION COEFFICIENTS FOR CALIBRATION CURVES OF MAJOR NEUTRAL LIPIDS AND PHOSPHOLIPIDS

Compound*	Fluorescence		Absorption	
	Regression equation	Correlation coefficient	Regression equation	Correlation coefficient
INT	$Y = 0.73X + 5.12$	0.998	$Y = 0.75X + 5.08$	0.995
UC	$Y = 0.50X + 4.89$	0.987	$Y = 0.53X + 4.94$	0.981
EC	$Y = 0.61X + 4.75$	0.991	$Y = 0.71X + 4.76$	0.996
TG	$Y = 0.61X + 4.47$	0.974	$Y = 0.74X + 4.57$	0.989
PE	$Y = 0.61X + 4.73$	0.971	$Y = 0.70X + 4.83$	0.994
PC	$Y = 0.63X + 4.39$	0.988	$Y = 0.71X + 4.65$	0.977
SPM	$Y = 0.55X + 4.35$	0.991	$Y = 0.61X + 4.56$	0.973

*For abbreviations see legend to Fig. 4.

Internal standard correction

The internal standard technique is used to correct for inevitable slight losses of material during the extraction, sample application and chromatography. Various internal standards have been tested:

Cholesteryl formate. This standard was well separated from all naturally occurring lipids (Figs. 1 and 2). Replicate analyses of a mass of 0.1 μg applied as a series of spots to one HPTLC plate showed that the coefficient of variation (C.V.) was $< 0.9\%$ for repeated measurement of one track and $< 5.4\%$ for all tracks on one HPTLC plate.

[^{14}C] Cholesteryl oleate and [^{14}C] phosphatidylcholine. The added radioactive material was of sufficiently high specific activity not to increase the mass of the naturally occurring lipids. In preliminary experiments, the C.V. for these standards was determined using two different amounts of radioactivity (1100 dpm per spot, and 5500 dpm per spot) and counting for different times between 180 and 900 sec. It was demonstrated that the addition of 5500 dpm per sample and a counting time above 200 sec gave a C.V. of 5.8—6.0% which is in the range of that obtainable with the cholesteryl formate mass standard.

Effect of different scanning directions

With both scanners it is possible to scan separated spots either along the length of a single track (track-by-track), or across tracks (cross-track). The C.V. for track-by-track measurement (0.7—1.0%) was significantly better than for cross-track (1.2—4.0%). Hence, in subsequent experiments, track-by-track scanning was always used.

Reproducibility

When the lipids of a single sample were scanned repeatedly by the track-by-track method (see above), the C.V. for all substances was $< 1\%$. When a single lipid extract was chromatographed on twelve separate tracks on one plate, the C.V. for all lipids was between 2.1 and 5.0%. If the single lipid extract was chromatographed on separate plates, however, the C.V. lay between 4.7 and 7.6%.

In order to test the precision for the complete method of extraction and analysis, five aliquots of a single serum sample were delipidated and analysed. The C.V. was between 7.2 and 9.1%.

Correlation of HPTLC method with other analytical techniques

The HPTLC method described here was compared with conventional enzymatic analyses for cholesterol and triglyceride and with large-plate TLC and phosphorus analysis for phospholipids. The following results were obtained. HPTLC versus CHOD—PAP method: for total cholesterol $r = 0.95$, for unesterified cholesterol $r = 0.91$. HPTLC versus enzymatic triglyceride method: for triglycerides $r = 0.92$. HPTLC versus TLC phosphorus method: for phosphatidylcholine $r = 0.90$, for phosphatidylserine $r = 0.91$, for sphingomyelin $r = 0.89$.

Application to cultured cells

As an example of tissue culture lipid analysis (shown in Fig. 7), mouse peritoneal macrophages were preincubated for 24 h at 37° C with human acetyl-

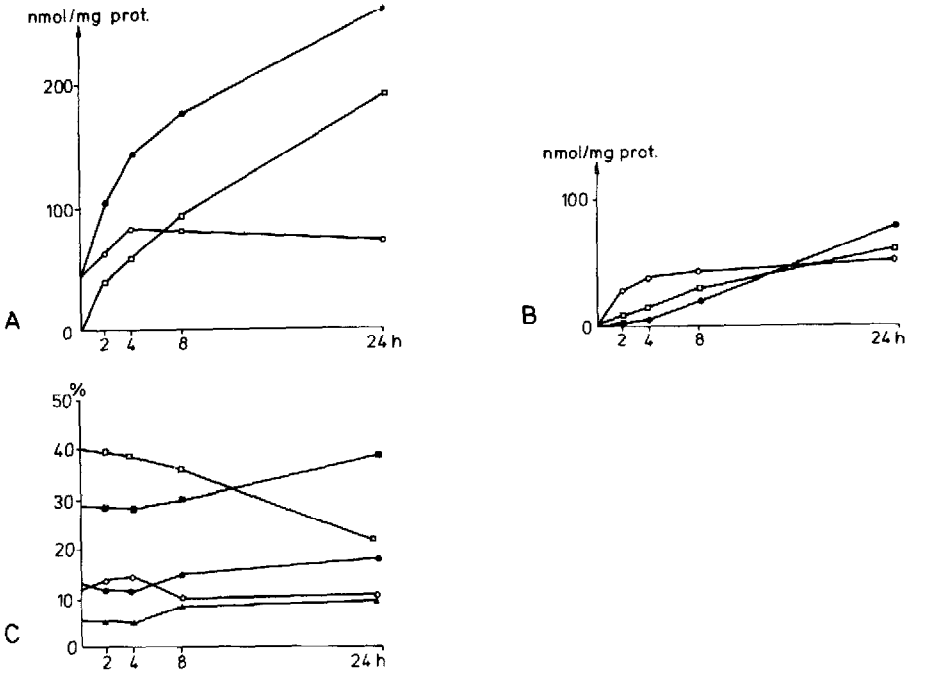

Fig. 7. Effect of incubation time on the lipid content of mouse peritoneal macrophages incubated with acetyl-LDL. Mouse peritoneal macrophage monolayers (60-mm dishes) were washed with DMEM and incubated at 37°C with 3 ml of DMEM containing 35 μg/ml acetyl-LDL for the indicated time. After incubation, two dishes were washed and harvested, and their lipid content measured. Panel A: • = total cholesterol, ○ = unesterified cholesterol, □ = esterified cholesterol. Panel B: • = cholesteryl oleate, □ = cholesteryl stearate, ○ = cholesteryl linoleate. Panel C: ■ = PC, □ = SPM, • = PE, ○ = PS, △ = PI.

LDL (35 μg protein per ml) to enrich cells with cholesterol [24, 25]. Total cholesterol increased in the cells during the incubation period due to a massive accumulation of esterified cholesterol (Fig. 7A). During the first 4 h of incubation, influx of cholesteryl linoleate was responsible for the increase in cellular cholesteryl esters (Fig. 7B). During further incubation the content of cholesteryl linoleate remained nearly constant. Whereas only small amounts of cholesteryl oleate were detectable in the first 4 h, this cholesteryl ester moiety increased and became predominant at the end of the incubation period. Free cholesterol increased in the cells in the first 4 h and did not change much in the following 20 h of incubation. In comparing the relative distribution of the phospholipids (Fig. 7C), it is evident that there was a relative increase in phosphatidylcholine and a decrease in sphingomyelin. Phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol remained nearly constant.

DISCUSSION

A method is presented for the rapid separation and estimation of major tissue and lipoprotein lipids. Separation and detection is uncomplicated and reliable, densitometry and subsequent calculation are fully automated [20]. Besides the classical solvent systems for neutral and polar lipids [26—28], we

have tested numerous published solvents with various internal standards on HPTLC plates.

For the separation of neutral lipids, due to the use of an internal standard, it was necessary to find a one-dimensional chromatography system which revealed uncontaminated spots and a good resolution of the internal standard from the naturally occurring lipids. The described system represents a suitable compromise for both these problems.

For the one-step differentiation between cholesteryl linoleate (90% of the unhydrolysed cholesteryl esters from extracellular origin) and cholesteryl oleate (most of the cholesteryl esters synthesized within the cell), the one-step solvent system used revealed optimal results. However, in complex mixtures cholesteryl palmitate can partially overlap with cholesteryl stearate.

For the phospholipid separation, the solvent system published by Vitiello and Zanetta [18] gave by far the best resolution, accuracy and precision of all one-step separations tested for cellular phospholipids. This method is sufficiently sensitive to monitor the major cellular phospholipids. Splitting of the sphingomyelin spot according to differences in the fatty acid composition did not occur using the Camag HPTLC linear chamber. The individual lysophospholipids do not contaminate the major phospholipid spots.

In our experience, one-dimensional separation of neutral lipids and phospholipids on the same plate [14] can cause contamination of the separated spots by other lipids (e.g. cholesterol contaminated by diglycerides). In addition, there are calibration problems due to large differences in concentration between individual lipid classes (for example, when cholesteryl esters are on the upper limit of the calibration curve, the phospholipids may only reach the lower limit of the calibration curve). We conclude, therefore, that the quantitative analysis of neutral and phospholipids in natural mixtures using one-dimensional HPTLC should be done on separate plates to reduce overlapping of lipid compounds.

The use of either the mass or radioactive internal standard technique improves the precision of the method if absolute values of individual lipids must be compared from different samples on different tracks and different HPTLC plates. However, this standardization is not necessary for the comparison of relative mass distribution of lipids between individual tracks as described by Touchstone et al. [15]. The advantage of a radioactive internal standard is that the ratio of standard to sample can vary over a much wider range than a mass standard without affecting the accuracy and precision of the method. A mass standard has to be present in approximately the same amount as the lipids of the sample. The advantage of a mass internal standard, however, is that it does not require a radiochromatogram scanner and can be measured with the scanning densitometer.

Chromatography was compared between ordinary tanks and the linear development system. Using the linear development system we found more stable conditions, better linearity and paralellism of sample tracks, and very low reagent consumption due to the application of a defined amount of solvent (4.5 ml per plate). For the detection of the separated lipids, we immersed the total HPTLC plate in the liquid detection reagent. We have tested various detection systems, including the ammonia vapour technique [21] which is very

time-consuming, and various spraying reagents [15, 26, 29, 30]. The manganese chloride—methanolic sulphuric acid reagent had the highest sensitivity both in reflectance and transmission mode and plates scanned after immersion revealed a much better C.V. when compared to spraying which leads to inevitable inhomogeneities of the spots.

As shown in Fig. 4, the in situ fluorescence and absorption characteristics of the various lipids are practically identical, all showing absorption and excitation maxima around 366 nm and emission maxima around 410 nm. It has been suggested [31] that fluorescence measurement is more sensitive than absorption. This is correct for measurements of the natural fluorescence of a compound, but the fluorescence of derivatives formed by reaction with a detection reagent lead to non-specific background fluroscence and hence to reduced sensitivity. We have compared the transmission and reflectance scanning mode and found a lower signal-to-noise ratio for the reflectance mode compared to the transmission mode. The transmission measurement is very sensitive to differences in layer thickness, whereas in reflectance the signal-to-noise ratio is basically determined by the surface quality of the layer [13]. In addition, we found in our studies that the transmission mode was ten times less sensitive than the fluorescence emission mode and had a smaller linear portion in the calibration curves.

Comparison of fluorescence and absorption measurements (Fig. 6 and Table I) indicates that the absorption curves have a slightly higher slope, but the useful range of the absorption measurement is more limited. The coefficients of variation for absorption and fluorescence measurements are comparable. The minimum amount detectable by the described fluorescence method is ten times lower compared with data from the literature [14]; 100—150 μg of cell protein were sufficient to obtain a complete lipid analysis.

The method described here allows the rapid separation and quantification of the major tissue and lipoprotein lipids with a high degree of sensitivity, precision, and accuracy. In particular, the method is suitable for the quantification of lipids in tissue culture studies. The idea of these studies was to give refinements and extensions (especially for cholesteryl esters) to information already published on the use of quantitative HPTLC for microlipid analysis. A computer program written in BASIC, using linear regression of logarithmically transferred data for the reduction and calculation of the data produced by this method, is described elsewhere [20].

NOTE TO READERS

The BASIC program [20] is available from the authors free of charge when a 5.25 inch standard discette is sent, together with a prepaid envelope, to our laboratory.

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