Journal of Chromatography, 143 (1977) 473–490
Biomedical Applications

CHROMBIO. 056

METHODS FOR THE RAPID SEPARATION AND ESTIMATION OF THE MAJOR LIPIDS OF ARTERIES AND OTHER TISSUES BY THIN-LAYER CHROMATOGRAPHY ON SMALL PLATES FOLLOWED BY MICRO-CHEMICAL ASSAYS

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(First received December 1st, 1976; revised manuscript received February 15th, 1977)

SUMMARY

Methods are described for the rapid separation of the major individual phospholipids and neutral lipids of tissues by thin-layer chromatography on small glass plates (75 × 75 mm), and for the specific microchemical estimation of separated lipids and for determination of fatty acid composition and radioactivity. The overall method, involving tissues extraction, thin-layer chromatographic separation and assay has been evaluated using pure standards and biological samples and gives good reproducibility and almost complete recovery of lipids.

INTRODUCTION

There is a need in many studies of tissue lipids for rapid separation and subsequent estimation of small amounts of material. This has been particularly important in our work on atherosclerosis, involving analytical and metabolic studies of individual atherosclerotic lesions from experimental animals and also organ and cell cultures. With these applications in mind, we have developed a set of methods for separation of the major individual phospholipids and neutral lipids by 2- and 3-dimensional thin-layer chromatography (TLC) on small glass plates. Separations may be made in less than 30 min and subsequent specific micro-chemical assays permit measurement of microgram amounts of each lipid, as would be obtained from a typical arterial sample of approximately 10 mg of wet tissue. The overall method has been shown to give good recovery and reproducibility for representative phospholipids and neutral lipids. It has given highly consistent separations, in our hands on over 2,000 tissue samples over a period of 4 years.

Micro thin-layer chromatography

TLC [1] is probably now the most frequently used method for the separation of lipids prior to estimation, determination of radioactivity or analysis of their constituent fatty acids. A disadvantage of commonly used 20 × 20 cm glass plates is that they may take about 1 h to run in one dimension and when two-dimensional separations are used to improve resolution, for example for phospholipid separation, the overall time for chromatography of one sample may be some hours. Much more rapid separation may be achieved by the use of smaller plates.

TLC, with plates the size of microscope slides, was first used in the early studies of Izmailov and Shraiber [2] and by Meinhard and Hall [3] and Kirchner et al. [4] which are reviewed by Mangold [5]. Since then they have found increasing use for a number of compounds, for example amino acids [6,7], peptides such as pentagastrin [8], tocopherols [9] and lipids [10—21]. Indeed for separation of lipids, Hofmann [11] recommended the technique in preference to large plates on account of its rapidity, convenience, economy and sensitivity. Despite this suggestion the small plates are still not widely used for lipid analysis. This may be partly due to the belief that resolution is compromised by small size and by the high speed of chromatography. On the contrary, the speed of migration of solvents ensures that there is minimal spreading of the spot by diffusion, giving good resolution. Furthermore, the small area occupied by the spot produces a useful increase in the sensitivity of detection, because of the higher concentration of substance per unit area. A more serious objection has been the lack of development of sufficiently sensitive methods of assay of separated substances.

When our studies were begun, Van Gent [14] had described separation of neutral lipids on small plates, and their estimation by charring, followed by scanning densitometry in situ. In extensive preliminary investigations, we found that estimation by charring either in situ or in the test tube [22—24] did not give sufficiently precise results. The charring was dependent upon the constituent fatty acids of the lipids and also upon the presence of small amounts of impurities. A similar finding has been reported for the ultrasensitive fluorimetric methods using rhodamine [18]. In view of such potential disadvantages we abandoned non-specific charring methods and developed published microchemical techniques for the tiny quantities of lipids separated on the small TLC plates.

This paper reports the details of the methods which we have developed for TLC separation and analysis and an evaluation of the recovery and reproducibility for major lipid classes.

MATERIALS AND METHODS

Reagents and glassware

The methods described are very sensitive and therefore, every precaution must be taken to ensure that glassware is clean and reagents pure. Sample vials and test tubes for assays were washed in concentrated sulphuric acid after use and reserved specially for these procedures. On no account should plastic stoppered tubes or bottles be used for samples or reagents; plasticizers are extracted by lipid solvents and interfere with chromatographic separations and analyses.

Specially made glass stoppered vials (45 mm × 5 mm I.D., vol. 2 ml, with C10 stoppers, from Camlab Glass, Cambridge, Great Britain) were used for samples of extracted lipids. Chromatography tanks were circular specimen jars or beakers with loose-fitting lids. All reagents used were AnalaR from BDH (Poole, Great Britain) or Hopkin & Williams (Chadwell Heath, Great Britain). Chloroform, methanol and petroleum ether (b.p. 40—60°) were further purified by redistillation. Diethyl ether was run through a short column of alumina (100 mesh, for chromatography, from BDH) immediately before use in order to remove peroxides. Silica gel was Camag Type DO without binder (Camag, 4132 Muttenz, Switzerland).

Pure lipids for standardisation of chromatograms were obtained as follows: Lyso-lecithin, sphingomyelin, lecithin were from Koch Light (Colnbrook, Great Britain); lyso-phosphatidyl ethanolamine was a kind gift from Dr. R.M.C. Dawson; cholesterol, cholesteryl oleate, oleic acid, glyceryl trioleate were from Sigma (London, Great Britain).

Radioactive lipid precursors for radio-labelling of lipids, to aid identification on chromatograms, were obtained from Radiochemical Centre (Amersham, Great Britain). Radioactivity was measured using a Nuclear Chicago Mk. II scintillation counter.

Preparation of silica gel for phospholipid separation

Separation of phospholipids on TLC may be improved by the addition of various substances to silica gel. In this method we have used ammonium sulphate [11,25] as suggested by Kaulen [26] for the improved resolution of phosphatidyl inositol from phosphatidyl serine. The silica gel is impregnated as follows: 200 g of silica gel are slurried in 2 l of 0.2% w/v aqueous ammonium sulphate solution and centrifuged at 60 *g* for 5 sec and the supernatant containing finings is decanted off. The silica gel is then dried by repeating the slurrying process and centrifuging at 60 *g* for 5 min three times with 500 ml of AnalaR methanol and once with 500 ml of AnalaR chloroform.

Preparation of silica gel for neutral lipid separation

The separation of neutral lipids is improved and made more reproducible by the addition of sodium carbonate to the silica gel to provide a stable, basic pH of the adsorbent: 200 g of silica gel are slurried with 2 l of 0.015 *M* (0.16% w/v) aqueous sodium carbonate solution, then allowed to stand for at least 2 h and the supernatant containing finings is decanted off. The silica gel is then dried by slurrying with methanol and chloroform as described for phospholipid plates.

Preparation of plates

Glass plates (75 × 75 mm; Hoslab, London, Great Britain) are wiped with absolute ethanol. The appropriate silica gel is slurried in chloroform and the plates coated with 0.25 mm layers using a spreader and template (Quickfit & Quartz, Stone, Great Britain). The plates are allowed to dry in air for 10 min, before being washed by running in chloroform—methanol (2 : 1). The plates are allowed to dry and stored in a dust-free environment. Immediately before use the plates are activated by heating on a hot plate at 100° for 2 min.

Extraction of tissue lipids

Tissue lipids are extracted by a modification of the Folch procedure [27,28]. The majority of lipids are removed from tissue in a "first" extraction with chloroform—methanol (2:1) at room temperature for 1 h and the residual lipid which is mainly free fatty acid, in a "second" extraction at 40° for 1 h. For tissues containing phosphatidyl inositol phosphates it is necessary to use an acidified solvent such as chloroform—methanol—1 *N* HCl (100 : 50 : 2). This is best done in the second extraction, after most of the lipids have already been extracted, because this minimises the artifactual trans-methylation of complex lipids to give methyl ester of their fatty acids [29].

In the experiments reported here both extractants were chloroform—methanol (2:1). The tissue (volume V) is disrupted, if necessary by freeze pressing [30] and placed in a weighed glass fibre filter disc (Whatman GF/A). The filter disc is previously extracted with chloroform—methanol (2:1) for 1 h at 40°, in order to remove any lipid contaminants, dried in air and stored in a desiccator over silica gel. The tissue on the filter disc is then extracted with 20 volumes (20 V) chloroform—methanol (2:1) for 1 h at room temperature; the methanol must be added separately, first. The extract is then transferred to a tap funnel, the tap of which has been previously moistened with methanol to prevent leakage of solvent. The filter disc with tissue is extracted with a second 20 volumes (20 V) of chloroform—methanol (2:1) for 1 h at 40°. The filter disc is then squeezed gently with forceps and washed with 20 volumes (20 V) chloroform—methanol (2:1) and squeezed gently again. The filter disc is then dried and re-weighed, giving the weight of dry defatted tissue. The dry residue is subsequently analysed for DNA.

The chloroform—methanol extracts are then "Folch-washed" with 0.2 volumes (0.2 × 60 V) of saline (0.9% w/v NaCl, pH 7.4). The chloroform lower phase and aqueous upper phase are separated by standing at 4° overnight or by centrifugation and the chloroform phase removed. The aqueous phase is then extracted with 40 volumes chloroform—methanol—saline (86 : 14 : 1) in order to retain fatty acid. The chloroform phases are then pooled and evaporated to dryness in a rotary evaporator in vacuo at 40°. The evaporator is always vented to nitrogen. The sample is then transferred to a small glass stoppered vial with chloroform—methanol—saline (86 : 14 : 1), and the solvent evaporated to dryness in a stream of nitrogen. This process is greatly facilitated by a special apparatus which allows evaporation of solvent from a number of samples simultaneously. These are available commercially, but we use one made in the laboratory. It consists of two separable modules of perspex. The lower module has a solid base-plate and an upper support-plate which is drilled to accept the sample vials. The upper module also has two plates. The lower holds twelve 19-gauge hyperdermic needles exactly above the middle of each sample vial. Each needle is connected to a brass gas manifold mounted on the top by means of a short length of PVC tube. Each tube passes through a hole in the upper plate where it may be restricted by a thumb screw tapped into the plastic, thus permitting independent control of the gas flow on each needle. Samples are stored in benzene at —20° until required.

Pipetting of samples onto TLC plates

Before the sample is spotted onto the plates, two lines are drawn across the silica gel, 1 cm from the top and right-hand edges to act as "stop-lines" for the solvents. The benzene is then evaporated from the sample in a stream of nitrogen, and the sample redissolved in a known volume of chloroform—methanol (2 : 1) containing ammonium hydroxide (1 ml of 0.880 sp.gr. ammonium hydroxide is added to 400 ml of solvent). A known volume of sample is measured by holding the sample in a syringe, usually 100 μl total capacity (Type 710; Hamilton Micromesure, Bonaduz, Switzerland). The sample vial is washed out with a further portion of chloroform—methanol (2 : 1) containing ammonium hydroxide, which is held in a second syringe. Samples are quantitatively transferred to plates by ejecting the required volume from the main and wash syringes with press button dispensers (Type PB 600, Hamilton Micromesure).

During pipetting of samples, the plate is held in a holder (Fig. 1) which allows a stream of nitrogen to be blown over the surface, thus aiding evaporation of the solvent and preventing oxidation of the lipids.

Two-dimensional separation of phospholipids (Fig. 2)

Chromatography tanks are lined with filter paper saturated with chromatography solvent; the papers should be changed after running about 5 plates. The solvents used are: for the first dimension, chloroform—methanol—acetic acid—water (55 : 35 : 3 : 2); and for the second dimension, chloroform—acetone—methanol—acetic acid—water (45 : 16 : 15 : 11 : 6).

The plates are run in the first dimension until the solvent reaches the stop

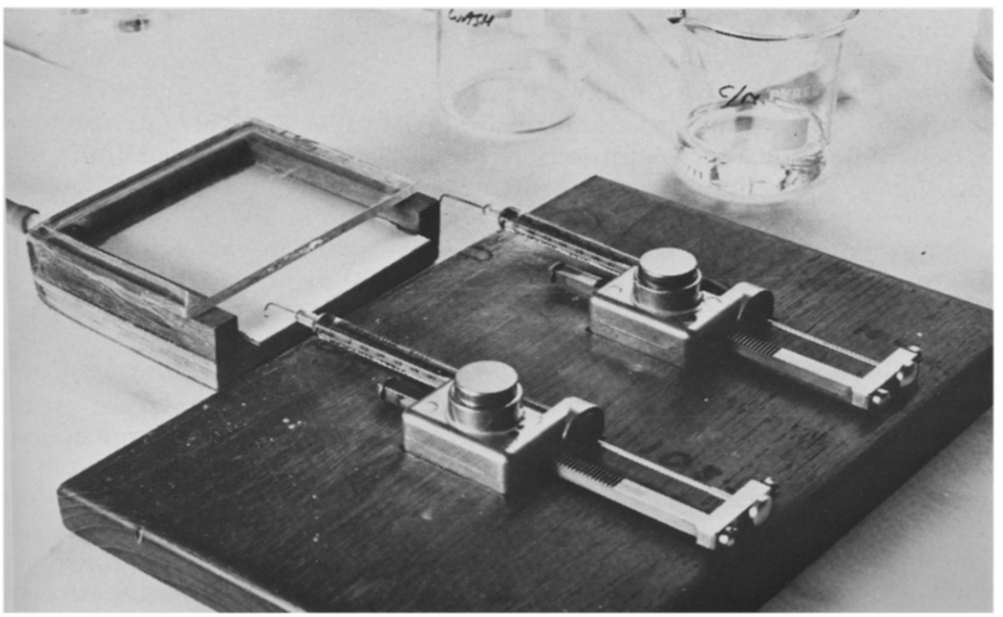

Fig. 1. On the left, a holder for a TLC plate which allows a stream of nitrogen to be passed over the plate during application of the sample, and, on the right, Hamilton syringes with dispensers for the "main" sample and "wash".

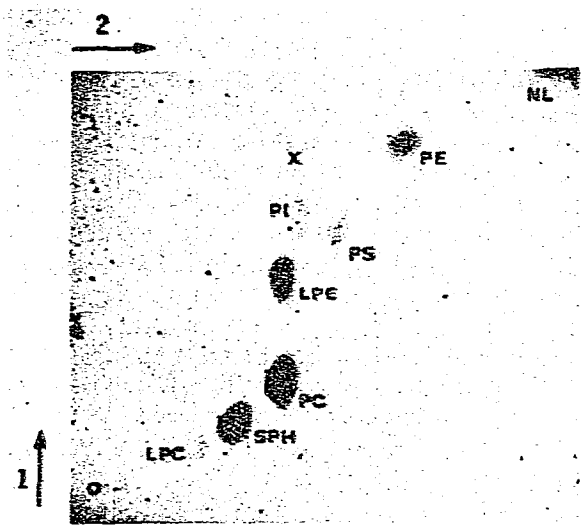

Fig. 2. Two-dimensional separation of phospholipids. See text for details. NL = neutral lipids; PE = phosphatidyl ethanolamine; X = unknown; PI = phosphatidyl inositol; PS = phosphatidyl serine; LPE = lysophosphatidyl ethanolamine; PC = lecithin; SPH = sphingomyelin; LPC = lyso-lecithin; O = origin.

line (ca. 12 min), then dried in a stream of nitrogen in a closed box for ca. 20 min. They are then run in the second dimension for an overall time of 20 min; this includes a period of ca. 10 min of "over-run" after the solvent has reached the stop line.

Two-dimensional separation of neutral lipids (Fig. 3)

Neutral lipid separations are done in unlined tanks. The solvents used are: for the first dimension, petroleum ether—diethyl ether—acetic acid (40 : 60 : 0.1); for the second dimension, petroleum ether—ethyl acetate—acetic acid (95 : 2 : 2).

Solvents are run to 1 cm from the edge of the plate in each dimension. In each case this takes about 5 min. Plates are dried as above for ca. 1 min.

"Three-dimensional" separation of neutral lipids (Fig. 4)

Occasionally it is expedient to use a "3-dimensional" separation of neutral lipids in order to improve the resolution of cholesteryl esters from methyl esters and hydrocarbons, especially when a sample containing a large concentration of these compounds is chromatographed. The third solvent is run in the opposite direction to the first as illustrated in Fig. 4. Chromatography is done in unlined tanks. The solvents used are: for the first dimension, petroleum ether—diethyl ether—acetic acid (35 : 55 : 0.1); for the second dimension, petroleum ether—ethyl acetate (90 : 4); for the third dimension, petroleum ether—ethyl acetate (90 : 2). As with the 2-dimensional separation, the solvents are run to 1 cm from the edge of the plate (ca. 5 min) for each dimension, and the plates are dried between solvents.

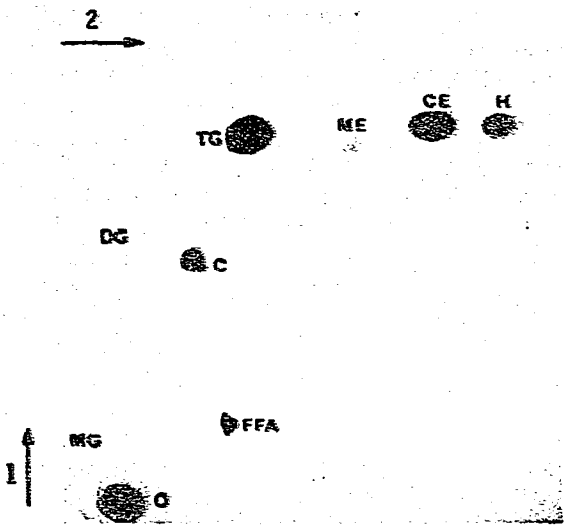

Fig. 3. Two-dimensional separation of neutral lipids. See text for details. H = hydrocarbons; CE = cholesteryl esters; ME = methyl esters; TG = triglycerides; DG = diglycerides; C = cholesterol; FFA = free fatty acids; MG = monoglycerides; O = phospholipids.

Visualisation

Charring. Because the plates do not contain any binder they do not stand up very well to spraying with aqueous reagents. They are best charred therefore, following vapour deposition of acid [31]. A plate is held in steam for 30 sec and then placed in a jar containing sulphuryl chloride vapour for 1 min. It is then heated at 180° on a hot plate for 5 min. This technique may be used prior to estimation of phospholipids by assay of phosphorus, but when measurement of neutral lipids or determination of fatty acid composition or radioactivity is required, the following non-destructive method must be used.

Iodine vapour. Plates are stood in iodine vapour for about 1 min at room temperature. The areas of lipids revealed fade rapidly on removal from the iodine vapour and should be outlined immediately with a needle.

Fluorescence. When lipids are to be analysed for fatty acid composition by gas–liquid chromatography (GLC), iodine vapour cannot be used, because it complexes with double bonds of unsaturated fatty acids. In this case the lipids are revealed by impregnating the plates with a fluorescent dye, 2,5-di-(5-*tert.*-butyl-2-benzoxazolyl)-thiophene (BBOT), which is normally used in liquid scintillation counting [32]. For both phospholipid and neutral lipid plates, BBOT (Ciba-Geigy, Duxford, Great Britain) is dissolved in the chromatography solvent (10 mg per 100 ml). After chromatography the plate is placed in a transparent plastic box under an atmosphere of nitrogen and viewed under UV light (3650 Å).

Photographic recording of separation

Charred and iodine stained plates are recorded by photography in transmitted light, using a small light box containing two 5-W fluorescent tubes. Plates

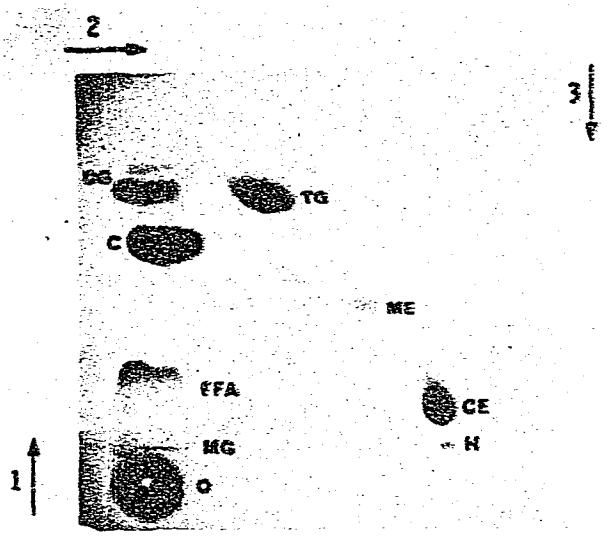

Fig. 4. "Three-dimensional" separation of neutral lipids. See text for details. Spot identification as in Fig. 3.

impregnated with BBOT are recorded by photography under UV light using a Wratten 6B lens filter.

Assays

Where assays are to be performed on samples separated by TLC, the appropriate area of the plate is scraped dry into a test tube by means of a small piece of razor-blade held in artery forceps.

Phosphorus assay. This assay is based on the malachite green method of Itaya and Ui [33]. The tubes used for this assay are 5 ml with C14 glass stoppers. They are reserved exclusively for this sensitive method. Glass distilled water is used throughout. To each tube is added 0.2 ml 60% perchloric acid and the material digested at 170—180° for 1 h. The tubes are cooled and 1.0 ml water added to each. With small spots from the plate 0.1 ml 60% perchloric acid and 0.5 ml water may be used to provide increased sensitivity, but for larger spots such as lecithin, the larger volumes must be used to ensure complete digestion of the phospholipid. The tubes are vortexed and then centrifuged at 1000 *g* for 5 min. 0.5 ml of the supernatant is sampled using an Eppendorff pipette, 1.5 ml of colour reagent (see below) is added and the tube shaken. The extinction at 660 nm is read against a reagent blank between 5 and 30 min later. A standard curve is prepared using a solution of potassium dihydrogen phosphate in the range of 0.05—1.2 μg phosphorus (1.6—38.8 nmoles).

The colour reagent is prepared as follows: one volume of ammonium molybdate (4.2% w/v in 5 *N* HCl) is mixed with 3 volumes of 0.2% (w/v) malachite green in water. After 30 min the mixture is filtered and kept at room temperature. It may be kept for 3 weeks.

Fluorimetric assay of cholesterol and cholesteryl esters. This method is based on that of Bondjers and Björkerud [34]. Lipid is extracted from the silica gel

with 2 lots of 1 ml of chloroform. The extracts are pooled, evaporated to dryness and 0.5 ml chloroform and 1.5 ml of freshly prepared reaction mixture (see below) are added. The tubes are stoppered and heated at 60° for 30 min then cooled immediately in crushed ice. The sample is measured at room temperature in a fluorimeter at an excitation wavelength of 528 nm, fluorescence wavelength of 565 nm between 40 and 80 min after heating. A standard curve of cholesterol or cholesteryl oleate is prepared in the range of 0.1—10 μg cholesterol (0.26—25.9 nmoles).

The reaction mixture is prepared as follows: a stock solution of zinc chloride is made by heating 4 g of zinc chloride (dried over phosphorus pentoxide) in 15 ml of glacial acetic acid at 80° for 2 h. This stock solution will last for 2 weeks at room temperature provided it is kept dry. A working solution is made immediately before use by adding 0.5 ml of stock solution to 10 ml of AnalaR acetyl chloride.

Glyceride assays. The glycerides are assayed using the autoanalyser method of Leon et al. [35] following elution from the silica gel: To each tube is added 1.0 ml *tert.*-butanol—isopropanol—water (50 : 50 : 4). The tubes are vortexed, stood for 1 h, vortexed again and then centrifuged at 1000 *g* for 5 min. The supernatant is decanted into a stoppered autoanalyser cup. A standard curve is prepared with diolein or triolein in the range of 5—60 μg triolein (5.7—67.8 nmoles).

Free fatty acid assay. This is based on a novel autoanalyser method of Bowyer et al. [36]. To each tube is added 1.2 ml of di-*n*-butyl ether and 1.0 ml of copper reagent. The tubes are vortexed for 2 min and centrifuged at 1000 *g* for 5 min. 1.0 ml of the upper phase is taken, 1.0 ml of colour reagent added and the colour allowed to develop for 15 min. The extinction is read at 540 nm. A standard curve is prepared using oleic acid in the range 1—6 μg (3.9—23.4 nmoles).

The copper reagent contains 0.1 *M* aqueous cupric acetate—water—glycerol—pyrrolidine (10 : 45 : 45 : 2). The colour reagent is a saturated solution of diphenyl carbazide in 0.5% (v/v) acetic acid in *n*-butanol, which is filtered before use.

Measurement of radioactivity. Silica gel from the plates is placed directly into the counter vials and 10 ml of scintillator fluid added. The solution which is based on that described by Patterson and Greene [37] contains: 1000 ml toluene; 500 ml Triton X-100, reagent grade; 6 g 2,5-diphenyloxazole (PPO), Packard, scintillation grade; 0.3 g 1,4-bis-(5-phenyloxazolyl-2)-benzene (POPOP), Packard, scintillation grade; 150 ml distilled water.

Preparation of methyl esters of fatty acids for GLC and estimation of fatty acid concentrations by an internal standard method. This is based on the method of Bowyer et al. [38]. Following chromatography, separated lipids are located by fluorescence using BBOT under nitrogen (see above). The required fractions are scraped into tubes and 1 ml of transmethylation reagent added. The tubes are stoppered and heated at 80° for 1 h except for sphingomyelin which is heated for 16 h. The tubes are then cooled, the solution transferred with 1 ml of distilled water to a tap funnel and extracted with 2 portions of 10 ml petroleum ether. The petroleum extracts are then combined, washed with 10 ml water and dried over ca. 0.5 g of anhydrous sodium bicarbonate for

5 min. The extract is then decanted off into a clean tube and evaporated to a small volume in a stream of nitrogen. This is then transferred to a new disposable glass sample vial and evaporated to dryness. The sample is dissolved in 1 μl of carbon disulphide and injected into the chromatograph (Pye Series 104, with column of 10% polyethylene glycol adipate on Celite, run isothermally at 190°; carrier gas, nitrogen; detector, hydrogen flame ionisation).

Extreme care must be taken to use ultra-clean glassware and redistilled solvents throughout. A blank of silica gel from a plate is run through the whole procedure before any samples are prepared to ensure that all of the reagents and glassware are clean.

The methylating solution is prepared specially in order to reduce contamination as follows: AnalaR methanol is redistilled before use from one pellet of sodium hydroxide using a redistillation apparatus closed with a drying tube containing calcium chloride. Concentrated sulphuric acid (3 ml) is then added to 100 ml of the redistilled methanol. The mixture is then heated at 70° for 1 h and extracted with 50 ml of petroleum ether and the extract discarded.

Where quantitation of the fatty acids of lipid fractions is required, an internal standard of margaric acid (C17:0) is added at a convenient stage [39]. In the estimation of free fatty acid the standard is added to the tissue before extraction; for other lipids it is included in the transmethylation step and it is assumed that the percentage conversion of the standard to methyl esters is the same as the conversion of the fatty acids of the complex lipids. A convenient weight of margaric acid is 10 μg for the lipids from a sample of 0.2 ml plasma or 50 mg of arterial tissue. This is added as a solution (100 μg/ml) of margaric acid in chloroform.

The weights of the separated fatty acids are determined by comparing the peak area for each fatty acid with the area of the added internal standard.

RESULTS AND DISCUSSION

Separation of phospholipids

A typical separation of phospholipids from atherosclerotic rabbit aorta is shown in Fig. 2. Such resolution has been consistently obtained in over 2000 separations and is only compromised if the plates are not properly activated or the sample is not clean, being contaminated by inorganic ions. Thus, plates should not be left in a humid environment, nor should they be heated for more than 1 min at 100° as this causes the ammonium sulphate to break down.

During preliminary investigations of optimum conditions of chromatography, the addition of materials to the silica gel to improve resolution of the phospholipids was investigated. Various substances had been suggested, for example magnesium silicate and magnesium acetate [40], sodium acetate [41,42], borax [43,44], sodium oxalate [45], and ammonium ions as ammonium sulphate or ammonium nitrate [11,25,26]. The use of the appropriate concentrations of ammonium sulphate as suggested by Kaulen [26], gave consistently good separation as shown here. The improved resolution apparently occurs because the bound hydrated ammonium ion increases the strength of hydrogen bonding to the stationary phase. Mobility of the acid phospholipids is also altered, because of the formation of their ammonium salts. A similar effect is

achieved if ammonium hydroxide is added to the chromatography solvent [25].

The separated phospholipids have been identified by co-chromatography with pure standards. In addition, the lyso-phosphatidyl ethanolamine (LPE) spot was isolated and kindly analysed by Dr. R.M.C. Dawson by hydrolysis and separation of the constituent bases [46]. Glyceryl-phosphoryl ethanolamine was the only base found, confirming the identity as LPE.

The identity of the phosphatidyl choline, phosphatidyl serine, phosphatidyl inositol and phosphatidyl ethanolamine (respectively PC, PS, PI and PE) spots was also confirmed by separation of radiolabelled lipids. These were synthesised from radiolabelled choline, serine, inositol or ethanolamine by incubation with minced arterial tissue in Krebs—Ringer bicarbonate buffer (pH 7.4) for 1 h. In each case 85% of the radioactivity was found in the expected spot. No label was found in lyso-phospholipids. Residual activity was associated with the origin of the plate, but its identity was not established.

Pure standards of phosphatidic acid and cardiolipid have also been chromatographed in this system and migrate near to the solvent front. One minor component of arterial lipids (X) remains unidentified.

In our experience, the method described for the separation of phospholipids gives much better separation than the single dimension separations, which have been described for the resolution of PC, PI, PS [47—51]. We have also found it to be as good as many of the 2-dimensional systems on large plates [52—58], but having the very important advantage of high speed of separation, good sensitivity and economy of materials. The only other published method [18] for the 2-dimensional separation of phospholipids using small plates, failed to separate PI from PS.

Assay of phospholipids

In preliminary investigations, it was found that the techniques of analysis of phosphorus [59—61] which have been widely used for phospholipids separated on large TLC plates were not sufficiently sensitive for the small amounts of material from small plates. On the other hand, the method of Itaya and Ui [33] using malachite green had sufficient sensitivity except where a spot was barely visible following charring (less than 1 μg of lipid). Other methods of similar sensitivity using malachite green have recently been published [62,63]. An even more sensitive fluorescence method using rhodamine G [18] might allow the measurement of phospholipid in even the smallest spots. That technique has the disadvantage, however, that the lipid must be eluted from the silica gel, a time consuming and potentially inaccurate technique and that a standard curve must be prepared with a pure standard for each lipid class.

The method of Itaya and Ui [33] was modified slightly in our procedure. Originally it required the addition of Tween 20 to stabilise the reaction product. We found that the Tween absorbed strongly at 660 nm, the wavelength used to measure the extinction of the reaction product. Thus, small errors in pipetting the Tween lead to large errors in the results. Its omission did not lead to rapid fading of the colour by precipitation of complexes, as suggested by Itaya and Ui, and the colour was quite stable at room temperature up to 30 min after addition of the malachite green reagent.

Using the techniques of charring on the plate and digestion of lipid in the

presence of silica gel we found no interference with the assay. This is in contrast to some reports concerning analysis by the phosphomolybdate complex methods, where the presence of silica gel may alter the pH [64] or lead to the formation of a silico-molybdate complex [65]. In our technique the very small amount of silica gel produced no effect.

In some samples, when there was a large difference in the concentration between the smallest and largest components, it was necessary to sample only a portion of the digest of the larger spots. This was simply achieved by the use of a different size Eppendorff pipette and did not affect the precision. With a little experience it was easy to judge from the intensity of charring on the plate, whether the whole sample or only a portion would be required for analysis.

In order to assess the overall recovery of phospholipids after TLC, 6 samples each of lyso-lecithin (LPC), PC and PE were assayed directly and 6 of each following TLC. The 6 samples were chosen to form a series of increasing amount within the usual range of the assay and the best straight line through the points computed by least squares. The mean recoveries for the chromatographed samples were: LPC, 98.5%; PC, 100.6%; PE, 95.0%.

Separation of neutral lipids

Typical separations of neutral lipids in 2 and 3 dimensions are shown in Figs. 3 and 4. Good resolutions in both systems have been consistently obtained. Impregnation of the silica gel with sodium carbonate markedly improved the reproducibility of separation of the free fatty acids (FFA); this was presumably due to stabilisation of the pH of the layer.

"Three-dimensional" separation was useful when a sample, such as atherosclerotic arterial tissue, contained a high concentration of free cholesterol and cholesteryl esters. The separated neutral lipids have been identified by co-chromatography with pure standards.

Assay of neutral lipids

The specific chemical methods of analysis had sufficient sensitivity for analysis of the amounts of materials encountered in most samples.

The fluorimetric analysis for cholesterol and cholesteryl esters [34] was sufficiently sensitive for measurement even of the low concentrations of cholesteryl esters in undiseased arterial tissue.

The method was also tested for interference by di- and triglycerides (DG, TG) and squalene; none was found. The recoveries after TLC for 6 samples each of free cholesterol (CHOL) and cholesteryl oleate (CE) in the usual range of assay, were: CHOL, 98.7%; CE, 94.8%.

The fluorimetric analysis of glycerides [35] by autoanalyser was always sufficiently sensitive for TG estimation, but in general the amount of DG was too small (<5 μg) to be measured. The recovery for 6 samples of TG was 97.5%.

Although the concentration of FFA in arterial tissue is small, the amount present in relation to the other major neutral lipids of a sample, could be measured by the novel method of Bowyer et al. [36]. Like other methods [66,67], this technique is based on the extraction of copper soaps of fatty acids into organic solvent, followed by estimation of copper. It is novel,

however, in two respects. Firstly, the di-*n*-butyl ether used as organic solvent forms an upper phase in the biphasic mixture and thus allows a cleaner and easier separation from the aqueous copper reagent. Secondly, the sensitivity of assay of copper is increased by the use of diphenyl carbazide. The recovery for 6 samples of FFA (oleic acid) was 93.6%.

General methods of analysis of phospholipids and neutral lipids separated by TLC

In the past few years many people have attempted to estimate materials which have been separated by TLC, directly on the plates, either by charring, followed by densitometry with a scanning spectrophotometer or by staining with a fluorescent dye, followed by scanning with a fluorimeter. It has been suggested that these methods have the advantage of speed over chemical analyses whilst providing good reproducibility. We have also investigated charring in situ using sulphuric acid-sodium dichromate sprays and vapour phase acid impregnation [31], followed by densitometry. In the first place small streaks of lipid were chromatographed in 1 dimension as suggested by Van Gent [14] and scanned using the Joyce Loebel scanning densitometer. When separated components were the same width as standards, good reproducibility was obtained. When, however, chromatography was not perfect because of a poor plate or poor sample application and hence the streaks were not uniform, the results were more variable. This obviously arises because the density of charred materials within the spectrophotometer window depends upon the width of the streak. In order to overcome this problem and to permit scanning of spots on 2-dimensional separation, we developed [68] a flying spot television scanner, connected to a PDP-7 computer which produced a digitised image of a plate. By the use of object recognition programs [69], it was possible almost instantaneously to present a table of integrated optical densities and hence amounts of each material on the plate. This approach thus overcame the difficulties inherent in 2-dimensional scanning. Similar techniques using a flying spot micro-densitometer and batch computation on an IBM 360 of the digitised image have been described [8].

Despite the undoubted improvement provided by this approach over conventional scanning, it has been our experience that the methods are less reproducible than chemical assays. Furthermore, the charring and fluorescence depend upon the fatty acid composition of the separated lipids, and for the most precise work a standard of the same composition as that of the unknown is required; a most improbable practicality. The difficulty of establishing reliable and stable standards to which assays done anywhere in the world can be related on an absolute scale is almost insurmountable. We have thus preferred to go back to precise chemical assays which can be reproduced anywhere. In addition, the method does not require specialised and expensive apparatus.

Measurement of radioactive samples

Various methods for measurement of radioactivity of lipids separated on TLC have been described. These include elution and direct counting of adsorbent either in scintillation cocktail or as suspension in Cabosil. The

direct method in scintillator is to be preferred from the point of view of simplicity and also because no loss of material is incurred in elution. There is a potential disadvantage, however, that if lipid remains bound to the silica gel and is not dissolved in scintillator, self-absorbtion losses of activity will occur, especially with weak β-emitting isotopes such as ^{3}H. In preliminary experiments, we investigated various scintillation mixtures and found that whereas non-polar lipids such as cholesteryl esters could be counted without self-absorbtion losses in a scintillator containing only toluene, a polar scintillator containing water was required for counting the polar phospholipids. The most effective and economical system was the heterogeneous system of Patterson and Greene [37] with the addition of 10% water. A similar system using a commercial scintillator mixture, Aquasol, with 10% water has been described by Webb and Mettrick [70].

In order to test for complete elution of representative lipids into the scintillator, samples of [7-^{3}H]cholesteryl oleate, [9,10-^{3}H]palmitic acid and 1-palmitoyl, 2-[9,10,12,13-^{3}H]linoleyl-glyceryl-phosphorylcholine, i.e. [^{3}H]-lecithin were counted, after TLC, in the toluene, Triton X-100 scintillator with and without water. The samples were shaken and the count rate determined for a period of 30 min. The results, summarised in Fig. 5, show that in scintillator without water, for lecithin and to a lesser extent for palmitic acid, the count rate falls as the silica gel sediments. On the other hand, in the scintillator containing water, although the absolute count rate is lower, it does not fall as the silica gel sediments, because the lipids are in solution. When it is required to measure counting efficiencies, either External Standard Channels Ratio (ECR) or Sample Channels Ratio (SCR) may be used, because complete elution of lipid into solution produces a homogeneous sample [71]. In the determination of efficiencies by ECR using counters such as the Nuclear Chicago, in which the external standard is positioned underneath the vial, it has been shown that the presence of more than 50 mg of silica gel on the bottom of the vial may lead to erroneous results [72]. In the method described we have shown that the small amount of silica gel introduced into the vial, even with the largest sample spot, is too small (<10 mg) to cause any interference.

Elimination of losses of lipid during the Folch extraction procedure and transfer to TLC

In view of the small amount of material which may be measured by this method and the number of stages and transfers during extraction, it was decided to investigate the recovery of lipid through the extraction procedures. Any stages at which losses or variability occurred were discovered by sequential investigations and methods were devised to overcome problems.

Firstly, an important reason for low recovery was found to be incomplete transfer of lipids through the various pieces of glassware used. Transfer from the small sample vial was improved by the use of two Hamilton syringes (one containing the sample, the other a wash of the sample vial). An unexpected loss was also found in transfer of the sample from the flasks used for evaporation of solvents. Originally chloroform—methanol (2:1) was used. Although quantitative transfer of relatively non-polar lipids such as cholesterol and cholesteryl esters was achieved, there was incomplete recovery of the more polar phopholip-

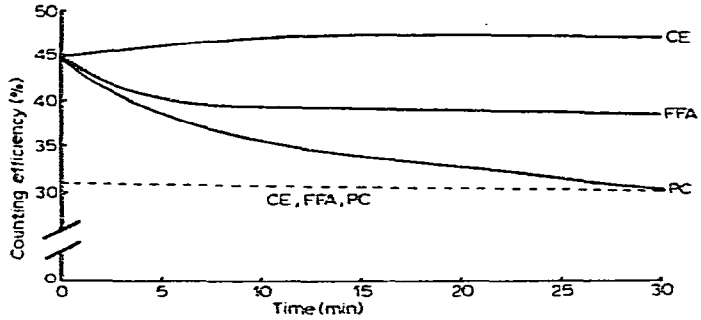

Fig. 5. Graph showing counting efficiency of [^{3}H]cholesteryl oleate (CE), [^{3}H]palmitic acid (FFA) and [^{3}H]lecithin (PC) in toluene—Triton X-100 scintillator, without (—) or with (— — —) 10% water.

ids such as LPC (80.0%) and PC (80.8%). This problem was overcome by the use of chloroform—methanol—saline (86 : 14 : 1). Lipids were dissolved in chloroform—methanol (2 : 1) and samples assayed directly. The solvent was evaporated to dryness and the sample transferred to small vials with three washes of either chloroform—methanol (2 : 1) or chloroform—methanol—saline (86 : 14 : 1). The samples were then re-assayed. Only the latter solvent gave complete transfer of polar lipids. It is probable that some of the polar phospholipids are bound to the glass by virtue of their surface active properties unless a wet solvent such as chloroform—methanol—saline (86 : 14 : 1) is used for transfers.

Secondly, careful analysis also showed that loss of extract could occur on the glass fibre filter disc. This was overcome by the procedure of squeezing the disc with forceps, washing with a further portion of solvent and then squeezing the disc again.

Thirdly, we considered the effectiveness of the extraction procedure. Although the method of Folch et al. [27,28] is well established, certain steps require careful attention or incomplete and variable extraction occurs. We have found that it is important during addition of the first extraction solvent, that the methanol be added before the chloroform. If a mixture of solvents is used, putty-like globules are formed and the lipid is only partially extracted. When methanol is added first, recovery of lipid is complete. Table I summarises the results of an experiment demonstrating this effect. Twelve 0.2-ml samples of plasma from a Cynomolgus monkey were extracted as described in the methods section, using either chloroform—methanol (2 : 1) mixture for the first solvent or methanol and chloroform sequentially. Six further samples were extracted with isopropanol as for the autoanalyser estimation [35] of cholesterol and triglyceride. The extracts were analysed for cholesterol as described [34]. When chloroform—methanol (2 : 1) was used, the recovery of cholesterol was only 56% compared with the isopropanol extract, but when methanol and chloroform were added in sequence, the recovery was 96%.

In the Folch procedure it is also important to minimise the loss of polar compounds, such as lyso-phospholipids and free fatty acids into the wash phase. In our procedure this was achieved by re-extracting the upper aqueous wash

phase with chloroform—methanol—saline (86 : 14 : 1). The use of a saline wash acidified with 0.01 *N* HCl as recommended by Bjerve et al. [73] for preventing loss of lyso-phosphatides was also investigated. This produced however, an insignificant improvement in lyso-lecithin recovery. It also had the serious disadvantage of causing hydrolysis of plasmalogens during an overnight wash even at 4° and was, therefore, not used.

The recovery of lipid through the whole procedure was checked using free cholesterol, cholesteryl oleate, triolein, lyso-lecithin, lecithin and phosphatidyl ethanolamine in separate experiments. For each lipid, standards containing 6 points in the working range of the assay were set up and the samples assayed directly. Similar amounts were then run through the whole procedure involving extraction, filtration and "Folch-washing" and then assayed. Recoveries were as follows: cholesterol, 95.0; cholesteryl oleate, 102.2; triolein, 95.7; oleic acid, 95.4; lyso-lecithin, 92.0; lecithin, 97.2; phosphatidyl ethanolamine, 88.6%.

Precision of the method for biological samples

In order to measure the precision of the methods, 6 samples of human plasma were analysed. Table II shows the results and the coefficient of variation

TABLE I

RECOVERY OF CHOLESTEROL FROM PLASMA AFTER EXTRACTION WITH DIFFERENT SOLVENTS

	Isopropanol	Methanol and chloroform in sequence	Chloroform—methanol (2 : 1)
Concentration (mg/100 ml)	385.3	367.0	198.6
± S.D.	± 14.60	± 14.09	± 9.06
Coefficient of variation (%)	3.79	3.84	4.56
Recovery compared with isopropanol extract (%)		95.9	55.7

TABLE II

REPRODUCIBILITY FOR REPLICATE ANALYSIS OF THE MAJOR LIPIDS OF HUMAN PLASMA

Concentration m*M* ± % coefficient of variation (% CV). Abbreviations as in Figs. 2 and 3.

	LPC	SPH	PC	FFA	CHOL	CE	TG
One lipid extract separated by TLC and analysed 6 times							
Mean	0.140	0.369	0.640	0.446	0.683	1.815	0.616
± % CV	9.09	3.44	8.76	9.01	9.13	5.59	3.44
6 samples of plasma extracted, separated by TLC and analysed							
Mean	0.161	0.365	0.660	0.438	0.661	1.788	0.580
± % CV	12.42	4.66	8.33	4.80	9.53	6.94	5.35

for the major lipids. In each case the errors are of the same order as commonly associated with pipetting.

ACKNOWLEDGEMENTS

We wish to thank Dr. R.M.C. Dawson for analysing and identifying LPE and for pointing out the hydrolysis of plasmalogens by acidified solvents. We are also grateful to Mrs. L.M. Wright for secretarial assistance and to Mr. P. Curtis for preparation of photographs and figures, and to the Tobacco Research Council of Great Britain, Nattermann, Köln, G.F.R. and May & Baker, Dagenham, Great Britain for financial support.

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