QUANTITATIVE THIN LAYER CHROMATOGRAPHY OF LIPIDS

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Thin layer chromatography is routinely used for the separation of all classes of lipids and has now been variously adopted to provide the basis for quantitative analysis of separated lipid[1]. Thus, CLAUSEN, LOU AND ANDERSEN[2] and SIAKOTOS AND ROUSER[3] have described methods of analysis based on densitometric measurement of spots visualised under standard conditions. Other authors prefer to scrape off the spot and extract the lipid repeatedly with methanolic solvent mixtures[4,5] or each area of separated lipid may be directly ashed and the released phosphate determined[6–8].

A previous communication from this laboratory[9] gave a preliminary account of both the principles and application of an alternative method providing for the complete recovery of intact lipids and their estimation after prior separation on thin layer chromatograms. In this paper further details of the present improved technique are given together with the analysis of various tissue lipids. Special attention has been given to the separation of certain minor lipid compounds.

METHODS

Lipid extracts

Protein free, washed extracts of human cerebral white matter (internal capsule), human erythrocyte ghosts and rat liver were prepared in chloroform–methanol (2:1, v/v) by the method of FOLCH, LEES AND SLOANE-STANLEY[10] and WEBSTER AND FOLCH[11].

Thin layer chromatography

The technique of thin layer chromatography was as previously described[9]. Chromatographic glass plates (20 × 20 cm) were layered with 0.5 mm thick Kieselgel G (Merck). Lipid extract (0.2–0.5 ml) containing not more than 5 mg was placed as a strip (3 cm long) at the origin and the chromatogram developed at room temperature. The various solvent mixtures employed for developing the chromatograms are shown in Table I. After drying the plates, spots or zones were generally made visible by exposure to iodine vapour[4] except where iodine could interfere in subsequent analysis. For identification purposes, more specific detection methods used were similar to those reported by SKIPSKI *et al.*[4] and SKIDMORE AND ENTENMAN[13]; in addition chromatograms of pure authentic lipid were run as markers.

After staining lipids with iodine, a rectangular zone enclosing each spot was marked off. The plates were air dried to remove most of the iodine and the adsorbent containing each lipid was transferred to a glass column (1 × 10 cm) with a reservoir (3 × 5 cm), which held up to 50 ml of eluting solvent. Traces of silicic acid and any

TABLE I

THIN LAYER CHROMATOGRAPHY OF LIPIDS

Authentic samples of lipid were run on silica gel plates with ascending developing solvents. Mixture A contained chloroform–methanol–ammonia 32.5% w/v in aqueous solution (17:7:1, by vol.); mixture B contained chloroform–methanol–ammonia 20.2% w/v in aqueous solution (17:7:1, by vol.); mixture C contained chloroform–methanol–water (14:6:1, by vol.); solvent D was 1,2-dichloroethane. The temperature of the chromatographic tank was maintained at 18–20°. The identity of each separated lipid was checked by use of appropriate spray reagents and was compared with R_F values of authentic samples of known lipids. The mean R_F values indicate the order of separation and allow comparison of the three systems.

Lipid	*R_F values*			
	A	*B*	*C*	*D*
Cholesterol ester	0.99	—	—	0.8
Triglyceride	—	—	—	0.55
Cholesterol	0.95	0.97	0.95	0.20
Cerebrosides	0.64 0.55	0.70 0.61	0.79 0.72	
Cardiolipin	0.55	0.50	0.84	
Ethanolamine phospholipid	0.45	0.40	0.58	
Sulphatide	0.45	0.40	0.45	
Lecithin	0.34	0.33	0.33	0
Sphingomyelin	0.18	0.15	0.20	
Ethanolamine lysophospholipid	0.18	0.15	0.26	
Phosphatidyl inositol	0.11	0.15	0.20	
Lysolecithin	0.11	0.15	0.15	
Serine phospholipid	0.06	0.07	0.10	
Serine lysophospholipid	0.03	—	—	
Phosphatidic acid[12]	0	0	0	

TABLE II

ELUTION SOLVENT MIXTURES

Each silicic acid zone containing a single lipid should be treated with solvents as described below. C = Chloroform; E = ethanol; M = methanol; W = water; N = 32.5% aqueous ammonia (w/v).

Lipid	*Solvent composition*	*Volume (ml)*
Cholesterol	C	20
Cerebrosides	C–M–W (7:7:1)	30
Cardiolipin	C–M–W (7:7:1)	30
Ethanolamine phospholipid	C–M–N (18:6:1)	30
Sulphatide	C–M–W (7:7:1)	30
Lecithin	C–M (1:1)	30
	E–C–W (5:2:2)	5
Sphingomyelin	C–M (1:1)	30
Ethanolamine lysophospholipid	E–C–W (5:2:2)	20
Phosphatidyl inositol	E–C–W (5:2:2)	20
Lysolecithin	C–M (1:4)	20
Serine phospholipid	E–C–W (5:2:2)	20
Serine lysophospholipid	E–C–W (5:2:2)	15

lipid remaining on the area of exposed glass plate were removed by cleaning with fat free cotton wool damped with chloroform and transferring this pad to the top of the columns. The columns were so packed as to allow slow passage of solvent mixtures, and elution overnight proved to be most convenient (Table II).

Plasmalogen

For the estimation of individual plasmalogen lipids, plates were not stained with iodine. Lipid samples and markers were spotted on the middle two and outside two lanes respectively of the thin layer plate. After the plate was developed and dried, the middle lanes were covered with a plate of glass or a wide strip of cellophane and the thin layer chromatogram stained with iodine (1 % w/v in petroleum ether b.p. 100–120°). Visible lipid zones were marked off and the iodine allowed to fade. The covering was removed and using the outer lanes as guides, the lipids in the middle lanes of the TLC were marked off. The plasmalogen lipid areas were removed, transferred to columns, eluted and evaporated under nitrogen at room temperature. The plasmalogen content was immediately determined. The method was checked by separating human white matter lipids and determining the plasmalogen content of the ethanolamine phospholipid zone. All the phospholipid could be recovered as phosphatidal ethanolamine indicating that no breakdown had occurred; in addition the results agreed with those reported previously[14]. Other samples of rat myelin were analysed by quantitative thin layer chromatography and the reliability of the technique again confirmed.

Second stage chromatography on alumina

The same type of glass columns were packed with 6–10 g of alumina (alumina for chromatography—BDH, Poole, England) and the powdered silicic acid–lipid zones to be rechromatographed were transferred to the top of the alumina. The lipids were then fractionated by stepwise elution, using the solvents shown in Table III.

Analytical methods

Details of analytical methods used are described in previous papers. Phospholipid phosphorus was determined by the method of MARTLAND AND ROBINSON[15], cholesterol and its esters by the method of SPERRY AND WEBB[16] and the sphingosine content of cerebroside and sulphatide was estimated using the method of LAUTER AND TRAMS[17]. Plasmalogens were determined by the iodine absorption method of WILLIAMS, ANDERSON AND JASIK[18], primary amino groups by the ninhydrin method of RHODES AND LEA[19] and ester groups, using the hydroxylamine reagent of SHAPIRO[20]. Total galactose content of lipid extracts was determined by a method based on those of EDGAR[21] and SVENNERHOLM[22] and the choline content of lecithin was determined by the method of SMITS[23].

Inositol was released from lipid samples by acid hydrolysis and assayed with *Kloeckera brevis*, using the method of CAMPLING AND NIXON[24]. Lipid samples were hydrolyzed in sealed tubes with 4 ml of 50 % v/v HCl at 110° for 6 h. The tops were broken off the sealed tubes, which were subjected to a stream of air in a boiling water bath until the volume was reduced to about 1 ml. This removed a large proportion of the HCl and the hydrolysate was then completely neutralized with 1*N* NaOH. The samples were then added to inositol assay medium KB (Difco Laboratories) and assayed with *K. brevis*.

TABLE III

FRACTIONATION OF LIPID SUBFRACTIONS ON ALUMINA AFTER THIN LAYER CHROMATOGRAPHY

Mixtures of pure lipids were analysed before and after separation on thin layer chromatography. Duplicate samples run on chromatograms were transferred as silicic acid areas to the top of pre-packed alumina columns. Solvent mixtures used were E = chloroform–methanol (1:1, v/v); solvent F = chloroform–methanol (1:4, v/v); solvent G = chloroform–methanol–water (7:7:1, by vol.); and solvent H = ethanol–chloroform–water (5:2:2, by vol.).

No.	*Lipid*	*Total lipid applied (μmoles)*	*Lipid recovered from thin layer plate (μmoles)*	*Lipid recovered after transferring silicic acid zone to alumina column and eluting with the respective solvents (μmoles)*			
				A (100 ml)	*F (80 ml)*	*G (80 ml)*	*H (100 ml)*
1	Pure lysophosphatidyl ethanolamine	0.383	0.380	0.004			0.380
2	Pure sphingomyelin	0.830	0.811	0.800			
1 + 2				0.795			0.382
3	Pure lysolecithin	—	0.500		0.473		
4	Pure phosphatidyl inositol	0.275	0.270		0		0.274
3 + 4					0.438		0.275
5	White matter sulphatide	—	0.240			—	
6	White matter ethanol-amine phospholipid	—	0.625			—	—
5 + 6						0.207	0.586

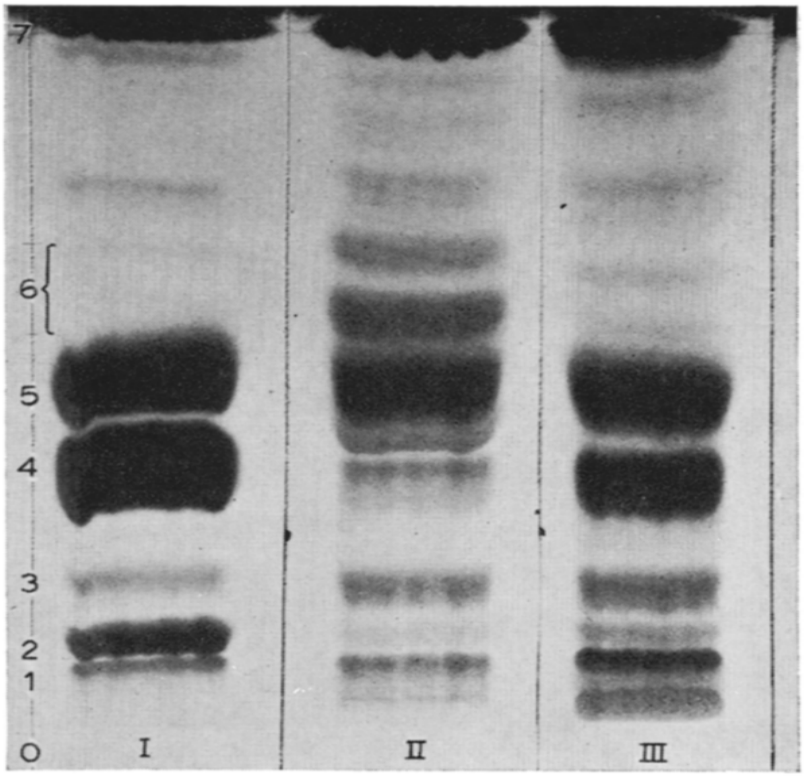

Fig. 1. Thin layer chromatograms of (I) rat liver, (II) human white matter, and (III) erythrocyte ghost lipids. Developing solvent, solvent A: chloroform–methanol–ammonia 32.5% w/v in aqueous solution (17:7:1, by vol.). O = Origin; 1 = serine phospholipid; 2 = phosphatidyl inositol; 3 = sphingomyelin; 4 = lecithin; 5 = ethanolamine phospholipid; 6 = cerebrosides; 7 = cholesterol. Quantity of lipid applied: 5 mg.

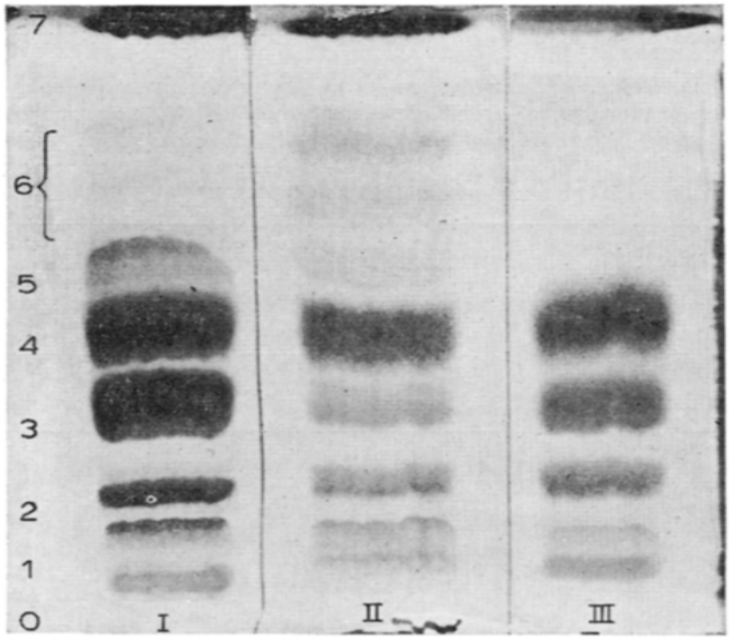

Fig. 2. Thin layer chromatograms of (I) rat liver, (II) human white matter, and (III) erythrocyte ghost lipids. Developing solvent, solvent B: chloroform–methanol–ammonia 20.2 % w/v in aqueous solution (17:7:1, by vol.). O = Origin; 1 = serine phospholipid; 2 = sphingomyelin and phosphatidyl inositol; 3 = lecithin; 4 = ethanolamine phospholipid; 5 = cardiolipin; 6 = cerebrosides; 7 = cholesterol. Quantity of lipid applied: 5 mg.

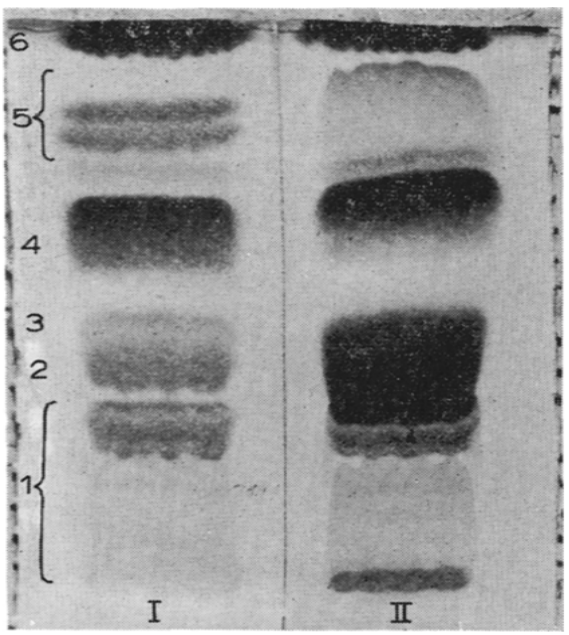

Fig. 3. Thin layer chromatograms of (I) human white matter and (II) rat liver. Developing solvent, solvent C: chloroform–methanol–water (14:6:1, by vol.). 1 = Phospholipids; 2 = lecithin; 3 = sulphatide; 4 = ethanolamine phospholipid; 5 = cardiolipin and cerebrosides; 6 = cholesterol. Quantity of lipid applied: 5 mg.

RESULTS

In preliminary studies (Table I) mixtures of chloroform and methanol with water or aqueous ammonia in different proportions were compared as developing solvents for individual standard lipids and for mixed phospho and galacto-lipids on Kieselgel G thin layer plates. 1,2-Dichloroethane was used as developing solvent for non-polar lipids. Solvent mixtures A and B were found to be particularly reliable for the separation of individual phospholipids and cerebrosides and were both employed in the analysis of the lipids of varied tissue fractions, such as myelin, microsomal membrane and erythrocyte ghosts. The second system (B) which resolved cardiolipin at the expense of monophosphoinositide, was used for the separation of mitochondrial lipid, which contains cardiolipin as a major component. The chloroform–methanol–water system (C) was particularly reliable for the separation of both cerebrosides and sulphatides, but frequently clear separation of phospholipid was not seen.

Purity and identity of separated lipids

Lipids separated by chloroform–methanol–32.5 % w/v aqueous ammonia (17:7:1) were eluted and found to run as single spots in different solvent systems. The individual lipids were identified firstly using specific chromatographic sprays[13]. The R_F values of the identified lipids were checked, using pure lipid markers, obtained from many sources. Each separated lipid was analysed for its chemical constitution. The analysis recorded in Table IV shows evidence for the identity and purity of separated lipids.

Secondary separation of mixed lipids

Where the amount of lipid was only just sufficient for one thin layer plate it was necessary to devise an additional fractionation step to obtain complete separation; this may be done by re-running eluted lipids on suitable thin layer plates or by column chromatography.

Nonpolar lipids. Triglycerides, cholesterol and its esters may be separated by further stepwise elution chromatography on alumina[25], on florisil[26] or on thin layer plates using carbon tetrachloride or 1,2-dichloroethane for development.

Minor phospholipids. In analysis of complex lipid mixtures, even with optimum solvent systems some overlapping of spots may occur. Three examples of second stage chromatography are shown in Table III. Samples of pure lipid standards (which run together in solvent mixture A) were applied to thin layer plates and after visualization the lipid zone from the chromatogram transferred to a pre-packed alumina column. Resolution of the mixed lipids was then obtained by stepwise elution of the alumina column.

Quantitative recovery of mixed lipids

Bulk extracts of human white matter and rat liver were prepared. Repeated analysis of each sample was achieved by the procedure described above (Table V). The method was further tested by mixing known volumes of the analyzed extracts and determining the composition of the resultant mixture (Table V). Lipid extracts, whose compositions are well established, were next prepared and analyzed in order to test the general reliability of the technique. Table VI shows the analysis of such lipid

TABLE IV

ANALYSIS OF SINGLE LIPIDS SEPARATED ON KIESELGEL G THIN LAYER PLATES

Lipid extracts of human white matter were separated and individual lipids recovered by elution of silicic acid zones on columns using appropriate solvents.

Separated lipid	*Molar ratio*									
	Phosphorus	*Ester*	*Serine*	*Choline*	*Ethanol-amine*	*Amine*	*Plasmal-ogen*	*Inositol*	*Galactose*	*Sphingosine*
Spot 1										
Serine phospholipid	1	1.9	0.9	—	0	0.9	—	—	—	—
Spot 2										
Phosphatidyl inositol	1	2.3	—	—	—	—	—	1.2	—	—
Spot 4										
Lecithin	1	2.2	—	0.94	—	—	—	—	—	—
Spot 5										
Ethanolamine phospholipid	1	1.5	—	—	0.95	1.2	1.04	—	—	—
Spot 6 + 7										
Cerebroside	0	—	—	—	—	—	—	—	1.08	1

lipid extracts in comparison with the most widely accepted figures available in the literature.

DISCUSSION

Methods of quantitative thin layer chromatography generally employed necessitate the destruction of separated lipid whether by charring or by digestion of separated silicic acid zones. Alternatively it is possible to recover intact lipid by repeatedly shaking silicic acid zones with solvent, centrifuging and re-extracting until lipid recovery is complete. This procedure is time consuming and moreover losses can easily occur during shaking and transference of eluting solvent.

TABLE V

QUANTITATIVE RECOVERY OF MIXED LIPID EXTRACTS FROM THIN LAYER CHROMATOGRAM (μmoles/mg lipid)

Samples of lipid extract or mixed extract were run on thin layer chromatograms using chloroform–methanol–32.5 % w/v ammonia (17:7:1, by vol.). Analytical methods are as described in the text.

Lipid	*Human white matter (3–6 runs)*	*Rat liver (3–5 runs)*	*Mixture of human white matter and rat liver (2–3 runs)*
Cholesterol	0.687 ± 0.025	0.151	0.790
Cerebroside	0.305 ± 0.033	—	—
Total phospholipid	0.508 ± 0.052	0.833 ± 0.022	—
Serine phospholipid	0.092 ± 0.002	0.031 ± 0.006	0.118
Phosphatidyl inositol	0.010	0.052 ± 0.007	0.061
Lysolecithin	0.004	0.008	0.010
Sphingomyelin	0.089 ± 0.004	0.022 ± 0.001	0.116
Lysophosphatidyl ethanolamine	0.020	0.016	0.037
Lecithin	0.122 ± 0.013	0.481 ± 0.018	0.570
Ethanolamine phospholipid	0.167 ± 0.015	0.206 ± 0.012	0.360
Cardiolipin	0.012	0.035	0.046
	101.5 % recovery	102 % recovery	

The methods described in this paper are designed to provide a rapid and reproducible means of quantitative separation of complex lipid mixtures. Recovery of lipids from silicic acid zones by elution in columns has four advantages: (1) it is time saving, in avoiding repeated shaking and extraction of silicic acid; (2) it results in the recovery of intact lipid and hence does not limit the use of analytical methods; (3) silicic acid is eliminated from the recovered lipid and hence it cannot interfere with colour development, or add to blank values; and (4) recovery of intact silicic acid-free lipid enables the method to be used for separation of labelled lipid. Previously completeness of recovery of phospholipid has been checked with ^{32}P labelled lipid[9]. In this paper fresh data gives detailed evidence of recovery based on repetitive chemical analyses.

The use of columns has also been adopted for a further subfraction akin to a two-dimensional chromatogram. ROUSER *et al.*[35] have employed diethylaminoethyl (DEAE) cellulose column chromatography as a primary step followed by quantitative thin layer chromatography. Spots produced are charred with sulphuric acid–potassium dichromate and measured by quantitative densitometry. The method described in this paper utilises the principle of column separation after thin layer chromatography.

TABLE VI

ANALYSIS OF VARIOUS TISSUE LIPIDS

The tissue extracts were prepared and lipids analysed by the method given in the text. Total phospholipid has been equated to a figure of 100 moles % and the molar ratios of all other lipids calculated on this basis.

Lipid	*Lipid molar ratios (phospholipid = 100)*						
	Human erthrocyte ghosts		*Rat liver mitochondria*		*Ox myelin*		
	Present study (3–6 runs)	*Literature*[27–30] *(2 runs)*	*Present study (1 run)*	*Literature*[31,32] *(4 runs)*	*Present study (3 runs)*	*Literature*[33,34] *(4–6 runs)*	*(5 runs)*
Cholesterol	89.5	89*	11.1	11	108	132[33]	110[34]
Cerebroside	—				49	51	48
Sulphatide	—				—	7	
Total phospholipid	100	100	100	100	100	100	100
Serine phospholipid	11.2	14.3	5.3	2.7	18.8	14	
Phosphatidyl inositol	4.7	1.4	7.5	6.0	3.1	2.0	
Sphingomyelin	27.2	20.1		4.6	15.4	15.5	
Lecithin	32.8	34.7	44.2	51.6	19.3	24	
Ethanolamine phospholipid	24.8	28	31.5	30.4	35.5	43	32
Cardiolipin	1.7	0	5.4	5.4	—	—	
Phosphatidic acid	0	2.2	—	—	—	—	

* Mean figures calculated from VAN DEENEN AND DE GIER[27].

This second stage separation has been successfully applied to resolution and analysis of lysophosphatides from other phospholipids, similarly it is possible to separate cholesterol, cholesterol esters and triglycerides on columns or by further running on thin layer plates.

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SUMMARY

A method for the quantitative separation of tissue lipids is described. The technique is based on preliminary separation of total lipids on thin layer plates of silicic acid with, when necessary, a second stage column chromatography. This simple procedure allows the complete recovery of intact single lipids from naturally occurring mixtures.

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